Sepsis-associated cholestasis is critically dependent on P-selectin-dependent leukocyte recruitment in mice

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\textbf{Running head:} Endotoxin-induced cholestasis and leukocyte recruitment

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Abstract

Cholestasis is a major complication in sepsis although the underlying mechanisms remain elusive. The aim of this study was to evaluate the role of P-selectin and leukocyte recruitment in endotoxemia-associated cholestasis. C57BL/6 mice were challenged intraperitoneally with endotoxin (0.4 mg/kg) and six hours later the common bile duct was cannulated for determination of bile flow and biliary excretion of bromosulfophthalein. Mice were pretreated with an anti-P-selectin antibody or an isotype-matched control antibody. Leukocyte infiltration was determined by measuring hepatic levels of myeloperoxidase. Tumor necrosis factor-α and CXC chemokines in the liver was determined by ELISA. Liver damage was monitored by measuring serum levels of alanine aminotransferase and aspartate aminotransferase. Apoptosis was quantified morphologically by nuclear condensation and fragmentation using Hoechst 33342 staining. Endotoxin induced a significant inflammatory response with increased TNF-α and CXC chemokine concentrations, leukocyte infiltration, liver enzyme release and apoptotic cell death. This response was associated with pronounced cholestasis indicated by a >70% decrease of bile flow and biliary excretion of bromosulfophthalein. Immunoneutralization of P-selectin significantly attenuated endotoxin-induced leukocyte infiltration reflected by a >60% reduction of hepatic myeloperoxidase levels. Interference with P-selectin decreased endotoxin-mediated hepatocellular apoptosis and necrosis, but did not affect hepatic levels of tumor necrosis factor-α and CXC chemokines. Of interest, inhibition of P-selectin restored bile flow and biliary excretion of bromosulfophthalein to normal levels in endotoxin-challenged animals. Our study demonstrates for the first time that P-selectin-mediated recruitment of leukocytes, but not the local production of pro-inflammatory mediators, is the primary cause of cholestasis in septic liver injury.

Key words: Apoptosis, bile, chemokines, inflammation, liver,
Introduction

Cholestasis is frequently observed in patients with bacterial infections, particularly in gram-negative sepsis (18,28). For example, the postoperative incidence of jaundice has been reported to be higher than 60% in cases with septic intraabdominal complications (29). Lipopolysaccharide (LPS) constitutes the dominant component of the outer membrane of most clinically relevant gram-negative bacteria found in human infections (19). LPS binds to the cell surface receptor CD14 and activates Toll-like receptor-4 (4), initiating complex signaling cascades that converge on specific transcription factors regulating gene expression of pro-inflammatory mediators (20). In the liver, the primary target of LPS is the Kupffer cell, which upon activation secretes tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and CXC chemokines. These pro-inflammatory mediators, in turn, bind to their receptors on various cell-types in the liver, including endothelial cells and hepatocytes resulting in an inflammatory response and leukocyte recruitment in the liver (3,15,25). In general, it is widely held that sepsis-induced cholestasis is related to direct actions exerted by intrahepatic cytokines on hepatocytes, which may modulate expression of transport proteins important in the regulation of bile formation (27,28). Indeed, numerous \textit{in vitro} studies have shown that inflammatory cytokines, including TNF-α, can decrease important hepatocyte transporters, such as bile salt export transporter (bsep) and multidrug resistance-associated protein 2 (mrp-2) (7-9). However, the potential role of indirect effects of pro-inflammatory mediators, including leukocyte recruitment, in endotoxin-induced cholestasis \textit{in vivo} is not known.

