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PO Box 117
221 00 Lund
+46 46-222 00 00

ON LEUKOCYTE RECRUITMENT IN CHOLESTATIC LIVER INJURY

Matthias W. Laschke

Academic Thesis

With permission from the Medical Faculty at Lund University
for the presentation of this PhD thesis in a public forum in the CRC,
Entrance 72, Malmö University Hospital, Malmö, on 28th November

Faculty opponent: Professor Thomas Minor, University of Bonn

Supervisor: Henrik Thorlacius, MD, PhD, Associate Professor



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| Abstract <p>Cholestasis is a frequent clinical syndrome, which is caused by a dysfunction in bile formation of the hepatocyte or from obstruction of the biliary tract. This is associated with inflammation of the liver tissue, resulting in severe liver injury. In this inflammatory process, leukocyte recruitment has emerged as a key feature. Therefore, the aim of this thesis was to analyze the detailed mechanisms behind intrahepatic leukocyte accumulation and its regulation in the pathophysiology of sepsis-associated or obstructive cholestasis and their impact on hepatocellular function and damage. For this purpose, cholestatic conditions were induced in C57BL/6 mice in the well established experimental models of LPS sepsis and obstructive cholestasis following bile duct ligation. Analyses included intravital fluorescence microscopy, histology, ELISA, RT-PCR, flow cytometry, determination of bilirubin and liver enzyme levels as well as measurement of bile flow and secretion. In doing so, it was found that P-selectin-mediated recruitment of leukocytes, but not the local production of pro-inflammatory mediators, is the primary cause of sepsis-associated cholestasis. Moreover, obstructive cholestasis is associated with P-selectin-mediated intrahepatic platelet accumulation, which crucially contributes to leukocyte recruitment and liver injury. Besides, LFA-1 mediates firm leukocyte adhesion in the liver microcirculation during obstructive cholestasis. Finally, inhibition of rhokinase attenuates cholestasis-induced CXC chemokine formation, leukocyte recruitment and hepatocellular damage in the liver. Thus, the results of this thesis clearly demonstrate that leukocyte recruitment in the liver plays a key role in the pathophysiology of cholestasis. Accordingly, it may be concluded that targeting leukocyte recruitment may be an effective strategy to preserve bile flow under septic conditions and to reduce cholestasis-induced liver injury.</p> | | |
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Academic Thesis



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To my family

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1. LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which are referred in the text to by their Roman numerals:

- I. **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* 2007; 292(5):G1396-402.

- II. **Laschke MW**, Dold S, Menger MD, Jeppsson B, Thorlacius H. Platelet-dependent accumulation of leukocytes in sinusoids mediates hepatocellular damage in bile duct ligation-induced cholestasis. *Br J Pharmacol* 2008; 153(1):148-56.

- III. Dold S*, **Laschke MW***, Lavasani S, Menger MD, Thorlacius H. Cholestatic liver damage is mediated by lymphocyte function antigen-1-dependent recruitment of leukocytes. *Surgery* 2008; 144(3):385-93.
**These authors contributed equally to this work*

- IV. **Laschke MW**, Dold S, Jeppsson B, Schilling MK, Menger MD, Thorlacius H. Rho-kinase inhibitor attenuates cholestasis-induced CXC chemokine formation, leukocyte recruitment and hepatocellular damage in the liver. *J Surg Res* 2008; *in press*

2. ABBREVIATIONS

| | |
|-------|---|
| ANOVA | analysis-of-variance |
| ALT | alanine aminotransferase |
| AST | aspartate aminotransferase |
| ATP | adenosine triphosphate |
| BDL | bile duct ligation |
| BSEP | bile salt export pump |
| BSP | bromosulfophthalein |
| DNA | deoxyribonucleic acid |
| DSS | dextran sodium sulfate |
| EGF | epidermal growth factor |
| ELISA | enzyme-linked immunosorbent assay |
| FITC | fluorescein isothiocyanate |
| GAL | galactosamine |
| GDP | guanosine diphosphate |
| GTP | guanosine triphosphate |
| HEV | high endothelial venule |
| HPF | high-power field |
| 5-HT | 5-hydroxytryptamine |
| IL | interleukin |
| i.p. | intraperitoneal |
| i.v. | intravenous |
| JAM | junctional adhesion molecule |
| KC | cytokine-induced neutrophil chemoattractant |
| LFA-1 | lymphocyte function antigen-1 |
| LPS | lipopolysaccharide |
| MAC-1 | macrophage antigen-1 |
| MIP-2 | macrophage inflammatory protein-2 |
| MMP-2 | matrix metalloproteinase-2 |
| MPO | myeloperoxidase |
| MRP-2 | multidrug resistance protein-2 |
| NA | numerical aperture |

| | |
|---------------|---|
| NTCP | sodium-dependent taurocholate cotransporter |
| OATP | organic anion transport protein |
| PBS | phosphate-buffered saline |
| PECAM-1 | platelet endothelial cell adhesion molecule-1 |
| PF-4 | platelet factor-4 |
| PSGL-1 | P-selectin glycoprotein ligand-1 |
| RNA | ribonucleic acid |
| ROCK | Rho-kinase |
| RT-PCR | real-time polymerase chain reaction |
| SEM | standard error of the mean |
| sLeX | sialyl Lewis X |
| TNF- α | tumor necrosis factor- α |
| VCAM-1 | vascular cell adhesion molecule-1 |

3. INTRODUCTION

Cholestasis is a frequent clinical syndrome characterised by an impaired bile flow, which is associated with clinical manifestations such as jaundice, pruritus, diarrhea, fat-soluble vitamin deficiency, acholic stool and dark urine. Chronic cholestatic conditions may even progress towards liver cirrhosis. In general, cholestasis either results from a dysfunction in bile formation and secretion of the hepatocyte, for example due to septic conditions, or from obstruction of the biliary tract. The accumulation of bile acids in the liver induces an inflammatory response with activation of intracellular signaling pathways, increased production and release of pro-inflammatory mediators by Kupffer cells and recruitment of circulating leukocytes. This reaction is associated with liver injury, which is characterized by hepatocellular apoptosis, necrosis and microvascular perfusion failure. In the small intestine in turn, decreased concentrations of bile acids and immunoglobulin A favour bacterial translocation and endotoxin absorption further aggravating hepatic inflammation. In this vicious cycle, leukocyte recruitment has emerged as a key feature. Leukocyte recruitment is a multi-step process, which is characterized by the coordinated sequence of ligand-receptor interactions between leukocytes and the microvascular endothelium. Besides, other circulating cell types, such as platelets, are considered to be involved in microvascular leukocyte recruitment. Activation of different cell populations is thereby tightly regulated by complex intracellular signaling pathways, such as the Rho-kinase pathway. However, the detailed mechanisms behind intrahepatic leukocyte accumulation during sepsis-associated or obstructive cholestasis and their impact on hepatocellular function and damage remain elusive. A more sophisticated understanding and capability to control these mechanisms may help to improve the therapy of patients suffering from cholestatic syndromes. Therefore, the purpose of this thesis was to analyze the process of leukocyte recruitment and its regulation in the pathophysiology of cholestatic liver injury.

4. BACKGROUND

4.1. Physiology of bile formation

Bile secretion plays an important role in liver physiology, because it serves as a central excretory route for many different metabolites and agents, including bilirubin, bile acids, cholesterol, phospholipids and drugs. Besides, secretion of bile acids is necessary for the digestion and absorption of lipids from the intestinal lumen, which is dependent on a tightly regulated interaction between the liver and the gastrointestinal tract. Accordingly, bile formation is a complex process that involves a variety of specific uptake and export transport systems within the hepatocyte (Zollner and Trauner, 2006) (*Figure 1*). For a better understanding of the pathogenesis of cholestasis, it is useful to know these systems, which are briefly outlined in the following.

Bile is formed by the inflow of water along osmotic gradients produced by secretion of bile acids into the hepatic canaliculi (Kubitz and Häussinger, 2007). In fact, bile acids, such as cholic and chenodeoxycholic acid, are the main solutes in bile and are considered to be the major osmotic driving force in the generation of bile flow. These bile acids undergo an enterohepatic circulation, which consists of their continuous hepatocellular secretion, active reabsorption in the terminal ileum and subsequent hepatic reuptake (Zollner and Trauner, 2006). Accordingly, bile acids circulate 6-10 times per day in humans, whereas less than 10% are eliminated in the feces (Meier and Stieger, 2002; Scotti *et al.*, 2007). At the sinusoidal membrane of the hepatocyte, conjugated bile acids are taken up by the sodium-dependent taurocholate cotransporter (NTCP). The principal driving force for this sodium-dependent sinusoidal uptake is the Na-K-ATPase pump, which maintains an inwardly directed sodium gradient. In contrast, unconjugated cholic acid, bromosulphothalein (BSP) and other lipophilic compounds are primarily taken up by the sodium-independent organic anion transport proteins (OATPs) (Trauner *et al.*, 1998). The bile acids are then transported from the sinusoidal membrane to the canalicular membrane of the hepatocyte bound on cytosolic transporter proteins or by vesicular transcytosis (Chand and Sanyal, 2007). In addition, bile acids are newly biosynthesized from cholesterol within the hepatocyte (Kevresan *et al.*, 2006). Finally, bile acids are secreted into the bile via ATP-dependent transport-

ers on the canalicular membrane including the bile salt export pump (BSEP) and multidrug resistance protein-2 (MRP-2).

Besides bile acids, bilirubin is an important component of the bile. It is the end product of the degradation of hemoglobin. In its unconjugated form bilirubin is highly hydrophobic and circulates reversibly bound to albumin in the blood plasma. At the sinusoidal membrane of the hepatocyte, bilirubin dissociates from albumin and is taken up by OATPs (Chand and Sanyal, 2007). Inside the hepatocyte, bilirubin is bound by a group of cytosolic proteins that prevent its efflux from the cell. In a further step, the enzyme uridine diphosphate-glucuronosyl-transferase conjugates bilirubin to glucuronides, which makes it hydrophilic (Jansen *et al.*, 1992). Subsequently, it can be excreted ATP-dependently into the bile by MRP-2 at the canalicular membrane of the hepatocyte (Tanaka *et al.*, 2002).

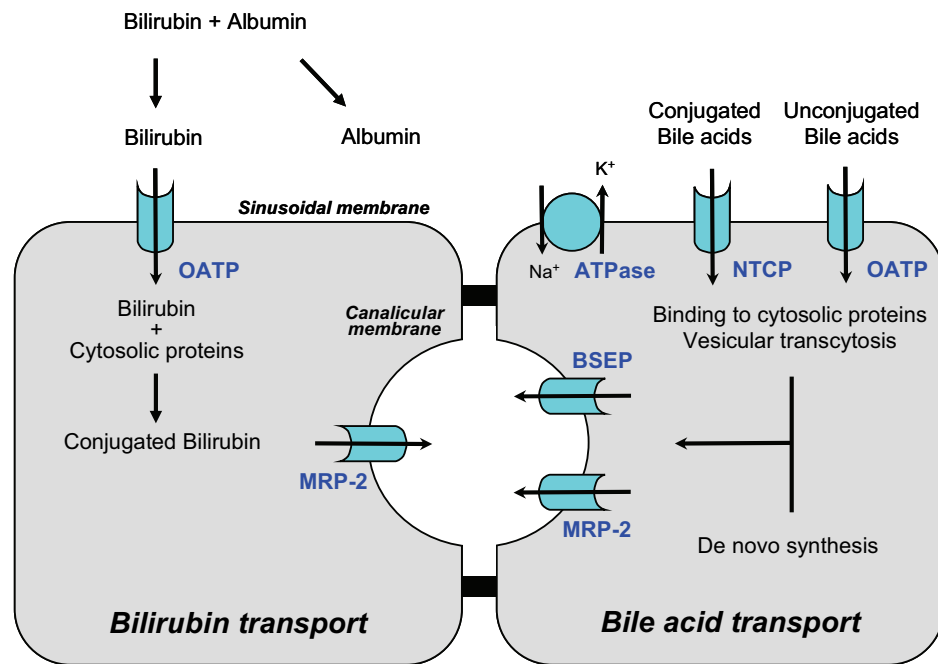


Figure 1: Important hepatocellular uptake and export systems for transport of bilirubin and bile acids, as described in chapter 4.1.

4.2. Cholestatic liver diseases

4.2.1. The cholestatic syndrome

Cholestasis is a frequent clinical syndrome, which is defined by an impaired bile flow. Its clinical manifestation results from an accumulation of substances in the liver, blood and other tissues that are normally excreted into the bile, and malabsorption of fat and fat-soluble vitamins as a result of inadequate postprandial bile acid concentrations in the upper small intestine (Pérez Fernández *et al.*, 2004). Accordingly, typical symptoms of cholestasis are jaundice, pruritus, diarrhea, fat-soluble vitamin deficiency, acholic stool and dark urine.

The etiology of cholestasis is multifactorial. However, from a pathophysiological point of view, the cholestatic syndrome can be either assigned to diseases, which result in a dysfunction in bile formation and secretion of the hepatocyte. These diseases include for example hereditary transporter defects, drug intoxication, hepatitis and sepsis (Pérez Fernández *et al.*, 2004; Zollner and Trauner, 2006). On the other hand, cholestasis may be induced by obstruction or destruction of the biliary tract, for instance due to gallstone impaction, sclerosing cholangitis, primary biliary cirrhosis or compression by tumors or enlarged lymph nodes. In both cases, cholestasis is sooner or later associated with hepatic inflammation, resulting in liver injury.

4.2.2. Sepsis-associated cholestasis

Cholestasis and hepatic dysfunction is a frequent complication in patients with bacterial infections, particularly in gram-negative sepsis (Moseley, 1999; Chand and Sanyal, 2007). For instance, the postoperative incidence of jaundice has been reported to be higher than 60% in cases with septic intraabdominal complications (te Boekhorst *et al.*, 1988). Although the primary site of infection is most often intraabdominal, there are also other types of infection that can be associated with this complication. These include for example urinary tract infection, pneumonia, meningitis or endocarditis (Vermillion *et al.*, 1969; Moseley, 1999; Chand and Sanyal, 2007).

In most cases, the primary cause of sepsis-associated cholestasis is not the direct invasion of the liver by bacteria, but the release of the bacterial endotoxin

lipopolysaccharide (LPS) and LPS-induced pro-inflammatory cytokines (Nolan, 1989; Trauner *et al.*, 1999). This finding is supported by studies demonstrating a reduction in bile formation of isolated perfused livers following LPS application (Trauner *et al.*, 1997; Lund *et al.*, 1999). LPS is the dominant component of the outer membrane of most clinically relevant gram-negative bacteria found in human infections (Opal and Cohen, 1999). It binds to the cell surface receptor CD14 and activates Toll-like receptor-4, activating complex signaling cascades, which regulate the gene expression of several pro-inflammatory mediators (Chow *et al.*, 1999; Pålsson-McDermott and O'Neill, 2004).

In the liver, the primary target of LPS is the Kupffer cell. Kupffer cells make up 80-90% of the fixed tissue macrophages of the reticuloendothelial system and represent terminally differentiated macrophages. Upon activation by LPS, they secrete a variety of pro-inflammatory cytokines including tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β and IL-6 as well as the CXC chemokines macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC) (Hirata *et al.*, 2001; Jirillo *et al.*, 2002; Li *et al.*, 2004a; Tukov *et al.*, 2007). During the last years, it has become evident that LPS and some of these cytokines decrease the sinusoidal and canalicular transport of bile acids and organic anions (Whiting *et al.*, 1995; Moseley, 1999). In fact, several studies showed an endotoxin-induced inhibition of the sinusoidal membrane Na-K-ATPase activity (Green *et al.*, 1996; Moseley *et al.*, 1996; Utili *et al.*, 1997). Moreover, it has been demonstrated that TNF- α and other pro-inflammatory cytokines downregulate the expression of transport proteins regulating bile formation, such as NTCP, BSEP and MRP-2 (Bolder *et al.*, 1997; Kubitz *et al.*, 1999; Hartmann *et al.*, 2002; Geier *et al.*, 2003, 2005). Accordingly, it is widely held that sepsis-associated cholestasis is based on these direct actions on the bile transport system of hepatocytes (Trauner *et al.*, 1998, 1999).

