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DIFFERENTIAL REGULATION OF HEPATIC TRANSPORTERS IN THE ABSENCE OF TNF-α, IL-1β, IL-6 AND NF-κB IN TWO MODELS OF CHOLESTASIS

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Abbreviatons: NF-κB, Nuclear Factor-κB; LPS, lipopolysaccharide; BDL, bile duct ligation; WT, wild type; IL, Interleukin; TNF-α, Tumor Necrosis Factor-α; Oat, Organic anion transporter; Ntcp, Sodium taurocholate co-transporting polypeptide; Oatp, Organic anion transporting polypeptide; Mdr, Multidrug-resistant; Bsep, Bile salt excretory protein; Multidrug resistance-associated protein, Mrp; TNFR1(-/-), Tumor Necrosis Factor Receptor-1 knockout; IL-1(-/-), 1Interleukin-1 knockout; IL-6(-/-), Interleukin-6 knockout; IKK-β(f/f), Inhibitor of κB Kinase β-floxed; IKKβΔhep, liver specific Inhibitor of κB Kinase β-deficient; bDNA, branched DNA; RLU, relative light unit.

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ABSTRACT

Hepatic transporters are responsible for uptake and efflux of bile acids and xenobiotics as an essential aspect of liver function. When normal vectorial transport of bile acids by the apical uptake and canalicular excretion transporters is disrupted, cholestasis ensues, leading to accumulation of toxic bile constituents and considerable hepatocellular damage. The purpose of this study was to assess the role of cytokines and Nuclear Factor-κB (NF-κB) in the transcriptional regulation of transporters in two models of cholestasis, lipopolysaccharide (LPS) administration and bile duct ligation (BDL). In wild type (WT) and knockout mouse strains lacking TNF-Receptor-1 (TNFR1), Interleukin (IL)-1-Receptor I (RI), IL-6 or IκB kinase β, transporter mRNA levels in liver were determined using branched DNA signal amplification 16h following LPS administration or 3 days after BDL. In WT mice, LPS administration tended to decrease mRNA levels of Oat2, Ntcp, Oatp1, Oatp4, Bsep, Mrp2 and Mrp6 compared to saline treatment, whereas it increased Mrp1, 3 and 5 levels. Similar changes were observed in each knockout strain following LPS administration. Conversely, BDL decreased only Oatp1 expression in WT mice, meanwhile increasing expression of Mrp1, 3, 5, and Oatp2 expression in both WT and knockout strains. Because the transcriptional effects of BDL- and LPS-induced cholestasis reflect dissimilarity in hepatic transporter regulation, we conclude that these disparities are not due to the individual activity of TNFα, IL-1, IL-6 or NF-κB, but to the differences in the mechanism of cholestasis.

INTRODUCTION

Cholestasis is generally defined as a condition in which bile flow is impaired. Consequent accumulation of bile constituents in hepatocytes produces oxidative stress, plasma membrane disruption, severe hepatocellular injury, necrotic and/or apoptotic cell death, likely due to the detergent-like properties of bile salts (Kasahara et al., 2002). Accordingly, maintaining bile flow and enterohepatic circulation of bile acids are among the most important functions of hepatocyte membrane transport systems. At the sinusoidal membrane, Na^+-taurocholate co-transporting polypeptide (Ntcp) is a secondary active transporter that is predominantly responsible for the uptake of bile salts from portal blood into hepatocytes. At the canalicular membrane, the ATP-dependent transporters Bile salt excretory protein (Bsep) and Multidrug resistance-associated protein 2 (Mrp2) mediate secretion of bile salts and glutathione. Bsep and Mrp2 are thereby responsible for the generation of bile salt-dependent and bile salt-independent bile flow, respectively.

Besides maintaining bile flow, a number of other hepatic transporters aid in uptake and biliary excretion of a wide variety of both endogenous and exogenous compounds. Many such transporters are constitutively expressed in the liver, including secondary active transporters such as the Organic anion-transporting polypeptides 1, 2 and 4 (Oatp1, 2 and 4). These transporters are responsible for the uptake of a wide variety of organic anions from sinusoidal blood into hepatocytes and contribute to the first-pass effect. Aside from vectorial

disposition of substrates into bile, several members of the Mrp family of transporters (Mrp1, Mrp3 and Mrp4) have been identified at the sinusoidal membrane where, conversely, they appear to mediate the sinusoidal efflux of a variety of substrates (Slitt et al., 2003;Rius et al., 2003).

Bile duct ligation (BDL) is used as a model to study extrahepatic, obstructive cholestasis. In addition to impaired canalicular and common bile duct flow, BDL produces a loss of bile salt secretory polarity and the accumulation of bile salt, bilirubin and other bile constituents in hepatocytes. To study intrahepatic cholestasis, LPS administration is a useful model. Upon entering the liver in portal blood, LPS, a component of the outer cell wall of Gram-negative bacteria, stimulates Kupffer cells to release proinflammatory cytokines such as TNF-α, IL-1β and IL-6 (Busam et al 1990). Previous studies have supported the notion that these three cytokines are essential in the regulation of transporter expression in models of intrahepatic cholestasis (Whiting et al., 1995;Green et al., 1996;Geier et al., 2003;Siewert et al., 2004). However, data regarding the activation and/or influence of cytokines in hepatic transporter regulation during obstructive cholestasis is not entirely clear (Bohan et al., 2003;Geier et al., 2005). *In vivo* studies have shown that bile acids are capable of inducing Kupffer cells to release proinflammatory cytokines and subsequently effect transcriptional alterations in the neighboring parenchymal cells (Miyake et al., 2000).

Whereas alterations in transporter regulation following extrahepatic obstruction are the result of toxic stress from accumulating bile, LPS induces alterations in transporter expression and function that appear to be the direct cause of cholestasis. The LPS model thus makes it possible to study transporter regulation directly, rather than the secondary response to the accumulation of bile constituents that results from biliary obstruction.

Among the molecular mediators in cholestasis that may possess key regulatory roles are the proinflammatory cytokines TNF-α, IL-1β and IL-6. Three lines of evidence have suggested that expression of hepatic transporters during cholestasis is dependent on intact cytokine signaling. First, it has been demonstrated that LPS-induced reduction in bile flow can be prevented by administration of TNF-α antibodies (Whiting et al., 1995). Second, recombinant TNF-α produces a down-regulation of Ntcp and Mrp2 mRNA levels (Green et al., 1996;Kim et al., 2000). Third, administration of dexamethasone, an anti-inflammatory steroid, blocks release of cytokines and prevents down-regulation of transporters by LPS (Roelofsen et al., 1995;Kubitz et al., 1999;Cherrington et al., 2004).

Currently, the understanding of the critical molecular mediators that regulate transporter expression during intrahepatic and extrahepatic cholestasis remains incomplete. The purpose of this study was to perform a comprehensive assessment of the role of the TNF-α, IL-1β, IL-6 and Nuclear Factor-κB (NF-κB)

in the transcriptional regulation of transporters in two models of cholestasis, lipopolysaccharide (LPS) administration and bile duct ligation (BDL).

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METHODS

Chemicals. Lipopolysaccharide (LPS; from *Escherichia coli* serotype 011:B4) and all other chemicals, unless otherwise indicated, were purchased from Sigma-Aldrich (St. Louis, MO).

Animals and treatments. Male C57BL6/J (wild type, WT) mice, as well as mice homozygous for the targeted mutation of Tumor Necrosis Factor Receptor-1 (TNFR1), Interleukin (IL)-1 Receptor I (RI) (IL-1RI), and IL-6, were purchased from Jackson Laboratory (Bar Harbor, ME). Liver-specific IκB kinase β (IKK-β)-deficient [IKKβΔhep] male and IKK-β-floxed [IKK-β(f/f)] mice were generated as described (Maeda et al., 2003). All animals were allowed water and standard chow *ad libitum*. Housing and experimental procedures were in accordance with the US National Institutes of Health and the American Association for Laboratory Animal Science guidelines.