An accumulating body of evidence suggests that leukocyte recruitment is a rate-limiting step in endotoxemic liver damage (10,11,13). Extravascular recruitment of leukocytes is a multistep process, comprising initial leukocyte rolling along the microvascular endothelium followed by subsequent firm leukocyte adhesion and transendothelial migration (1,24). The host response to endotoxin challenge provokes up-regulation of specific endothelial cell
adhesion molecules. Interestingly, it has been shown that P-selectin supports leukocyte
rolling and lymphocyte function antigen-1 mediates firm adhesion of leukocytes in
postsinusoidal venules in septic liver injury (14,15). Moreover, hepatic accumulation of
leukocytes in response to endotoxin challenge is dependent on CXC chemokines, including
macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil
chemoattractant (KC), which trigger tissue extravasation of leukocytes (16).

Based on the considerations above, we therefore hypothesized herein that endotoxin-
provoked cholestasis may rather be dependent on the recruitment of leukocytes into the liver
than direct actions on hepatocyte transport functions exerted by locally generated pro-
inflammatory mediators.
Materials and Methods

Animals

Adult male C57/BL/6 mice (23-27 g) were kept on a 12-12 hour light-dark cycle with free access to food and tap water. Animals were anesthetized by intraperitoneal (i.p.) administration of 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 mg body weight. Test substances, fluorescent dyes and additional anesthesia were administered intravenously (i.v.) via retroorbital injection. The local ethics committee at Lund University approved all the experiments of this study.

Experimental protocol

Six hours prior to surgery and collection of bile samples, mice were pre-treated i.p. with a combination of LPS (0.4 mg/kg, LPS was from Escherichia coli serotype 0111:B4, Sigma Chemical Co., St. Louis, MO, USA) and D-galactosamine (Gal, 720 mg/kg, Sigma Chemical Co.). Additionally, mice were pre-treated i.v. with phosphate-buffered saline (PBS; 0.2 ml, n = 4), an anti-P-selectin antibody (RB40.34, 1.5 mg/kg, Pharmingen, San Diego, California, USA; n = 5) or an isotype-matched control antibody (IgG, R3-34, 1.5 mg/kg, Pharmingen; n = 5). Five additional mice which were not exposed to LPS/Gal served as controls.

Bile flow and secretion

Bile flow and bile secretion analysis were performed 6 hours after LPS/Gal challenge. For assessment of bile secretion, anesthetized mice were injected i.v. with bromosulfophthalein (BSP, 0.1 mg/g body weight, Sigma Chemical Co.) as a bolus. BSP is an organic anion excreted into the bile via mrp-2, a hepatocyte transport protein, located in the canalicular membrane of hepatocytes. After midline laparotomy, the common bile duct was cannulated
with a polyethylene catheter (PE-10) and bile samples were collected in pre-weighed tubes for 30 min. Bile flow was expressed as µl/min/g liver weight, assuming a bile density of 1 g/ml. For analysis of BSP excretion, bile samples were diluted (1:200) in 0.1 N NaOH, and the absorbance at 580 nm was recorded and quantified against known standards. After collection of bile samples, animals were killed and the liver was removed, weighed and stored for subsequent analyses.

**Blood sampling and assays**

At the end of the experiments, blood was drawn from the inferior vena cava for analysis of liver enzymes, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), using standard spectrophotometric procedures. Systemic leukocyte counts, including polymorphonuclear leukocytes (PMNL) and mononuclear leukocytes (MNL) were determined with a hematocytometer.

**Apoptosis**

Hepatocyte apoptosis was determined morphologically by fluorescence microscopy after topical application of the fluorochrome Hoechst 33342 (0.02 ml, 0.2 µg/ml, Molecular Probes, Leiden, the Netherlands) onto the surface of the liver tissue sample. Hoechst 33342, which stains hepatocyte DNA, is a fluorescent dye that has been widely used for analysis of nuclear morphology, e.g. nuclear condensation and fragmentation in cultured hepatocytes and endothelial cell (21). For microscopic analysis, a modified Olympus microscope (BX50WI, Olympus Optical Co. GmbH, Hamburg, Germany) was used equipped with an x63 water immersion lens (NA 0.9). The images were televised using a charge-coupled device video camera (FK 6990 Cohu, Pieper GmbH, Schwerte, Germany) and recorded on CD-ROM for subsequent off-line evaluation. Five microscopical fields were recorded for
off-line quantification of hepatocyte nuclei showing signs of apoptosis (chromatin condensation and fragmentation). Hepatocyte apoptosis was given as the percentage of the number of hepatocyte nuclei showing apoptotic features from the total number of hepatocyte nuclei observed.

