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Interleukin-6 regulates hepatic transporters during acute-phase response

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Abstract

Cholestasis develops during inflammatory conditions characterized by the release of cytokines like interleukin-6 (IL-6), which is the major player in the hepatic acute-phase response. However, the exact contribution of IL-6 to transporter down-regulation is unclear. Therefore, we compared wild-type and IL-6-deficient mice after IL-6-injection and induction of an aseptic (turpentine-injection) or septic (LPS-injection) acute-phase response. Down-regulation of basolateral (Ntcp, Oatp1, and Mrp3) and canalicular (Mrp2, Bsep) transporter mRNA occurred after treatment with IL-6, turpentine, and LPS. In IL-6-deficient mice, turpentine failed to decrease mRNA-levels of basolateral and canalicular transporters, whereas LPS-mediated down-regulation of Ntcp, Mrp3, and Mrp2 was abolished at later time points (24 h). In conclusion, induction of an aseptic and septic acute-phase response leads to the down-regulation of basolateral and canalicular organic anion transporters. IL-6 is required for transporter down-regulation during aseptic inflammation. Furthermore, IL-6 also contributes to transporter regulation during LPS-induced cholestasis at more delayed time points.

Keywords: Cholestasis; Organic anion transporter; Gene regulation; Inflammation; Cytokines; Interleukin-6; Acute-phase response

Cholestasis, defined as an impaired hepatocellular excretion of various constituents of bile, occurs in response to a variety of inflammatory stimuli [1]. Conditions such as bacterial or viral sepsis and aseptic inflammation (e.g., tissue injury/trauma, surgery, and burns) are characterized by an increased production of cytokines which mediate a wide range of biological effects including induction of hepatic acute-phase protein synthesis [2,3]. These inflammation-associated cytokines comprise, among others, tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and interleukin-6 (IL-6), the latter known to be the major stimulator of hepatic acute-phase proteins, whereas the other above-mentioned cytokines influence subgroups of acute-phase proteins [3]. In vitro studies using hepatocytes as well as in vivo studies in rats have shown that inflammation induced by either endotoxin (also known as lipopolysaccharide, LPS) or by isolated cytokines (TNFα, IL-1β, and IL-6) decreases the uptake and secretion of bile constituents [4–7].

Studies on the pathophysiology of cholestasis and the molecular regulation of hepatocellular transport systems have been facilitated by the cloning and functional characterization of several hepatic organic anion transport systems [1,8]. Physiologically, organic anions are taken up from portal blood by a sodium-dependent taurocholate cotransporter (Ntcp, *Slc10a1*; TCDB: 2.A.28.1.1 organic acid/(conjugated) bile acid (taurocholate):Na^+ symporter) and several members of a growing family of sodium-independent organic anion transporters including Oatp1 (*Slco1a1*; TCDB 2.A.60.1.1). After rapid

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transhepatic transport to the canalicular membrane, organic anions are secreted into the bile by two ATP-dependent export pumps, the bile salt export pump (*Abcb11*; TCDB 3.A.1.201.2), and the multidrug resistance associated protein 2 (*Abcc2*; TCDB 3.A.1.208.2 hepatic canalicular conjugate exporter). During obstructive cholestasis, multispecific organic anion transporters Mrp3 (*Abcc3*; TCDB 3.A.1.208.9) and Mrp4 (*Abcc4*; TCDB 3.A.1.208.7), which also belong to the Mrp-family but are exclusively expressed at the basolateral membrane, are induced as a rescue system to transport potentially toxic bile acids back into the sinusoidal blood [9,10]. Molecular studies using several animal models of cholestasis including bile duct ligation and treatment with estrogen and endotoxin have demonstrated a selective down-regulation of organic anion transporters at the basolateral and canalicular membranes [6,11–16].