In each model of cholestasis, six groups of age-matched male mice (25-35 g) underwent treatment. BDL surgery was performed under pentobarbital (Associated Medical Supply, Scottsdale, AZ) anesthesia (75 mg/kg, *ip*) and sterile surgical conditions, with the common bile duct being ligated at two locations. Sham controls underwent laparotomy, without ligation of the bile duct. For the LPS model, LPS (4 mg/kg, 5 mL/kg) or saline vehicle was administered to mice by intraperitoneal injection. Livers were collected and snap-frozen in

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liquid nitrogen (16 hours after LPS administration or 72 hours after BDL surgery) and stored at -80°C until RNA isolation was performed.

RNA isolation. Total RNA was isolated from liver using RNA Bee reagent (Tel-Test Inc., Friendswood, TX) according to the manufacturer's instructions. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. The integrity of each RNA sample was confirmed by gel electrophoresis before analysis.

Development of specific oligonucleotide probe sets for bDNA analysis. Probe sets for mouse Oatp1, 2, 4, 5, 9, 11, 12 and 14; Oat2, 3; Ntcp; Bsep, and Mrp1-7 and 9 were used as described previously (Cheng et al., 2005;Buist et al., 2004;Aleksunes et al., 2005;Maher et al., 2005). The probe set for mouse Mdr1b is described in Table 1. These target sequences were analyzed by ProbeDesigner™ Software Version 1.0 (Genospectra, Fremont, CA). Oligonucleotide probes designed were specific to a single mRNA transcript (*e.g.,* Mrp1). All oligonucleotide probes were designed with a T_m of approximately 63°C enabling hybridization conditions to be held constant (*i.e.,* 53°C) during each hybridization step and for each oligonucleotide probe set. Each probe developed in ProbeDesigner™ was submitted to the National Center for Biotechnological Information (NCBI) for nucleotide comparison by the basic logarithmic alignment search tool (BLASTn), to ensure minimal cross-reactivity with other known mouse sequences and expressed sequenced tags.

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Branched DNA assay. All reagents for analysis (*i.e.,* lysis buffer, capture hybridization buffer, amplifier/label probe buffer, and substrate solution) were supplied in the Quantigene Discovery Kit. Specific oligonucleotide probes were diluted in lysis buffer supplied in the Quantigene™ HV Signal Amplification Kit (Genospectra, Fremont, CA). Total RNA (1 μg/μl; 10 μl) was added to each well of a 96-well plate containing capture hybridization buffer and 50 μl of each diluted probe set. Total RNA was allowed to hybridize to each probe set overnight at 53°C. Subsequent hybridization steps were carried out as per the manufacturer's protocol, and luminescence was measured with a Quantiplex™ 320 bDNA luminometer (Bayer Diagnostics, East Walpole, MA) interfaced with Quantiplex™ Data Management Software Version 5.02 (Bayer Diagnostics) for analysis of luminescence from 96-well plates.

Statistics. Statistical differences were determined by two-way ANOVA followed by Duncan's Multiple Range post-hoc test [$p \leq 0.05$, n = 3-5 for WT, TNFR1(**-/-**), IL-1RI(-/-), IL-6(**-/-**)]. Data obtained from experiments performed in IKKβΔhep and IKK-β(f/f) mice (n = 2-3) were subjected to the Student's t-test.

RESULTS

Effects of BDL surgery and LPS administration on hepatic transporter expression in wild type (WT), TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice. In Figures 1 through 8, significant down-regulation in mRNA levels will be described as a percent decrease compared to the control, whereas significant up-regulation will be described as a fold increase. Figure 1 illustrates the effect of BDL surgery and LPS administration on Oat2, Oat3 and Ntcp mRNA levels in livers of WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice. In WT and TNFR1(-/-) mice, BDL decreased Oat2 mRNA levels in liver by 66% and 80% relative to sham-operated controls. Although not statistically significant, BDL reduced Oat2 levels in livers of IL-1R(-/-) mice by 72%. BDL significantly decreased Ntcp levels by 70% in livers of TNFR1(-/-), but had no significant effect on Ntcp mRNA levels in livers of WT, IL-1R(-/-) or IL-6(-/-) mice. BDL did not significantly affect Oat3 mRNA levels in livers of any of the four mouse strains. In sham-operated TNFR1(-/-) mice, the mRNA expression levels of Ntcp were significantly elevated (2.5-fold) above the sham-operated WT.

Figure 1 also shows that, in WT mice, mRNA expression of the organic anion transporter (Oat) family members Oat2 and Oat3 did not undergo significant changes following LPS administration, while Ntcp was down-regulated significantly by 58% compared to saline control. The Oat2 mRNA levels in each of the cytokine knockouts, TNFR1(-/-), IL-1RI (-/-) and IL-6(-/-), were significantly decreased by 68%, 74% and 66% of saline control, respectively. In contrast, LPS

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increased Oat3 mRNA in the IL-6(-/-) mice (2-fold). Messenger RNA levels of Ntcp in livers of WT, TNFR1(-/-), IL-1R(-/-), IL-6(-/-) mice following LPS administration were decreased by 73%, 93%, 86% and 90%, respectively. In saline-treated TNFR1(-/-) mice, the mRNA expression levels of Ntcp were significantly elevated (2.6-fold) above the saline-treated WT group.

Figure 2 shows the consequence of BDL surgery to Oatp1, 2, 4, 5, 9, 11, 12, and 14 mRNA levels in livers of WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice. BDL resulted in a significant decrease in Oatp1 mRNA expression in each of the four strains, with 84%, 88%, 81% and 74% reductions in WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice, respectively. In contrast, Oatp2 mRNA levels were increased in all four strains, with significant increases being detected in the IL-1R(-/-) (5.4-fold) and IL-6(-/-) (3.6-fold). Similar to Oatp1, Oatp4 mRNA expression was decreased by BDL in all 4 strains, but this decrease was significant in only the TNFR1(-/-) strain (57%). Bile duct ligation did not have any significant effect upon the mRNA levels of Oatp5 or 9. The overall effect of BDL on Oatp11 mRNA expression was similar to that observed for Oatp2, i.e. increased in all 4 strains but to a significant extent in only the WT (3.9-fold) and TNFR1(-/-) mice (2.3-fold). Interestingly, it was only in the IL-6(-/-) mice that Oatp12 and 14 mRNA expression levels were significantly up-regulated, 2.3- and 12-fold, respectively. In sham-operated mice, the mRNA expression levels of Oatp4 in TNFR1(-/-) and Oatp11 in IL-6(-/-) mice were significantly elevated 1.7- and 2.7-fold compared to WT. Conversely, mRNA expression levels of Oatp14 in sham-operated mice

were significantly lower than WT in the TNFR1(-/-) (by 94%), IL-1R(-/-) (by 94%) and IL-6(-/-) mice (by 86%).

Figure 2 further demonstrates that LPS administration results in overall decreases in Oatp1 and 4 mRNA levels in each of the four mouse strains. Importantly, these are the three most highly expressed Oatp isoforms found in mouse liver (Cheng, Maher, Chen, and Klaassen, 2005). Specifically, Oatp1 mRNA levels were decreased by 90%, 89%, 88% and 87% in WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice, respectively. Oatp2 expression underwent 28%, 76%, 59% and 50% reductions in WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice, respectively, which were significant in the TNFR1(-/-) and IL-6(-/-) mice. Oatp4 mRNA underwent significant decreases of 65%, 79%, 59% and 83% in WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice, respectively. While there was significant up-regulation of Oatp5 mRNA expression (1.8-fold) in WT mice in response to LPS administration, Oatp9 mRNA levels were significantly decreased in TNFR1(-/-) mice (37%). Conversely, mRNA expression of Oatp11 and 14 was significantly up-regulated in all four strains following LPS administration. Namely, Oatp11 mRNA levels were increased 2.8-, 4.1-, 2.8- and 16-fold, respectively; and Oatp14 mRNA expression was increased 8-, 10-, 12- and 12-fold, respectively, in WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice. Oatp12 mRNA expression was significantly up-regulated (2.2-fold) in only the IL-6(-/-) mice. In saline-treated TNFR1(-/-) mice, mRNA expression levels of Oatp9 were significantly increased (1.6-fold) relative to WT.