**Myeloperoxidase (MPO)**

Liver tissue was collected, weighed and homogenized in 10 ml 0.5% hexadecyltrimethylammonium bromide. Next, the sample was freeze-thawed, after which the MPO activity of the supernatant was assessed. The MPO activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance occurring in the redox reaction of $\text{H}_2\text{O}_2$ (460 nm, 25°C). Values are expressed as MPO units per g tissue.

**ELISA**

The right liver lobe was weighed, washed and homogenized in PBS containing 1% penicillin and streptomycin and fungizone (100 U/ml) and then kept cool in cold serum-free Dulbecco’s modified Eagle’s medium. Centrifugation was completed with 10 min from harvest and supernatants were collected and stored for up to one week in -20°C until analysis of TNF-$\alpha$, KC and MIP-2 by using double antibody Quantikine ELISA kits (R & D Systems) using recombinant murine TNF-$\alpha$, KC and MIP-2 as standards. The minimal detectable protein concentrations were less than 0.5 pg/ml.

**Histology**

Samples were taken from the left lobe of liver and fixed in 4% formaldehyde phosphate buffer over night. Dehydrated, paraffin embedded 6 µm sections were stained with hematoxylin and eosin and analyzed under light microscopy. The number of extravascular
leukocytes was randomly quantified in 40 high-power fields and expressed as number of cells/mm².

**Quantitative PCR**

Liver samples were harvested and kept in a RNA stabilization reagent (RNAlater, Quiagen-Nordic, Sweden) at -20ºC. Total RNA was isolated using RNeasy® Mini Kit (Qiagen, West Sussex, Great Britan) following the manufacturer’s protocol and then treated with RNasea-free DNase (DNase I; Amersham Pharmacia Biotech, Sollentuna, Sweden) in order to remove potential genomic DNA contaminants. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. Each cDNA was synthesized by reverse transcription from 10 µg of total RNA using StrataScript® First-Strand Synthesis System and random hexamers primers (Stratagene, AH diagnostics AB, Stockholm, Sweden). Real-time PCR was performed using a Brilliant® SYBR® Green QPCR Master Mix and MX 3000P™ Detection System (Stratagene). The specific primers were as follows: mrp-2 (f) 5’-CTG AGT GCT TGG ACC AGT GA -3’; (r) 5’-CAA AGT CTG GGG GAG TGT GT-3’; oatp1 (f) 5’-TGG GGA GAA AAA TGT CCT TG-3’; (r) 5’-ATG GCT GCG AGT GAG AAG AT-3’; bsep (f) 5’-GTG GAG GAA ACG GAG CGT GGT TGC-3’; (r) 5’-AAG CTG CAC TGT CTT TTC AC-3’; -actin (f) 5’-ATG TTT GAG ACC TTC AAC ACC-3’; (r) 5’-TCT CCA GGG AGG AAG AGG AT-3’. Standard PCR curves were generated for each PCR product to establish linearity of the RT-PCR reaction. PCR amplifications were performed in a total volume of 50 µl, containing 25 µl SYBR Green PCR 2 x master mix, 2 µl 0.15 µM each primer, 0.75 µl reference dye and one 1 µl cDNA as a template adjusted up to 50 µl with water. PCR reactions were started with 10 min denaturing temperature of 95ºC, followed by a total of 40 cycles (95ºC for 30 s and 55ºC for 1 min) and 1 min of elongation at 72ºC. The relative differences in expression between groups were expressed using cycling
time (Ct) values. Ct values for the specific target genes were first normalized with that of β-actin in the same sample, and then relative differences between groups were expressed as percentage of control.