However, the cytokine-mediated mechanisms underlying transcriptional regulation of these hepatocellular transport systems during the acute-phase response remain controversial. It could be shown that transporter expression was maintained after inactivation of TNFα and IL-1β in toxic and cholestatic models of liver injury [17]. Treatment with IL-6 did not significantly affect Ntcp mRNA in one study investigating the effect of isolated cytokines on transporter expression [6], whereas differences were observed for Oatp 1, Oatp2, Mrp2, and Bsep in a consecutive report [18]. Recent data on the mRNA expression of hepatobiliary transport proteins during the turpentine-induced acute-phase response further support a role of IL-6 in the regulation of hepatic organic anion transporters, since the turpentine-induced acute-phase response is mainly characterized by an increased IL-6 production [19] and the mRNA levels of different organic anion transporters including Ntcp are decreased even in the absence of an increased production of TNFα in this animal model [20].

Since cytokines operate both as initiators of linear signal cascades and as a part of complex interacting networks in regulating the production of acute-phase proteins, it is still unclear to which extent IL-6, located "downstream" of TNFα and IL-1β, actually contributes to the down-regulation of hepatobiliary organic anion transporters during the acute-phase response. To clarify this question, we induced an acute-phase response by either turpentine or LPS in both wild-type and IL-6-deficient mice to evaluate the contribution of IL-6 compared to other cytokines acting upstream of IL-6 (e.g., IL-1β) as direct effectors of transporter down-regulation.

Materials and methods

Cytokines and reagents. Recombinant human IL-6 with a specific activity of 5×10^6 B-cell stimulatory factor 2 U/mg protein was produced in the *Escherichia coli* expression system, purified to homogeneity by gel filtration and ion exchange high-performance liquid chromatography as described [21]. Endotoxin content was less than 3.5 pg/mg as measured in the Limulus assay, which is far below the concentration of LPS needed to induce an acute-phase protein production [22]. Steam distilled turpentine was purchased from Roth (Karlsruhe, Germany).

Animal models. Animal experiments were performed at the RWTH Aachen animal facility. As described previously [23], 8- to 12-week-old female C57BL/6 wild-type (IL-$6^{+/+}$) and homozygous IL-6 knock-out (IL-$6^{-/-}$) mice (20–23 g), generated as described earlier [24], were obtained from H. Bluethmann (Roche, Basel/Switzerland) via the Biotechnology and Animal Breeding Division of RCC (Fuellinsdorf, Switzerland) and bred under specific pathogen-free conditions with a 12 h light–dark cycle and free access to standard chow and water. Livers were harvested 14 and 24 h after injection of either 100 μl of turpentine subcutaneously, 125 μg LPS intraperitoneally, and 0.9% NaCl intraperitoneally, and subcutaneously or 14 h after repetitive intraperitoneal injection of 5 μg of recombinant human IL-6 (rhIL-6) every 5 h. We used the experimental setup of repetitive injections of IL-6 as described [23] because in previous studies [22] maximal effects of a single injection of IL-6 on mRNA synthesis of acute-phase genes occurred after 4 h, but effects were quantitatively rather limited. Thereafter, the livers were immediately frozen in liquid nitrogen for RNA-analysis. All study protocols were approved by the local Government's Animal Care Committee.