However, besides their effects on hepatocytes, intrahepatic cytokines activate other cell types, including endothelial cells, which in turn express specific adhesion molecules mediating leukocyte recruitment into the liver (Klintman *et al.*, 2004; Li *et al.*, 2004b). Meanwhile it is well known that leukocyte recruitment is a rate-limiting step in the pathogenesis of endotoxemic liver injury (Jaeschke *et al.*,

1991; Holman and Saba, 1998; Klintman *et al.*, 2002). In fact, inhibition of leukocyte-endothelial cell interaction by blockade of the adhesion molecules P-selectin or lymphocyte function antigen-1 (LFA-1) could significantly reduce hepatocellular damage in endotoxemic mice (Klintman *et al.*, 2004; Li *et al.*, 2004b). Thus, it may be speculated that sepsis-associated cholestasis is dependent on the recruitment of leukocytes rather than on direct effects of locally released pro-inflammatory mediators on hepatocyte transport functions. The aim of study I of the present thesis was to further examine this hypothesis.

4.2.3. Obstructive cholestasis

Obstruction of the biliary tract results in an increase of bile acids in the hepatocyte and an absence of bile acids in the small intestine. Both events contribute to cholestatic liver injury.

Increased bile acid concentration in the liver leads to direct destruction of hepatocytes. However, the exact mechanism of cell death is still controversially discussed. On the one hand, it is known that hydrophobic bile acids, such as deoxycholic acid or glycochenodeoxycholic acid, induce apoptosis in primary hepatocytes (Patel *et al.*, 1994; Rodrigues *et al.*, 1998). On the other hand, other bile acids exert an anti-apoptotic effect by inhibiting the development of mitochondrial membrane permeability transition (Rodrigues *et al.*, 1998). Moreover, recent morphological studies of liver tissues after obstructive cholestasis demonstrated that most of the hepatocytes, which had been defined in former studies as apoptotic cells by TUNEL-staining, were necrotic (Gujral *et al.*, 2003a). Therefore, Jaeschke *et al.* (2004) postulated that the predominant mode of cell death during obstructive cholestasis is oncotic necrosis.

In addition, accumulation of bile acids in the liver leads to Kupffer cell activation with increased production and release of pro-inflammatory mediators (Saito and Maher, 2000; Abe *et al.*, 2004). In the small intestine in turn, decreased concentrations of bile acids and immunoglobulin A favour bacterial translocation and endotoxin absorption, further aggravating this hepatic inflammation (Trauner *et al.*, 1999). The final result of these events may be a vicious cycle of endotoxin absorption caused by impaired bile secretion, which enhances endotoxin absorption

and subsequent Kupffer cell activation in the liver (Figure 2). Accordingly, the initial bile acid-induced liver injury may be complicated by this inflammatory process.

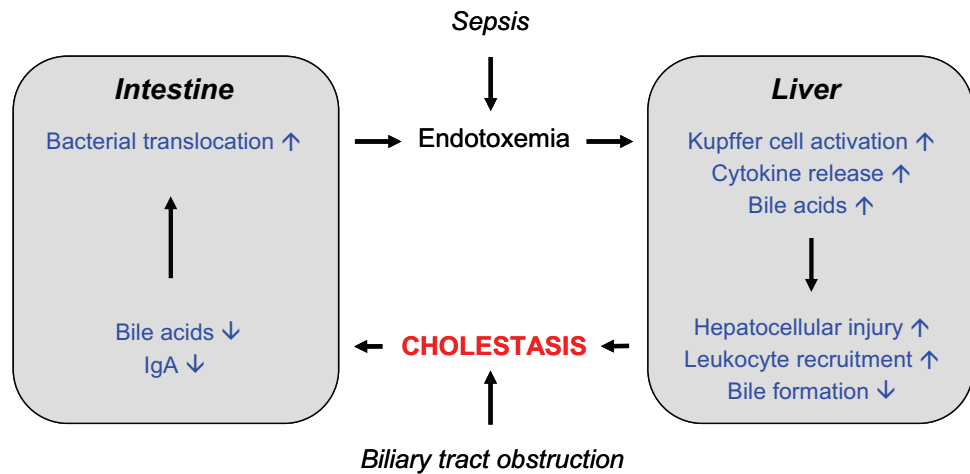


Figure 2: Vicious cycle of cholestasis. Cholestasis is associated with decreased intestinal concentrations of bile acids and immunoglobulin A, which favours translocation of bacteria and endotoxin absorption. In the liver, endotoxemia induces an inflammatory response, resulting in hepatocellular damage and reduced bile formation, further aggravating cholestasis. This vicious cycle can either be activated by obstruction of the biliary tract or septic conditions.

In rodents, the pathophysiological mechanisms of obstructive cholestasis can be analyzed in the well established experimental model of bile duct ligation (BDL) (Georgiev *et al.*, 2008). In the past, this model has been used to study cholestatic liver injury (Miyoshi *et al.*, 1999; Gujral *et al.*, 2003b, 2004a; Wang *et al.*, 2005; Bergheim *et al.*, 2006), fibrinogenesis (Bataller *et al.*, 2003; Isayama *et al.*, 2006) and the impact of obstructive cholestasis on a second hit such as hepatic ischemia (Georgiev *et al.*, 2007) or infection (Abe *et al.*, 2004; Georgiev *et al.*, 2007). Investigations of time-related changes in the liver after BDL in mice have shown that the first hours to days after BDL reflect a phase of acute cholestatic injury, which is followed by a phase of chronic injury, resulting in liver fibrosis (Georgiev *et al.*, 2008). The acute phase is characterized by bile duct epithelial cell proliferation and immune cell infiltration, whereby neutrophils represent the predominant infiltrating cell type (Georgiev *et al.*, 2008). These inflammatory cells accumulate mainly into and around biliary infarcts, i.e. clusters of injured hepatocytes, and

have been shown to aggravate acute liver injury by reactive oxygen species formation (Gujral *et al.*, 2003b; Georgiev *et al.*, 2008). Accordingly, leukocyte recruitment is considered to be a key feature in the pathogenesis of cholestatic liver injury. The aim of study II-IV of the present thesis was therefore to highlight and clarify important signaling pathways and adhesion molecules that may mediate this leukocyte recruitment in obstructive cholestasis.

4.3. Leukocyte recruitment

4.3.1. Leukocyte recruitment cascade

Tissue infiltration of leukocytes from the blood stream across the endothelial cell monolayer of postcapillary venules into the surrounding tissue is a key feature in the pathogenesis of inflammation. Leukocyte recruitment is a multi-step process that involves leukocyte tethering, rolling, adhesion and transmigration (Butcher, 1991; Kubes and Kerfoot, 2001; Chen and Geng, 2006) (*Figure 3*). All of these interdependent steps are characterized by the close interaction between leukocytes and microvascular endothelial cells, and are tightly regulated by specific adhesion molecules expressed on the surfaces of both cell types. These belong to the major adhesion molecule families of selectins and integrins as well as to the immunoglobulin superfamily (Smith, 2008).

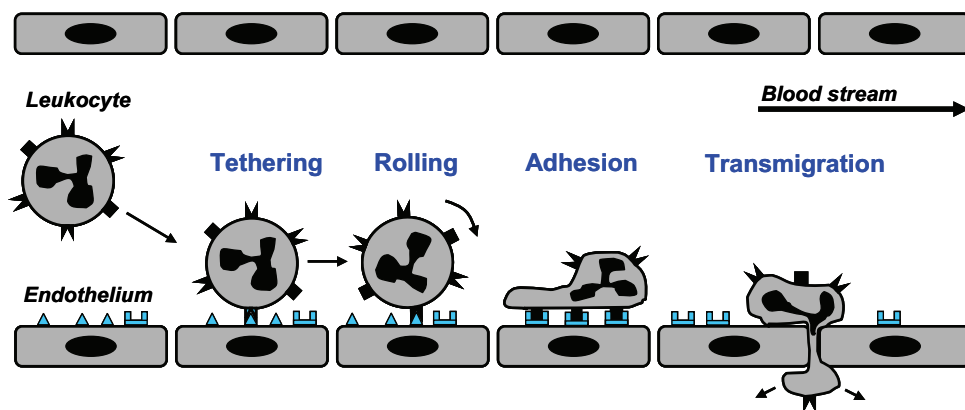


Figure 3: The leukocyte recruitment cascade. Leukocyte recruitment is a multi-step process, which comprises leukocyte tethering/rolling, firm adhesion and transendothelial migration.

4.3.2. Leukocyte rolling

Leukocytes moving at very high velocity in the blood stream have to tether to and roll along the endothelium before they can migrate to sites of inflammation. In fact, the reduction of flow velocity enables leukocytes to detect chemoattractant molecules either exposed on activated endothelial cells or leaking through the endothelium from the surrounding inflamed tissue (Jung *et al.*, 1998). In the past, several studies suggested that this first contact between leukocytes and endothelial cells is a major prerequisite for subsequent firm adhesion and transmigration (von Andrian *et al.*, 1991; Lindbom *et al.*, 1992; Månsson *et al.*, 2000). However, some organs may not require rolling to recruit leukocytes due to their unique microcirculatory architecture. For instance, liver sinusoids are sufficiently narrow so that leukocytes can tether and immediately adhere without apparent rolling (Fox-Robichaud and Kubes, 2000). Similar results have been reported for the lung (Mizgerd *et al.*, 1996). Whether this observation is due to a simple mechanical trapping of activated, more rigid leukocytes or mediated by molecular adhesive events has not been clarified so far.

Leukocyte rolling is mediated by the selectin family of adhesion molecules, which consists of three closely related members, i.e. L-selectin (LAM-1, CD62L), E-selectin (ELAM-1, CD62E) and P-selectin (PADGEM, CD62P) (Tedder *et al.*, 1995). All of the selectins have a unique extracellular region composed of an NH₂-terminal lectin domain, an epidermal growth factor (EGF)-like domain, 2-9 short consensus repeat units homologous to domains found in complement binding proteins, a single transmembrane region and a cytoplasmic tail (Tedder *et al.*, 1995). Each selectin is selectively expressed on different cell types and recognizes and binds to specific carbohydrate determinants on selectin ligands in a calcium-dependent manner (Vestweber and Blanks, 1999).

L-selectin is constitutively expressed on most classes of leukocytes at some stages of differentiation (Tedder *et al.*, 1995). The dominant ligands for L-selectin are P-selectin glycoprotein ligand-1 (PSGL-1), a sialomucin expressed on most leukocytes, and glycoproteins found on high endothelial venules (HEVs) of lymphatic tissue (Smith, 2008). Accordingly, L-selectin is involved in two major processes. It is critical for the interaction of lymphocytes with HEVs of lymph nodes

and the spleen, mediating lymphocyte homing to lymphatic organs (Gallatin *et al.*, 1983; Arbonés *et al.*, 1994). Furthermore, it enables the formation of leukocyte aggregates by adhesion of flowing leukocytes to adherent leukocytes on activated endothelial cells via its interaction with PSGL-1, thus amplifying and exaggerating inflammatory processes (Chen and Geng, 2006).

E-selectin is a marker for endothelial cell activation. It is only expressed on endothelial cells following stimulation with pro-inflammatory mediators such as TNF- α , IL-1 or LPS with maximal levels of cell surface expression after 4-6 hours (Bevilacqua *et al.*, 1987, 1989). Subsequently, its expression rapidly decreases to basal levels after another 12-16 hours. E-selectin recognizes several glycoproteins decorated with sialyl Lewis X (sLeX)-related carbohydrates, including PSGL-1 (Smith, 2008), and mediates rolling of different immune cells, including neutrophils and T lymphocytes (Abbassi *et al.*, 1993; Olofsson *et al.*, 1994; Diacovo *et al.*, 1996). However, L- or P-selectin must be present to guarantee efficient capture of free-flowing leukocytes before they can then roll on E-selectin (Kunkel and Ley, 1996). Moreover, E-selectin mediates leukocyte rolling at a significantly lower velocity than either L- or P-selectin. Thus, it was suggested that this slow E-selectin-mediated leukocyte rolling may serve to target an inflammatory response by increasing the transit time of leukocytes in an inflamed tissue and, thus, the probability of leukocyte activation by locally released chemoattractants (Kunkel and Ley, 1996).

P-selectin is stored in Weibel-Palade bodies of endothelial cells and in α -granules of platelets (Hsu-Lin *et al.*, 1984; McEver *et al.*, 1989). Upon thrombogenic and inflammatory challenges, P-selectin is rapidly expressed by exocytosis (Stenberg *et al.*, 1985; Takano *et al.*, 2002). According to L- and E-selectin, it binds to PSGL-1 to mediate leukocyte rolling on stimulated endothelial cells (Smith, 2008). During the last years, numerous studies could demonstrate that P-selectin-mediated leukocyte rolling plays a central role for leukocyte recruitment in various models of inflammation, including LPS-induced sepsis (Klintman *et al.*, 2004; Mangell *et al.*, 2007) and dextran sodium sulfate (DSS)-colitis (Vowinkel *et al.*, 2007). Besides, P-selectin contributes to hemostasis and thrombosis by mediating the heterotypic aggregation of platelets on stimulated endothelial cells and

on adherent leukocytes, the homotypic aggregation of activated platelets and the transfer of microparticles containing both tissue factor and PSGL-1 to thrombi (Chen and Geng, 2006). Finally, it should be mentioned that P-selectin does not only bind leukocytes and platelets, but also several human cancer cell types, indicating that P-selectin may also play an important role in the growth and metastasis of tumors (Aruffo *et al.*, 1992; Goetz *et al.*, 1996; Pottratz *et al.*, 1996).

Taken together, the three members of the selectin family of adhesion molecules have been shown to play a key role in inflammation. They mediate the first contact of free flowing leukocytes to the endothelium and, thus, set the stage for subsequent firm adhesion and transendothelial migration of leukocytes into inflamed tissue.

4.3.3. Firm adhesion of leukocytes

In inflamed tissue, endothelial cells are stimulated by pro-inflammatory mediators to synthesize chemokines that are presented on their luminal surface. Moreover, they transport chemokines, such as IL-8, from their abluminal to the luminal side (Middleton *et al.*, 1997). When leukocytes then roll along the endothelium, they are activated by the interaction of endothelial selectins with leukocytic PSGL-1 on the one hand and the interaction of chemokine receptors on the leukocyte surface with the secreted chemokines of the endothelium on the other hand (Smith, 2008). This results in the activation and upregulation of a group of leukocytic adhesion molecules, the integrins, which mediate firm leukocyte adhesion.

Integrins are heterodimeric transmembrane glycoproteins, which are composed of a α -subunit noncovalently linked to a β -subunit (Carlos and Harlan, 1994; Smith, 2008). Each subunit is characterized by a large extracellular domain, a single transmembrane domain and a short cytoplasmatic domain. In leukocytes, integrins are existent in different states of activation. Inactive integrins are folded with their extracellular domain near the cell membrane, whereas inside-out signaling results in conformational changes that extend the extracellular domain with its binding site to a distance above the membrane about 4- to 5-fold of that of the inactive state (Wegener and Campbell, 2008). Subsequently, ligand binding and low venular shear forces pulling on the bonds result in the adhesion of leukocytes

to endothelial cells. Meanwhile, 18 α -subunits and 8 β -subunits combining to 24 integrins could be identified in vertebrates (Smith, 2008). Four of the β -subunits, i.e. β_1 , β_2 , β_3 and β_7 , are expressed on leukocytes, where the β_2 -integrins are the most important integrins for leukocyte-endothelial cell interaction. They are composed of the common β_2 -subunit (CD18), which is linked to one of four α -subunits (CD11a-d) (Carlos and Harlan, 1994; Springer, 1994). Accordingly, the β_2 -integrin subfamily includes CD11a/CD18 (LFA-1), CD11b/CD18 (macrophage antigen (MAC)-1), CD11c/CD18 (P150,95) and CD11d/CD18 ($\alpha_d\beta_2$).

β_2 -integrins interact with members of the immunoglobulin superfamily expressed on the endothelial cell surface. This group of transmembrane glycoproteins can be subdivided into the family of intercellular adhesions molecules (ICAM-1 to ICAM-5), junctional adhesion molecules (JAMs) and vascular cell adhesion molecule-1 (VCAM-1) (Smith, 2008). ICAM-1 is constitutively expressed on venular endothelium and upregulated after stimulation with pro-inflammatory cytokines, such as TNF- α (Kim *et al.*, 2008). It is one of the most important endothelial molecules mediating leukocyte adhesion under various inflammatory conditions (Basit *et al.*, 2006; Ramudo *et al.*, 2007; Martinesi *et al.*, 2008). Interestingly, it has recently been shown that ICAM-1 may also play a role in leukocyte recruitment and liver injury during obstructive cholestasis (Gujral *et al.*, 2004b). ICAM-1 binds to the β_2 -integrin LFA-1 expressed on leukocytes. Although LFA-1 has been reported to support leukocyte accumulation in several models of liver inflammation, including alcoholic liver disease (Ohki *et al.*, 1998), viral hepatitis (Matsumoto *et al.*, 2002), endotoxemia (Li *et al.*, 2004b), and graft-versus-host disease (Kimura *et al.*, 1996), the specific role of LFA-1 in obstructive cholestasis-induced liver injury has not been analyzed so far.

