Towards treatment of cholestatic liver disease in children via interference with transcriptional regulation of hepatic transport systems
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CHAPTER 4

THE LXR-AGONIST T0901317 ATTENUATES ENDOTOXIN-INDUCED CHANGES IN HEPATOBILIARY TRANSPORTER GENE EXPRESSION IN MICE

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Under revision

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Abstract

Background & Aims: During sepsis-associated cholestasis, circulating endotoxins (e.g. LPS) activate resident liver macrophages, Kupffer cells, leading to pro-inflammatory cytokine production, activation of hepatocellular cell-signaling cascades and rapid suppression of hepatobiliary transporter expression. We sought to determine if LPS-associated suppression of transporter genes in mice could be attenuated by T0901317, a liver X receptor (LXR) agonist, recently shown to exert anti-inflammatory actions in LPS-stimulated peritoneal macrophages.

Methods: In vivo, mice were gavage-fed T0901317 (1-100mg/kg/day) for three days prior to intraperitoneal LPS injection. In vitro, mouse and rat primary hepatocytes were treated with TNFα or IL-1β after pre-treatment with varying doses of T0901317. Transporter and cytokine expression were assessed by real-time PCR and immunoblotting, plasma cytokine levels by Luminex assay, and nuclear transcription factors by EMSA and immunoblotting.

Results: In vivo, T0901317 attenuated LPS-induced suppression of 3 transporter genes—Na/taurocholate co-transporting polypeptide (Ntcp), multidrug resistance-associated protein 2 (Mrp2) and Mrp3, while up-regulation of multidrug resistance gene 1b (Mdr1b) was blocked. These effects were differentially reflected in protein expression with partially preserved Mrp2. Surprisingly, cytokine expression was not reduced by T0901317 pre-treatment, suggesting that T0901317 has direct anti-inflammatory activities in hepatocytes. In vitro, cytokine-induced suppression of Mrp2 expression, and nuclear NF-κB binding, were both attenuated by T0901317 pre-treatment.

Conclusions: LXR agonist T0901317 attenuates the effects of LPS on hepatic transporter gene expression in vivo, most likely via direct, cytokine-independent signaling pathways in hepatocytes. LXR agonists may provide a novel and effective means to reduce the deleterious consequences of hepatic inflammation.
**INTRODUCTION**

Sepsis-associated cholestasis is a frequently occurring clinical phenomenon in both adults and children, particularly in premature infants. The putative mechanism underlying this inflammation-induced cholestasis involves endotoxin-mediated (e.g., LPS) activation of cell signaling pathways, primarily in the resident liver macrophages, commonly referred to as Kupffer cells. LPS-activated Kupffer cells produce pro-inflammatory cytokines, including TNFα, IL-1β and IL-6. These, in turn, activate hepatocellular signaling pathways that change expression of many genes involved in bile formation, mainly affecting those centrally responsible for hepatobiliary transport. In rodent models, endotoxemia leads to down-regulation of bile acid transporters at RNA and protein levels, as well as to alterations of their subcellular localization and functional activity. Among the best studied hepatobiliary transporters that respond to endotoxemia at transcriptional and post-transcriptional levels are the basolateral Na+/taurocholate co-transporting peptide (Ntcp, Slc10A1), the canalicular bile salt exporting protein (Bsep, Abcb11) and the multi-drug resistance related protein 2 (Mrp2, Abcc2). The latter is primarily involved in transport of di-anionic bile salt metabolites and glutathione, whose secretion into the canalicular lumen along with that of bile acids exported by Bsep provides the driving force for bile formation in rodents.

A central role of Kupffer cells and their release of pro-inflammatory cytokines in the cascade leading to reduced transporter gene expression has been shown in various rodent models of sepsis-associated cholestasis. In vivo, inactivation or depletion of Kupffer cells not only reduced whole liver cytokine expression and secretion, but also attenuated the hepatocellular responses to endotoxin challenge. Moreover, treatment of primary hepatocytes with medium derived from LPS-treated Kupffer cells mimicked *in vivo* LPS-responses, while LPS itself had no apparent direct effect on hepatocytes, while addition of anti-cytokine antibodies to conditioned medium led to reduction of hepatocellular LPS-responses. This indicates that cytokines are indeed key mediators of this process, which was confirmed by *in vivo* studies in several models, in which administration of anti-TNFα and anti-IL-1β antibodies reduced LPS-induced effects on transporter expression and nuclear binding activity of key transcription factors. Conversely, treatment of rodents with TNFα or IL-1β mimicked the effects of LPS treatment.