Northern blot analysis. RNA was isolated from whole liver by the Ultraspec phenol chloroform extraction procedure (Biotecx Laboratories, Houston, TX) according to the manufacturer's instruction manual, quantified spectrophotometrically at 260 nm, and stored at −70 °C. Total RNA (10–20 μg) was denatured, electrophoresed on a 1% agarose/formaldehyde gel, transferred to a nylon membrane (Nytran 0.2; Schleicher & Schüll, Dassel, Germany) by overnight capillary blotting, and UV-crosslinked (UV Stratalinker 1800, Stratagene, La Jolla, CA). Ethidium bromide staining of 18S and 28S bands was used to ensure equal loading for each sample. The membranes were prehybridized for 30 min at 60 °C in ExpressHyb solution (Clontech, Palo Alto, CA). After replacement with fresh ExpressHyb solution, hybridization was performed at 60 °C for 1 h after addition of specific complementary DNA (cDNA) probes labeled with [^{32}P]dCTP (specific activity 10^8 cpm/μg) by a random primed method (High Prime, Boehringer–Mannheim, Mannheim, Germany). Blots were washed twice with 2× SSC/0.05% SDS for 10 min at room temperature, followed by 2× SSC/1% SDS for 20 min at 50 °C. Specific mRNA levels were detected after exposure of membranes to a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) and quantified using the ImageQuant software (Bio-Rad). Mouse-specific cDNA probes for *Oatp1*, *Mrp2*, *Mrp3*, *Bsep*, and *Gapdh* were cloned from the corresponding cDNAs after RT-PCR or derived from EST clones with >95% homology to the previously identified genes, as described in [25]. For *Ntcp*, a cross-reacting rat cDNA was employed [6].

Statistical analysis. Statistical analysis between controls and IL-6-, turpentine-, or LPS-treated animals was performed using multivariate ANOVA with post-testing (Bonferroni). Statistical significance was considered at P values of <0.05. Data represent means ± SD of 3–4 animals per group.

Results

Transporter mRNA expression in IL-6-treated wild-type mice

To determine whether an inflammatory response induced by IL-6 affects the mRNA expression of hepatobiliary organic anion transporters in wild-type mice in vivo, we quantified the steady-state mRNA levels of

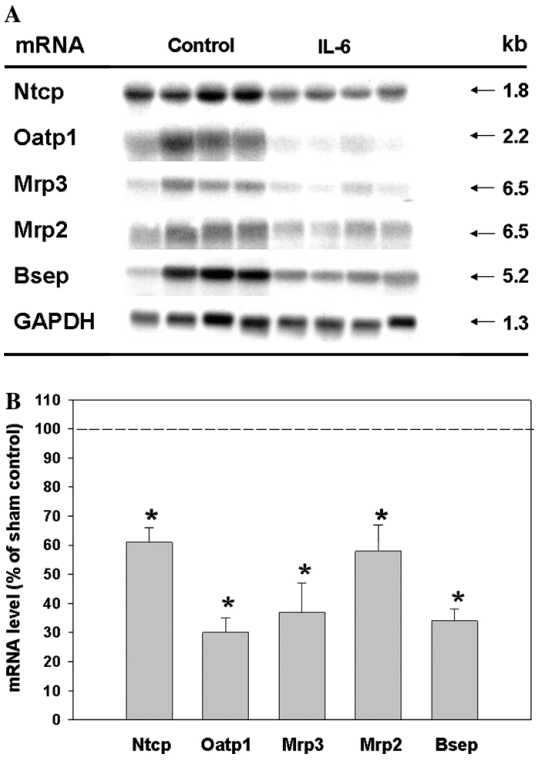

Fig. 1. IL-6 reduces steady-state mRNA levels of hepatic organic anion transporters in wild-type mice. Total RNA was isolated from livers of wild-type mice ($n = 4$) 14 h after repetitive intraperitoneal injection of IL-6 at 0, 5, and 10 or 14 h after injection of 0.9% NaCl (control animals; $n = 4$), as described in Materials and methods. mRNA levels of various hepatic organic anion transporters were analyzed by Northern blotting using specific [^{32}P]-labeled cDNAs. (A) Representative autoradiographs of four independent samples are shown. (B) Densitometric analysis of transporter mRNA expression after treatment with IL-6. Data are given as means ± SD ($n = 4$ for IL-6 treated mice; $^*P < 0.05$).

transport proteins exclusively localized either at the basolateral (Ntcp, Oatp1, and Mrp3) or canalicular (Bsep, Mrp2) plasma membrane of hepatocytes by Northern blotting (Figs. 1A and B). There was a reduction in the mRNA levels of all basolateral transporters analyzed: Ntcp mRNA declined to 61 ± 5%, Oatp1 mRNA to 30 ± 5%, and Mrp3 to 37 ± 10% of control levels. Similarly, mRNA levels of the canalicular export pumps also declined: Bsep (33 ± 4% of controls) was more sensitive to treatment with IL-6 than Mrp2 (58 ± 9% of controls), respectively ($P < 0,05$ each) (Figs. 1A and B). The decrease of the organic anion transporter mRNAs was specific since mRNA expression of the housekeeping gene GAPDH remained unchanged.