4.3.4. Transendothelial migration of leukocytes

After leukocytes have been captured from the blood stream to the vessel wall, they crawl along the luminal surface of the endothelium to reach a point for transendothelial migration (diapedesis) into the surrounding tissue. While leukocyte extravasation has classically been considered to occur at contacts between adjacent endothelial cells (Hurley and Xeros, 1961; Marchesi, 1961), it is now obvious

that next to this so-called paracellular pathway leukocytes can also directly move through the endothelial cell body, i.e. transcellular migration (Engelhardt and Wolburg, 2004; Hordijk, 2006; Vestweber, 2007). However, the factors that determine the choice of leukocytes for one or the other route remain to be further elucidated. This may depend on the type of leukocyte (Yang *et al.*, 2005; Nieminen *et al.*, 2006), the source of the endothelium (microvascular vs. macrovascular) (Millán *et al.*, 2006) or the state of activation of the endothelium and the leukocyte (Cinamon *et al.*, 2004; Yang *et al.*, 2005; Nieminen *et al.*, 2006). Moreover, it has to be clarified whether both pathways are of equal physiological relevance. At present, most studies report that the contribution of the transcellular pathway is only 10-30% to the total transmigration events. In addition, several endothelial membrane proteins that participate in leukocyte transmigration have been found to be mainly localized at endothelial junctions (Vestweber, 2007). These findings indicate that the paracellular pathway may be of greater relevance under physiological conditions.

Within the last few years, many endothelial membrane proteins have been shown to participate in leukocyte transendothelial migration. These include platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31), E-selectin, JAMs, ICAM-1, ICAM-2, VCAM-1 and CD99 (Hordijk, 2006; Vestweber, 2007). Interestingly, *in vitro* studies blocking the function of some of these molecules could identify at least three consecutive steps of the diapedesis process. Blocking the first immunoglobulin domain of PECAM-1 arrests leukocytes on the apical surface of endothelial cells (Liao *et al.*, 1995, 1997). In contrast, blocking of CD99 results in arresting monocytes between endothelial cells (Schenkel *et al.*, 2002). Finally, the lack of PECAM-1 as well as antibodies directed against PECAM-1 cause an accumulation of leukocytes between the basal side of endothelial cells and the basement membrane (Liao *et al.*, 1995; Wakelin *et al.*, 1996; Dangerfield *et al.*, 2002). Thus, similar to the capture of free flowing leukocytes to endothelial cells, leukocyte transmigration through the endothelium has to be considered as a multi-step process, which is tightly regulated by the interaction of leukocytes with a variety of endothelial adhesion molecules.

4.3.5. Role of platelets for leukocyte recruitment

Platelets are small anuclear cell fragments that are derived from megakaryocytes in the bone marrow (Italiano and Shivdasani, 2003). Under physiological conditions, platelets circulate for approximately 10 days in the blood stream before they are cleared by macrophages in the spleen and the liver. While in the past, platelets have been considered predominantly to be essential for hemostasis and thrombosis, more recent data also suggest their important role in inflammation and tissue injury (von Hundelshausen and Weber, 2007). In this context, some recent studies reported that platelets may be involved in the process of leukocyte recruitment (Salter *et al.*, 2001; Singbartl *et al.*, 2001). Indeed, platelet aggregates accumulate in inflammatory lesions concomitantly with leukocytes (Schneider, 1974; Jeffery *et al.*, 1989). Accordingly, depletion of platelets has been shown to decrease leukocyte accumulation in models of allergic inflammation (Pitchford *et al.*, 2003, 2004, 2005), hydrochloric acid-induced lung damage (Zarbock *et al.*, 2006) or ischemia-reperfusion injury (Salter *et al.*, 2001). These observations may be due to the fact that platelets express a multitude of adhesion molecules on their surface allowing them to interact with the endothelium on the one hand and leukocytes on the other hand. These receptors include PSGL-1, P-selectin, GPIIb/IIIa, ICAM-2 and JAM-A (von Hundelshausen and Weber, 2007).

PSGL-1 allows platelets to roll on endothelial P-selectin (Frenette *et al.*, 1995, 1998), whereas the integrin GPIIb/IIIa mediates arrest of activated platelets to the adhesion molecules ICAM-1 and $\alpha_v\beta_3$ on the endothelium by forming a bridge to adhesive proteins, such as fibrinogen, von Willebrand factor or fibronectin (von Hundelshausen and Weber, 2007). Adherent platelets on endothelial cells in turn may serve as an adhesive P-selectin substrate and directly capture circulating leukocytes from the blood stream. Alternatively, platelets and leukocytes can also directly interact via P-selectin/PSGL-1-binding in the blood stream, resulting in the formation of platelet-leukocyte aggregates. These aggregates may subsequently be trapped mechanically in narrow segments of the microcirculation, such as in lung capillaries or liver sinusoids. In addition, P-selectin/PSGL-1-mediated adhesion between platelets and leukocytes results in reciprocal cell activation. In platelets, this activation leads to increased secretion of matrix metalloproteinase-2

(MMP-2), which further enhances platelet-leukocyte aggregation (Abou-Saleh *et al.*, 2005). The activated leukocytes have been shown to upregulate the surface expression of CD11b, which may prime them for firm adhesion to the endothelium (Pitchford *et al.*, 2003).

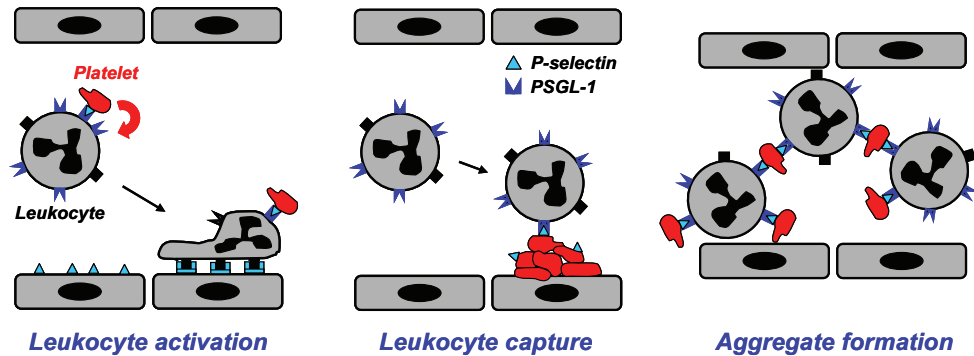


Figure 4: Mechanisms of platelet-mediated leukocyte recruitment. Leukocytes may be activated by P-selectin/PSGL-1-mediated adhesion between platelets and leukocytes or by platelet secretion of pro-inflammatory cytokines, resulting in leukocyte firm adhesion to the endothelium. Moreover, adherent platelets on endothelial cells may serve as an adhesive P-selectin substrate and directly capture circulating leukocytes from the blood stream. Finally, platelets and leukocytes may directly interact via P-selectin/PSGL-1-binding in the blood stream, resulting in the formation of platelet-leukocyte aggregates, which are then trapped in narrow blood vessels.

Besides these specific receptor-ligand interactions between platelets and leukocytes, platelets may contribute to leukocyte recruitment by secretion of several pro-inflammatory cytokines, such as platelet factor-4 (PF-4) (von Hundelshausen and Weber, 2007). Although PF-4 has been found in other cell types, it appears to be most relevant in platelets, because of the most substantial expression levels achieved. In the presence of appropriate costimuli, such as TNF- α , PF-4 induces firm leukocyte adhesion to the endothelium (Petersen *et al.*, 1996; Kasper *et al.*, 2004).

Taken together, platelets are ideally suited to react with sites of activated endothelium via multiple adhesion molecules expressed on their surface. However, the same adhesion molecules also enable platelets to support leukocyte recruitment into inflamed tissue. Moreover, platelets may activate leukocytes by secretion of several pro-inflammatory mediators. Nonetheless, the role of platelets in leukocyte recruitment and cholestatic liver injury has not been determined so far and, thus, was analyzed in study II of the present thesis.

4.4. Rho-kinase signaling pathway

During the last decade, advances in molecular biology have elucidated the substantial role of intracellular signaling pathways mediated by small GTP-binding proteins (Fukata *et al.*, 2001; Takai *et al.*, 2001; Shimokawa and Takeshita, 2005). Important members of this group are the Rho GTPase family proteins, such as Rho, Rac1 and Cdc42 (Hall, 2005). Like all regulatory GTPases, these proteins exist in an inactive GDP-bound conformation and an active GTP-bound conformation (Hall, 2005). They control a variety of fundamental cellular processes, including cell proliferation, differentiation, adhesion, migration and apoptosis (Narumiya *et al.*, 1997; Etienne-Manneville and Hall, 2002; Shi and Wei, 2007). One of the best characterized effectors of Rho are the Rho-kinases (ROCKs), of which two members, i.e. ROCK-I and ROCK-II, could be identified (Leung *et al.*, 1995; Ishizaki *et al.*, 1996; Matsui *et al.*, 1996). They are ubiquitously expressed across human, rat and mouse tissues, where ROCK-I is more pronounced in liver, testes and kidney, and ROCK-II is more prominent in brain and striated muscle (Ishizaki *et al.*, 1996; Leung *et al.*, 1996; Nakagawa *et al.*, 1996). Both ROCKs consist of an N-terminal catalytic kinase domain, followed by a central coiled-coil domain including a Rho-binding domain and a C-terminal pleckstrin-homology domain with an internal cysteine-rich domain (Shi and Wei, 2007). ROCK inhibitors, such as fasudil or Y27632, bind to the kinase domain and inhibit both ROCKs with similar potency (Ishizaki *et al.*, 2000; Breitenlechner *et al.*, 2003). In doing so, they have been shown to be effective against myocardial reperfusion injury (Bao *et al.*, 2004), cerebral ischemia (Sato *et al.*, 2001) and pulmonary hypertension (Abe *et al.*, 2004).

In the past, the role of the Rho/ROCK pathway has been primarily investigated in cardiovascular disease (Shimokawa and Takeshita, 2005; Shimokawa and Rashid, 2007). This is mainly due to the fact that this intracellular signaling pathway is substantially involved in the effects of various vasoactive substances, including angiotensin II, 5-hydroxytryptamine (5-HT), thrombin or platelet-derived growth factor (PDGF). However, meanwhile it has become increasingly evident that the Rho/ROCK pathway is also involved in inflammatory processes, such as chemokine expression, leukocyte-endothelial cell interaction and LPS-induced

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platelet capture to the endothelium (Slotta *et al.*, 2006, 2008a, 2008b; Thorlacius *et al.*, 2006). Accordingly, inhibition of Rho-kinase has been shown to be protective against liver injury in models of ischemia-reperfusion (Shiotani *et al.*, 2004) and sepsis (Thorlacius *et al.*, 2006; Slotta *et al.*, 2008b) as well as carbon tetrachloride-induced hepatic fibrosis (Murata *et al.*, 2003) and cell death (Ikeda *et al.*, 2007). Recently, a study of Zhou *et al.* (2006) reported that Rho and ROCK is upregulated in BDL rats, indicating that this signaling pathway may also play a crucial role in hepatic inflammation during obstructive cholestasis. Therefore, the aim of study IV of the present thesis was to analyze the effect of the ROCK inhibitor Y-27632 on hepatic CXC chemokine formation, leukocyte recruitment and hepatocellular damage under cholestatic conditions.

5. AIMS OF THE THESIS

- I. To analyze the role of P-selectin-dependent leukocyte recruitment in sepsis-associated cholestasis.

- II. To analyze the role of platelets and the adhesion molecule P-selectin in liver injury caused by obstructive cholestasis.

- III. To analyze the role of the adhesion molecule LFA-1 in cholestatic liver injury.

- IV. To analyze the role of Rho-kinase on CXC chemokine formation, leukocyte recruitment and hepatocellular damage in the liver during obstructive cholestasis.

6. MATERIALS AND METHODS

6.1. Animals

Adult male C57BL/6 mice with a body weight of 22-27g were used for the studies. The animals were housed one per cage on a 12-12h light-dark cycle and had free access to standard pellet food and tap water throughout the experiments.

All animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, WA, USA) and were approved by the local ethics-committee at Lund University.

6.2. Model of obstructive cholestasis

In order to induce obstructive cholestasis (II-IV), animals underwent BDL. BDL was performed under ketamine/xylazine anesthesia via a midline laparotomy. By means of a surgical stereomicroscope, the common bile duct was prepared and carefully ligated with a 7.0 prolene suture. Subsequently, the laparotomy was closed again by a 5.0 running suture and the animals were allowed to recover from anesthesia and surgery for 12h. Sham-operated animals received phosphate-buffered saline (PBS) i.v. and underwent an identical laparotomy and liver manipulation without BDL.

6.3. Intravital fluorescence microscopy

In studies II-IV, the hepatic microcirculation was examined by intravital fluorescence microscopy 12h after BDL. For this purpose, a transverse subcostal incision was made in anesthetized animals and the ligamentous attachments from the liver to the diaphragm and the abdominal wall were gently released. Subsequently, the mice were positioned on their left side and the left liver lobe was carefully exteriorized onto an adjustable stage for microscopic analysis. An equilibration period of 5min was allowed before starting the microscopical observation.

For intravital fluorescence microscopy, a modified Olympus microscope (BX50WI, Olympus Optical Co. GmbH, Hamburg, Germany) was used, which was equipped with different water immersion lenses (x40 numerical aperture (NA) 0.75; x63 NA 0.9). The microscopic images were recorded by a charge-coupled

device video camera (FK 6990 Cohu, Pieper GmbH, Schwerte, Germany) and transferred to CD-ROM for off-line evaluation. Blood perfusion within individual microvessels was studied after i.v. injection of 0.1 ml 5% fluorescein isothiocyanate (FITC)-labeled dextran 150,000 (contrast enhancement by intravascular staining of plasma; Sigma Chemical Co., St Louis, MO, USA) (Figure 5A). *In vivo* labelling of leukocytes and platelets with 0.1% rhodamine-6G (0.1 ml i.v., Sigma Chemical Co.) enabled quantitative analysis of leukocyte (II-IV) and platelet-flow (II) behaviour in both sinusoids and postsinusoidal venules (Figure 5B). Five postsinusoidal venules with connecting sinusoids were evaluated in each animal. Microcirculatory analysis included determination of sinusoidal perfusion by measuring the number of non-perfused sinusoids given as a percentage of the total number of sinusoids observed. Within sinusoids and postsinusoidal venules, leukocyte and platelet adhesion were measured by counting the number of cells adhering along the endothelium and remaining stationary during an observation period of 20s. Cell adhesion was expressed as number of cells per 10 high-power fields (HPFs) and cells/mm², respectively. In addition, platelet aggregates (that is more than three platelets) in sinusoids and postsinusoidal venules were determined in each animal and were expressed as cells per 10 HPFs and cells/mm², respectively (II).

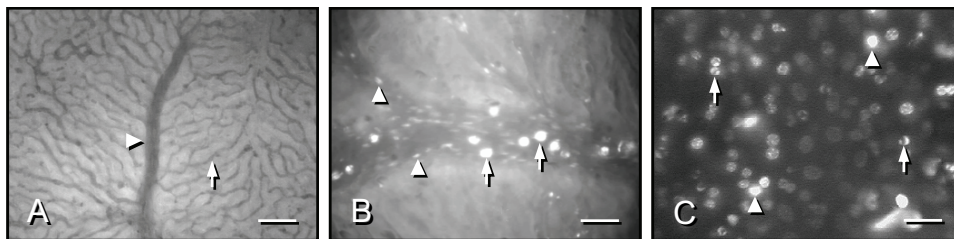


Figure 5: Intravital fluorescence microscopy of a mouse liver. A: Blue light epi-illumination with contrast enhancement by 5% FITC-labeled dextran 150,000 i.v. enables the visualization of the liver microcirculation with sinusoids (arrow) and postsinusoidal venules (arrowhead). B: Flow behaviour of leukocytes (arrows) and platelets (arrowheads) is analyzed in green light epi-illumination by direct *in vivo* staining of these cells by 0.1% rhodamine 6G i.v.. C: Hepatocyte apoptosis is determined morphologically in ultraviolet epi-illumination after topical application of Hoechst 33342 onto the surface of the liver for staining of nuclear chromatin. Apoptotic cells can be identified by their increased nuclear chromatin condensation (arrowheads) and fragmentation (arrows). Scale bars: A = 100µm; B = 25µm; C = 16µm.