Better understanding of the inflammatory cascade leading to cholestasis has provided several potential targets for intervention. These include members of the nuclear receptor (NR) superfamily, which have been shown to serve as key regulators of hepatic transporter gene expression and to be centrally involved in inflammatory pathways. Two type II members of the NR superfamily have recently been identified as potential anti-inflammatory targets—the peroxisome proliferator-activated receptor (PPAR)-γ and the liver X receptor (LXR). Recent evidence suggests that rosiglitazone, an agonist of the PPARγ (Nr1c3), acts as an anti-inflammatory agent.
in liver, and can interfere with endotoxin-mediated suppression of hepatobiliary transporter genes. LXR has two isoforms, LXRα and LXRβ (Nr1h3 and Nr1h2, respectively). LXRα is primarily expressed in the liver, intestine, kidney and adipose tissue, while LXRβ is ubiquitously expressed, albeit at a lower level. The natural ligands for LXR are oxysterols and LXR is primarily involved in control of cholesterol and fatty acid homeostasis, as it serves as a sterol sensor with feed-forward characteristics. Upon activation, LXR enhances the reverse transport of cholesterol, cholesterol esterification and biliary cholesterol excretion, as well as conversion of cholesterol to bile acids in rodents.

Recently, it was found that activation of LXR has potent anti-inflammatory effects in macrophages in mouse models of atherosclerosis and dermatitis. Furthermore, Joseph et al. showed that LPS injection in LXRα/β double knock-out mice led to increased hepatic expression of the genes encoding inducible nitric oxide synthase (iNOS) and TNFα, as well as to increased plasma levels of IL-6. Considering the crucial role of Kupffer cells, the resident hepatic macrophage population, in the pathogenesis of inflammation-associated cholestasis and that these cells express both LXR isoforms in rats, we regarded LXR a potentially attractive target for pharmacological intervention in the inflammatory cascade leading to cholestasis. In this study, we examined the effects of the synthetic LXR agonist, T0901317, as an anti-inflammatory agent in liver, employing both in vivo and in vitro models of sepsis-associated cholestasis.

**Materials and Methods**

**Animals and treatments**

Eight week old, male C57BL/6 (Charles River, Laboratories Inc., Wilmington, MA) mice were housed at our facility at constant room temperature, humidity and light-dark cycle, and had free access to both water and standard rodent chow (Pico-lab Rodent Diet 20, Purina Mills, St. Louis, MO) throughout experiments. Animal protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Mice were administered T0901317 (1-100 mg/kg/d, Cayman Chemical, Ann Arbor, MI) or equi-volume amounts of vehicle (corn oil) by gavage for three consecutive days. Over this range of doses, T0901317 had previously been shown to dose-dependently increase liver phospholipid transfer protein in mice. Fifteen minutes after the third dose, mice were injected intraperitoneally with a non-lethal dose (2 μg/g bodyweight) of LPS (Salmonella typhimurium, Sigma-Aldrich, St. Louis, MO) or vehicle (0.9% saline) alone, as described previously. Mice were sacrificed at indicated time-points after brief inhalation anesthesia by isoflurane. Livers were excised, weighed and snap-frozen in liquid nitrogen. Blood was collected at time of sacrifice in EDTA-containing vials. Liver tissue and plasma were stored at -80°C until further use.
Primary hepatocyte culture

Primary hepatocytes were isolated from mouse and rat using an in situ collagenase-perfusion method. Mouse and rat hepatocytes were isolated according to protocols described previously and purified using multiple centrifugation steps (purity > 99%, viability > 80%). Mouse hepatocytes were plated on Primaria plates (BD Biosciences, Franklin Lakes, NJ) and rat hepatocytes on collagen-coated plates in William's medium E containing fetal bovine serum (10%), penicillin/streptomycin (100 U/L), glutamine/gentamycin (50 mg/L). After 3-4 hours, medium was changed to serum-free medium. After 16-24 hours, medium was changed again. After 90 minutes, cells were pre-treated with T0901317 (0.01-20 μM) or vehicle (DMSO, 0.25% final concentration) alone for 30-60 minutes, after which TNFα (20 ng/ml) or vehicle (0.1% bovine serum albumin in phosphate buffered saline) alone was added. Cells were harvested at indicated time-points.

Quantitative Real-Time PCR

RNA was isolated from frozen mouse livers using the RNeasy Midikit (Qiagen, Valencia, CA) according to manufacturer’s instructions with the addition of on-column RNase-free DNase digestion. RNA was isolated from cultured hepatocytes using Tri-reagent (Sigma, St. Louis, MO) according to manufacturer’s instructions. RNA-concentrations were assessed by spectrophotometry or RiboGreen RNA Quantitation kit (Molecular Probes, Inc., Eugene, OR). RNA-integrity was confirmed by gel electrophoresis. RNA was reversed transcribed using the PROSTAR First Strand RT-PCR kit (Stratagene, La Jolla, CA) or Moloney-murine leukemia virus reverse transcriptase (Sigma) according to manufacturers’ instructions. cDNA was stored at -20°C until further usage. RNA-expression levels were determined using Taqman real-time PCR method with the ABI Prism 7000 Gene Detection system (Applied Biosystems, Foster City, CA) as described previously. Target gene expression was normalized to cyclophilin expression for each individual sample. Primer and dual-labeled probes (5’-FAM, 3’-TAMRA) were synthesized by Sigma Genosys (The Woodlands, TX), Synthegen (Houston, TX) or Eurogentec (Seraing, Belgium). Sequences are shown in Tables 1a (mouse) and 1b (rat). Primers/probe sets for cyclophilin and iNOS were newly designed using Primer Express software (Applied Biosystems). Those sets not shown in Table 1 were obtained directly from Applied Biosystems (Assay-on-Demand).