Figure 3 illustrates the effect of BDL surgery and LPS administration on Mdr1b and Bsep mRNA levels in livers of WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice. Although BDL appeared to increase Mdr1b mRNA levels in all four strains, the up-regulation was significant in only the IL-6(-/-) mice (10-fold). Interestingly, BDL resulted in a significant reduction (46%) in Bsep mRNA expression in only the TNFR1(-/-) mice. While LPS had no significant effect on Mdr1b gene expression, Bsep mRNA levels were significantly decreased in WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice by 59%, 88%, 70% and 91%, respectively.

Figure 4 illustrates the effect of BDL surgery on Mrp1-7 and 9 mRNA levels in livers of wild type (WT), TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice. In general, the mRNA expression of Mrp1, 3, 4, 5 and 7 was increased in each of the four mouse strains following bile duct ligation. For Mrp1, this up-regulation of mRNA expression was significant in only the IL-6(-/-) mice (10-fold). Significant up-regulation of both Mrp3 and Mrp5 mRNA levels was observed following BDL of all four strains. Mrp3 mRNA levels were up-regulated 4.5-, 4.8-, 3.4- and 3.4-fold in WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice, respectively. Mrp5 mRNA levels were up-regulated 4.5-, 4.8-, 3.4- and 3.4-fold in WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice, respectively. Generally, Mrp4 and Mrp7 mRNA were also increased in response to BDL, but the increases were significant only in the case of Mrp4 expression in IL-1R(-/-) (8-fold) and IL-6(-/-) (2.2-fold) mice. Mrp6 expression, in contrast, was significantly reduced in TNFR1(-/-) (79%) and IL-

1R(-/-) mice (70%). In sham-operated TNFR1(-/-) mice, there was a significant elevation in Mrp2 (1.7-fold) and Mrp6 (1.5-fold) mRNA expression levels compared to sham-operated WT. Also in sham-operated TNFR1(-/-) mice, mRNA expression levels of Mrp9 were significantly decreased (by 72%) compared to the sham-operated WT. In sham-operated IL-1R(-/-) mice, Mrp4 (by 88%) and Mrp9 (by 68%) mRNA levels were significantly decreased relative to the sham-operated WT. Finally, in IL-6(-/-) sham-operated mice, mRNA expression levels of Mrp4 were significantly decreased (by 30%) relative to the sham-operated WT.

Figure 4 also displays the effect of LPS administration on Mrp1-7 and 9 mRNA levels. As occurred following BDL, LPS administration generally induced up-regulation of Mrp1, 3, 5 and 7 mRNA levels in all four strains of mice. Up-regulation of Mrp1 mRNA expression was significant in WT (3.6-fold), TNFR1(-/-) (2.4-fold), IL-1R(-/-) (5.7-fold) and IL-6(-/-) mice (5.8-fold); Mrp3 up-regulation was significant in only the IL-1R(-/-) mice (3.4-fold); Mrp5 up-regulation was significant in IL-1R(-/-) (2-fold) and IL-6(-/-) mice (2.1-fold); and Mrp7 up-regulation was significant in only the IL-1R(-/-) mice (1.9-fold). In contrast, mRNA levels of Mrp2 and Mrp6 were generally decreased by LPS administration. Mrp2 down-regulation was significant in TNFR1(-/-) (80%), IL-1R(-/-) (59%) and IL-6(-/-) (69%). Mrp6 down-regulation was significant in WT (65%), TNFR1(-/-) (79%), IL-1R(-/-) (70%) and IL-6(-/-) mice (56%). In saline-treated TNFR1(-/-) mice, Mrp3 mRNA expression levels were significantly increased (2.3-fold), whereas Mrp5

levels were significantly decreased (by 51%). In saline-treated IL-1R(-/-) mice, Mrp5 mRNA expression levels were significantly decreased (by 45%) below the saline-treated WT. Finally in saline-treated IL-6(-/-) mice, mRNA levels of Mrp3 were elevated (2.5-fold) over WT, while Mrp5 (by 42%) and Mrp6 (by 41%) were decreased below the saline-treated WT.

Effect of BDL surgery and LPS on hepatic transporter expression in IκB kinase β-deficient, IKKβΔhep and IκB kinase β-floxed, IKK-β(f/f) mice. Figure 5 shows that BDL surgery did not significantly alter Oat2 or Oat3 mRNA levels. Although BDL surgery did not affect Ntcp mRNA levels in IKK-β(f/f) mouse liver, it did produce significant down-regulation of Ntcp in the IKKβΔhep mice (35%). Generally, LPS administration resulted in down-regulation of Oat2, Oat3 and Ntcp mRNA levels in both IKK-β(f/f) and IKKβΔhep mouse liver. The down-regulation of Oat2 mRNA expression was significant in IKK-β(f/f) mouse liver (85%); Oat3 down-regulation was significant in both IKK-β(f/f) (63%) and IKKβΔhep (58%) mouse liver; and Ntcp down-regulation was likewise significant in both strains (66%, 73%). In sham-operated IKK-βΔhep mice, Oat3 mRNA expression levels were significantly decreased (by 84%) below the sham-operated IKK-β(f/f) group.

Figure 6 illustrates the effects of BDL and LPS administration on mRNA levels of Oatp family members. BDL produced up-regulation of Oatp2 (2-fold) and Oatp5 (2.1-fold). Importantly, this effect was observed in only IKK-β(f/f) mice. There was

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no significant change in the levels of Oatp1, 2, 4, 9, 12 and 14 mRNA following BDL. As was observed in WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice (Figure 2), LPS administration generally resulted in down-regulation of Oatp1, 2, 4 and 5 mRNA levels in both IKK-β(f/f) and IKKβΔhep mice. Thus, following LPS administration, Oatp1 underwent significant reductions of 57% and 60% in mRNA expression in IKK-β(f/f) and IKKβΔhep mice, respectively; Oatp2 underwent 96% and 80% reductions in IKK-β(f/f) and IKKβΔhep mice, respectively; and Oatp4 underwent 75% and 41% reductions in IKK-β(f/f) and IKKβΔhep mice, respectively. In IKKβΔhep mice, Oatp5 (48%), Oatp12 (17%) and Oatp14 (58%) mRNA each underwent significant decreases. Conversely, Oatp11 was significantly induced by LPS in both IKK-β(f/f) (4.6-fold) and IKKβΔhep mice (6-fold), an effect also observed in WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice (Figure 2). In sham-operated IKK-βΔhep mice, Oatp2 and Oatp5 mRNA levels were significantly increased above the sham-operated IKK-β(f/f) by 2.2- and 2.1-fold. In saline-treated IKK-bDhep mice, Oatp14 mRNA levels were also significantly elevated (2.2-fold) above the saline-treated IKK-β(-/-) mice.

Figure 7 demonstrates the effect of BDL surgery and LPS administration on Mdr1b and Bsep mRNA expression. BDL had no significant effect on Mdr1b mRNA, while Bsep mRNA was significantly decreased only in IKKβΔhep mice (48%). Following LPS administration, Mdr1b mRNA levels were significantly decreased only in IKKβΔhep mice (77%). As was observed in WT, TNFR1(-/-),

IL-1R(-/-) and IL-6(-/-) mice (Figure 3), LPS administration decreased Bsep mRNA expression in IKK-β(f/f) (70%) and IKKβΔhep mice (82%).