6.4. Apoptosis

In study I, hepatocyte apoptosis was determined morphologically by fluorescence microscopy after topical application of the fluorochrome Hoechst 33342 (0.02 ml, 0.2 g/ml, Molecular Probes, Leiden, the Netherlands) onto the surface of a liver tissue sample (*Figure 5C*). Hoechst 33342, which stains desoxyribonucleic acid (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 cells (Rauen *et al.*, 1999). For microscopic analysis, a modified Olympus microscope (see 6.3.) was used. 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.

6.5. Bile flow and bile secretion

In study I, bile flow and bile secretion were measured under septic conditions. For assessment of bile secretion, anesthetized mice were injected i.v. with BSP (0.1 mg/g body wt, Sigma Chemical Co.) as a bolus. BSP is an organic anion secreted 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 30min. Bile flow was expressed as $\mu\text{l}/\text{min} \times \text{g}$ liver weight, with bile density assumed to be 1 g/ml. For analysis of BSP secretion, bile samples were diluted (1:200) in 0.1N NaOH, and the absorbance at 580 nm was recorded and quantified against known standards.

6.6. Blood sampling and assays

After the *in vivo* experiments, animals were killed and blood was drawn from the inferior vena cava for standard spectrophotometric analyses of bilirubin and liver enzymes, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In addition, systemic platelet and leukocyte counts, including poly-

morphonuclear leukocytes and mononuclear leukocytes, were determined with a hematocytometer.

6.7. Histology

For histology, liver samples were fixed in 4% formaldehyde phosphate buffer overnight. Dehydrated, paraffin embedded 6µm sections were stained with hematoxylin and eosin and analyzed under light microscopy. The number of extravascular leukocytes was randomly quantified in 40 HPFs and expressed as number of cells/mm² (I). For histological quantification of hepatocellular injury ('biliary infarcts') (III, IV), five random HPFs were evaluated by use of a semiautomatic image analysis system (NIS-Elements Advanced Research, Nikon Corporation, Kanagawa, Japan) and findings were given as percentage of liver surface, as described previously (Georgiev *et al.*, 2008).

6.8. Myeloperoxidase activity

For assessment of myeloperoxidase (MPO) activity (I, II), liver tissue was collected, weighed, and homogenized in 10ml 0.5% hexadecyltrimethylammonium bromide. Subsequently, 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 H₂O₂ (460 nm, 25°C). Values were expressed as MPO units per g tissue.

6.9. Enzyme-linked immunosorbent assay

The right liver lobe was weighed, washed and homogenized in PBS containing 1% penicillin and streptomycin and fungizone (100U/ml) and then kept cool in cold serumfree Dulbecco's modified Eagle's medium. After centrifugation, supernatants were collected and stored in -20°C until analysis of TNF-α and the CXC chemokines, including MIP-2 and KC, by the use of double ab Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems Europe, Abingdon, Oxon, UK) using recombinant murine KC and MIP-2 as standards. The minimal detectable protein concentrations were less than 0.5 pg/ml.

6.10. Real-time polymerase chain reaction

For real-time polymerase chain reaction (RT-PCR) (I), liver samples were harvested and kept in a ribonucleic acid (RNA) stabilization reagent (RNAlater) at -20°C. Total RNA was isolated using 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 260nm spectrophotometrically. Each cDNA was synthesized by reverse transcription from 10µg of total RNA using StrataScript First-Strand Synthesis System and random hexamer primers (Stratagene, AH diagnostics, Stockholm, Sweden). RT-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 AGT GA-3'; reverse 5'-CAA AGT CTG GGG GAG TGT GT-3'; OATP-1 forward 5'-TGG GGA GAA AAA TGT CCT TG-3'; reverse 5'-ATG GCT GCG AGT GAG AAG AT-3'; BSEP forward 5'-GTT CAG TTC CTC CGT TCA AA-3'; reverse 5'-AAG CTG CAC TGT CTT TTC AC-3'; actin forward 5'-ATG TTT GAG ACC TTC AAC ACC-3'; reverse 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 50µl, containing 25µl of SYBRgreen PCR 2 x master mix, 2µl of 0.15µM each primer, 0.75µl of reference dye and 1µl cDNA as a template adjusted up to 50µl with water. PCR reactions were started with 10min denaturing temperature of 95°C, followed by a total of 40 cycles (95°C for 30s and 55°C for 1min) and 1min 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 to that of actin in the same sample and then relative differences between groups were expressed as percentage of control.

6.11. Flow cytometrie

For analysis of surface expression of LFA-1 on neutrophils (III), blood was collected via cardiac puncture into heparinized syringes at the end of the experi-

ments. Erythrocytes were lysed using red blood cell lysing buffer (Sigma-Aldrich, Stockholm, Sweden) and the leukocytes recovered after centrifugation. Cells were incubated with anti-CD16/CD32 to block Fcγ III/II receptors and reduce non-specific labeling for 5min and stained at 4°C for 30min simultaneously with PE-conjugated anti-Gr-1 (clone RB6-8C5) and FITC-conjugated anti-CD11a/LFA-1 (clone M17/4) monoclonal antibodies (all purchased from BD Biosciences Pharmingen, San Jose, CA, USA). Flow-cytometric analysis was performed according to standard settings on a FACSort flow cytometer (Becton Dickinson, Mountain View, CA, USA). A viable gate was used to exclude dead and fragmented cells.

6.12. Drugs, antibodies and reagents

Animals were anesthetized by i.p. administration of 7.5mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100g body weight. Test substances, antibodies and fluorescent dyes were administered i.v. via retroorbital injection.

To analyze the role of P-selectin in sepsis-associated cholestasis (I), mice were pretreated i.p. 6h before the measurements with a combination of LPS (0.4mg/kg; *Escherichia coli* serotype 0111:B4, Sigma Chemical Co.) and D-galactosamine (GAL, 720 mg/kg, Sigma Chemical Co.). Additionally, mice were pretreated i.v. with PBS (0.2 ml), an anti-P-selectin antibody (RB40.34, 1.5 mg/kg, Pharmingen, San Diego, CA, USA) or an isotype-matched control antibody (R3-34, 1.5 mg/kg, Pharmingen).

To delineate the role of platelets and P-selectin in the pathogenesis of obstructive cholestasis (II), animals were pretreated i.v. 2h prior to BDL with an anti-GP1β antibody (rat IgG, 1mg/kg, Emfret Analytics GmbH & Co., Eibelstadt, Germany), which depletes mice of platelets, an anti-P-selectin antibody (RB40.34, 1.5mg/kg) or an isotype-matched control ab (R3-34, 1.5mg/kg).

To analyze the role of LFA-1 in leukocyte recruitment during obstructive cholestasis (III), mice were pretreated 15min prior to BDL with an anti-LFA-1 antibody (M17/4.4.11.9, rat IgG, 1.5mg/kg, Novartis Pharma AG, Preclinical Research, Basel, Switzerland) or with an isotype-matched control antibody (R3-34, 1.5mg/kg).

On leukocyte recruitment in cholestatic liver injury

To delineate the role of Rho-kinase in hepatic inflammation associated with obstructive cholestasis (IV), mice were pretreated 15min before BDL with 1mg/kg or 10mg/kg of the Rho-kinase inhibitor Y-27632 (Sigma Chemical Co.).

6.13. Statistical analysis

All data are presented as mean values \pm SEM. Statistical evaluations were performed using Kruskal-Wallis one-way ANOVA on ranks followed by the appropriate post hoc comparison test (SigmaStat; Jandel Corporation, San Rafael, CA, USA). Statistical significance was accepted for a value of $P < 0.05$.

7. RESULTS AND DISCUSSION

7.1. Leukocyte recruitment in sepsis-associated cholestasis

During sepsis, circulating LPS leads to an activation of hepatic Kupffer cells, which release a variety of pro-inflammatory mediators, including TNF- α , IL-6 and CXC chemokines (Hirata *et al.*, 2001; Jirillo *et al.*, 2002; Li *et al.*, 2004a; Tukov *et al.*, 2007). These mediators have been reported to decrease the expression of hepatocyte transport proteins, which are important for the formation of bile, such as BSEP or MRP-2 (Geier *et al.*, 2003, 2005; Hartmann *et al.*, 2002). Accordingly, increased levels of circulating LPS and intrahepatic pro-inflammatory cytokines have been suggested to be the main reason for sepsis-associated cholestasis. On the other hand, convincing experiments have demonstrated that leukocyte recruitment is a rate-limiting step in endotoxemic liver injury (Jaeschke *et al.*, 1991; Holman and Saba, 1998; Klintman *et al.*, 2002), whereby P-selectin supports leukocyte rolling (Klintman *et al.*, 2004) and LFA-1 mediates leukocyte firm adhesion in postsinusoidal venules (Li *et al.*, 2004b). Moreover, transendothelial migration and tissue infiltration of leukocytes in LPS-treated mice is critically dependent on the release of the CXC chemokines MIP-2 and KC (Li *et al.*, 2004a). However, the potential role of leukocyte recruitment for the development of sepsis-associated cholestasis has not been clarified so far, and, thus, was analyzed in study I of the present thesis.

Challenge of mice with LPS/GAL caused severe liver injury, as indicated by increased serum levels of ALT and AST, enhanced numbers of apoptotic cells as well as massive panlobular hemorrhage and necrosis in liver tissue. This was associated by an increased production of TNF- α , MIP-2 and KC and a significant reduction in bile flow and BSP secretion. In addition, measurement of hepatic MPO activity revealed that LPS/GAL treatment provoked the accumulation of leukocytes in the liver. Interestingly, immunoneutralization of P-selectin did not only effectively inhibit this leukocyte recruitment, but also markedly decreased the cholestatic effect of endotoxin. In fact, bile flow and BSP secretion were restored in endotoxemic animals pretreated with the anti-P-selectin antibody, indicating that P-selectin-dependent leukocyte recruitment is crucially involved in the pathogenesis of sepsis-provoked cholestasis. In order to clarify the underlying mecha-

nisms, gene expression of important hepatocellular bile transport proteins was analyzed by RT-PCR. According to previous studies (Hartmann *et al.*, 2002), LPS/GAL challenge markedly reduced gene expression of OATP-1, BSEP and MRP-2 in the liver. However, inhibition of P-selectin abolished the endotoxin-induced transcriptional suppression of these hepatocyte transporters. This demonstrates that P-selectin-dependent leukocyte recruitment directly affects the gene expression of hepatocyte transporters. In this context, it is important to note that inhibition of P-selectin had no effect on endotoxin-induced increases of TNF- α , MIP-2 and KC, which have been reported to be potent inhibitors of hepatic transport proteins *in vitro* (Geier *et al.*, 2003, 2005; Hartmann *et al.*, 2002). Accordingly, it may be concluded that under *in vivo* conditions the accumulation of leukocytes in endotoxemic livers is relatively more important for provoking significant cholestasis than the local production of pro-inflammatory mediators. Besides, immunoneutralization of P-selectin also decreased sepsis-induced hepatocellular damage and apoptosis, which may help to explain the protective effect against cholestasis with the assumption that intact hepatocytes are required for bile formation and secretion.

In summary, study I demonstrates that leukocyte recruitment is a key component in the pathophysiology of sepsis-associated cholestasis. In fact, inhibition of leukocyte accumulation in septic livers abolishes the reduction in bile flow, BSP secretion and expression of hepatobiliary transport proteins, despite high intrahepatic levels of pro-inflammatory mediators. Thus, inhibition of P-selectin-mediated leukocyte recruitment may represent a promising therapeutic strategy in order to preserve bile flow and secretion in septic liver injury.

7.2. Platelet-dependent leukocyte accumulation in obstructive cholestasis

Leukocyte recruitment is a rate-limiting step in the pathophysiology of different inflammation-associated conditions in the liver, such as ischemia-reperfusion, endotoxemia or alcoholic liver disease (Adams, 1994; Sawaya *et al.*, 1999; Klintman *et al.*, 2004). Moreover, several studies reported that obstructive cholestasis induces neutrophil infiltration, aggravating cholestatic liver injury (Gujral *et al.*, 2003b, 2004b; Georgiev *et al.*, 2008). However, the adhesive mechanisms behind

leukocyte accumulation in obstructive cholestasis have not been analyzed so far. According to other inflammatory conditions, leukocyte recruitment in cholestatic liver disease may be characterized by the classical leukocyte recruitment cascade, including leukocyte rolling, firm adhesion to the endothelium and subsequent transendothelial migration into the surrounding tissue (Butcher, 1991; Kubes and Kerfoot, 2001; Chen and Geng, 2006). On the other hand, depletion of platelets has been shown to decrease leukocyte accumulation in models focusing on inflammatory processes (von Hundelshausen and Weber, 2007). Thus, it has been suggested that platelets may also exert an important function in microvascular leukocyte recruitment (Salter *et al.*, 2001; Singbartl *et al.*, 2001). Therefore, the aim of study II was to analyze the role of platelets and the adhesion molecule P-selectin in liver injury caused by obstructive cholestasis.

To induce cholestatic liver injury in this study, animals underwent BDL, which is a well established rodent model of obstructive cholestasis (Georgiev *et al.*, 2008). In fact, already 12h after BDL, animals exhibited systemic bilirubin levels, which were threefold increased when compared to sham-operated controls. This was associated with a more than 26-fold increase of liver enzymes, indicating that BDL caused substantial hepatocellular damage. Moreover, determination of MPO levels in the liver demonstrated that BDL provoked extravascular recruitment of leukocytes. Of interest, depletion of platelets decreased MPO levels in BDL mice by 44%. This clearly demonstrates that platelets support hepatic leukocyte accumulation in obstructive cholestasis. A more detailed analysis of the blood cell-endothelium interaction in liver sinusoids and postsinusoidal venules by intravital fluorescence microscopy revealed that systemic depletion of platelets significantly decreased BDL-induced leukocyte adhesion in sinusoids by 48% without affecting leukocyte adhesion in postsinusoidal venules. Thus, the sinusoid seems to be the dominant site of platelet-dependent leukocyte recruitment in the liver.

Furthermore, study II showed for the first time that platelets play a crucial role in the development of cholestatic liver injury. In fact, platelet depletion decreased cholestasis-induced hepatocellular damage by more than 83%. Because depletion of platelets simultaneously decreased leukocyte recruitment and hepatocellular damage, the present findings suggest a mechanistic link between platelet-

mediated leukocyte recruitment on the one hand and BDL-induced liver injury on the other hand. This adds the liver to the lung and the kidney as an organ, in which platelet-mediated leukocyte recruitment plays an important role in distinct disease states (Pitchford *et al.*, 2003, 2004, 2005; Singbartl *et al.*, 2001; Kuligowski *et al.*, 2006).

Study II further demonstrated that administration of anti-P-selectin antibodies decreased BDL-induced leukocyte adhesion in both sinusoids and postsinusoidal venules, suggesting that P-selectin is critically involved in cholestasis-induced leukocyte recruitment in the liver. This result is in contrast to earlier studies, suggesting that selectin-mediated functions may play only a minor role in hepatic leukocyte recruitment (Wong *et al.*, 1997). For instance, Essani *et al.* (1998) reported that inhibition of P-selectin had no effect on sinusoidal accumulation of leukocytes under septic conditions. Nonetheless, more recent studies found that P-selectin is indeed an important adhesion molecule regulating leukocyte recruitment in the liver under various conditions, such as ischemia-reperfusion and endotoxemia (Sawaya *et al.*, 1999; Klintman *et al.*, 2004). However, P-selectin is not expressed on sinusoidal endothelium (Steinhoff *et al.*, 1993; Essani *et al.*, 1998; Massaguer *et al.*, 2002) and intravital microscopic studies have shown that there is no leukocyte rolling in sinusoids (Wong *et al.*, 1997; Klintman *et al.*, 2004). Therefore, the observation of study II that immunoneutralization of P-selectin inhibited sinusoidal leukocyte accumulation has to be considered as an indirect effect. In this context, it is important to note that treatment with the anti-P-selectin antibody reduced sinusoidal accumulation of platelets by 37%, which was similar in magnitude to the 48% reduction in sinusoidal recruitment of leukocytes. This indicates that P-selectin-dependent accumulation of leukocytes in hepatic sinusoids may be mediated by platelet-leukocyte interactions. For instance, leukocytes may be activated by platelet secretion of pro-inflammatory cytokines, resulting in leukocyte firm adhesion to the sinusoidal endothelium. Moreover, adherent platelets on endothelial cells may serve as an adhesive P-selectin substrate and directly capture circulating leukocytes from the blood stream. Alternatively, platelets and leukocytes may directly interact via P-selectin/PSGL-1-binding in the

blood stream, resulting in the formation of platelet-leukocyte aggregates, which are then trapped in the narrow liver sinusoids.