Statistical Analyses

Data are presented as mean values ± SEM. Statistical evaluations were performed using Kruskal-Wallis one-way analysis of variance on ranks followed by multiple comparisons versus control group (Dunn’s method). P < 0.05 was considered significant and n represents the number of animals.
Results

Hepatocellular injury and apoptosis

It was found that challenge with LPS caused a serious injury to the liver, illustrated by a marked increase in liver enzymes released into the circulation. Thus, administration of LPS enhanced ALT and AST by more than 39- and 30-fold, respectively (Table 1, $P < 0.05$ vs. control, $n = 5$). Notably, pre-treatment with the anti-P-selectin antibody significantly decreased ALT from $20.2 \pm 8.7$ to $0.5 \pm 0.06$ µkat/l and AST from $17.0 \pm 5.1$ to $1.8 \pm 0.3$ µkat/l in endotoxemic mice, corresponding to a more than 89% reduction in LPS-induced increases in liver enzymes (Table 1, $P < 0.05$ vs. IgG + LPS/Gal, $n = 5$). Indeed, it was found herein that administration of LPS significantly increased the number of apoptotic cells from baseline at $1.1 \pm 0.4\%$ up to $14.1 \pm 2.9\%$ in septic animals (Table 1, $P < 0.05$ vs. control, $n = 5$). Interestingly, immunoneutralization of P-selectin reduced LPS-induced apoptosis by 84% down to $2.0 \pm 0.6\%$ (Table 1, $P < 0.05$ vs. IgG + LPS/Gal, $n = 5$).

Moreover, morphologic examination showed normal microarchitecture in livers from control animals (Figure 1a), whereas administration of LPS resulted in severe destruction of the liver tissue structure characterized by massive panlobular hemorrhage and necrosis as well as infiltration of neutrophils (Figure 1b). In line with the aforementioned data on liver enzymes and apoptosis, it was found that immunoneutralization of P-selectin almost completely protected against endotoxin-induced destruction of tissue architecture, hepatocellular damage and neutrophil infiltration in the liver (Figure 1c).

Leukocyte recruitment

Global accumulation of leukocytes was determined by quantifying the MPO activity in the liver. It was found that hepatic MPO activity increased from $0.07 \pm 0.01$ U/g in control mice up to $1.24 \pm 0.26$ U/g in endotoxemic animals (Figure 2, $P < 0.05$ vs. control, $n = 5$).
Notably, we observed that pre-treatment with the anti-P-selectin antibody decreased LPS-induced MPO activity down to 0.39 ± 0.06 U/g, corresponding to a 62% reduction in MPO activity (Figure 2, *P* < 0.05 vs. IgG + LPS/Gal, *n* = 5). On top of that, quantification of extravascular polymorphonuclear leukocytes in the liver showed that LPS challenge increased leukocyte recruitment from 200 ± 78 cells/mm² in controls to 1998 ± 356 cells/mm² (Figure 3, *P* < 0.05 vs. control, *n* = 5). Strikingly, immunoneutralization of P-selectin significantly decreased LPS-provoked leukocyte accumulation in the liver down to 480 ± 78 cells/mm² (Figure 3, *P* < 0.05 vs. IgG + LPS/Gal, *n* = 5), corresponding to a 70% reduction. As shown in Table 2, there was no significant difference in systemic leukocyte differential counts between the experimental groups.

**Expression of pro-inflammatory mediators**

LPS challenge significantly increased hepatic production of TNF-α, MIP-2 and KC (Table 3). However, immunoneutralization of P-selectin did not decrease expression of TNF-α and CXC chemokines in the liver of endotoxemic animals (Table 3, *P* < 0.05 vs. IgG + LPS/Gal), suggesting that inhibition of P-selectin-dependent leukocyte recruitment has no effect on the actual levels pro-inflammatory mediators in endotoxin-induced liver injury and cholestasis.

