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

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Laschke MW, Menger MD, Wang Y, Lindell G, Jeppsson B, Thorlacius H. Sepsis-associated cholestasis is critically dependent on P-selectin-dependent leukocyte recruitment in mice. Am J Physiol Gastrointest Liver Physiol 292: G1396–G1402, 2007. First published January 25, 2007; doi:10.1152/ajpgi.00539.2006.—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 6 h 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 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 proinflammatory mediators, is the primary cause of cholestasis in septic liver injury.

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 proinflammatory 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 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 proinflammatory mediators, including leukocyte recruitment, in endotoxin-induced cholestasis 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 upregulation 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).

On the basis of the considerations above, we therefore hypothesized herein that endotoxin-provoked cholestasis may be dependent on the recruitment of leukocytes into the liver rather than on direct actions on hepatocyte transport functions exerted by locally generated proinflammatory mediators.

MATERIALS AND METHODS

Animals. Adult male C57/BL/6 mice (23–27 g) were kept on a 12:12-h light-dark cycle with free access to food and tap water. Animals were anesthetized by intraperitoneal administration of 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight. Animals were used in accordance with the guidelines for the care and use of laboratory animals of the European Community. One week after surgery, male mice were divided into five groups. Animals were pretreated with an anti-P-selectin antibody or an isotype-matched control antibody and 6 h later the common bile duct was cannulated for determination of bile flow and biliary excretion of bromosulfophthalein.
mg body wt. Test substances, fluorescent dyes, and additional anesthesia were administered intravenously via retroorbital injection. The local ethics committee at Lund University approved all the experiments of this study.

Experimental protocol. Six hours before surgery and collection of bile samples, mice were pretreated intraperitoneally with a combination of LPS (0.4 mg/kg; LPS was from Escherichia coli serotype 0111:B4, Sigma Chemical, St. Louis, MO) and d-galactosamine (Gal, 720 mg/kg, Sigma Chemical). Additionally, mice were pretreated intravenously with phosphate-buffered saline (PBS; 0.2 ml, n = 4), an anti-P-selectin antibody (RB40.34, 1.5 mg/kg, Pharmingen, San Diego, CA; 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 the LPS/Gal combination served as controls.

Bile flow and secretion. Bile flow and bile secretion analysis were performed 6 h after LPS/Gal challenge. For assessment of bile secretion, anesthetized mice were injected intravenously with bromosulfophthalein (BS, 0.1 mg/g body wt, Sigma Chemical) 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 preweighed tubes for 30 min. Bile flow was expressed as microliters per minute per gram liver weight, with bile density assumed to be 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), by standard spectrophotometric procedures. Systemic leukocyte counts, including polymorphonuclear leukocytes and mononuclear leukocytes, 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, Hamburg, Germany) was used equipped with an ×63 water immersion lens (NA 0.9). The images were televised using a charge-coupled device video camera (FK 6990 Cuh, Pieper, Schwerte, Germany) and recorded on CD-ROM for subsequent off-line evaluation. Five microscopic 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.

MPO. Liver tissue was collected, weighed, and homogenized in 10 ml 0.5% hexadecyltrimethylammonium bromide. Next, the sample was freeze-thawed, after which the myeloperoxidase (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 H₂O₂ (460 nm, 25°C). Values are expressed as MPO units per gram 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 within 10 min from harvest, and supernatants were collected and stored for up to 1 wk in −20°C until analysis of TNF-α, KC, and MIP-2 by using double antibody Quantikine ELISA kits (R & D Systems) using recombinant murine TNF-α, KC, and MIP-2 as standards. The minimal detectable protein concentrations were <0.5 pg/ml.

Histology. Samples were taken from the left lobe of liver and fixed in 4% formaldehyde phosphate buffer overnight. 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 per square millimeter.

Quantitative PCR. Liver samples were harvested and kept in a RNA stabilization reagent (RNeasy Mini Kit (Qiagen, West Sussex, UK) following the manufacturer’s protocol and then treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech, Sollentuna, Sweden) 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 Stratagene First-Strand Synthesis System and random hexamers primers (Stratagene, AHI diagnostics, Stockholm, Sweden). Real-time PCR was performed using a Brilliant SYBRgreen QPCR master mix and MX 3000P detection system (Stratagene). The specific primers were as follows: mrp-2 forward 5′-CTG AGT GCT TGG ACC GT-3′; reverse 5′-CAA AGT GGG GAG GAG GT-3′; organic anion transporting polypeptide 1 ( oatp1) forward 5′-TGG GGA GAA AAA TGT CCT TG-3′; reverse 5′-ATG GCT GGC AGT GAG AA-3′; bsep forward 5′-GGT CAG TTC CTC GTG CTA AA-3′; reverse 5′-AAAG CTG CAC TGT CTT TTC AC-3′; β-actin forward 5′-ATG TTT GAG ACC TTC AAC ACC-3′; reverse 5′-TCT CCA GGA 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 of SYBRgreen PCR 2 × master mix, 2 μl of 0.15 μM each primer, 0.75 μl of 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 95°C, followed by a total of 40 cycles (95°C for 30 s and 55°C for 1 min) and 1 min of elongation at 72°C. The relative differences in expression between groups were expressed by using cycling time values. Cycling time 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 were presented as means ± SE. Statistical evaluations were performed by using Kruskal-Wallis one-way analysis of variance on ranks followed by multiple comparisons vs. control group (Dunn’s method), P < 0.05 was considered significant, and n represents the number of animals.