Finally, study II revealed that BDL results in an increased hepatic formation of the CXC chemokines MIP-2 and KC. These chemokines have previously been shown to regulate leukocyte extravasation in septic liver injury (Li *et al.*, 2004a). Interestingly, systemic depletion of platelets significantly decreased CXC chemokine production by more than 63% in cholestatic livers. Similarly, immunoneutralization of P-selectin also abolished the BDL-induced formation of MIP-2 and KC. Taken together, these findings suggest that platelets constitute an early component in the pathophysiology of obstructive cholestasis upstream of MIP-2 and KC production in the liver. The link between platelets and CXC chemokine formation has to be analyzed in future studies. However, it may be speculated that pro-inflammatory mediators secreted from activated platelets and leukocytes stimulate tissue-resident Kupffer cells in the liver to upregulate their chemokine production.

In summary, study II demonstrates that platelets crucially contribute to leukocyte recruitment and liver injury during obstructive cholestasis. Moreover, P-selectin mediates platelet and leukocyte as well as platelet-dependent leukocyte recruitment, suggesting that P-selectin plays a key role in cholestatic liver damage.

7.3. Role of LFA-1 for leukocyte recruitment in obstructive cholestasis

LFA-1 (CD11a/CD18) is an important member of the integrin family of adhesion molecules that mediates firm leukocyte adhesion (Carlos and Harlan, 1994; Springer, 1994). Interestingly, Gujral *et al.* (2004b) recently reported that the LFA-1 receptor molecule ICAM-1 is of importance in the mechanism of neutrophil-induced liver injury in BDL mice. Therefore, the aim of study III was to analyze the role of LFA-1 in cholestatic liver injury.

Using flow cytometry, it could be demonstrated that BDL significantly increased surface expression of CD11a on circulating leukocytes, indicating that LFA-1 may indeed constitute a potential trigger in cholestatic liver injury. In fact, administration of an anti-LFA-1 antibody markedly decreased serum activities of ALT and AST by 46% to 70% and reduced BDL-induced biliary infarcts by 62%.

In line with these findings, intravital fluorescence microscopic analyses revealed that inhibition of LFA-1 function decreased BDL-provoked venular attachment of leukocytes by more than 90% without affecting leukocyte rolling. Thus, LFA-1 represents the dominant adhesion molecule for firm leukocyte adhesion in postsinusoidal venules during obstructive cholestasis.

Moreover, it was found that treatment with the LFA-1-antibody could protect the liver against BDL-induced sinusoidal perfusion failure. This observation may be due to the tissue protection exerted by blocking accumulation of leukocytes in the microcirculation of the liver. For example, extravascular edema formation and endothelial cell swelling associated with tissue injury appear to be major determinants in causing microvascular compression and decreased organ perfusion (Menger *et al.*, 1997). In contrast, inhibition of LFA-1 had no effect on the intrahepatic production of MIP-2 and KC, suggesting that the decrease of leukocyte accumulation in BDL mice is not related to changes in CXC chemokine production after immunoneutralization of LFA-1.

In summary, study III demonstrates the important role of LFA-1 in supporting cholestasis-induced leukocyte recruitment in the liver. Thus, targeting LFA-1 may help to protect against pathologic inflammation and liver injury in cholestatic liver diseases.

7.4. Rho-kinase inhibition in obstructive cholestasis

ROCK-mediated signaling has mainly been described in conjunction with a variety of essential actomyosin-based cellular events, such as cell migration, cytokinesis and contraction (Narumiya *et al.*, 1997). However, during the last years it has become increasingly evident that this signaling pathway is also involved in several pathological processes. Interestingly, two recent studies have reported that ROCK activity is increased during obstructive cholestasis and that ROCK regulates portal pressure in rats with secondary biliary cirrhosis (Zhou *et al.*, 2006; Anegawa *et al.*, 2008). The aim of study IV was therefore to evaluate for the first time the role of ROCK in cholestasis-induced inflammation.

To inhibit the activity of ROCK, mice were treated with the pyridine derivative Y-27632, which according to fasudil inhibits the kinase activity of both ROCK-I

and -II by competing with ATP for binding to their catalytic sites (Ishizaki *et al.*, 2000). During the last years, these ROCK inhibitors have been successfully used in numerous studies to analyze the involvement of ROCK in pathological processes, such as ischemia-reperfusion damage of the liver and heart (Bao *et al.*, 2004; Shiotani *et al.*, 2004), septic liver injury (Thorlacius *et al.*, 2006; Slotta *et al.*, 2008b), cerebral ischemia (Yagita *et al.*, 2007; Yamashita *et al.*, 2007) and pulmonary hypertension (Oka *et al.*, 2007). The present study showed that Y-27632 dose-dependently protects against cholestasis-induced hepatic injury. In fact, administration of 10mg/kg of Y-27632 decreased BDL-provoked increases in the serum levels of ALT and AST by more than 87% as well as the percentage of biliary infarcts by 71%. In contrast, animals treated with only 1mg/kg of the ROCK inhibitor did not markedly differ from PBS-treated control animals.

As shown by intravital fluorescence microscopy of the liver microcirculation, inhibition of ROCK additionally reduced BDL-induced leukocyte rolling and adhesion in postsinusoidal venules as well as leukocyte trapping in liver sinusoids of cholestatic mice. This observation may be due to the fact that treatment with Y-27632 also reduced the release of the CXC chemokines MIP-2 and KC in cholestatic livers, which have been shown to regulate leukocyte trafficking into inflamed liver tissue (Li *et al.*, 2004a).

Moreover, inhibition of the ROCK signaling pathway markedly reduced the microvascular perfusion failure observed in BDL mice. Since leukocytes trapped in sinusoids may interfere with the blood flow in these narrow vessels, it may be suggested that the reduced sinusoidal accumulation of leukocytes may partially explain the restored microvascular perfusion in cholestatic livers. In addition, recent studies have shown that ROCK is not only upregulated during obstructive cholestasis but also controls contraction of microvessels in the liver and accordingly portal pressure in secondary biliary cirrhosis (Zhou *et al.*, 2006; Anegawa *et al.*, 2008). This may be a complementary explanation for the improved sinusoidal perfusion observed in cholestatic mice treated with Y-27632. Anegawa *et al.* (2008) reported that ROCK activation in chronic cholestasis causes defective endothelial nitric oxide function and subsequently increased intrahepatic vascular resistance. Thus, inhibition of the ROCK signaling pathway may improve hepatic

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blood perfusion in two distinctly different ways. Inhibition of ROCK on the one hand attenuates leukocyte-dependent obstruction and on the other hand normalizes intrahepatic vascular resistance, which may be in particular of benefit for patients with cholestatic liver disease.

In summary, study IV demonstrates for the first time that inhibition of ROCK markedly reduces CXC chemokine formation, leukocyte recruitment, perfusion failure and hepatocellular damage in cholestatic mice, indicating that ROCK plays a key role in the pathophysiology of cholestatic liver injury. Thus, targeting the ROCK signaling pathway may represent a novel therapeutic strategy in the management of inflammation and liver injury associated with obstructive cholestasis.

8. MAJOR CONCLUSIONS

1. P-selectin-mediated recruitment of leukocytes, but not the local production of pro-inflammatory mediators, is the primary cause of sepsis-associated cholestasis.
2. P-selectin mediates intrahepatic platelet and leukocyte accumulation during obstructive cholestasis.
3. Platelets crucially contribute to leukocyte recruitment and liver injury during obstructive cholestasis.
4. LFA-1 mediates firm leukocyte adhesion in the liver microcirculation during obstructive cholestasis.
5. Inhibition of Rho-kinase attenuates cholestasis-induced leukocyte recruitment and hepatocellular damage in cholestatic liver injury.

9. SWEDISH SUMMARY

Syntes och sekretion av galla utgör en viktig och integrerad del av magtarm systemet. Gallan är inte bara viktig för upptag av födoämnen utan också för utsöndringen av toxiska ämnen och läkemedel. Dessutom underhåller gallan funktionen och integriteten av ytepitelet i tarmen och hämrat gallflöde kan öka risken för infektioner. Gallflödet kan hindras (kolestas) av sjukdomsprocesser, tex sepsis, som skadar levercellerna och deras utsöndringsförmåga eller processer såsom tumörer som fysiskt blockerar gallflödet i gallgångarna till tarmen, vilket kan leda till ikterus och infektioner i gallvägarna. Eftersom mekanismerna bakom kolestas är delvis okända och tillståndet är associerat med inflammatoriska förändringar i levern var avsikten med den aktuella avhandlingen att studera betydelsen av leukocyter och mekanismerna bakom leukocytrekrytering till levern i samband med olika former av kolestas. I delarbete I studeras betydelsen av lokalt bildade cytokiner (TNF- α) vid sepsis och huruvida dessa substanser direkt orsakade nedsatt sekretions förmåga i levercellerna. Vi fann att blockering av P-selektin inte bara hämmade leukocytrekryteringen till levern utan också återställde intakt gallflöde utan att påverka nivåerna av TNF- α i levern. Detta betyder att proinflammatoriska substanser såsom TNF- α verkar indirekt via cirkulerande leukocyter för att orsaka kolestas vid sepsis. I delarbete II studerades betydelsen av trombocyter i samband med extrahepatisk kolestas vilket uppnåddes genom ligation av gallgångarna. Det visade sig att möss i vilka trombocyterna var borttagna mha en specifik antikropp hade mycket mindre infiltration av leukocyter och uppvisade samtidigt mindre inflammation och skada i levern. Dessa data indikerar att cirkulerande trombocyter spelar en viktig roll för kolestas-inducerad leukocytrekrytering och levercellsskada. I delarbete III undersöktes betydelsen av LFA-1 som är en adhesionsmolekyl på ytan av leukocyter. Vi fann att blockering av LFA-1 reducerade leukocytinfiltrationen och skyddade levern som samtidig cellskada. I delarbete IV undersöktes betydelsen av Rho-kinase som är ett protein som är viktigt för intracellulär signalering i vissa sammanhang. Rho-kinase-medierad signalering blockerades vilket skyddade levern mot kolestas-inducerad inflammation och cellskada. Således har den här avhandlingen lyckats kartlägga nya och viktiga mekanismer orsakande leverskada i samband med störd gallsekretion

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I

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

Matthias W. Laschke,^{1,2} Michael D. Menger,² Yusheng Wang,
Gert Lindell,¹ Bengt Jeppsson,¹ and Henrik Thorlacius¹

¹Department of Surgery, Malmö University Hospital, Lund University, Malmö, Sweden and

²Institute for Clinical and Experimental Surgery, University of Saarland, Homburg/Saar, Germany

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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 bromosulphophthalein. 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 bromosulphophthalein. 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 bromosulphophthalein 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.

apoptosis; bile; chemokines; inflammation; liver

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 intra-abdominal 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 proinflammatory mediators (20).

Address for reprint requests and other correspondence: H. Thorlacius, Dept. of Surgery, Malmö Univ. Hospital, Lund Univ., S-205 02 Malmö, Sweden (e-mail: henrik.thorlacius@med.lu.se).

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

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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 bromsulphthalein (BSP, 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 hemacytometer.

Apoptosis. Hepatocyte apoptosis was determined morphologically by fluorescence microscopy after topical application of the fluorochrome Hoechst 33342 (0.02 ml, 0.2 μ g/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 $\times 63$ water immersion lens (NA 0.9). The images were televised using a charge-coupled device video camera (FK 6990 Cohu, Pieper, 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.

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 H_2O_2 (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- μ m 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 (RNAlater) at -20°C . Total RNA was isolated using 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 μ g of total RNA using StrataScript First-Strand Synthesis System and random hexamers primers (Stratagene, AH 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 AGT GA-3'; reverse 5'-CAA AGT CTG GGG GAG TGT GT-3'; organic anion transporting polypeptide 1 (oatp1) forward 5'-TGG GGA GAA AAA TGT CCT TG-3'; reverse 5'-ATG GCT GCG AGT GAG AAG AT-3'; bsep forward 5'-GTT CAG TTC CTC CGT TCA AA-3'; reverse 5'-AAG CTG CAC TGT CTT TTC AC-3'; β -actin forward 5'-ATG TTT GAG ACC TTC AAC ACC-3'; reverse 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 μ l, containing 25 μ l of SYBRgreen PCR 2 \times master mix, 2 μ l of 0.15 μ M each primer, 0.75 μ l of reference dye, and one 1- μ l cDNA as a template adjusted up to 50 μ l 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 are presented as means \pm 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.

Table 1. Endotoxin-induced hepatitis

| | ALT, μ kat/l | AST, μ kat/l | Apoptosis, % |
|------------------|------------------|------------------|-----------------|
| Control | 0.58 \pm 0.1 | 0.82 \pm 0.2 | 1.1 \pm 0.4 |
| PBS + LPS/Gal | 22.8 \pm 12.3* | 24.9 \pm 8.2* | 14.1 \pm 2.9* |
| IgG + LPS/Gal | 20.2 \pm 8.7* | 17.0 \pm 5.1* | 12.9 \pm 4.9* |
| Anti-P + LPS/Gal | 0.48 \pm 0.1† | 1.8 \pm 0.3† | 2.0 \pm 0.6† |

Data are means \pm 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 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 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

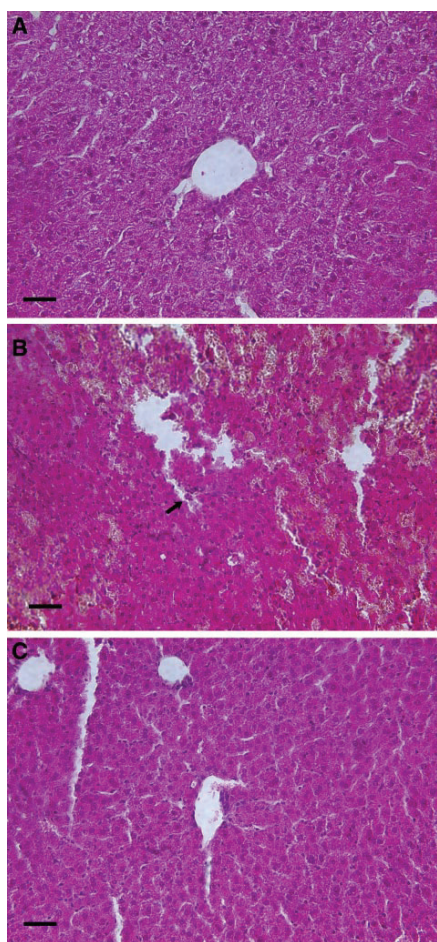


Fig. 1. Representative hematoxylin and eosin cross sections of liver tissue harvested 6 h after treatment with a combination of lipopolysaccharide (LPS, 0.4 mg/kg) and D-galactosamine (Gal, 720 mg/kg) (LPS/Gal). Mice were pretreated with a control antibody (IgG, 1.5 mg/kg, B) or an antibody directed against P-selectin (1.5 mg/kg, C) 15 min before LPS/Gal challenge. Control animals without LPS/Gal challenge received only PBS (A). Arrow indicates area of neutrophil infiltration; scale bar, 100 μ m.