**Bile flow and BSP secretion**

Bile flow was determined by dividing the volume of bile per min collected from the common bile duct by the liver weight. It was found that baseline bile flow was 420 ± 3 nl/min/g liver tissue and that challenge with LPS decreased bile flow down to 120 ± 6 nl/min/g liver tissue (Figure 4, *P* < 0.05 vs. control, *n* = 5-7). We observed that the bile flow was restored to normal levels in endotoxemic mice pre-treated with the anti-P-selectin antibody (Figure 4, *P* < 0.05 vs. IgG + LPS/Gal, *n* = 5-7). Mice were injected with BSP in order to analyze the
excretory function of the liver in vivo (23). We found that BSP excretion in control mice was 7.6 ± 0.3 mg/ml and that LPS treatment decreased excretion of BSP down to 1.3 ± 0.5 mg/ml, corresponding to an 83% reduction (Figure 5, \( P < 0.05 \) vs. control, \( n = 5-7 \)). Notably, we observed that immunoneutralization of P-selectin restored BSP excretion in endotoxemic mice towards baseline levels, i.e. 5.4 ± 0.7 mg/ml (Figure 5, \( P < 0.05 \) vs. IgG + LPS/Gal, \( n = 5-7 \)). Thus, inhibition of P-selectin significantly reduced LPS-provoked downregulation of BSP excretion by 52%.

**Hepatobiliary transport proteins**

In order to examine the molecular mechanisms behind the restoration of BSP excretion and bile flow in endotoxemic animals receiving the anti-P-selectin antibody, we quantified the gene expression of oatp1, mrp-2 and bsep in the liver. We found that challenge with LPS decreased hepatic mRNA levels of oatp1, mrp-2 and bsep by more than 70% compared to controls (Figure 6, \( P < 0.05 \) vs. control, \( n = 5 \)). Interestingly, we found that immunoneutralization of P-selectin protected against decreased mRNA levels of oatp1, mrp-2 and bsep in mice challenged with LPS (Figure 6, \( n = 5 \)). In fact, administration of the anti-P-antibody restored gene expression of oatp1, mrp-2 and bsep towards normal levels (70-80% of controls), corresponding to a more than 209% increase in mRNA levels of hepatobiliary transport proteins in endotoxemic animals (Figure 6, \( P < 0.05 \) vs. IgG + LPS/Gal, \( n = 5 \)).
Discussion

This study demonstrates that leukocyte recruitment constitutes a key component in endotoxin-induced cholestasis *in vivo*. We found that inhibition of P-selectin-dependent leukocyte accumulation abolished the reduction in bile flow, BSP excretion and expression of hepatobiliary transport proteins in septic liver injury. In fact, this protective action against leukocyte recruitment and cholestasis by interfering with P-selectin was observed in spite of intact increases in pro-inflammatory mediators, including TNF-α and CXC chemokines in endotoxemic liver damage. Thus, these findings not only demonstrate that leukocyte recruitment is an integral part of the pathophysiology in sepsis-induced cholestasis but also that the generation of pro-inflammatory mediators is not sufficient alone for provoking significant cholestasis in endotoxemic liver injury *in vivo*. Taken together, these novel findings contribute to the understanding of the regulatory mechanisms in sepsis-associated cholestasis and suggest that targeting leukocyte recruitment may be an important strategy for the development of effective therapies aiming to maintain efficient bile flow and excretion in the liver.