Transporter mRNA expression in response to aseptic inflammation induced by turpentine in wild-type and IL-6-deficient mice

We next studied whether mRNA down-regulation of the above-mentioned hepatobiliary organic anion transporters during inflammation due to aseptic tissue injury induced by turpentine is similar to their regulation in response to IL-6. Whereas repetitive injection of IL-6 with peak effects on mRNA synthesis as early as 4 h post-injection could lead to measuring indirect effects, we made use of the turpentine model eliciting delayed maximal effects not before 14 h [22] so that indirect signaling events are postponed to later time points. Steady-state mRNA levels of basolateral (Ntcp, Oatp1, and Mrp3) and canalicular (Bsep, Mrp2) transport proteins were again quantified by Northern blotting (Figs. 2A and B). Similar to IL-6 treatment (Fig. 1), turpentine-mediated inflammation led to a variable reduction of basolateral transporter mRNAs, ranging from almost absent mRNA levels of Oatp1 to moderate changes (−40%) for Ntcp and Mrp3, the latter with a rather low constitutive expression in untreated control animals (Fig. 2B) as described earlier [9]. mRNA levels of the canalicular

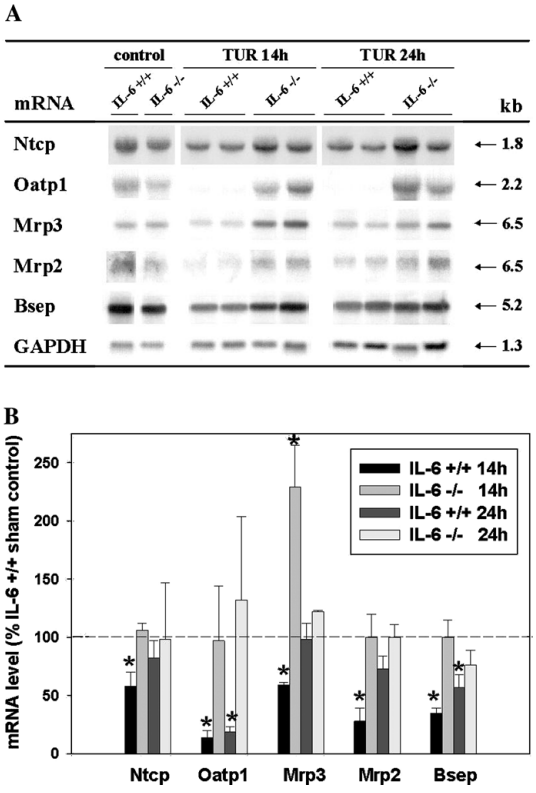

Fig. 2. Turpentine induces a down-regulation of hepatic organic anion transporter mRNA levels in wild-type but not in IL-6$^{-/-}$ mice. Total RNA was isolated from livers of turpentine-treated wild-type ($n = 3$) or IL-6-knock-out mice ($n = 3$) as well as 0.9% NaCl-injected controls ($n = 4$). mRNA levels of various hepatic organic anion transporters were analyzed by Northern blotting using specific [^{32}P]-labeled cDNAs. (A) Representative autoradiographs of two independent samples are shown. (B) Densitometric analysis of transporter mRNA expression after treatment with turpentine. Data are given as means ± SD ($n = 3$ for turpentine in IL-6$^{+/+}$ and IL-6$^{-/-}$ mice, respectively; $^*P < 0.05$).

export pumps Mrp2 and Bsep also declined to 30–40% after 14 h (Fig. 2B). All transporters except for Oatp1 (20% of controls) and Bsep (60% of controls) returned to baseline 24 h after turpentine-injection (Fig. 2B).