Plasma analysis

Plasma levels of IL-1β, IL-6 and TNFα were determined simultaneously using xMAP technology (Luminex Corporation, Austin, TX) with a commercially available kit (Linco Research, St.Charles, MO).
Elecreophoretic mobility shift assay (EMSA)

Nuclear extracts from liver tissue were prepared using a method described by Timchenko et al.\textsuperscript{38} with some modifications\textsuperscript{28}. Nuclear extracts from primary hepatocyte cultures were prepared using the method described by Hoppe-Seyler et al.\textsuperscript{39} with some modifications\textsuperscript{40}. Protein concentration of nuclear extracts was determined using BCA protein assay (Pierce, Rockford, IL). EMSAs were performed using 5-10 μg of nuclear extract protein according to the previously described protocol\textsuperscript{28}. Briefly, nuclear extracts were incubated with 2.5 x 10⁴ cpm of radiolabeled probe. For competitor-studies, a 100-fold excess of cold specific and non-specific oligonucleotides were added immediately preceding the addition of the probe. Samples were incubated on ice for 30 minutes and electrophoresed through a non-denaturing 6% polyacrylamide gel at 4°C. Gels were dried on filter paper at 80°C for 35-40 minutes and autoradiographed or analyzed using a phosphorimager (Cyclone, PerkinElmer, Boston, MA).

Table 1 Primers and probe sequences for mouse (A) and rat (B) genes

Table 1a. Mouse primers and probe sequences

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<tr>
<th>Gene</th>
<th>Acc. nr.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
<th>Ref.</th>
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<tr>
<td>Cyclophilin</td>
<td>XM_130275</td>
<td>CAGATCGGAG-GGATGAGATTCAG</td>
<td>TCACCACCTGTACAC-CCTCATT</td>
<td>CTCCTCCACATTGGA-GACAGAGATGCA</td>
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<td>Ntcp</td>
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<td>ATGCCCACTGGCTC-CAGCTT</td>
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<td>Bsep</td>
<td>NM_021022</td>
<td>CTGCCAAAGAT-GTACTGCA</td>
<td>CGATGCTGAT-GGCCACCTTG</td>
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<td>Mrp2</td>
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<td>GCTGGAGAATAT-GGAGATGTC</td>
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<td>Mrp3</td>
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<td>TCCCACTTTTGG-GAGAGCTAAC</td>
<td>ACTGAGGACCTT-GAAGCTTGG</td>
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<td>Mdr1b</td>
<td>M14757</td>
<td>GCTGGGAACACGT-CTGCATAG</td>
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<td>Srebp1c</td>
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<td>GAGGCGATGAT-GGCACATT</td>
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<td>IL-1b</td>
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<td>CAGCACAAGGT-GATATCTCCAT</td>
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<td>TNFa</td>
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<td>sIL-1Ra</td>
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<td>CTCCTCTCATCCT-TCTGTTCAT</td>
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Table 1b. Rat primers and probe sequences

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<th>Gene</th>
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<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
<th>Ref.</th>
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<td>Mrp2</td>
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<td>iNOS</td>
<td>NM_012611</td>
<td>CTATCTCGGACATGCA-TACTCCGACAT</td>
<td>CCGGGTGATGCTG-CCCTGAT</td>
<td>CTCCTCCACATTGGA-GACAGAGATGCA</td>
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Immunoblotting

Liver total membrane fractions were prepared using a protocol modified from Klett et al.\textsuperscript{41}. Briefly, \(~\)100mg of liver tissue was homogenized in ice-cold buffer (5mM Heps, 250mM sucrose, pH 7.4) containing protease-inhibitors (Complete (Roche, Almere, the Netherlands), 1mM PMSF, 1mM DTT) and phosphatase-inhibitors (1mM activated Na3VO4, 1mM NaF). After 15 minute incubation, homogenates were centrifuged at 200 x g during 5 minutes. The supernatant was re-centrifuged at 1,500 x g for 5 min. The supernatant of this second spin was then subjected to ultracentrifugation at 100,000 x g during 60 minutes. The resulting pellet was subsequently resuspended in lysis-buffer (10mM Tris, 2mM EDTA, 2mM EGTA, 1.0% Triton X-100, 0.5M NaCl, 0.25% deoxycholate) with protease- and phosphatase-inhibitors, incubated for 30 minutes on ice and finally centrifuged at 16,000 x g for 10min. The resulting supernatant containing the solubilized membranes was snap-frozen and stored at -80\degree C until further use. Whole cell lysates were prepared from primary hepatocytes using the following protocol. Hepatocytes were washed with ice-cold PBS and incubated with the same lysis buffer described above for 30 minutes on ice. Plates were scraped, lysates were spun 20,000 x g for 5 minutes and supernatants were used for further analysis. Protein content of total membrane fractions and cell lysates was determined by BCA protein assay.