Figure 8 illustrates the effect of BDL surgery and LPS administration on Mrp1-7 and 9 mRNA levels in livers of IKK-β(f/f) and IKKβΔhep mice. BDL induced up-regulation of Mrp3 mRNA expression in IKK-β(f/f) (2-fold) but not IKKβΔhep mouse liver. In IKKβΔhep mice, Mrp5 mRNA expression was significantly decreased (67%) by BDL. Also in IKKβΔhep mice, Mrp7 was increased (2.5-fold) by BDL. After LPS administration, Mrp1 mRNA expression was increased in both IKK-β(f/f) (16-fold) and IKKβΔhep mice (2.3-fold), as seen also in WT, TNFR1(-/-), IL-1R(-/-) and IL-6(-/-) mice (Figure 4). LPS also induced Mrp7 up-regulation in IKKβΔhep mice (2.7-fold), while decreasing Mrp2 (49%) and Mrp5 (59%) mRNA levels in IKK-β(f/f) and IKKβΔhep mice, respectively. In sham-operated IKK-βΔ hep mice, mRNA levels of Mrp1 were increased (7.0-fold) while Mrp4 levels were decreased (52%) below the IKK-β(f/f) sham-operated mice. In saline-treated IKK-βΔhep mice, Mrp1 mRNA levels were similarly increased increased (7.3-fold) above the saline-treated IKKβΔhep mice. Conversely in saline-treated IKK-βΔhep mice, Mrp5 mRNA levels were decreased (28%) relative to the saline-treated IKK-β(f/f/) mice.

DISCUSSION

The current study investigated the liver expression of 21 transporter genes after BDL and LPS administration, models of extrahepatic and intrahepatic cholestasis, respectively. The present data confirm previous reports that demonstrate the ability of LPS to down-regulate mRNA levels of the sinusoidal uptake transporters Oatp1, Oatp2, Ntcp, and Oatp4 in mouse and rat liver (Trauner et al., 1998;Geier et al. 2003;Cherrington et al., 2004; Siewert et al. 2004). Further, the present study illustrates that Oat2 mRNA levels in liver tend to decrease after LPS administration, which is similar to the observation in rats (Cherrington et al., 2004). Our data also demonstrated that LPS decreased mRNA levels of Bsep and Mrp2 in mouse liver. This is consistent with other reports that observe down-regulation of Bsep and Mrp2 mRNA expression after LPS administration (Hartmann et al., 2002;Siewert et al. 2004). Together, these findings have important implications for understanding the pathogenesis of cholestasis because the protein products of these two transcripts are largely responsible for bile formation. Their down-regulation is likely a direct cause of cholestasis after LPS exposure or during sepsis. The finding that expression of the efflux transporters Mrp1, 3, 5 and 7 tended to increase in livers of WT mice following LPS administration is novel. However, these data are not consistent with earlier studies that demonstrated the lack of changes in Mrp1 and down-regulation of Mrp3 in mice (Hartmann et al., 2002;Siewert et al., 2004). In rats, however, up-regulation of Mrp1, Mrp3, and Mrp5 has likewise been reported (Vos et al., 1998;Cherrington et al., 2004;Donner et al., 2001). Importantly, the Mrp

transporters that were induced by LPS function to export organic anions back into the blood from the hepatocyte. Up-regulation of these transporters could play a hepatoprotective role during cholestasis.

As with LPS, BDL down-regulated expression of the uptake transporters Oat2 and Oatp1 in livers of WT mice. This is consistent with other studies that demonstrated down-regulation of Oatp1 and Oatp4 in rats and Oatp4 in mice following BDL (Ogawa et al., 2000). In contrast, BDL induced up-regulation of Mrp3 and Mrp5 in livers of WT mice. Mrp5 up-regulation is a new finding among mice and rats (Soroka et al., 2001;Bohan et al., 2003;Denk et al., 2004;Cherrington et al., 2004). The lack of alteration in liver Mrp2 mRNA expression has been a common observation in rats although decreased expression has also been reported (Ogawa et al., 2000;Paulusma et al., 2000;Hyogo et al., 2001).

The second component of this study was to determine whether the effects of BDL surgery and LPS administration on mRNA levels are dependent on TNF-α, IL-1β, IL-6 or NF-κB activity. In forms of extrahepatic cholestasis (primary sclerosing cholangitis, bile duct carcinoma, gallstones), the initial insult results from physical obstruction of the flow of bile, causing an accumulation of bile constituents in the liver, consequent hepatocellular damage, and recruitment of inflammatory mediators. By contrast, various forms of intrahepatic cholestasis (primary biliary cirrhosis, cholestasis of sepsis, alcoholic hepatitis), are initiated

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by and remain characterized by inflammation that produces disruption of hepatic transporter-mediated bile flow and consequent cholestasis. Importantly, each of the above clinical diagnoses of intrahepatic cholestasis has been documented as having elevated serum levels of TNF-α, IL-1β or IL-6 (Bird et al., 1990;Khoruts et al., 1991;O'Donohue et al., 1996;Simpson et al., 1997;Neuman et al., 2002). Additionally, NF-κB is a primary regulator of inflammatory responses and accordingly plays a central role in cholestatic liver diseases such as viral hepatitis and alcoholic liver disease (Barnes et al., 1997;Heyninck et al., 2001). These and other findings discussed in our introduction suggest that the cytokines TNF-α, IL-1β and IL-6 and the transcription factor NF-κB are integral components of signal transduction and gene regulation during cholestasis. Therefore, to determine the requirement of TNF-α, IL-1β, IL-6 and NF-κB for transcriptional regulation of hepatic transporters during cholestasis, mice homozygous for the corresponding targeted mutations [TNFR1(-/-), IL-1RI(-/-), IL-6(-/-),IKKβΔhep] were employed.

There were a number of perplexing discrepancies in basal mRNA expression levels of transporters in the six genotypes employed in this study. It is unclear how to account for such differences. Additionally, BDL and LPS treatment, respectively, appear to have had opposite effects on levels of a given mRNA transcript between the six genotypes studied. For example, BDL effected a significant change (increase) in Oatp5 mRNA levels in only IKK-β(f/f) mice. Conversely, LPS induced Oatp5 only in WT, meanwhile signaling down-regulation in the IKK-β(-/-) mice. The mechanism of transcriptional regulation of

Oatp5 during obstructive or LPS-induced cholestasis is not well-studied. Similar discrepancies were observed with Oatp11, Mrp5 and Mrp6. In each case, the mechanisms that underlie the differential regulation of the various genotypes are unclear. Thus, speculation with regard to mechanism will not be issued at this time. Studies including EMSA and supershift assay will be necessary to fully assess the role of NF-κB in the transcriptional regulation of these transporters.

Statistical analyses indicate that mRNA levels of Oat3, Oatp9, Mrp2 and Mrp9 following BDL are not dependent on the individual activity of TNF-α, IL-1β, IL-6 or NF-κB. In contrast to the lack of Mrp2 mRNA alterations shown currently, a previous report found Mrp2 mRNA expression to be down-regulated in both WT and TNFR1(-/-) after 14 days of BDL (Bohan et al., 2003), as opposed to 3 days (current study). Both findings nonetheless support the hypothesis that transcriptional regulation of Mrp2 during obstructive cholestasis is TNF-α-independent. Additionally, it is evident that bile duct ligation had a nearly equivalent effect on Oat2, Oatp1 and Mrp4 in all six strains. Oat2 and Oatp1 mRNA levels were generally down-regulated following BDL, regardless of the absence of TNF-α, IL-1β, IL-6 or NF-κB activity.