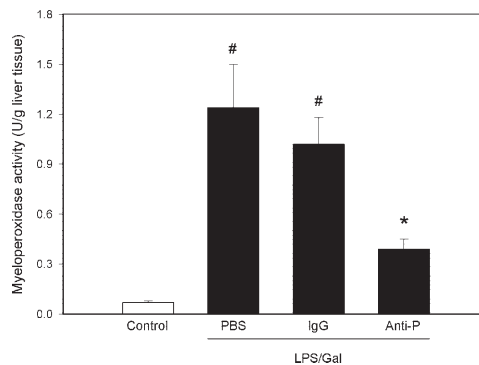


Fig. 2. Myeloperoxidase (MPO) activity in the liver 6 h after treatment with a combination of LPS (0.4 mg/kg) and Gal (720 mg/kg). Mice were pretreated with PBS, a control antibody (IgG, 1.5 mg/kg), or an antibody directed against P-selectin (Anti-P, 1.5 mg/kg) 15 min before LPS/Gal challenge. Control animals without LPS/Gal challenge received only PBS. Data represent means \pm SE; $n = 5$. [#] $P < 0.05$ vs. control and ^{*} $P < 0.05$ vs. IgG + LPS/Gal.

antibody significantly decreased ALT from 20.2 ± 8.7 to 0.5 ± 0.06 μ kat/l and AST from 17.0 ± 5.1 to 1.8 ± 0.3 μ kat/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 $1.1 \pm 0.4\%$ 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, 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 $1,998 \pm 356$ 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,

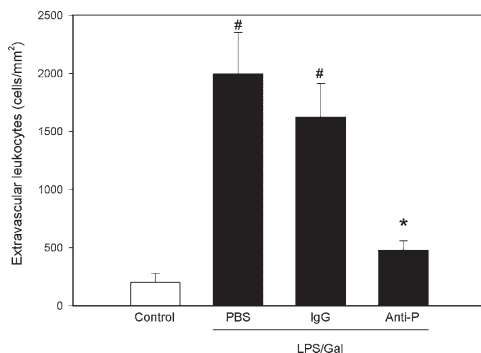


Fig. 3. Extravascular accumulation of leukocytes in the liver 6 h after treatment with a combination of LPS (0.4 mg/kg) and Gal (720 mg/kg). Mice were pretreated with IgG (1.5 mg/kg) or Anti-P (1.5 mg/kg) 15 min before LPS/Gal challenge. Control animals without LPS/Gal challenge received only PBS. Data represent means \pm SE; $n = 5-7$. [#] $P < 0.05$ vs. control and ^{*} $P < 0.05$ 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 $420 \pm 3 \text{ nl} \cdot \text{min}^{-1} \cdot \text{g liver tissue}^{-1}$ and that challenge with LPS decreased bile flow down to $120 \pm 6 \text{ nl} \cdot \text{min}^{-1} \cdot \text{g liver tissue}^{-1}$ (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

Table 2. Systemic leukocyte counts

| | PMNL | MNL | Total |
|------------------|---------------|---------------|---------------|
| Control | 0.4 \pm 0.1 | 2.2 \pm 0.2 | 2.7 \pm 0.3 |
| PBS + LPS/Gal | 0.3 \pm 0.1 | 2.0 \pm 0.2 | 2.3 \pm 0.2 |
| IgG + LPS/Gal | 0.3 \pm 0.1 | 2.0 \pm 0.3 | 2.3 \pm 0.3 |
| Anti-P + LPS/Gal | 0.4 \pm 0.1 | 2.1 \pm 0.4 | 2.5 \pm 0.3 |

Data are means \pm SE ($n = 5$) and represent 10^6 cells per ml. 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 by using a standard hemacytometer. Mice were challenged with LPS (0.4 mg/kg) and Gal (720 mg/kg) and pretreated with PBS, IgG (1.5 mg/kg), or Anti-P (1.5 mg/kg). Animals not exposed to LPS/Gal served as controls. No significant differences were found between the groups.

Table 3. Expression of proinflammatory mediators

| | TNF- α , pg/g | MIP-2, ng/mg | KC, ng/mg |
|------------------|----------------------------|------------------------------|------------------------------|
| Control | 218 \pm 23 | 0.5 \pm 0.08 | 3.0 \pm 1.3 |
| PBS + LPS/Gal | 541 \pm 103 [*] | 15.4 \pm 2.8 [*] | 22.0 \pm 2.5 [*] |
| IgG + LPS/Gal | 567 \pm 98 [*] | 16.8 \pm 4.7 [*] | 21.8 \pm 4.1 [*] |
| Anti-P + LPS/gal | 677 \pm 108 [*] | 36.9 \pm 23.8 [*] | 47.0 \pm 21.8 [*] |

Data are means \pm 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.

was $7.6 \pm 0.3 \text{ mg/ml}$ and that LPS treatment decreased excretion of BSP down to $1.3 \pm 0.5 \text{ mg/ml}$, corresponding to an 83% reduction (Fig. 5, $P < 0.05$ vs. control, $n = 5-7$). Notably, we observed that immunoneutralization of P-selectin restored BSP excretion in endotoxemic mice toward baseline levels, i.e., $5.4 \pm 0.7 \text{ mg/ml}$ (Fig. 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. 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 *bscp* in the liver. We found that challenge with LPS decreased hepatic mRNA levels of *oatp1*, *mrp-2*, and *bscp* by $>70\%$ compared with controls (Fig. 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 *bscp* in mice challenged with LPS (Fig. 6, $n = 5$). In fact, administration of the anti-P-antibody restored gene expression of *oatp1*, *mrp-2*, and *bscp* toward normal levels (70–80% of controls), corresponding to a $>209\%$ increase in mRNA levels of hepatobiliary transport proteins in endotoxemic animals (Fig. 6, $P < 0.05$ vs. IgG + LPS/Gal, $n = 5$).

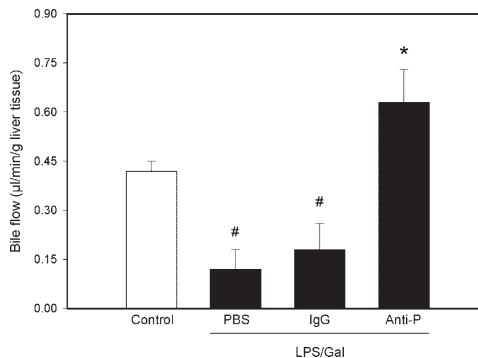


Fig. 4. Bile flow 6 h after treatment with a combination of LPS (0.4 mg/kg) and Gal (720 mg/kg). Mice were pretreated with PBS, IgG (1.5 mg/kg), or Anti-P (1.5 mg/kg) 15 min before LPS/Gal challenge. Control animals without LPS/Gal challenge received only PBS. Data represent means \pm SE; $n = 5-7$. [#] $P < 0.05$ vs. control and ^{*} $P < 0.05$ vs. IgG + LPS/Gal.

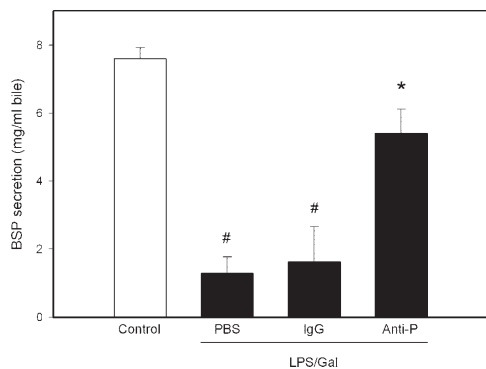


Fig. 5. Bromosulphophthalein (BSP) excretion 6 h after treatment with a combination of LPS (0.4 mg/kg) and Gal (720 mg/kg). Mice were pretreated with PBS, IgG (1.5 mg/kg), or Anti-P (1.5 mg/kg) 15 min before LPS/Gal challenge. Control animals without LPS/Gal challenge received only PBS. Data represent means \pm SE; $n = 5-7$. # $P < 0.05$ vs. control and * $P < 0.05$ vs. IgG + LPS/Gal.

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

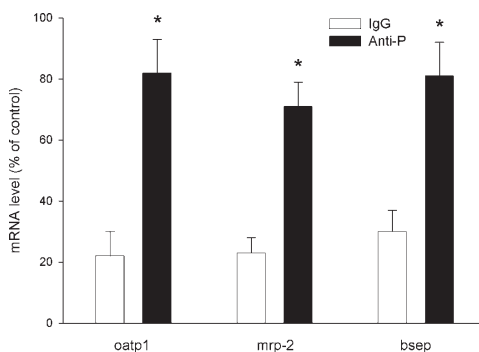


Fig. 6. Gene expression of organic anion transporting polypeptide 1 (oatp1), bile salt export transporter (bsep), and multidrug resistance-associated protein 2 (mrp-2) 6 h after treatment with a combination of LPS (0.4 mg/kg) and Gal (720 mg/kg). Mice were pretreated with PBS, IgG (1.5 mg/kg), or Anti-P (1.5 mg/kg) 15 min before LPS/Gal challenge. Control animals without LPS/Gal challenge received only PBS. Levels of oatp1, bsep, and mrp-2 mRNA were quantified by real-time polymerase chain reaction. The expression of mRNA levels for each transport protein was normalized as a ratio using β -actin mRNA as a housekeeping gene. Data represent means \pm SE; $n = 5$. # $P < 0.05$ vs. control and * $P < 0.05$ vs. IgG + LPS/Gal.

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 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 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.

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RESEARCH PAPER

Platelet-dependent accumulation of leukocytes in sinusoids mediates hepatocellular damage in bile duct ligation-induced cholestasis

MW Laschke^{1,2,3}, S Dold^{1,2,3}, MD Menger², B Jeppsson¹ and H Thorlacius¹

¹Department of Surgery, Malmö University Hospital, Lund University, Malmö, Sweden and ²Institute for Clinical and Experimental Surgery, University of Saarland, Homburg/Saar, Germany

Background: Although it is well known that extrahepatic cholestasis induces liver damage, the mechanisms are still not completely understood. The aim of the present study was to evaluate the role of platelets and P-selectin in cholestasis-induced liver injury.

Experimental approach: C57BL/6 mice underwent bile duct ligation (BDL) and pretreatment with an anti-GP1b α antibody, which depletes platelets, an anti-P-selectin antibody or a control antibody. Hepatic platelet and leukocyte recruitment as well as microvascular perfusion were determined by intravital fluorescence microscopy.

Key results: BDL caused significant liver damage and sinusoidal perfusion failure. BDL further induced hepatic platelet accumulation with widespread intravascular platelet aggregates, increased platelet adhesion in postsinusoidal venules and massive platelet accumulation in liver sinusoids. Administration of the anti-GP1b α antibody reduced systemic platelet count by 90%. Depletion of platelets in BDL mice not only abolished accumulation and adhesion of platelets in sinusoids and venules but also restored sinusoidal perfusion and reduced liver enzymes by more than 83%. Platelet depletion further reduced BDL-associated sinusoidal leukocyte accumulation by 48% although leukocyte–endothelium interactions in venules were not affected. Immunoneutralization of P-selectin also inhibited hepatic microvascular accumulation of platelets and leukocytes, and protected against cholestasis-provoked hepatocellular damage.

Conclusions and implications: Platelets play an important role in BDL-induced liver injury by promoting leukocyte recruitment and deteriorating microvascular perfusion. Moreover, our findings demonstrate that cholestasis-induced accumulation of platelets is mediated by P-selectin. Thus, targeting platelet accumulation may be a useful strategy against liver damage associated with obstructive jaundice.

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Keywords: adhesion; chemokines; cholestasis; microcirculation; leukocytes; liver; P-selectin; platelets

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ab, antibody; BDL, bile duct ligation; FITC, fluorescein isothiocyanate; HPF, high-power field; KC, cytokine-induced neutrophil chemoattractant; MIP-2, macrophage inflammatory protein-2; MPO, myeloperoxidase

Introduction

Enterohepatic recirculation of bile is pivotal for homeostatic functions in the gastrointestinal tract (Zeuzem, 2000). Cholestasis triggers immediate liver injury and the absence of bile in the intestine facilitates bacterial translocation, which, in turn, may cause sepsis and further liver injury (Ding *et al.*, 1994; Chand and Sanyal, 2007). In this vicious cycle, leukocyte recruitment has emerged as a key feature in

the pathogenesis of cholestatic liver injury (Gujral *et al.*, 2003, 2004; Laschke *et al.*, 2007) although the adhesive mechanisms behind leukocyte accumulation in obstructive jaundice remain elusive. Leukocytes extravasate from the hepatic microcirculation, which consists of a mixed hepatic arterial and portal venous inflow system, a low-pressure sinusoidal perfusion and blood drainage by postsinusoidal venules. In general, early steps in the leukocyte extravasation process are mediated by the selectin family of adhesion molecules (L-, E- and P-selectin) and their respective glycoprotein counterligands (Vestweber and Blanks, 1999). Some investigators, however, have suggested that early leukocyte–endothelium interactions, at least in hepatic sinusoids, may be selectin independent (Wong *et al.*, 1997)

Correspondence: Dr H Thorlacius, Department of Surgery, Lund University, Malmö University Hospital, Malmö 20502, Sweden.
E-mail: henrik.thorlacius@med.lu.se

³These authors contributed equally to this work.

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due to the lack of P-selectin on sinusoidal endothelial cells (Steinhoff *et al.*, 1993; Essani *et al.*, 1998; Massaguer *et al.*, 2002). Nonetheless, P-selectin function appears to be critical in reperfusion- and endotoxin-mediated leukocyte recruitment, liver damage and intrahepatic cholestasis (Sawaya *et al.*, 1999; Klintman *et al.*, 2004; Laschke *et al.*, 2007). In contrast, the role of P-selectin in cholestasis-induced leukocyte accumulation, sinusoidal perfusion failure and hepatic tissue injury is not known.

Platelets have been considered to be essential for haemostasis although accumulating data also suggest a role in inflammation and tissue injury (von Hundelshausen and Weber, 2007). Of interest, some recent studies have reported that platelets may exert a role in microvascular leukocyte recruitment (Salter *et al.*, 2001; Singbartl *et al.*, 2001). Accordingly, depletion of platelets has been shown to decrease pulmonary leukocyte accumulation in models of allergic inflammation and hydrochloric acid-induced lung damage (Pitchford *et al.*, 2004, 2005; Zarbock *et al.*, 2006). The detailed mechanisms of this platelet-mediated accumulation of leukocytes in the lung are still under investigation but may be related to the formation of platelet-leukocyte aggregates within the systemic circulation. Adhesion between platelets and leukocytes results in reciprocal cell activation (Abou-Saleh *et al.*, 2005), which may facilitate subsequent interactions with the vessel wall. Alternatively, platelet-leukocyte aggregates may be trapped, mechanically, at the narrow sites in the organ microvasculature. Mechanistic studies have revealed that P-selectin is an adhesive link between platelets and leukocytes in aggregate formation. Interestingly, Singer *et al.* (2006) have recently reported that neutrophils can facilitate platelet adhesion in septic liver injury. However, a role of platelets in leukocyte recruitment and cholestatic liver injury remains to be demonstrated.

Based on the considerations above, the aim of the present study was to determine the role of platelets and P-selectin in cholestasis-induced leukocyte recruitment and hepatocellular damage. For this purpose, intravital fluorescence microscopy of the hepatic microcirculation was examined after ligation of the common bile duct in mice.

Methods

Animals

All animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, WA, USA), and were approved by the local ethics committee at Lund University.

Adult male C57BL/6 mice with a body weight of 22–27 g were used for the study. The animals were housed one per cage on a 12–12 h light–dark cycle and had free access to standard pellet food and tap water throughout the experiment. Animals were anaesthetized by 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 g body weight. Test substances and fluorescent dyes were administered i.v. via retroorbital injection.

Experimental protocol

Animals underwent bile duct ligation (BDL) to induce obstructive cholestasis. BDL was performed under ketamine/xylazine anaesthesia via a midline laparotomy. By means of a surgical microscope, the common bile duct was prepared and carefully ligated with a 7–0 prolene suture. Subsequently, the laparotomy was closed again by a 5–0 running suture and the animals were allowed to recover from anaesthesia and surgery for 12 h. Sham-operated animals received phosphate-buffered saline (PBS) i.v. and underwent an identical laparotomy and liver manipulation without BDL. To delineate the role of platelets and P-selectin in the pathogenesis of obstructive cholestasis, animals were pre-treated i.v. 2 h prior to BDL with an anti-GP1b α antibody (ab) (rat IgG, 1 mg kg⁻¹, Emfret Analytics GmbH & Co., Eibelstadt, Germany), which depletes mice of platelets, an anti-P-selectin ab (RB40.34, rat IgG, 1.5 mg kg⁻¹, Pharmingen, San Diego, CA, USA) or an isotype-matched control ab (rat IgG, R3–34, 1.5 mg kg⁻¹, Pharmingen).