In contrast to turpentine-treated wild-type animals, the same inflammatory stimulus caused almost no effect on both basolateral and canalicular transporter expression in IL-$6^{-/-}$ mice (Fig. 2B). Both types of mice showed similar transporter mRNA levels in the absence of inflammatory stimuli (NaCl-treated), suggesting that IL-6 does not play a critical role in the constitutive expression of basolateral and canalicular transporters (Fig. 2A, lanes 1 and 2). Interestingly, Mrp3 mRNA expression was significantly upregulated in turpentine-treated IL-$6^{-/-}$ mice (220% of controls), most likely by other acute-phase stimuli in the absence of the negative regulator IL-6 (Fig. 2B).

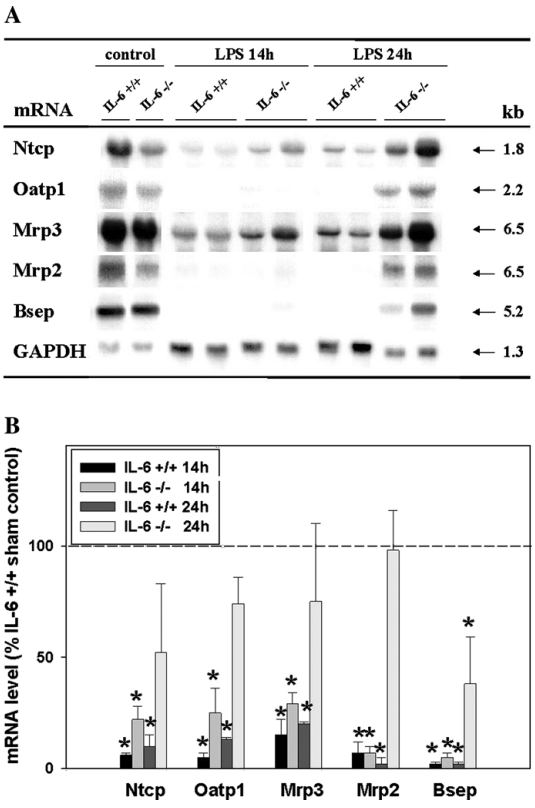

Fig. 3. Endotoxin mediates a down-regulation of hepatic biliary transporter mRNA in wild-type mice and at early time points in IL-$6^{-/-}$ mice. Wild-type and IL-$6^{-/-}$ mice were injected intraperitoneally with LPS or subcutaneously with 0.9% NaCl, livers were harvested, and total RNA was isolated. mRNA levels of various hepatic organic anion transporters were analyzed by Northern blotting using specific [^{32}P]-labeled cDNAs. (A) Representative autoradiographs of two independent samples are shown. (B) Densitometric analysis of transporter mRNA expression after treatment with endotoxin. Data are given as means ± SD ($n = 3$ for endotoxin in IL-$6^{+/+}$ and IL-$6^{-/-}$ mice, respectively; $^{*}P < 0.05$).

Transporter mRNA expression in response to septic cholestasis induced by endotoxin in wild-type and IL-6-deficient mice

Finally, the contribution of IL-6 to the down-regulation of hepatobiliary organic anion transporter mRNA expression in the presence of other cytokines, such as IL-1 and TNFα, during endotoxin-induced cholestasis was assessed using IL-$6^{-/-}$ mice. Steady-state mRNA levels of the transport proteins were again quantified by Northern blotting (Figs. 3A and B). As described previously [1,8], endotoxin-induced cholestasis led to a down-regulation of all basolateral and canalicular transporters. Expression of Bsep mRNA after administration of LPS was almost absent and all other transporters were decreased by 80–90% after both 14 and 24 h (Fig. 3B).