Twenty-five µg of membrane fractions or cell lysates were separated by SDS-PAGE (7.5% or 12% gel respectively), transferred to a nitrocellulose membrane and incubated with antibodies for Ntcp\textsuperscript{42}, Mrp2\textsuperscript{43} (both gifts from Dr. Bruno Stieger, University Hospital, Zurich), IκBα (sc-371, Santa Cruz Biotechnology, Santa Cruz, CA) or phospho-NF-κB p65 (#3031, Cell Signaling Technology, Beverly, MA), followed by species-specific secondary antibodies. Detection was performed either Tropix substrate (Applied Biosystems) and the Image Station 2000R (Kodak, Rochester, NY) or SuperSignal West Pico substrate (Pierce) and the Gel-Doc XR (Bio-Rad, Hercules, CA)

Statistical analysis

Statistical analysis of results was performed using SPSS 14.0 (SPSS Inc., Chicago, IL). Data are reported as mean ± S.D. Due to the relatively small sample-sizes, non-parametric tests were used: Kruskal-Wallis H-test followed by pair-wise comparisons with the Mann-Whitney test. P-values <0.05 were considered significant.
**Results**

**Pre-treatment with T0901317 attenuates LPS-effects on hepatic gene expression in mice in a dose-dependent and gene-specific manner**

We determined the effects of T0901317 (1-100mg/kg/day) on LPS-mediated regulation of 5 hepatobiliary transporter genes and the LPS/LXR responsive gene encoding the sterol regulatory element binding protein-1c (Srebp1c). Alterations in basal RNA levels by LPS of each of these genes were attenuated to variable degrees, in a dose-dependent fashion, for each individual gene.

Among the most severely affected genes was Ntcp. Eight hours after LPS administration, Ntcp gene expression was profoundly suppressed (to ~13% of control levels) (Figure 1A), while pre-treatment with T0901317 significantly attenuated this LPS-induced suppression in a dose-dependent manner. Ntcp gene expression rose to 40-45% of control (vehicle-treated) levels at the highest doses of T0901317 (50 and 100 mg/kg/day).

![Graph showing gene expression levels](image)

**Figure 1.** T0901317 attenuates LPS-induced alterations of hepatic transporter and Srebp1c RNA levels. Mice were treated with T0901317 (1-100 mg/kg/d) or vehicle alone for 3 days. Fifteen minutes after the third dose, mice received LPS (2 μg/g BW) or saline alone by intraperitoneal injection. Mice were sacrificed after 8 hours. RNA expression was determined by Taqman quantitative real-time PCR and normalized to cyclophilin gene expression for each individual animal (N = 4 per group). Vehicle/saline expression was set to 1. Error bars denote SD. Differences were analyzed pair-wise using the Mann-Whitney test: * P < 0.05 vs. vehicle/saline; #, P < 0.05 vs. vehicle/LPS (only analyzed vs. other LPS-groups).
Mrp2, Mrp3 (Abcc3) and Bsep gene expression were all suppressed by LPS-treatment, albeit to different extents (Figure 1B-D). Mrp2 expression showed a similar pattern as that of Ntcp, i.e. LPS-induced suppression to 8% of control levels, which rose more than 3-fold to 30% in mice pre-treated with the highest dose of T0901317. (Figure 1C.) Mrp3 gene expression on the other hand returned to control levels at the highest T0901317 dose. This, however, appeared to be the result of a substantial direct effect of T0901317 on Mrp3 gene expression (Figure 1D).

Interestingly, T0901317 exhibited anti-inflammatory effects on hepatic genes activated by LPS. T0901317 pre-treatment prevented up-regulation of the expression of the multi-drug resistance-1b (Mdr1b, Abcb1) gene. Mdr1b, one of two rodent homologues of the human MDR1 (ABCB1) gene, is up-regulated after LPS-treatment of rats.10, 44 LPS-treatment led to a 4-fold up-regulation of Mdr1b expression in mice, which was dose-dependently reduced by T0901317 pre-treatment, to the point that Mdr1b expression was indistinguishable from control levels at doses of 50 and 100 mg/kg/day (Figure 1E). Interestingly, higher doses of T0901317 also significantly reduced Mdr1b gene expression in saline-injected animals, suggesting that its basal expression is regulatable by this compound.

Taken together, these results indicate that T0901317 interferes with the inflammatory signaling that leads to altered hepatic gene expression during endotoxemia in a dose-dependent and gene-specific manner.

Pre-treatment with T0901317 distinctly affects LPS-mediated suppression of Ntcp and Mrp2 protein levels

To determine whether the effects of T0901317 pre-treatment on LPS-altered hepatic transporter gene expression were also reflected by changes at the protein level, Mrp2 and Ntcp protein abundances were analyzed using immunoblotting. LPS-treatment led to suppressed expression of both transporter proteins, although the degree of LPS-induced suppression of protein expression is somewhat less than that seen for each transporter gene’s RNA (Figure 2).