Intravital fluorescence microscopy

Twelve hours after BDL, the hepatic microcirculation was examined by intravital fluorescence microscopy. For this purpose, in anaesthetized animals, a transverse subcostal incision was made and the ligamentous attachments from the liver to the diaphragm and the abdominal wall were gently released. Subsequently, the mice were positioned on their left side and the left liver lobe was carefully exteriorized onto an adjustable stage for microscopic analysis. An equilibration period of 5 min was allowed before starting the microscopical observation. For intravital fluorescence microscopy, we used a modified Olympus microscope (BX50WI, Olympus Optical Co. GmbH, Hamburg, Germany) equipped with different water immersion lenses ($\times 40$ NA 0.75/ $\times 63$ NA 0.9). The microscopic images were recorded by a charge-coupled device video camera (FK 6990 CoHu, Pieper GmbH, Schwerte, Germany) and transferred to CD-ROM for off-line evaluation. Blood perfusion within individual microvessels was studied after i.v. injection of 0.1 ml 5% fluorescein isothiocyanate-labeled dextran 150 000 (contrast enhancement by intravascular staining of plasma; Sigma Chemical Co., St Louis, MO, USA). *In vivo* labelling of leukocytes and platelets with 0.1% rhodamine-6G (0.1 ml i.v., Sigma Chemical Co.) enabled quantitative analysis of leukocyte and platelet-flow behaviour in both sinusoids and postsinusoidal venules. Five postsinusoidal venules with connecting sinusoids were evaluated in each animal. Microcirculatory analysis included determination of sinusoidal perfusion by measuring the number of non-perfused sinusoids given as a percentage of the total number of sinusoids observed. Within sinusoids and postsinusoidal venules, leukocyte and platelet adhesion were measured by counting the number of cells adhering along the venular endothelium and remaining stationary during an observation period of 20 s. Cell adhesion is expressed as number of cells per 10 high-power field (HPF) and cells mm⁻², respectively. In addition, platelet aggregates (that is more than three platelets) in sinusoids and postsinusoidal venules were determined in each animal and are expressed as cells per

10 HPF and cells mm^{-2} , respectively. After intravital microscopic observations, animals were killed and blood was drawn from the inferior vena cava for standard spectrophotometric analysis of bilirubin and liver enzymes, including alanine aminotransferase and aspartate aminotransferase. In addition, systemic platelet and leukocyte counts, including polymorphonuclear leukocytes, were determined with a haematocytometer.

Measurement of myeloperoxidase activity

Liver tissue was collected, weighed and homogenized in 10 ml 0.5% hexadecyltrimethylammonium bromide. Subsequently, 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-catalysed change in absorbance occurring in the redox reaction of H_2O_2 (460 nm, 25 °C). Values are expressed as MPO units per g liver tissue.

Enzyme-linked immunosorbent assay for chemokines

The right liver lobe was weighed, washed and homogenized in PBS containing 1% penicillin and streptomycin and fungizone (100 U ml^{-1}) and then kept cool in cold serum-free Dulbecco's modified Eagle's medium. After centrifugation, supernatants were collected and stored in -20°C until analysis of CXC chemokines, including macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC), by the use of double ab Quantikine enzyme-linked immunosorbent assay kits (R & D Systems Europe, Abingdon, Oxon, UK) using recombinant murine KC and MIP-2 as standards. The minimal detectable protein concentrations were less than 0.5 ng ml^{-1} .

Statistics

All data are presented as mean values \pm s.e.mean. Statistical evaluations were performed using Kruskal–Wallis one-way ANOVA on ranks followed by multiple comparisons vs control group (Dunn's method) (SigmaStat; Jandel Corporation, San Rafael, CA, USA). Statistical significance was accepted for a value of $P < 0.05$.

Results

Hepatocellular damage

We observed that ligation of the bile duct significantly increased systemic bilirubin levels by more than threefold, suggesting that clear-cut cholestasis was induced in this model (Figure 1a). Notably, the bilirubin levels in animals depleted of platelets by administration of the anti-GP1b α ab and in mice pretreated with the anti-P-selectin ab were not different from that in positive control animals after BDL, suggesting that the degree of cholestasis was similar in all bile-duct ligated animals (Figure 1a). Moreover, BDL caused substantial hepatocellular damage as indicated by a more than 26-fold increase of liver enzymes (Figures 1b and c; $P < 0.05$ vs sham, $n = 7-8$). Administration of the anti-GP1b α

ab directed against platelets significantly reduced alanine aminotransferase and aspartate aminotransferase levels in mice with BDL (Figures 1b and c; $P < 0.05$ vs Control ab + BDL, $n = 7-8$). Furthermore, treatment with the anti-GP1b α ab decreased systemic platelets by more than 87% (Table 1), suggesting that this ab efficiently depleted mice of platelets. Further, immunoneutralization of P-selectin, which was not associated with a decrease of systemic platelet count, also reduced alanine aminotransferase and aspartate aminotransferase levels by 88 and 83%, respectively (Figures 1b and c; $P < 0.05$ vs Control ab + BDL, $n = 7-8$).

Leukocyte and platelet accumulation in the hepatic microcirculation

Accumulation of leukocytes is considered to be a rate-limiting step in BDL-induced liver injury (Gujral *et al.*, 2003, 2004). Extravascular recruitment of leukocytes was determined by analysing MPO levels in the liver. We found that BDL increased MPO levels from 0.03 ± 0.01 up to $0.18 \pm 0.03 \text{ U g}^{-1}$ in the liver (Figure 2, $P < 0.05$ vs sham, $n = 7-10$). Platelet depletion by anti-GP1b α decreased MPO levels to $0.10 \pm 0.01 \text{ U g}^{-1}$ in BDL mice, corresponding to a 44% reduction in MPO activity (Figure 2, $P < 0.05$ vs Control ab + BDL, $n = 7-10$). This suggests a significant role for platelets in the hepatic accumulation of leukocytes in cholestatic animals. Moreover, inhibition of P-selectin reduced hepatic MPO activity by 64% in BDL mice (Figure 2, $P < 0.05$ vs Control ab + BDL, $n = 7-10$). Neither the anti-GP1b α nor the anti-P-selectin ab altered the numbers of circulating leukocytes (Table 1). Having observed that platelets support hepatic leukocyte recruitment, we next wanted to analyse the role of platelets and P-selectin for leukocyte accumulation in cholestatic mice in more detail. For this purpose, we used intravital fluorescence microscopy, which allows detailed investigation of the blood cell-endothelium interactions in hepatic sinusoids and venules *in vivo*. We found that BDL enhanced platelet and leukocyte adhesion in liver sinusoids as well as in postsinusoidal venules (Figures 3 and 4, $P < 0.05$ vs sham, $n = 7-10$). As expected, systemic depletion of platelets markedly reduced platelet adhesion in both sinusoids and postsinusoidal venules (Figure 3). However, administration of the anti-GP1b α ab also significantly decreased BDL-induced leukocyte adhesion in hepatic sinusoids, that is from 28.7 ± 2.2 down to 14.9 ± 1.9 leukocytes per 10 HPE, corresponding to a 48% reduction (Figure 4a, $P < 0.05$ vs Control ab + BDL, $n = 7-8$). In contrast, platelet depletion had no effect on BDL-induced leukocyte adhesion in the hepatic postsinusoidal venules (Figure 4b, $P > 0.05$ vs Control ab + BDL, $n = 7-8$), suggesting that platelets support leukocyte accumulation in hepatic sinusoids but not in venules during cholestasis. Administration of the anti-P-selectin ab reduced BDL-induced platelet adhesion in sinusoids by 37% and in postsinusoidal venules by 71% (Figures 3a and b, $P < 0.05$ vs Control ab + BDL, $n = 7-8$). Moreover, immunoneutralization of P-selectin significantly inhibited BDL-induced leukocyte adhesion in sinusoids by 41% and postsinusoidal venules by 84% (Figures 4a and b, $P < 0.05$ vs Control ab + BDL, $n = 7-8$).

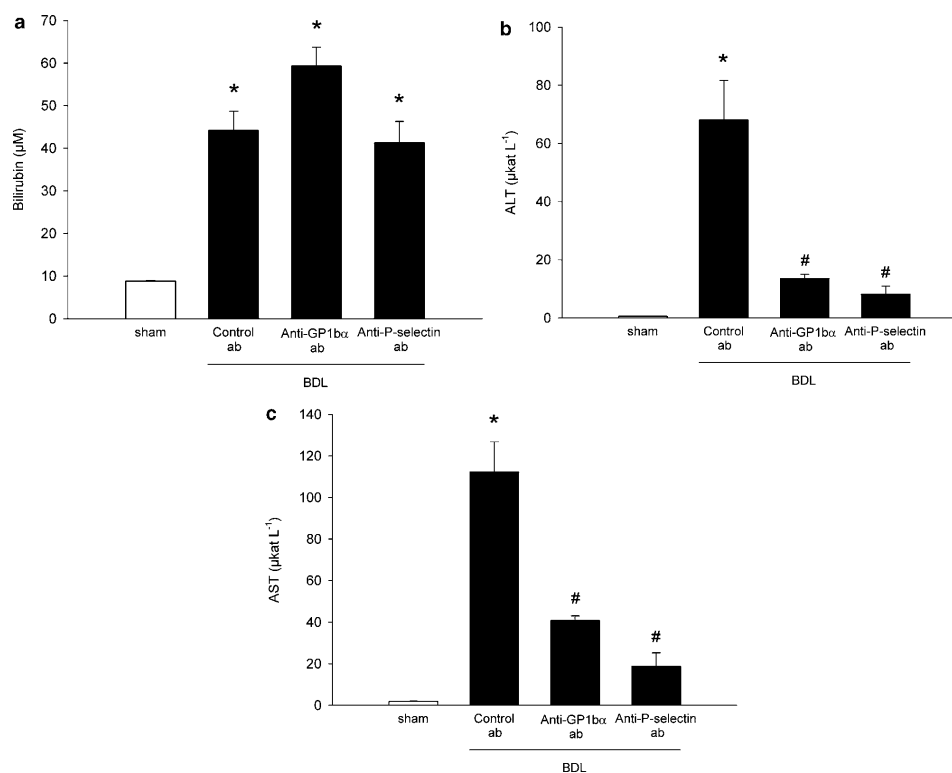


Figure 1 Bilirubin and liver enzymes 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1b α (anti-GP1b α ab) or against P-selectin (anti-P-selectin ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. The levels of (a) bilirubin (b) alanine aminotransferase (ALT) and (c) aspartate aminotransferase (AST) were determined spectrophotometrically. Data represent means \pm s.e.mean ($n = 7-8$). * $P < 0.05$ vs sham and # $P < 0.05$ vs Control ab + BDL.

Table 1 Systemic leukocyte and platelet counts

| | Platelets | Leukocytes |
|--------------------------|----------------|---------------|
| Sham | 296 \pm 34.6 | 1.7 \pm 0.3 |
| Control ab + BDL | 331 \pm 28.5 | 1.5 \pm 0.2 |
| Anti-GP1 ab + BDL | 40 \pm 5.9* | 1.2 \pm 0.2 |
| Anti-P-selectin ab + BDL | 330 \pm 22.9 | 1.7 \pm 0.1 |

Abbreviation: BDL, bile duct ligation. Blood samples were drawn from the inferior vena cava at the end of the experiments. Platelets and leukocytes were counted using a standard haematocytometer. Mice were treated with a control antibody (ab), an anti-GP1b α ab or an anti-P-selectin ab prior to induction of BDL. Animals not exposed to BDL served as sham. Data are means \pm s.e.mean ($n = 7-10$) and represent 10^6 cells ml^{-1} . * $P < 0.05$ vs Control ab + BDL.

Sinusoidal perfusion and platelet aggregates

Cholestatic liver injury is also characterized by a deterioration of microvascular perfusion (Koeppel *et al.*,

1997). Indeed, we found that the percentage of non-perfused sinusoids increased from 5.4 ± 0.9 up to $39.1 \pm 3.7\%$ in BDL mice (Figure 5, $P < 0.05$ vs sham, $n = 7-8$). The number of non-perfused sinusoids after BDL decreased to $15.7 \pm 3.2\%$ in platelet-depleted animals (Figure 5, $P < 0.05$ vs Control ab + BDL, $n = 7-8$). Moreover, administration of the ab directed against P-selectin reduced perfusion failure to $16.5 \pm 2.8\%$ in BDL mice (Figure 5, $P < 0.05$ vs Control ab + BDL, $n = 7-8$). We also noted numerous and widespread aggregates (that is more than three platelets) of platelets in the hepatic microvasculature after ligation of the common bile duct. The number of these platelet aggregates increased by 16- and 30-fold in sinusoids and postsinusoidal venules, respectively, in BDL mice (Figure 6, $P < 0.05$ vs sham, $n = 7-8$). Administration of the anti-GP-1b α and the anti-P-selectin ab abolished BDL-induced formation of platelet aggregates in the hepatic microcirculation (Figure 6, $P < 0.05$ vs Control ab + BDL, $n = 7-8$).

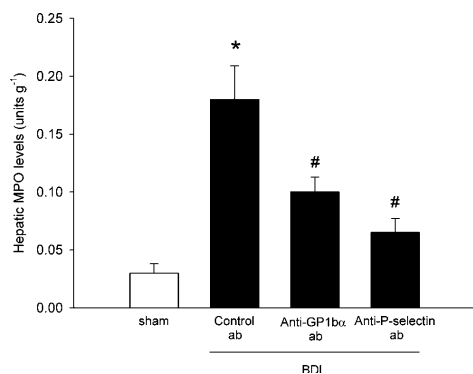


Figure 2 Hepatic levels of myeloperoxidase (MPO) 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1b α (anti-GP1b α ab) or against P-selectin (anti-P-selectin ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. Data represent means \pm s.e.mean ($n=7-10$). * $P<0.05$ vs sham and # $P<0.05$ vs Control ab + BDL.

CXC chemokines

Leukocyte extravasation into the liver parenchyma has been reported to be directed by secreted CXC chemokines (Li *et al.*, 2004). We observed that the hepatic levels of CXC chemokines in sham animals were low but detectable (Figure 7, $n=6-8$). In contrast, ligation of the common bile duct markedly increased hepatic levels of MIP-2 and KC (Figure 7, $P<0.05$ vs sham, $n=6-8$). Interestingly, pretreatment with the anti-GP-1b α ab reduced BDL-provoked expression of MIP-2 and KC. That is, depletion of platelets attenuated formation of CXC chemokines by more than 63% in cholestatic mice (Figure 7). Similarly, administration of the anti-P-selectin ab significantly reduced BDL-induced expression of MIP-2 by 73% and of KC by 66% (Figure 7, $P<0.05$ vs Control ab + BDL, $n=6-8$).

Discussion and conclusions

Surgical and endoscopic decompression is the principal treatment of biliary obstruction but may not be sufficient to prevent development of hepatic injury and septic complications. Thus, mechanistic studies are needed to delineate the pathophysiology of cholestasis-induced liver damage. This study demonstrates for the first time an important role of platelets in supporting BDL-mediated leukocyte recruitment in the liver. Our data show that platelets facilitate sinusoidal accumulation of leukocytes in cholestasis. Indeed, depletion of platelets not only reduced hepatic recruitment of leukocytes but also protected against liver injury in cholestatic mice. Moreover, inhibition of P-selectin prevented cholestasis-induced platelet and leukocyte recruitment as well as the associated hepatocellular damage. Taken together, our findings suggest that platelets

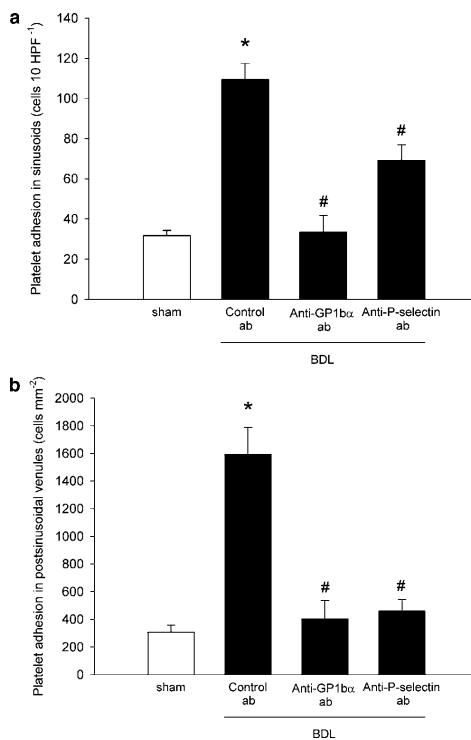


Figure 3 Platelet adhesion in (a) sinusoids and (b) postsinusoidal venules 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1b α (anti-GP1b α ab) or against P-selectin (anti-P-selectin ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. Data represent means \pm s.e.mean ($n=7-8$). * $P<0.05$ vs sham and # $P<0.05$ vs Control ab + BDL.

play an important role in cholestasis-induced leukocyte accumulation and liver injury and that P-selectin regulates platelet and leukocyte accumulation in cholestasis.