In contrast to the normalization of transporter expression in turpentine-treated IL-6 knock-out animals, decreased mRNA expression of both basolateral and canalicular transporters in endotoxemic IL-$6^{-/-}$ mice was unchanged at 14 h when compared with wild-type mice (Fig. 3B). However, abrogation of IL-6 led to a largely maintained expression of Oatp1, Mrp2, and Mrp3 mRNA expression 24 h after LPS-treatment (Fig. 3B). At this time point, a partial effect of IL-6 was also observed on Bsep and Ntcp mRNA expression since the respective transporter mRNA levels recovered to 40–50% of control levels in IL-6-knock-out mice (Fig. 3B).

Discussion

The acute-phase reaction is an orchestrated complex response to tissue injury, infection or inflammation leading either to the induction of acute-phase proteins involved in the restoration of homeostasis (positive acute-phase proteins) or down-regulation of acute-phase proteins not required for host defense (negative acute-phase proteins) [3,26]. Cytokines, particularly interleukin-6, are important mediators of the acute-phase response [2,3]. However, the patterns of cytokine production and of the acute-phase response differ depending on the causative inflammatory conditions. Molecular studies on endotoxin-induced systemic inflammation in rats have shown a selective down-regulation of both mRNA and protein of organic anion transporters at the basolateral and canalicular membrane which was mainly attributed to TNFα and IL-1β signaling [6,12,16,27]. Pathophysiological observations confirm these molecular data with an endotoxin-induced reduction in bile flow and hepatic bile salt excretion in isolated perfused rat livers [4]. However, data on the expression of hepatobiliary transport proteins during the acute-phase response and under the

influence of IL-6, the master cytokine in this pathophysiological state, are conflicting; particularly the contribution of IL-6 in the regulation of these transporters during cholestasis is unknown.

Turpentine-induced tissue injury mimics aseptic inflammatory processes and represents a well-established model of an acute-phase response mainly characterized by an IL-1β-dependent increase in IL-6 production [28,29]. Recent data on the expression of hepatobiliary transport proteins in this animal model showed a down-regulation of Ntcp, Oatp1, Oatp2, Mrp2, and Bsep at the mRNA level in rats [20]. Analysis of local cytokine mRNA levels in liver tissue revealed increased IL-6 levels in turpentine-treated animals, whereas TNFα, mainly involved in endotoxin-induced down-regulation, remained unchanged [20]. In our studies, application of IL-6 caused a decrease in mRNA levels of all basolateral and canalicular transporters to a variable extent, ranging from 40% to 70% (Fig. 1). Regarding *Mrp2* mRNA expression, our results confirm previous data in IL-6-treated hepatocytes [30,31] and rats [18,31]. Similarly, our results in mice treated with IL-6 meet well with observations in rats which exhibit a significant reduction in Oatp1 and Bsep expression [18]. Mrp3 expression has been found to be slightly but not significantly decreased in that series of experiments using a rat model.

In contrast to data in both IL-6-treated hepatocytes and mice [5,6] but in line with data in rats [18,31], *Ntcp* mRNA expression was significantly reduced to about 60% of controls in our study. Pathophysiological data are in line with a decrease in *Ntcp* expression since both TNFα and IL-6 have been shown to inhibit sodium-dependent taurocholate uptake by hepatocytes to a comparable extent [5,7]. Detection of significant IL-6-mediated effects could have been prevented in the in vitro studies by Green et al. [5] due to the observed rapid decrease of transporter mRNA even in untreated hepatocytes; likewise, significant Ntcp mRNA reduction might have been missed in vivo due to the limited number of animals used [6]. On the other hand, if IL-6 itself does indeed not affect the expression of organic anion transporters in turpentine-treated animals, signaling of other mediators released by IL-6 might account for their down-regulation.