Mrp2 protein levels (Figure 2, bottom panel) are induced by T0901317 pre-treatment and this effect persists in LPS-treated mice, indicating that the transcriptional effects are translated to the protein level. On the other hand, Ntcp protein levels are reduced by T0901317 pre-treatment both in saline-injected and LPS-injected mice (Figure 2, top panel). Thus, the higher Ntcp gene expression in T0901317-pre-treated LPS-injected mice compared to LPS-injected controls does not correspond to higher Ntcp protein levels, suggesting that post-transcriptional effects are at play. It appears that T0901317 has minimal effect on basal Ntcp and Mrp2 gene expression, but leads to changes in Ntcp and Mrp2 protein levels that suggest a distinct, post-translational target of action of T0901317. Thus, the combined effects on Ntcp and Mrp2 RNA and protein levels by T0901317 in the LPS model are complex, but appear to work favorably together to enhance adaptation.
Treatment of mice with T0901317 leads to increased liver weight

Liver weight increased after T0901317-treatment compared to vehicle-treatment in a dose-dependent manner. Liver weight in vehicle-treated mice was 51.4 mg liver/g bodyweight, while liver weight in mice treated with 100mg/kg T0901317 was 87.6 mg liver/g bodyweight (both groups saline-injected, P < 0.05). This agrees with previous reports and most likely related to enhanced lipogenesis. Of note, liver weight was not different between saline and LPS-injected mice receiving the same dose of T0901317. As anticipated, T0901317 treatment led to the induction of the established LXR target gene, Srebp1c (Figure 1F), a key regulator of de novo fatty acid synthesis. Thus, mice pre-treated with T0901317 appear to follow the expected physiological effects of this compound, indicated that the livers of these mice were sufficiently enriched with T0901317.

Attenuation of LPS-effects by T0901317 is not mediated by reduced hepatic cytokine expression

Given the role for cytokine-induced pathways in suppressing transporter gene expression in liver, and the reported suppression of macrophage-mediated cytokine gene expression by LXR agonists in cell culture, it was relevant to first determine if the effects on transporter gene expression were potentially mediated by \textit{in vivo} effects of T0901317 on liver cytokine gene expression. Thus, gene expression of 6 LPS-induced cytokines was measured at both 4 (peak) and 8 hours after LPS in livers from mice pre-treated with either vehicle or T0901317.

Surprisingly, hepatic gene expression of IL-1β, TNFα and IFNγ was elevated to similar levels in vehicle and T0901317 pre-treated mice at both 4 (Figure 3A, C, D) and 8 hours after LPS-injection (data not shown) even at doses that clearly reduced effects of LPS-signaling on transporter gene expression. Moreover, LPS-induced
T0901317 reduces LPS-modified hepatic gene expression

Figure 3. **T0901317 does not reduce LPS-induced cytokine RNA levels.** Mice were treated with T0901317 (50mg/kg/d) or vehicle alone for 3 days. Fifteen minutes after the third dose, mice received LPS (2 μg/g BW) by intraperitoneal injection or saline alone and were sacrificed after 4 hours. Total RNA was isolated from liver tissue and reverse transcribed. Cytokine gene expression was determined by Taqman quantitative real-time PCR and normalized to cyclophilin gene expression for each individual animal. N=5 per group. Vehicle/saline expression was set to 1. Error bars denote SD. Differences were analyzed pair-wise using the Mann-Whitney test. Expression levels of all cytokines were significantly higher in both LPS-injected groups than in the saline-injected group. *, P < 0.05 vs. vehicle/LPS.

Figure 4. **T0901317 does not reduce LPS-induced plasma levels of pro-inflammatory cytokines.** Mice were treated with T0901317 (50mg/kg/d) or vehicle alone for 3 days. Fifteen minutes after the third dose, mice received LPS (2 μg/g BW) by intraperitoneal injection or saline alone. Mice were sacrificed after 4 hours and blood was collected. Cytokine (IL-1β, TNFα, IL-6) levels were determined by Luminex assay. N=5 per group. Vehicle/saline expression was set to 1. Error bars denote SD. Differences were analyzed pair-wise using the Mann-Whitney test. Expression levels in both LPS-injected groups were significantly higher than in the saline-injected group.
IL-6 expression was even higher in T0901317-pre-treated mice compared to vehicle-treated controls (Figure 3B). Of note, T0901317 alone did not lead to changes in cytokine gene expression in saline-injected animals (data not shown).

To address potential effects through anti-inflammatory cytokines or modulation of pro-inflammatory cytokine action, we also determined expression of the anti-inflammatory cytokine IL-10 and the secreted form of the IL-1 receptor antagonist (sIL-1Ra) (Figure 3E-F). The latter was recently shown to be up-regulated in THP1-monocytic cells by PPARγ-agonists. Gene expression of both IL-10 and sIL-1Ra were, however, not affected by T0901317 pre-treatment.

Although hepatic cytokine gene expression after LPS-treatment has previously been shown to correspond well with actual cytokine release, we also measured serum cytokine protein levels as a more physiologically relevant metric of Kupffer cell activation. Analysis of plasma levels of IL-1β, IL-6 and TNFα showed that plasma levels of these cytokines corresponded well with their hepatic gene expression levels and that T0901317 did not reduce circulating protein levels of these three pro-inflammatory cytokines (Figure 4A-C). Hence, the effects of T0901317 on hepatic transporter gene expression cannot be attributed to effects on cytokine levels in these mice. Taken together, these results suggest that T0901317 does not exert its anti-inflammatory effects through changes in hepatic cytokine expression—rather, these effects appear to occur either through other inflammatory mediators or, perhaps, via direct effects on hepatocytes.