Cholestasis is frequently associated with sepsis (18,28). A vicious cycle may develop when endotoxin-induced cholestasis results in a decreased concentration of bile acids in the small intestine, which in turn appears to favor bacterial translocation (2) and endotoxin absorption (6), which then further aggravates the cholestasis. Our present data show that leukocyte recruitment is a critical component in endotoxin-induced cholestasis *in vivo*. Thus, we observed that immunoneutralization of P-selectin not only blocked leukocyte recruitment (62-70% reduction) but also markedly decreased the cholestatic effect of endotoxin, *i.e.* bile flow and BSP excretion was restored in endotoxemic mice pre-treated with the anti-P-selectin antibody. Indeed, this 62-70% reduction in leukocyte recruitment correlated to the 52% improved BSP excretion observed in endotoxemic mice pre-treated with the anti-P-
selectin antibody. This is the first study to show that hepatic recruitment of leukocytes is a prerequisite in endotoxin-provoked cholestasis in vivo. To a great extent, the prevailing opinion has been that LPS-inducible pro-inflammatory mediators, such as TNF-α, directly cause cholestasis by down-regulating expression of certain hepatocyte transporters, including bsep and mrp-2 (7-9). In this context, it is relevant to note that mrp-2 is the main transport protein of BSP. As shown previously by Hartman et al. (9), we found that LPS challenge markedly reduced gene expression of oatp1, bsep and mrp-2 in the liver. However, it was observed herein that inhibition of P-selectin abolished LPS-induced transcriptional suppression of these hepatocyte transporters, indicating a causal link between P-selectin-dependent leukocyte recruitment on one hand and gene expression of hepatocyte transporters on the other hand in septic liver injury. It is also interesting noting that cytokine-mediated transcriptional inhibition in vivo (>90%) is much stronger than suppression of gene expression in vitro of hepatocyte transporters (5,26), indicating that the mechanisms behind sepsis-associated cholestasis is more complex in a multicellular environment in vivo.

Concomitantly, we observed that inhibition of P-selectin had no effect on endotoxin-induced increases in pro-inflammatory compounds, such as TNF-α and CXC chemokines, in the liver, suggesting that immunoneutralization of P-selectin maintained expression of oatp1, bsep and mrp-2 by primarily blocking leukocyte recruitment. Thus, our in vivo data demonstrate that targeting P-selectin maintains intact bile flow, expression of hepatocyte transporters and excretory function in endotoxemic animals in spite of the concomitant presence of high levels of pro-inflammatory mediators, which in vitro are potent inhibitors of hepatic transport proteins (7-9). Indeed, we also observed herein that inhibition of P-selectin decreased endotoxin-induced hepatocellular damage and apoptosis, a key feature in sepsis (17), which may help explain the protective effect against cholestasis assuming that intact hepatocytes are required for bile formation and excretion. Thus, considered together, we
conclude that leukocyte recruitment is a relatively more important component compared to the generation of pro-inflammatory substances in mediating sepsis-associated cholestasis.

It is widely held that leukocyte recruitment is a rate-limiting step in septic liver injury (10,11,13). In general, the recruitment process of leukocytes is considered to be a multistep process, in which initial leukocyte rolling is a precondition for subsequent firm adhesion and transendothelial migration of leukocytes (1,24). Leukocyte rolling is mediated by the selectin family of adhesion molecules and serves to reduce the velocity of circulating leukocytes in order to allow time for detection of chemotactic substances released from the inflamed tissue (12). This paradigm has been challenged in the liver by a report suggesting only a minimal role of selectins in the recruitment process of leukocytes in the liver (30). Notably, we found in the present study that inhibition of P-selectin significantly decreased extravascular accumulation of leukocytes in the liver of endotoxemic mice, suggesting that P-selectin plays, in fact, a key role in the recruitment process of leukocytes in the liver. Our study did not address which stage in the transmigration process of leukocytes that P-selectin supports. However, a recent intravital microscopic study demonstrated that P-selectin supports LPS-induced leukocyte rolling in the liver (14), which helps explain our present findings showing that inhibition of P-selectin substantially blocks endotoxin-provoked accumulation of leukocytes in the extravascular space in the liver. Thus, it appears that the recruitment process of leukocytes in the liver is similar to that observed in other organs. This notion is also supported by several recent studies reporting that inhibition of P-selectin blocks hepatic recruitment of leukocytes not only in septic liver injury but also in ischemia-reperfusion injury and hemorrhage-reinfusion (22,31).