As was observed for some genes during extrahepatic cholestasis, transcriptional control of a number of liver transporters during intrahepatic cholestasis clearly does not have an individual requirement for TNF-α, IL-1β, IL-6 or NF-κB activity. These include Ntcp, Bsep, Oatp1, Oatp11, Mrp1 and Mrp9. The ability of LPS to

down-regulate Ntcp, Oatp1 and Bsep mRNA expression has been demonstrated previously *in vivo* in rats and mice (Trauner et al., 1998;Geier et al., 2003;Cherrington et al., 2004;Hartmann et al., 2002;Siewert et al., 2004;Geier et al., 2005). The current results in IL-6(-/-) mice also confirm a previous report in which LPS administration resulted in transcriptional down-regulation of Ntcp, Bsep and Oatp1 that was equally robust in WT and IL-6(-/-) mice (Siewert et al., 2004). In addition to these transporters, it is evident that mRNA levels of Oat2, Oatp2, Oatp4 and Mrp2 were also generally down-regulated by LPS, though not all significant, in all six strains of mice. Down-regulation of Oatp2 and Mrp2 has been reported previously in both rats and mice following LPS administration (Vos et al. 1998;Cherrington et al., 2004;Donner et al., 2001;Geier et al., 2003;Hartmann et al., 2002;Siewert et al., 2004). Furthermore, recombinant TNF-α, IL-1β and IL-6, like LPS, are each capable of producing down-regulation of Ntcp, Bsep, Oatp1, Oatp2 and Mrp2 mRNA expression in mice (Hartmann et al., 2002;Siewert et al., 2004;Geier et al., 2005). Given that both TNF-α and IL-1β have the ability to signal activation of NF-κB-mediated transcription, each likely constitute at least one facet of compensatory signaling in the acute phase response to LPS in TNFR1(-/-) and IL-1RI(-/-) mice. Given the redundant nature of cytokine signaling, it is reasonable to suggest that the remaining cytokines in these knockout mice are capable of mediating compensatory signaling within the acute phase response to LPS (Taga et al., 1992).

The current study indicates that the extrahepatic and intrahepatic models of cholestasis produce a variety of effects on transcriptional regulation of hepatic transporters in mice. We conclude that these effects reflect important differences in initiating events leading to cholestasis, rather than individual activity of TNF-α, IL-1β, IL-6 or NF-κB. Overall, the changes that occurred in the LPS model included decreased expression of uptake (Ntcp, Oatps) and excretion transporters (Bsep, Mrp2), thereby reducing further accumulation of organic anions in the liver meanwhile failing to eliminate them into the bile. The current findings thus indicate that LPS is capable of inducing intrahepatic cholestasis. Importantly, simultaneous up-regulation of sinusoidal efflux transporters (Mrps), was observed in both the LPS and BDL models. Both models therefore indicate a hepatoprotective response to cholestasis via up-regulation of sinusoidal efflux transporters, such that would enable the efflux of organic anions and thus minimize the organ's exposure to the toxic compounds.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Hepatic Oat2, Oat3 and Ntcp mRNA expression in WT, TNFR1(-/-), IL1-RI(-/-) and IL-6(-/-) mice following BDL and LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data expressed as relative light units (RLU) ± standard error of mean. Asterisk (*) indicates significance from sham operation/saline treatment ($p \leq 0.05$). Dagger (‡) indicates significantly different from sham operation/saline treatment ($p \leq 0.05$).

Figure 2. Hepatic Oatp family mRNA expression in WT, TNFR1(-/-), IL1-RI(-/-) and IL-6(-/-) mice following BDL and LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data expressed as relative light units (RLU) ± standard error of mean. Asterisk (*) indicates significance from sham operation/saline treatment ($p \leq 0.05$). Dagger (‡) indicates significantly different from sham operation/saline treatment ($p \leq 0.05$).

Figure 3. Hepatic Mdr1b and Bsep mRNA expression in WT, TNFR1(-/-), IL1-RI(-/-) and IL-6(-/-) mice following BDL and LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data expressed as relative light units (RLU) ± standard error of mean. Asterisk (*)

indicates significance from sham operation/saline treatment ($p \leq 0.05$). Dagger (‡) indicates significantly different from sham operation/saline treatment ($p \leq 0.05$).

Figure 4. Hepatic Mrp family mRNA expression in WT, TNFR1(-/-), IL1-RI(-/-) and IL-6(-/-) mice following BDL and LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data expressed as relative light units (RLU) ± standard error of mean. Asterisk (*) indicates significance from sham operation/saline treatment ($p \leq 0.05$). Dagger (‡) indicates significantly different from sham operation/saline treatment ($p \leq 0.05$).

Figure 5. Hepatic Oat2, Oat3 and Ntcp mRNA expression in IKK-β(f/f) and IKKβΔhep mice following BDL or LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data expressed as relative light units (RLU) ± standard error of mean. Asterisk (*) indicates significance from sham operation/saline treatment ($p \leq 0.05$). Dagger (‡) indicates significantly different from sham operation/saline treatment ($p \leq 0.05$).

Figure 6. Hepatic Oatp family mRNA expression in IKK-β(f/f) and IKKβΔhep mice following BDL or LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data expressed as relative light units

(RLU) ± standard error of mean. Asterisk (*) indicates significance from sham operation/saline treatment ($p \leq 0.05$). Dagger (‡) indicates significantly different from sham operation/saline treatment ($p \leq 0.05$).

Figure 7. Hepatic Oat2, Oat3 and Ntcp mRNA expression in IKK-β(f/f) and IKKβΔhep mice following BDL or LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data expressed as relative light units (RLU) ± standard error of mean. Asterisk (*) indicates significance from sham operation/saline treatment ($p \leq 0.05$). Dagger (‡) indicates significantly different from sham operation/saline treatment ($p \leq 0.05$).

Figure 8. Hepatic Mrp family mRNA expression in IKK-β(f/f) and IKKβΔhep mice following BDL or LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data expressed as relative light units (RLU) ± standard error of mean. Asterisk (*) indicates significance from sham operation/saline treatment ($p \leq 0.05$). Dagger (‡) indicates significantly different from sham operation/saline treatment ($p \leq 0.05$).

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TABLES

TABLE 1

Oligonucleotide probes generated for analysis of mouse Mdr1b expression by bDNA signal amplification

Function	Probe Sequence
CE	ttattaaaggtgatttggattcttctTTTTTctcttggaaagaaagt
CE	cttctctcttggtcttgctttctgTTTTTctcttggaaagaaagt
CE	gatttagatttaggatccgccaaTTTTTctcttggaaagaaagt
CE	tgaagccctgaaagaaatatgtaaTTTTTctcttggaaagaaagt
CE	ggactcgcttggtgaggatctTTTTTctcttggaaagaaagt
CE	atgactcctgtcccgaggtttTTTTTctcttggaaagaaagt
LE	tggacacttctgtaaattgatctccTTTTTaggcataggacccgtgtct
LE	aacaagtaaataaggccattcacttaTTTTTaggcataggacccgtgtct
LE	acactggttgtatgcacccattTTTTTaggcataggacccgtgtct
LE	caattctgtcgtttagtttcatggtTTTTTaggcataggacccgtgtct
LE	cgaaccagcttatatcctgtctcaTTTTTaggcataggacccgtgtct
LE	gctacattctgggtaactacagcaagTTTTTaggcataggacccgtgtct
LE	gccagccatagactaaggagaggTTTTTaggcataggacccgtgtct
LE	ttccgcccaatacaatgagcTTTTTaggcataggacccgtgtct
LE	ccagacaacagcttcatttcaataaTTTTTaggcataggacccgtgtct
LE	tcttcccagagatctcaagctgtTTTTTaggcataggacccgtgtct
LE	agttttcaattgcttctgtagcaaTTTTTaggcataggacccgtgtct
BL	catccacagcctctttcatactaagt

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BL	aaggaaaccagaggcacatctt
BL	tataacagcgcaaagtacgcc
BL	caatccttgaaaatactatggcaa
BL	catcatctcttgaaaaaacccta
BL	cagaaagaacagggaaaacaaatta
BL	caaaagaaatcagccccataac
BL	ctccggctttgccaaatg
BL	gcatggatttgaaaaccatgtatc
BL	gccagtgctgttcttatggtcat
BL	gcgagcctggtggtcagtga
BL	ccctttaacactagaagcatcactg
BL	cctggcgcccatcgc
BL	ggtataattactacaagtagaagtgtcagct
BL	ttcttgtccttcaaggcttgg

CE, capture extender; LE, label extender; BL, blocker probe.