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<th>Table 1. Endotoxin-induced hepatitis</th>
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<td>ALT, μkat/l</td>
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<td>Control</td>
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<tr>
<td>PBS + LPS/Gal</td>
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<td>IgG + LPS/Gal</td>
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<td>Anti-P + LPS/Gal</td>
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Data are means ± SE; n = 5. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically. Apoptosis was determined by counting the percentage of observed hepatocyte nuclei showing 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 pretreated 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. *P < 0.05 vs. control; †P < 0.05 vs. IgG + LPS/Gal.
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 ~39- and 30-fold, respectively (Table 1, P < 0.05 vs. control, n = 5). Notably, pretreatment with the anti-P-selectin antibody significantly decreased ALT from 20.2 ± 8.7 to 0.5 ± 0.06 μkat/l and AST from 17.0 ± 5.1 to 1.8 ± 0.3 μkat/l in endotoxemic mice, corresponding to a >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 ± 0.4% up to 14.1 ± 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 ± 0.6% (Table 1, P < 0.05 vs. IgG + LPS/Gal, n = 5). Moreover, morphological examination showed normal microarchitecture in livers from control animals (Fig. 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 (Fig. 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 (Fig. 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 ± 0.01 U/g in control mice up to 1.24 ± 0.26 U/g in endotoxemic animals (Fig. 2, P < 0.05 vs. control, n = 5). Notably, we observed that pretreatment 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 (Fig. 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 1,998 ± 356 cells/mm² (Fig. 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 (Fig. 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 proinflammatory 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 proinflammatory mediators in endotoxin-induced liver injury and cholestasis.

Bile flow and BSP secretion. Bile flow was determined by dividing the volume of bile per minute collected from the common bile duct by the liver weight. It was found that baseline bile flow was 420 ± 3 nL·min⁻¹·g liver tissue⁻¹ and that challenge with LPS decreased bile flow down to 120 ± 6 nL·min⁻¹·g liver tissue⁻¹ (Fig. 4, P < 0.05 vs. control, n = 5–7). We observed that the bile flow was restored to normal levels in endotoxemic mice pretreated with the anti-P-selectin antibody (Fig. 4, P < 0.05 vs. IgG + LPS/Gal, n = 5–7). Mice were injected with BSP 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 (Fig. 5, P < 0.05 vs. control, n = 5–7).

Table 3. Expression of proinflammatory mediators

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<th>TNF-α, pg/g</th>
<th>MIP-2, ng/mg</th>
<th>KC, ng/mg</th>
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<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>547±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>
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Data are means ± SE; n = 5. The expression of TNF-α, macrophage inflammatory protein 2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC) in the liver was determined by use of specific ELISA. Mice were challenged with LPS (0.4 mg/kg) and Gal (720 mg/kg) and pretreated 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. *P < 0.05 vs. control.
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 despite intact increases in proinflammatory 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 proinflammatory 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 aggravate 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 were restored in endotoxemic mice pretreated 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 pretreated 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 proinflammatory mediators, such as TNF-α, directly cause cholestasis by downregulating 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 Hartmann 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 to note 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 proinflammatory 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 despite the concomitant presence of high levels of proinflammatory 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 with the assumption that intact hepatocytes are required for bile formation and excretion. Thus, considering these facts together, we conclude that leukocyte recruitment is a relatively more important component compared with the generation of proinflammatory 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 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 extracellular 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 pathophysiology of sepsis-associated cholestasis in vivo. Indeed, the present results indicate that the leukocyte response is relatively more important than the local production of proinflammatory mediators. Moreover, this study suggests that P-selectin is important for hepatic accumulation of leukocytes in the extravascular space. Thus, on the basis of these findings, it may be concluded that targeting leukocyte recruitment may be an effective strategy to limit sepsis-induced cholestasis in the liver.

GRANTS

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