Taken together, our data suggest that leukocyte recruitment is a critical component in the patophysiology of sepsis-associated cholestasis \textit{in vivo}. Indeed, the present results indicate that the leukocyte response is relatively more important than the local production of
pro-inflammatory mediators. Moreover, this study suggests that P-selectin is important for hepatic accumulation of leukocytes in the extravascular space. Thus, based on these findings it may be concluded that targeting leukocyte recruitment may be an effective strategy to limit sepsis-induced cholestasis in the liver.
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Figure legends

Figure 1. Representative hematoxylin & eosin cross sections of liver tissue harvested 6 hours after treatment with a combination of lipopolysaccharide (LPS, 0.4 mg/kg) and D-galactosamine (Gal, 720 mg/kg). Mice were pre-treated with a control antibody (IgG, 1.5 mg/kg, B) or an antibody directed against P-selectin (1.5 mg/kg, C) 15 min prior to LPS/Gal challenge. Control animals without LPS/Gal challenge received only PBS (A). Arrow indicates area of neutrophil infiltration and a scale bar is inserted in the left corner indicating 100 µm.

Figure 2. Myeloperoxidase (MPO) activity in the liver 6 hours after treatment with a combination of lipopolysaccharide (LPS, 0.4 mg/kg) and D-galactosamine (Gal, 720 mg/kg). Mice were pre-treated with PBS, a control antibody (IgG, 1.5 mg/kg) or an antibody directed against P-selectin (Anti-P, 1.5 mg/kg) 15 min prior to LPS/Gal challenge. Control animals without LPS/Gal challenge received only PBS. Data represent means ± SEM and n = 5. #P < 0.05 vs. control and *P < 0.05 vs. IgG + LPS/Gal.

Figure 3. Extravascular accumulation of leukocytes in the liver 6 hours after treatment with a combination of lipopolysaccharide (LPS, 0.4 mg/kg) and D-galactosamine (Gal, 720 mg/kg). Mice were pre-treated with a control antibody (IgG, 1.5 mg/kg) or an antibody directed against P-selectin (Anti-P, 1.5 mg/kg) 15 min prior to LPS/Gal challenge. Control animals without LPS/Gal challenge received only PBS. Data represent means ± SEM and n = 5-7. #P < 0.05 vs. control and *P < 0.05 vs. IgG + LPS/Gal.

Figure 4. Bile flow 6 hours after treatment with a combination of lipopolysaccharide (LPS, 0.4 mg/kg) and D-galactosamine (Gal, 720 mg/kg). Mice were pre-treated with PBS, a
control antibody (IgG, 1.5 mg/kg) or an antibody directed against P-selectin (Anti-P, 1.5 mg/kg) 15 min prior to LPS/Gal challenge. Control animals without LPS/Gal challenge received only PBS. Data represent means ± SEM and n = 5-7. #P < 0.05 vs. control and *P < 0.05 vs. IgG + LPS/Gal.

Figure 5. BSP excretion 6 hours after treatment with a combination of lipopolysaccharide (LPS, 0.4 mg/kg) and D-galactosamine (Gal, 720 mg/kg). Mice were pre-treated with PBS, a control antibody (IgG, 1.5 mg/kg) or an antibody directed against P-selectin (Anti-P, 1.5 mg/kg) 15 min prior to LPS/Gal challenge. Control animals without LPS/Gal challenge received only PBS. Data represent means ± SEM and n = 5-7. #P < 0.05 vs. control and *P < 0.05 vs. IgG + LPS/Gal.

Figure 6. Gene expression of organic anion transporting polypeptide 1 (oatp1), bile salt export transporter (bsep) and multidrug resistance-associated protein 2 (mrp-2) 6 hours after treatment with a combination of lipopolysaccharide (LPS, 0.4 mg/kg) and D-galactosamine (Gal, 720 mg/kg). Mice were pre-treated with PBS, a control antibody (IgG, 1.5 mg/kg) or an antibody directed against P-selectin (Anti-P, 1.5 mg/kg) 15 min prior to LPS/Gal challenge. Control animals without LPS/Gal challenge received only PBS. Levels of oatp1, bsep and mrp-2 mRNA were quantified using real-time polymerase chain reaction. The expression of mRNA levels for each transport protein was normalised as a ratio using β-actin mRNA as a housekeeping gene. Data represent means ± SEM and n = 5. #P < 0.05 vs. control and *P < 0.05 vs. IgG + LPS/Gal.
Table 1. Endotoxin-induced hepatitis