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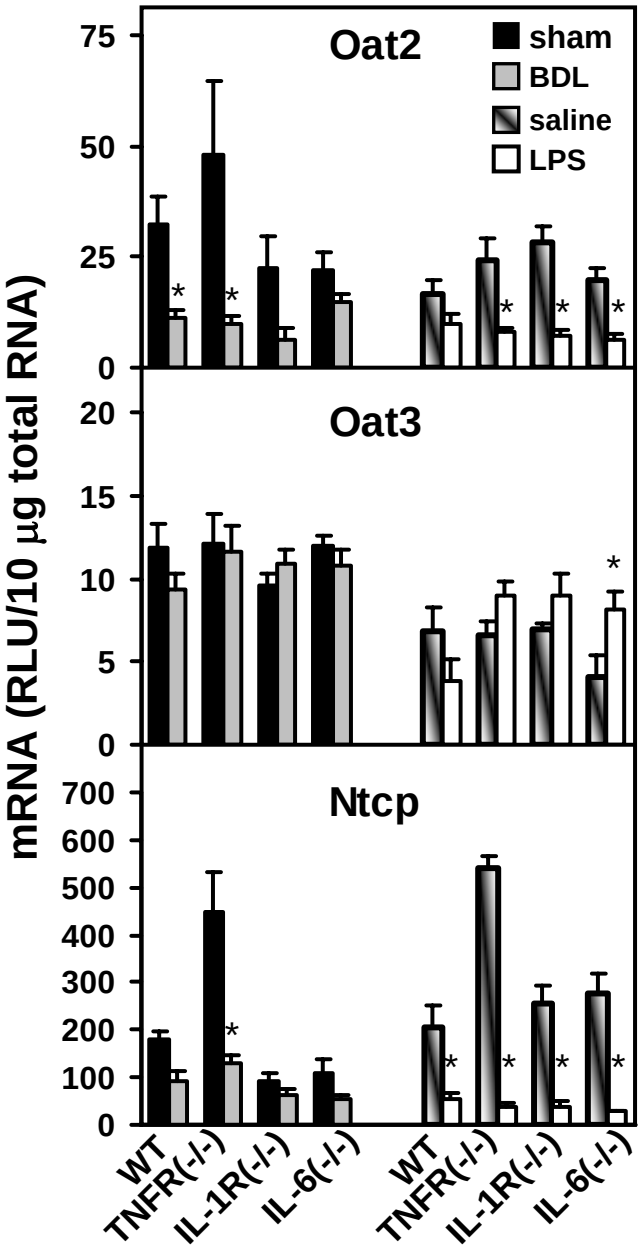

Figure 1

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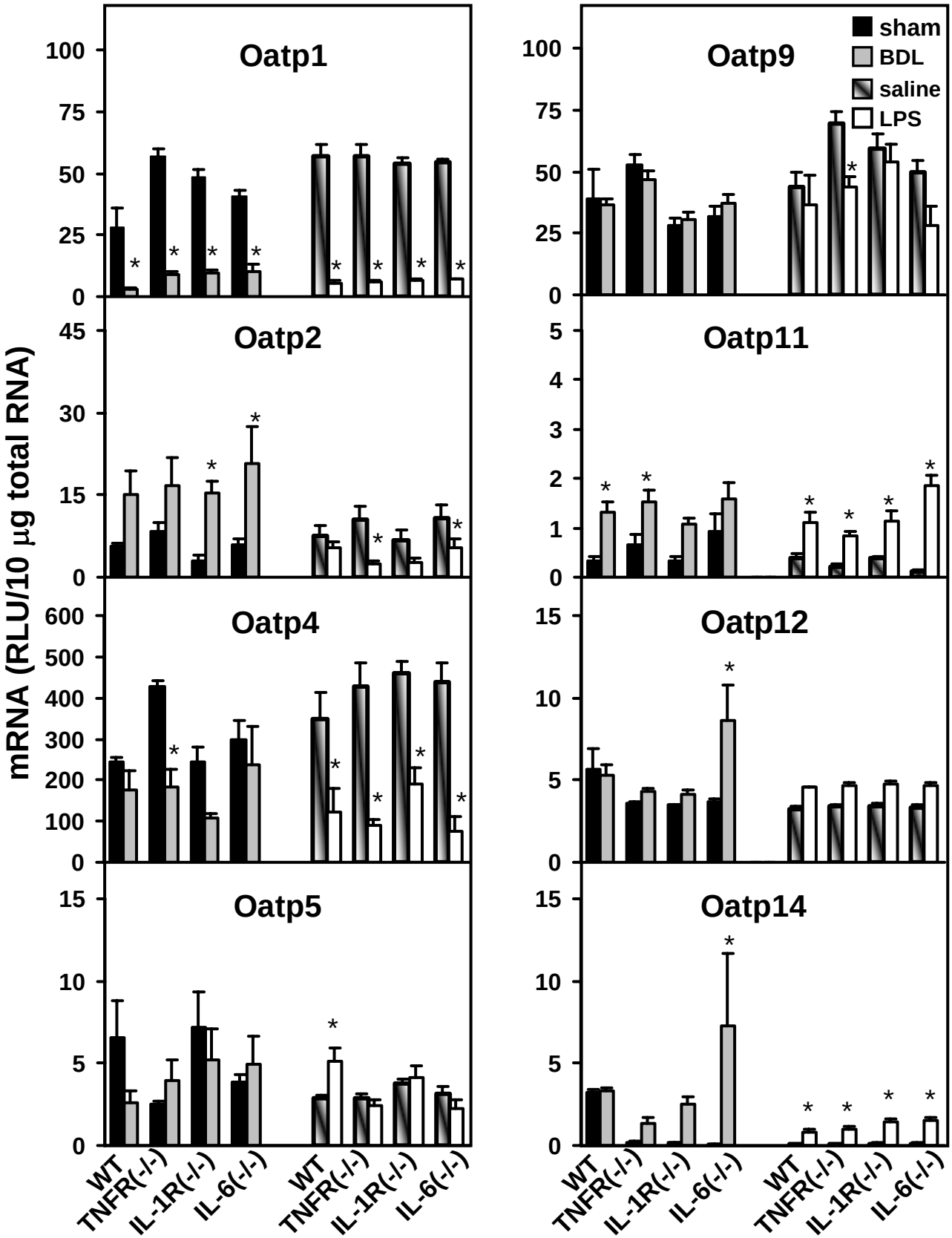

Figure 2

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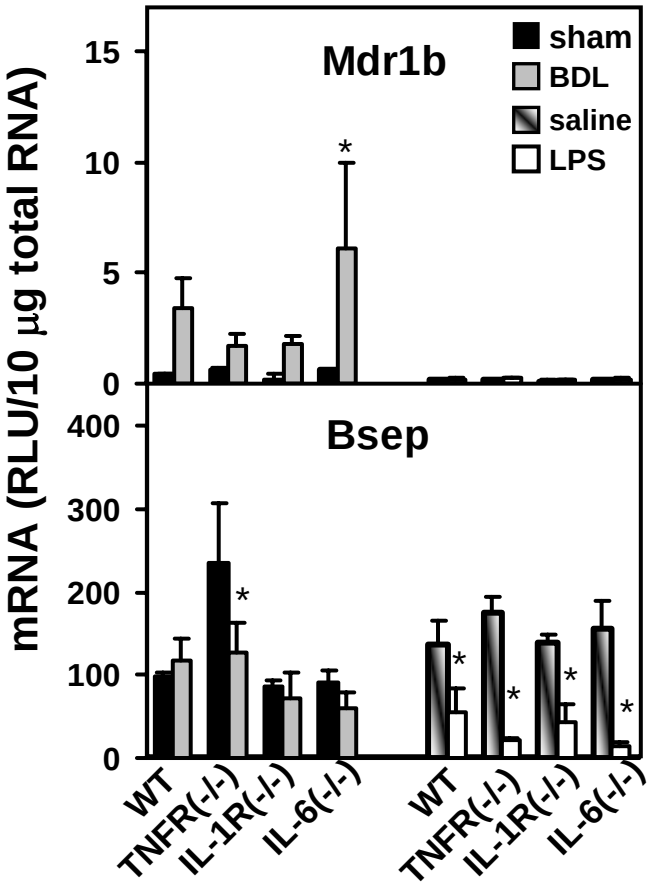