To further determine whether mRNA expression of the mentioned hepatocellular organic anion transporters during the acute-phase response is similar to their regulation by IL-6 and to exclude effects of other cytokines such as IL-1β, we treated wild-type mice with turpentine. Compared to untreated control animals, mRNA expression of Oatp1 was virtually absent during this acute-phase response while all other transporters including Ntcp, Mrp2, Mrp3, and Bsep still declined significantly but changes were less pronounced (Fig. 2). These data are in good agreement with previous observations [30]. In contrast to the down-regulation of basolateral transporters such as Ntcp or Oatp1, our finding of decreased Mrp3 mRNA levels is discordant to Mrp3 up-regulation during cholestasis in rats [9,32,33]. Consequently, clearance of toxic bile acids from hepatocytes should be reduced during the acute-phase response. Hence, it appears that the acute-phase reaction is not beneficial in this particular context, but a generally reduced production of proteins might be more important for the restoration of body homeostasis than possible local functional advantages.

However, in mice it is difficult to estimate the relevance of IL-6 as compared to IL-1β because those acute-phase response studies employed wild-type mice. Thus, the particular role of IL-6 in this pathophysiological condition was analyzed using IL-$6^{-/-}$ mice. Our results clearly show that, in contrast to turpentine-treated wild-type animals, the decrease in mRNA levels of basolateral and canalicular transporters was abolished in IL-$6^{-/-}$ mice (Fig. 2). Despite the complexity of cytokine networks regulating the synthesis of acute-phase proteins, our data indicate that IL-6 is the major player in the down-regulation of these hepatobiliary organic anion transporters during turpentine-induced aseptic systemic inflammation, while TNFα and IL-1β are of minor importance in this condition.

Finally and of major clinical relevance, a contribution of IL-6 to the down-regulation of hepatobiliary organic anion transporters during endotoxin-induced cholestasis was demonstrated using the same IL-$6^{-/-}$ approach. As described previously, endotoxin-induced cholestasis led to a down-regulation of all basolateral and canalicular transporters by 80–90% after both 14 and 24 h. However, transporter mRNA expression of Oatp1, Mrp2, and Mrp3 was largely maintained in endotoxemic IL-$6^{-/-}$ mice even in the presence of other cytokines during the later time course (Fig. 3). At this delayed time point, at least a partial effect of IL-6 on Bsep and Ntcp mRNA expression was also observed.

IL-6 has been shown to negatively regulate the expression of pregnane-X-receptor (PXR) and constitutively activated receptor (CAR), which induce *Mrp2* gene expression [34] and, thus, could account for the decrease in steady-state mRNA levels after treatment with IL-6 and turpentine. Farnesoid-X-receptor (FXR) mRNA levels are reduced after endotoxin-treatment in hamsters [35] but this regulation occurs in response to IL-1β and not IL-6 in Hep3B human hepatoma cells [36]. Similarly, IL-1β has been shown to down-regulate nuclear levels of retinoid-X-receptor:retinoic acid receptor (RXRα:RARα) heterodimers responsible for decreased promoter activity of both basolateral *Ntcp* and canalicular *Mrp2* gene in HepG2 cells [37].

Although IL-6-type cytokine signaling through the gp130/Jak/STAT pathway and its role in the regulation of complex cellular processes like gene activation, prolif-

eration, and differentiation have been investigated in detail (reviewed by Heinrich et al. [38,39]), its contribution to the regulation of organic anion transporters was widely questioned over the past decade. Our studies with IL-6 knock-out mice prove the importance of IL-6 in the down-regulation of hepatic organic anion transporters during both acute-phase response and cholestasis. In septic cholestasis, IL-6 regulatory events appear to be of major importance for transporter down-regulation during the chronic phase of inflammation. However, the particular molecular mechanisms of IL-6 in the regulation of organic anion transporters still remain unclear in detail and need to be further investigated employing the respective mouse gene promoters in the future.

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