T0901317 blocks cytokine-induced alterations of primary hepatocyte gene expression

To examine the down-stream consequences of the direct hepatocellular anti-inflammatory effects of T0901317, we determined its effects on transporter gene expression in primary rodent hepatocytes after treatment with TNFα or IL-1β. In primary rat hepatocytes, an well-studied model of Mrp2 gene regulation, TNFα and IL-1β suppressed Mrp2 gene expression by 44% and 32%, respectively, while pre-treatment with T0901317 dose-dependently reversed this (Figure 5A, B). This effect, however, may also in part be the result of direct induction of Mrp2 by T0901317 in vitro or a repression of endogenous activation of cell signaling pathways in cultured primary hepatocytes, since Mrp2 expression in rat hepatocytes pre-treated with T0901317 alone was also increased. Expression of iNOS, another inflammation-regulated gene, was highly induced after cytokine treatment. This induction was strongly and dose-dependently reversed by T0901317 (Figure 5C, D), indicating that T0901317 inhibits inflammatory signaling in hepatocytes.
T0901317 reduces LPS-modified hepatic gene expression

To explore possible direct anti-inflammatory hepatocellular effects of T0901317, we examined its effects on TNFα-treated hepatocytes. Since NF-κB is involved in the inflammation-mediated induction of both Mdr1b gene expression in vivo (Figure 1E,51) and iNOS gene expression by cytokines (Figure 5,52,53), we focused our studies on T0901317’s effects on TNFα induction of NF-κB. GW3965, a potent synthetic LXR agonist, has previously been shown to have LXR-dependent anti-inflammatory effects in cultured mouse macrophages, mediated by interference with NF-κB signaling31,54, yet the precise mechanism of action remains unknown. Thus, using the model of cultured mouse hepatocytes, TNFα-treatment led to increased nuclear NF-κB binding activity, which was substantially inhibited by short-term pre-treatment with T0901317 (Figure 6A). Interestingly, pre-treatment with T0901317 also led to slightly reduced binding activity in cultured hepatocytes not stimulated with TNFα. The effect of T0901317 on NF-κB binding by TNFα is dose-dependent (Figure 6B). Neither dissociation of NF-κB from a complex with the inhibitor of κB-α (IκBα), nor NF-κB phosphorylation, was targeted by T0901317

Figure 5. T0901317 attenuates effects of pro-inflammatory cytokines TNFα and IL-1β on primary rat hepatocyte Mrp2 and iNOS gene expression. Primary rat hepatocytes were treated with T0901317 (1-20 μM) for 1 hour prior to treatment with TNFα (20 ng/ml) or IL-1β (10 ng/ml). Cells were harvested after 12 hours. RNA isolation and cDNA synthesis were performed as described in Materials and Methods. Mrp2 (A, B) and iNOS (C, D) gene expression was determined by real-time quantitative RT-PCR. Target gene expression levels were normalized to cyclophilin expression levels for individual samples. Vehicle/saline expression was set to 1. Error bars denote SD. Differences were analyzed pair-wise using the Mann-Whitney test: * P < 0.05 vs. vehicle/saline; #, P < 0.05 vs. vehicle/cytokine (only analyzed vs. other cytokine-treated-groups).

T0901317 reduces TNFα-induced NF-κB binding activity in primary mouse hepatocytes

To explore possible direct anti-inflammatory hepatocellular effects of T0901317, we examined its effects on TNFα-treated hepatocytes. Since NF-κB is involved in the inflammation-mediated induction of both Mdr1b gene expression in vivo (Figure 1E,51) and iNOS gene expression by cytokines (Figure 5,52,53), we focused our studies on T0901317’s effects on TNFα induction of NF-κB. GW3965, a potent synthetic LXR agonist, has previously been shown to have LXR-dependent anti-inflammatory effects in cultured mouse macrophages, mediated by interference with NF-κB signaling31,54, yet the precise mechanism of action remains unknown. Thus, using the model of cultured mouse hepatocytes, TNFα-treatment led to increased nuclear NF-κB binding activity, which was substantially inhibited by short-term pre-treatment with T0901317 (Figure 6A). Interestingly, pre-treatment with T0901317 also led to slightly reduced binding activity in cultured hepatocytes not stimulated with TNFα. The effect of T0901317 on NF-κB binding by TNFα is dose-dependent (Figure 6B). Neither dissociation of NF-κB from a complex with the inhibitor of κB-α (IκBα), nor NF-κB phosphorylation, was targeted by T0901317.
signaling (Figure 6C). Thus, there is a T0901317-mediated reduction of the availability of NF-κB for binding to its cognate DNA recognition sites, perhaps by altering the macromolecular transcription complexes in the nucleus in ways to ultimately mediate its transrepressive and gene regulatory effects.