Figure 3

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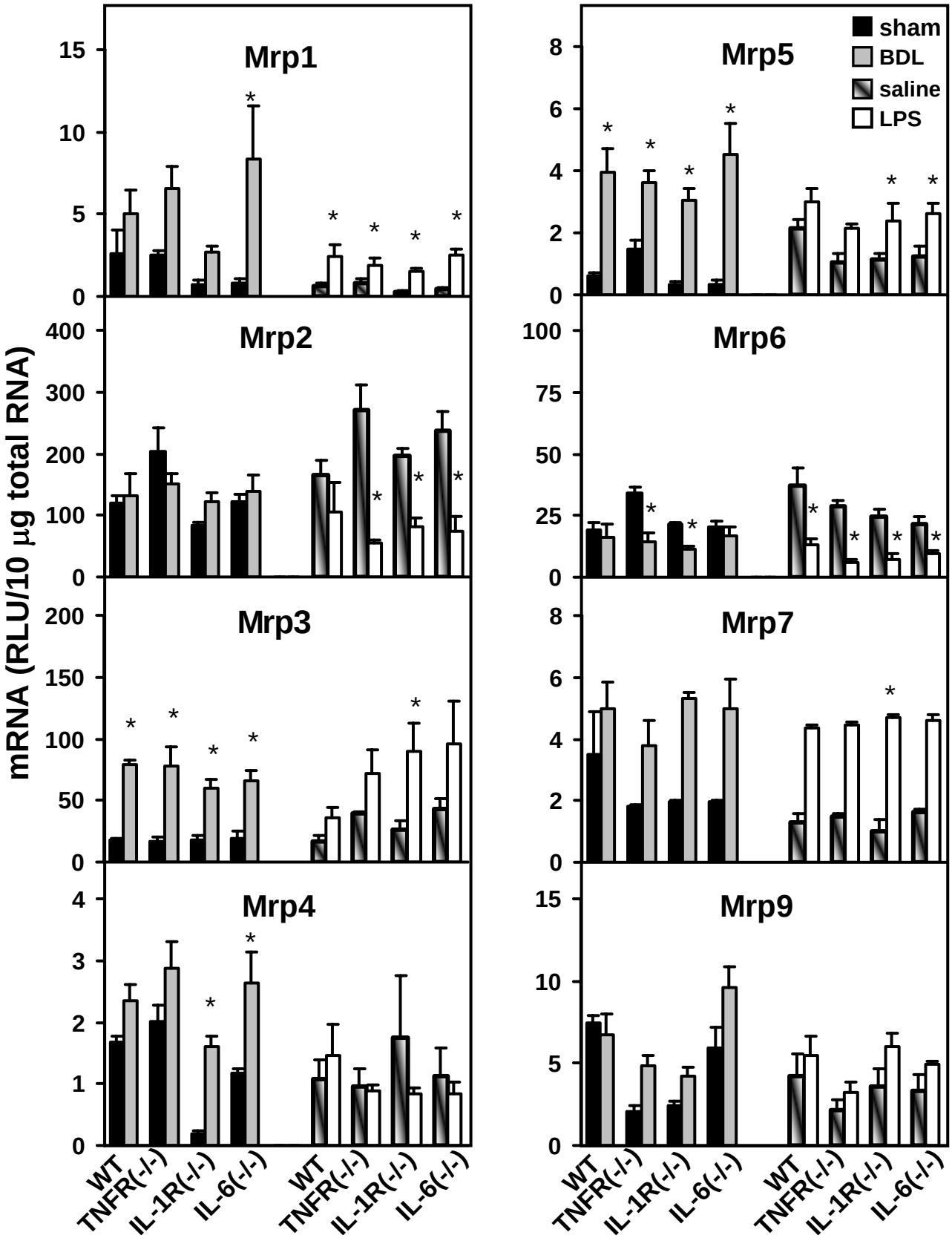

Figure 4

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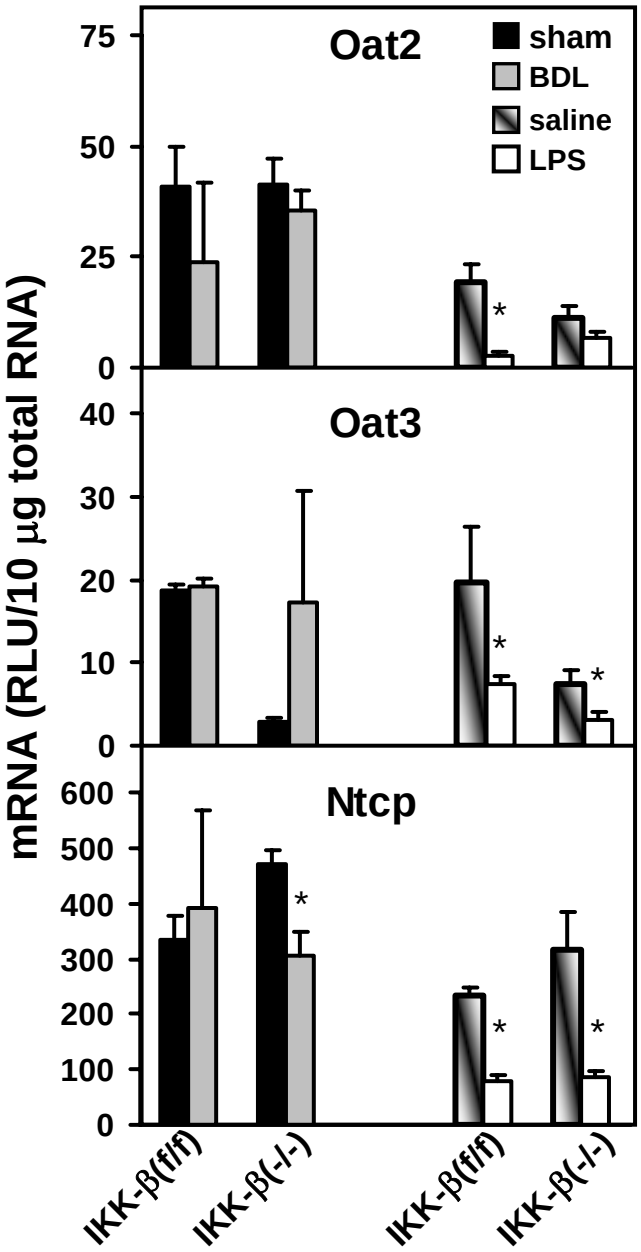