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<th>ALT (µKat/L)</th>
<th>AST (µKat/L)</th>
<th>Apoptosis (%)</th>
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<tr>
<td>Control</td>
<td>0.58 ± 0.1</td>
<td>0.82 ± 0.2</td>
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<td>Anti-P + LPS/Gal</td>
<td>0.48 ± 0.1*</td>
<td>1.8 ± 0.3*</td>
<td>2.0 ± 0.6*</td>
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Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically. Apoptosis was determined by counting the percentage of observed hepatocyte nuclei with morphological signs of nuclear condensation and fragmentation after administration of the fluorochrome Hoechst 33342. Mice were challenged with lipopolysaccharide (LPS, 0.4 mg/kg) and D-galactosamine (Gal, 720 mg/kg) and pre-treated with PBS, a control antibody (IgG, 1.5 mg/kg) or an antibody directed against P-selectin (Anti-P, 1.5 mg/kg). Animals not exposed to LPS/Gal served as controls. Data are means ± SEM and n = 5. *P < 0.05 vs. control and *P < 0.05 vs. IgG + LPS/Gal.
Table 2. Systemic leukocyte counts

<table>
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<th>PMNL</th>
<th>MNL</th>
<th>Total</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.4 ± 0.1</td>
<td>2.2 ± 0.2</td>
<td>2.7 ± 0.3</td>
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<td>PBS + LPS/Gal</td>
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<td>Anti-P + LPS/Gal</td>
<td>0.4 ± 0.1</td>
<td>2.1 ± 0.4</td>
<td>2.5 ± 0.3</td>
</tr>
</tbody>
</table>

Blood samples were drawn from the inferior vena cava at the end of the experiments. The number of polymorphonuclear (PMNL) and mononuclear (MNL) leukocytes was counted using a standard hematocytometer. Mice were challenged with lipopolysaccharide (LPS, 0.4 mg/kg) and D-galactosamine (Gal, 720 mg/kg) and pre-treated with PBS, a control antibody (IgG, 1.5 mg/kg) or an antibody directed against P-selectin (Anti-P, 1.5 mg/kg). Animals not exposed to LPS/Gal served as controls. Data are means ± SEM (n = 5) and represent 10⁶ cells per ml. No significant differences were found between the groups.
Table 3. Expression of pro-inflammatory mediators

<table>
<thead>
<tr>
<th></th>
<th>TNF-α (pg/g)</th>
<th>MIP-2 (ng/mg)</th>
<th>KC (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>218 ± 23</td>
<td>0.5 ± 0.08</td>
<td>3.0 ± 1.3</td>
</tr>
<tr>
<td>PBS + LPS/Gal</td>
<td>541 ± 103#</td>
<td>15.4 ± 2.8#</td>
<td>22.0 ± 2.5#</td>
</tr>
<tr>
<td>IgG + LPS/Gal</td>
<td>567 ± 98#</td>
<td>16.8 ± 4.7#</td>
<td>21.8 ± 4.1#</td>
</tr>
<tr>
<td>Anti-P + LPS/gal</td>
<td>677 ± 108#</td>
<td>36.9 ± 23.8#</td>
<td>47.0 ± 21.8#</td>
</tr>
</tbody>
</table>

The expression of TNF-α, MIP-2 and KC in the liver was determined by use of specific ELISA. Mice were challenged with lipopolysaccharide (LPS, 0.4 mg/kg) and D-galactosamine (Gal, 720 mg/kg) and pre-treated with PBS, a control antibody (IgG, 1.5 mg/kg) or an antibody directed against P-selectin (Anti-P, 1.5 mg/kg). Animals not exposed to LPS/Gal served as controls. Data are means ± SEM and n = 5. *P < 0.05 vs. control.