**Discussion**

This study establishes the ability of the LXR-agonist, T0901317, to attenuate effects of endotoxemia on hepatic transporter gene expression in mice. This capacity of T0901317 to modulate the effects of LPS is dose-dependent and gene-specific. This protective effect is (in part) reflected at the protein level as well. Surprisingly, it appeared that the actions of the LXR-agonist were not mediated by reduction of cytokine gene expression or secretion into the bloodstream of treated mice. Coincident with the attenuation of LPS’ effects on transporter gene expression, was a reduction in NF-κB signaling in liver. This suggests that, at least in part, the anti-inflammatory properties of T0901317 work directly in hepatocytes, perhaps by T0901317-LXR directed suppression of the macromolecular transcriptional machinery, including those involved in NF-κB activation.

The experimental set-up used allowed us to directly examine the potential of T0901317 as an anti-inflammatory agent in liver, since the consequences of a single
dose of LPS are primarily due to endotoxemia per se without extensive confounding due to initiation of adaptive mechanisms within hepatocytes. Furthermore, the relatively low doses of LPS used in this study did not lead to major hepatocellular damage, indicated by the lack of elevated alanine aminotransferase levels (ALT) (data not shown).

The most surprising result from this study was that the attenuation of LPS-induced changes in transporter gene expression by T0901317 occurred without affecting either gene expression or secretion of pro-inflammatory cytokines IL-1β, IL-6 and TNFα. The level of attenuation of inflammation-mediated gene expression by this NR agonist is substantially similar to the degree of inhibition mediated by the PPARγ-agonist rosiglitazone on LPS-mediated suppression of transporter genes, although the mechanism of action of these two NR ligands are distinct, since the T0901317 compound, unlike rosiglitazone, did not alter the LPS-mediated subcellular localization of RXRα (data not shown) 28. Together, these data strongly suggest that neither NR ligand works through inhibiting non-parenchymal cell cytokine expression, but via direct, and likely distinct, intracellular targets within hepatocytes. Although implied by work in macrophage cell culture, it appears that the effects of both NR ligands seen in vitro, do not correlate with effects in vivo on Kupffer cells. These results reveal potential novel opportunities to interfere with sepsis-associated cholestasis, and inflammation-mediated effects on liver function as a whole.

Previously, LXR had been shown to interfere with NF-κB signaling in cultured macrophages 31, 54, 56. Suppression of inflammation-induced matrix metalloproteinase-9 (Mmp9) gene expression by LXR-agonists T0901317 and GW3965 was found to be mediated by inhibition of NF-κB activity. This suppression was absent in macrophages derived from LXRα/β null-mice 54. In contrast to our data, these authors did not find reduced NF-κB binding activity upon LXR-activation. We can only speculate on the cause of this discrepancy, but it may be related to differences in the cell-types studied (macrophage vs. hepatocyte), the type of inflammatory stimulant used (LPS vs. TNFα) and duration of treatment. The doses of T0901317 used in our in vitro experiments were higher that those used by Castrillo et al. 54, but corresponded to actual hepatic concentrations after oral dosing 57.

The molecular mechanisms underlying the interaction between LXR and NF-κB signaling remain to be elucidated. Multiple mechanisms could be at play, for instance competition for co-factors as suggested by Terasaka et al. 56 or induction or regulation of additional mediators. The latter concept is supported by two recent publications showing that LXR-agonists induce factors that are able to enhance macrophage survival in different settings 58, 59. LXR-activation could perhaps also affect expression of anti-inflammatory mediators in a similar fashion. Thirdly, LXR could also directly interact physically with NF-κB. This principle had already been described to be the mechanism underlying the anti-inflammatory effect of PPARγ 60, but was very recently shown to be effective in LXR-mediated suppression of inflam-
matory signaling in cultured macrophages as well by Ghisletti et al.\textsuperscript{55}. Transrepression of inflammatory gene expression was shown to occur by ligand-dependent SUMOylation of PPAR\textgamma{} and LXR and subsequent inhibition of co-repressor complex removal and thus inhibition of gene expression\textsuperscript{55}. It was also shown that, although the general principle of transrepression by LXR and PPAR\textgamma{} may be comparable, the actual SUMOylation pathways are different\textsuperscript{55}. The latter may also explain (in part) differential effects of T0901317 and rosiglitazone on hepatic transporter gene expression after LPS\textsuperscript{28}. Whether or not T0901317 induced SUMOylation of LXR is playing a role in altering the broad transcriptional programs induced by LPS remains to be determined.

There are few \textit{in vivo} studies on the mechanistic targets for anti-inflammatory effects of LXR agonist but, recently, Wang et al.\textsuperscript{61} reported that pre-treatment of rats with GW3965 attenuated liver injury induced by co-administration of LPS and peptidoglycan. This effect was associated with reduced TNF\alpha{} plasma levels, which appears to contradict our findings. However, co-administration of LPS and peptidoglycan is known to cause extensive liver injury as can be deduced from the profound increase in ALT levels as well as histological evidence of focal hepatocellular injury\textsuperscript{61}. Notably, as in our studies with T0901317, GW3965 pre-treatment did not affect serum IL-6 and IL-10 levels in this study, suggesting that the \textit{in vivo} anti-inflammatory effects of LXR agonists may be more diverse and distinct form those identified in cell culture studies\textsuperscript{61}.