Figure 5

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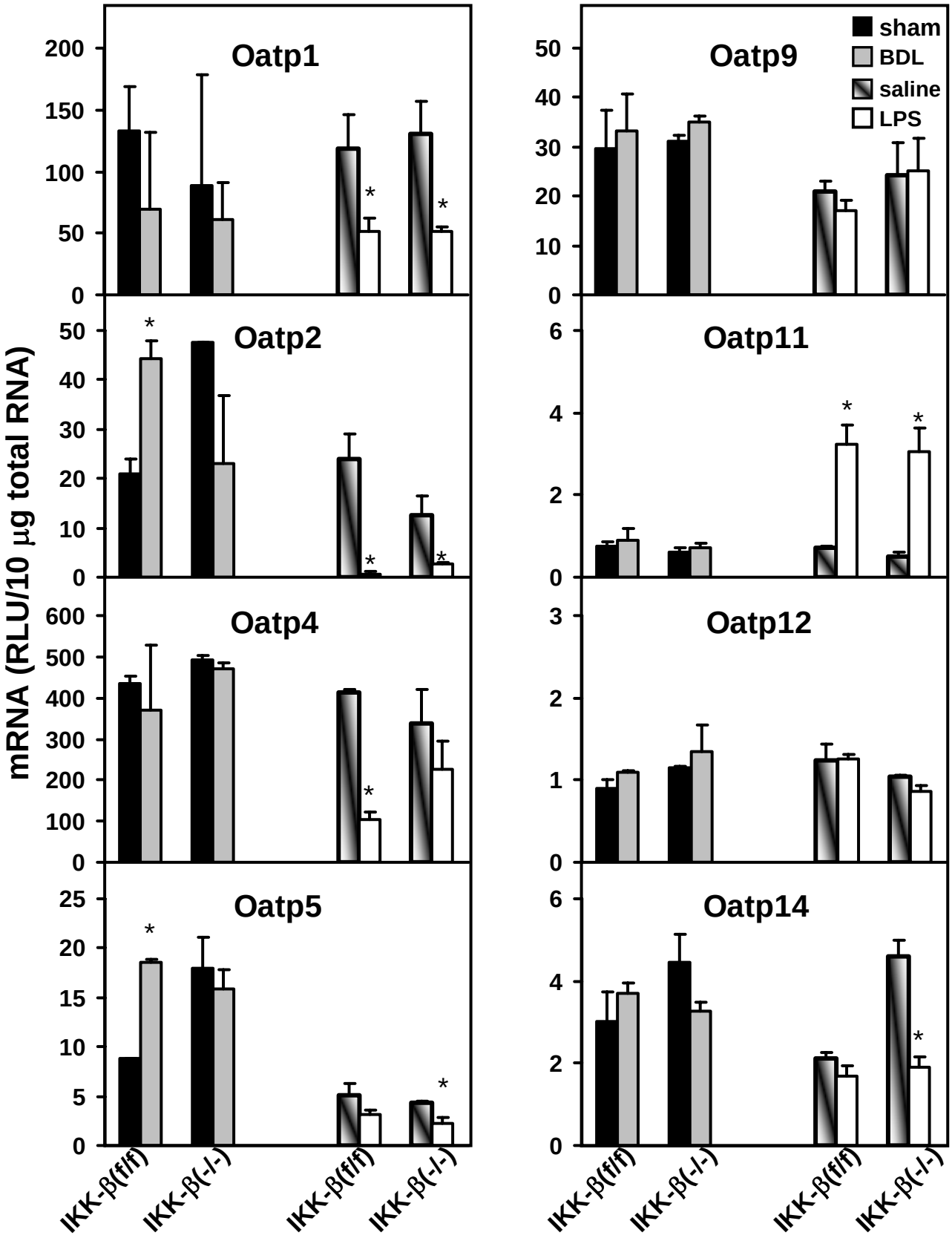

Figure 6

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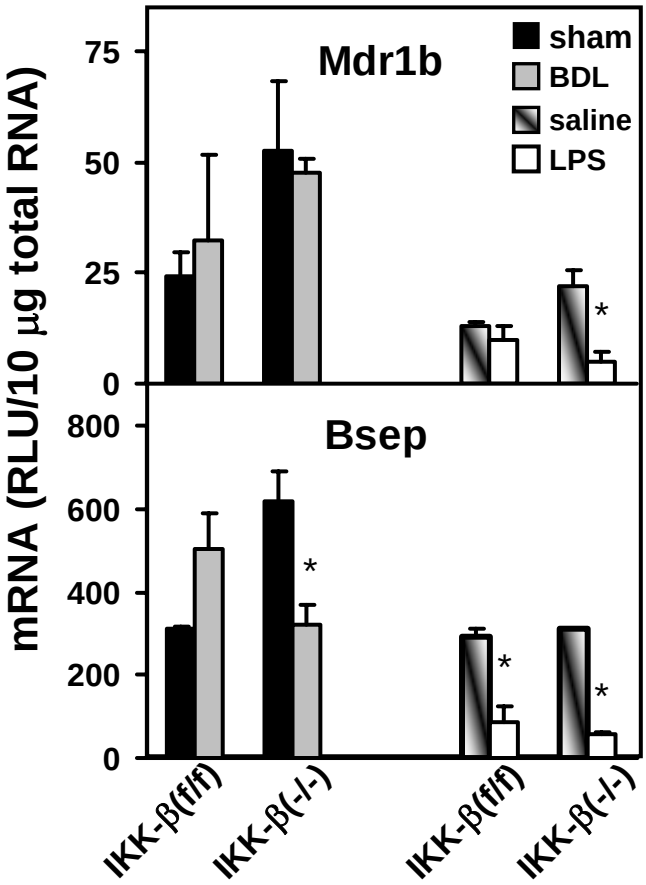

Figure 7

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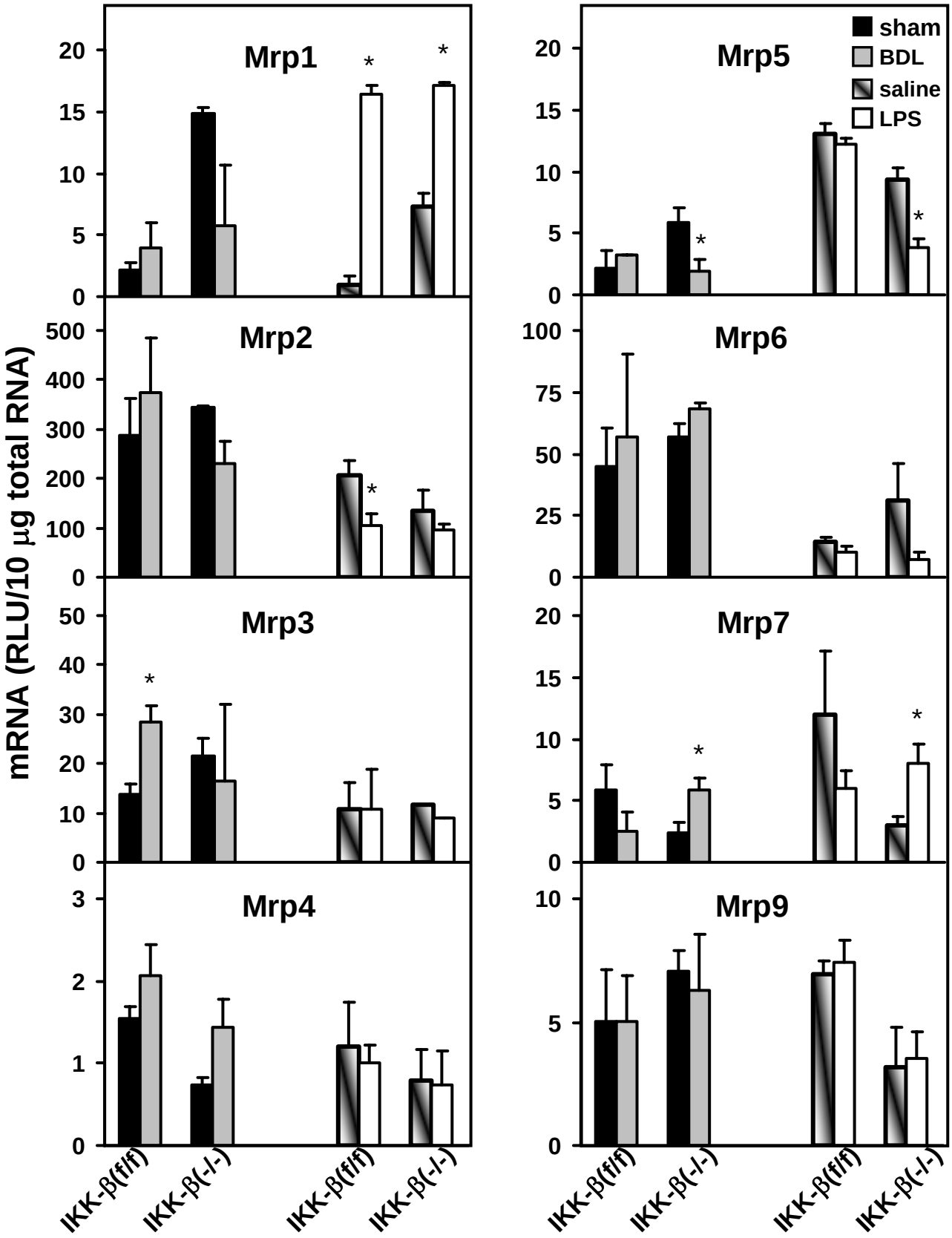

Figure 8