Multiple studies have shown that structurally unrelated LXR-ligands, both natural and synthetic, possess anti-inflammatory effects in macrophages as well as keratinocytes and that these are dependent on LXR expression\textsuperscript{31, 32, 54}. Preliminary results using the LXR-agonist GW3965 showed that this compound also inhibits iNOS up-regulation in hepatocytes. However, cytokine-induced Mrp2 suppression was not reversed by GW3965, which suggests that agonist functionality of T0901317 and GW3965 are distinct when it comes to Mrp2 regulation or that T0901317 affects Mrp2 expression through LXR-independent mechanisms. Activation of the pregnane-X-receptor (PXR) by T0901317 may be the likely contributor to this effect since Mrp2 is a known PXR target\textsuperscript{62} and it was recently shown that T0901317, but not GW3965, can activate both LXR and PXR\textsuperscript{63, 64}.

In addition, T0901317 has also been implicated as potential agonist of the farnesoid-X-receptor (FXR)\textsuperscript{65}. It is, however, unclear whether FXR-activation played a role in the current study: while Bsep expression was modestly induced by T0901317, expression of the small heterodimer partner (SHP), another classical target gene of FXR, was actually suppressed (data not shown). Thus, it is not likely that FXR is a relevant target of T0901317.

One intriguing finding is the reduction of TNF\alpha{}-induced NF-\kappa{}B DNA binding in cells treated with T0901317 and the question whether or not this is related to its anti-inflammatory effects in the absence of altered hepatic cytokine expression. Since
many of the hepatic transporter genes are themselves activated by RXR-containing heterodimers\textsuperscript{22}, one can envision several molecular targets in this pathway where reduced NF-κB signaling may directly or indirectly increase RXR-mediated gene expression. First, NF-κB and RXR bind to each other and mutually repress their abilities as transcriptional activators\textsuperscript{66, 67} so that any potential sequestration of NF-κB would derepress any interactions with RXR. Indirectly, if T0901317 induces binding of SUMOylated LXR to corepressors, this may allow for coactivators to be more available for RXR-containing heterodimers. Investigations of either of these possibilities are avenues for future research.

The induction of hepatic steatosis by T0901317, previously described\textsuperscript{45-47}, probably precludes clinical use of this particular compound. The anticipated arrival of LXR-agonists with less pronounced lipogenic effects might allow for more specific modulation of inflammatory processes\textsuperscript{68}. Moreover, if the apparent lack of effect at the level of Kupffer cells were due to pharmacokinetic mechanisms rather than an inherent insensitivity to T0901317, then targeting of LXR-agonists to these cells would be a feasible alternative to circumvent steatotic effects. Wang et al.\textsuperscript{61} showed that activation of isolated rat Kupffer cells can indeed be suppressed by the other synthetic LXR-agonist GW3965.

Cholestasis can be the result of inflammatory signaling, yet cholestatic liver disease by itself can also be considered as the cause of recurrent endotoxemia\textsuperscript{2-4}. This indicates that inflammation and cholestasis are intertwined phenomena and implies that treatments aimed at suppressing the response to endotoxemia may also be beneficial in cholestatic liver disease in general. Previously, various strategies have been employed in attempt to modulate the hepatic inflammatory response cascade leading to cholestasis. These were aimed at different levels of this cascade and included, amongst others, Kupffer cell depletion or inactivation\textsuperscript{12, 18-20}, use of glucocorticoids to inhibit cytokine secretion\textsuperscript{13, 69, 70}, and administration of anti-cytokine antibodies\textsuperscript{21, 22}. Although of use in experimental settings, this has of yet not led to clinically effective and accepted therapies in addition to removal of endotoxin-sources by effective antibiotic treatment. Therefore, there remains a dire need for anti-inflammatory treatments that are effective and safe under cholestatic conditions. Intriguingly, Uppal et al. recently found a protective role of LXR against bile acid toxicity and cholestasis per se\textsuperscript{71}. Although this was found to be limited to female mice for yet to be clarified reasons, it suggests that it may actually be LXR-agonists that fulfill this role of the desired anti-cholestatic and anti-inflammatory agent.

In conclusion, the presented study shows that the synthetic LXR-agonist T0901317 possesses anti-inflammatory characteristics that attenuate the effects of endotoxemia on hepatic transporter gene expression in mice, most likely through a Kupffer cell-independent mechanism. This is also partially reflected by altered transporter protein expression. This suggests that alternative routes can be taken in attempt to modulate the hepatic inflammatory response leading to cholestasis.
zation of treatment regimens, e.g. through the addition of other nuclear receptor modulators (e.g. rosiglitazone), the use of selective LXR-agonists or cell-specific targeting, may provide us with even more potent modulators of hepatic inflammation and ultimately lead to new treatment strategies for inflammation-induced cholestasis.

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REFERENCES