It is well accepted that neutrophil infiltration plays an important role in cholestatic liver injury (Gujral *et al.*, 2003, 2004). However, none of these studies have evaluated a potential role of platelets for leukocyte recruitment or hepatic damage. It is interesting to note that an accumulating body of evidence indicates that platelets exert numerous pro-inflammatory effects beyond their well-known haemostatic functions (von Hundelshausen and Weber, 2007). The present study is the first to demonstrate a role of platelets in hepatic accumulation of leukocytes. Indeed, we found that depletion of platelets decreased MPO levels, a marker of leukocyte recruitment, by 44% in the liver. This effect correlated well with our observation that platelet depletion reduced BDL-induced leukocyte adhesion in hepatic sinusoids by 48%. Depletion of platelets had no effect on

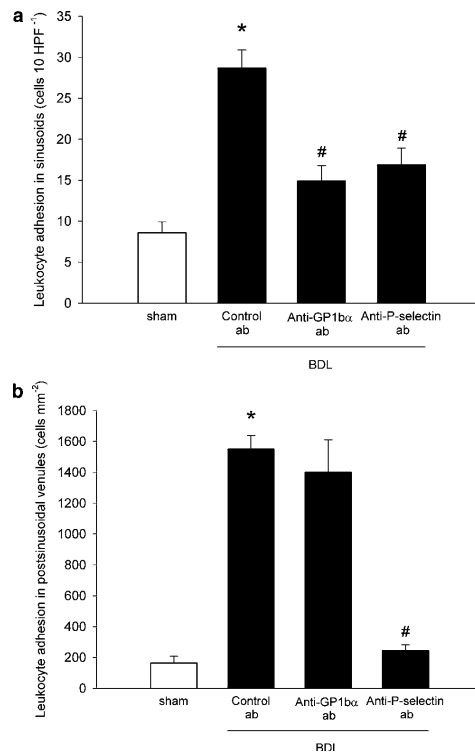


Figure 4 Leukocyte adhesion in (a) sinusoids and (b) postsinusoidal venules 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1b α (anti-GP1b α ab) or against P-selectin (anti-P ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. Data represent means \pm s.e.mean ($n = 7-8$). * $P < 0.05$ vs sham and # $P < 0.05$ vs Control ab + BDL.

leukocyte accumulation in postsinusoidal venules, suggesting that the sinusoid is the dominant site of platelet-dependent leukocyte recruitment in the liver. In addition, our work is also the first to show that platelets play a significant role in cholestatic liver injury. Thus, platelet depletion decreased cholestasis-induced hepatocellular damage by more than 83%. Considering previous work showing a critical role of neutrophils in cholestatic liver injury (Gujral *et al.*, 2003, 2004) and our observation that depletion of platelets simultaneously decreased leukocyte recruitment and hepatocellular damage, suggest a mechanistic link between platelet-mediated leukocyte recruitment on one hand and BDL-induced liver injury on the other. Moreover, the present findings add the liver to the lung (Pitchford *et al.*, 2004, 2005; Zarbock *et al.*, 2006) and kidney (Singbartl *et al.*, 2001; Kuligowski *et al.*, 2006) as organs in which platelet-mediated leukocyte recruitment appears to play a significant role in distinct disease states.

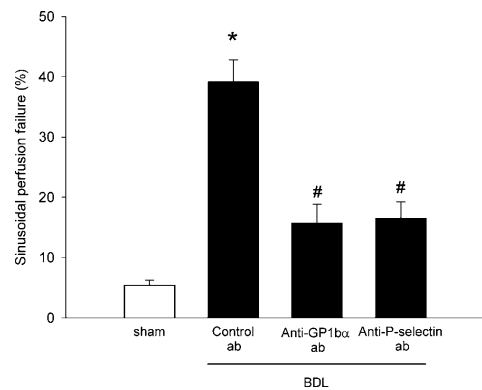


Figure 5 Sinusoidal perfusion failure 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1b α (anti-GP1b α ab) or against P-selectin (anti-P-selectin ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. Data represent means \pm s.e.mean ($n = 7-8$). * $P < 0.05$ vs sham and # $P < 0.05$ vs Control ab + BDL.

Early reports suggested that selectin-mediated functions may play only a minor role in leukocyte recruitment in the liver (Wong *et al.*, 1997). For example, Essani *et al.* (1998) reported that inhibition of P-selectin has no effect on sinusoidal accumulation of leukocytes in endotoxaemic mice. Herein, we observed that immunoneutralization of P-selectin decreased BDL-induced leukocyte adhesion in both sinusoids and postsinusoidal venules, suggesting that P-selectin indeed plays a fundamental role in cholestasis-induced leukocyte recruitment in the liver. This finding is in line with more recent studies postulating P-selectin as an important adhesion molecule regulating leukocyte recruitment in the liver in various conditions, such as ischaemia/reperfusion and endotoxaemia (Sawaya *et al.*, 1999; Klintman *et al.*, 2004; Laschke *et al.*, 2007). Moreover, considering our findings that inhibition of P-selectin decreases both platelet and leukocyte adhesion in BDL mice, targeting P-selectin may be of particular value in this case, because both platelets and leukocytes may cause tissue damage in cholestatic liver injury. In this context, it is important to underline that the inhibitory effect of the anti-P-selectin ab on BDL-induced accumulation of leukocytes in sinusoids is likely to be an indirect effect, that is convincing data have shown that P-selectin is not expressed in sinusoidal endothelium (Steinhoff *et al.*, 1993; Essani *et al.*, 1998; Massaguer *et al.*, 2002) and intravital observations have shown that leukocytes do not roll in sinusoids (Wong *et al.*, 1997; Klintman *et al.*, 2004). Notably, we observed that inhibition of P-selectin reduced sinusoidal accumulation of platelets by 37%, which was similar in magnitude to the 48% reduction in sinusoidal recruitment of leukocytes. In combination, it may be suggested that P-selectin-mediated accumulation of leukocytes in hepatic sinusoids is platelet dependent. P-selectin is not only expressed in Weibel-Palade bodies of endothelial cells but also in α -granules of platelets (Isenberg

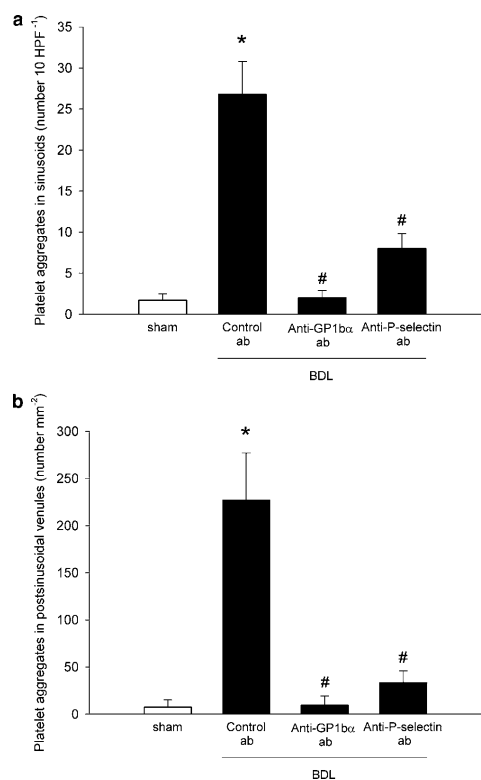


Figure 6 Platelet aggregates in (a) sinusoids and (b) postsinusoidal venules 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1b α (anti-GP1b α ab) or against P-selectin (anti-P-selectin ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. Platelet aggregates were defined as groups of more than three adherent platelets. Data represent means \pm s.e.mean ($n=7-8$). * $P<0.05$ vs sham and # $P<0.05$ vs Control ab + BDL.

et al., 1986). In fact, numerous studies have demonstrated that adhesive interactions between platelets and leukocytes are supported by platelet P-selectin binding to P-selectin glycoprotein ligand-1 expressed on leukocytes (Hamburger and McEver, 1990; Rinder *et al.*, 1991; Abou-Saleh *et al.*, 2005). The detailed role of P-selectin remains elusive and may be multiple. For example, adherent platelets on endothelial cells may serve as an adhesive P-selectin substrate and directly capture circulating leukocytes on the endothelium. However, platelets and leukocytes can also interact via P-selectin/P-selectin glycoprotein ligand-1 in the circulation resulting in aggregate formation, which might subsequently be trapped mechanically in the narrow liver sinusoids. In addition, leukocytes attached to platelets become activated and upregulate surface expression of

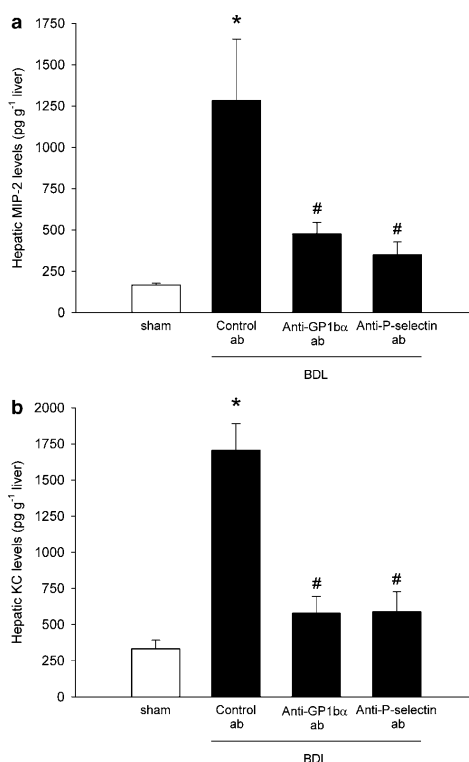


Figure 7 Hepatic levels of (a) macrophage inflammatory protein-2 (MIP-2) and (b) cytokine-induced neutrophil chemoattractant (KC) 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1b α (anti-GP1b α ab) or against P-selectin (anti-P-selectin ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. Data represent means \pm s.e.mean ($n=6-8$). * $P<0.05$ vs sham and # $P<0.05$ vs Control ab + BDL.

CD11b (Pitchford *et al.*, 2004), which may prime leukocytes for firm adhesion in sinusoids and tissue infiltration.

Activation and tissue navigation of leukocytes are coordinated by secreted chemokines (Campbell *et al.*, 2003). The CXC chemokines, MIP-2 and KC, are considered to attract predominately neutrophils and have been demonstrated to regulate leukocyte recruitment in septic liver injury (Li *et al.*, 2004). Herein, we observed that the hepatic formation of MIP-2 and KC was greatly increased after ligation of the common bile duct. Interestingly, platelet depletion significantly decreased CXC chemokine production in cholestatic livers. Similarly, inhibition of P-selectin function also abolished BDL-induced formation of MIP-2 and KC in the liver. These findings are somewhat surprising considering that CXC chemokines are largely secreted from Kupffer cells and hepatocytes in the liver (Hisama *et al.*, 1996; Mosher *et al.*, 2001; Li *et al.*, 2004). Nonetheless, these data suggest

that platelets constitute an early component in the pathophysiology of cholestasis upstream of MIP-2 and KC production in the liver. It is noteworthy to point out that platelet depletion markedly decreased hepatic injury in spite of unchanged levels of adherent leukocytes in the post-sinusoidal venules. This may be explained by the fact that extravasation of leukocytes is critically dependent on CXC chemokines in the liver (Li *et al.*, 2004), in combination with the present findings showing that platelet depletion reduced CXC chemokine formation in cholestatic liver injury. Thus, our results indicate that P-selectin-dependent platelet functions regulate subsequent CXC chemokine-induced leukocyte recruitment in the cholestatic liver injury. The link between platelets and CXC chemokine formation is speculative but may be related to pro-inflammatory compounds secreted from activated platelets and leukocytes, which in turn may activate tissue-resident cells in the liver.

In conclusion, this study demonstrates for the first time a functional role of platelets in supporting leukocyte recruitment in the liver. Our results show that depletion of platelets not only reduces accumulation of leukocytes but also ameliorates BDL-induced hepatocellular damage, implicating platelets in the pathogenesis of cholestatic liver injury. Moreover, the present findings demonstrate that P-selectin regulates platelet and leukocyte as well as platelet-dependent leukocyte recruitment, suggesting that P-selectin plays a key role in cholestatic liver damage. Thus, our findings document an important contribution of platelets and P-selectin in cholestatic liver injury, which may pave the way for more-specific therapeutic strategies to protect the liver in conditions with obstructed bile flow.

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Conflict of interest

The authors state no conflict of interest.

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III

Cholestatic liver damage is mediated by lymphocyte function antigen-1-dependent recruitment of leukocytes

Stefan Dold, MD,^{a,b,*} Matthias W. Laschke, MD,^{a,b,*} Shahram Lavasani, MD,^a Michael D. Menger, MD,^b and Henrik Thorlacius, MD, PhD,^a Malmö, Sweden, and Homburg/Saar, Germany

Background. The role of specific adhesion molecules in cholestasis-induced leukocyte recruitment in the liver is not known. Therefore, the aim of our experimental study was to evaluate the role of lymphocyte function antigen-1 (LFA-1) in cholestatic liver injury.

Methods. C57BL/6 mice underwent bile duct ligation for 12 hours. Mice were pretreated with an anti-LFA-1 antibody or control antibody. Subsequently, hepatic accumulation of leukocytes and sinusoidal perfusion were determined by means of intravital fluorescence microscopy. Hepatocellular damage was monitored by measuring serum levels of alanine aminotransferase and aspartate aminotransferase. CXC chemokines in the liver were determined by enzyme-linked immunosorbent assay.

Results. Bile duct ligation provoked clear-cut recruitment of leukocytes and liver damage, as indicated by increased serum activities of liver enzymes and sinusoidal perfusion failure. Neutrophils expressed greater levels of LFA-1 and inhibition of LFA-1 significantly decreased serum activity of alanine aminotransferase and aspartate aminotransferase levels in cholestatic mice. Immunoneutralization of LFA-1 reduced leukocyte adhesion in postsinusoidal venules that had been induced by bile duct ligation, whereas leukocyte rolling and sinusoidal accumulation were not changed. Moreover, blocking LFA-1 function restored sinusoidal perfusion in cholestatic animals.

Conclusion. These findings demonstrate an important role of LFA-1 in supporting cholestasis-induced leukocyte recruitment in the liver. Thus, targeting LFA-1 may help to protect against pathologic inflammation and liver damage in cholestatic liver diseases. (*Surgery* 2008;144:385-93.)

From the Department of Surgery, Malmö University Hospital, Lund University, Malmö, Sweden^a; and the Institute for Clinical and Experimental Surgery, University of Saarland, Homburg/Saar, Germany^b

OBSTRUCTIVE CHOLESTASIS represents a frequent manifestation of several clinical settings, such as gallstone impaction, sclerosing cholangitis, cholangiocarcinoma, or compression of the common bile duct by pancreatic tumors or enlarged lymph

nodes.^{1,2} The accumulation of bile acids in the portal tract induces a strong inflammatory reaction in the liver with activation of Kupffer cells leading to increased production and release of proinflammatory mediators, including interleukin-6 (IL-6), tumor necrosis factor- (TNF)- α , and CXC chemokines.^{3,4} Several studies have reported that this response is associated with recruitment of leukocytes in the liver, suggesting a key role of this inflammatory cell type in cholestatic liver injury.⁵⁻⁷ The molecular mechanisms mediating leukocyte attachment to endothelial cells in cholestatic liver injury, however, remain elusive.

Leukocyte recruitment in the liver is a multistep process characterized by a coordinated sequence of ligand-receptor interactions between leukocytes and the microvascular endothelium.^{8,9} On activation, leukocytes roll along the endothelium of postsinusoidal venules, mediated by the selectin family of adhesion molecules such as P-selectin.¹⁰ This rolling, adhesive interaction is a major prerequisite for the subsequent firm adhesion of leukocytes to the endothelium. Firm adhesion generally requires

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Reprint requests: Henrik Thorlacius, MD, PhD, Department of Surgery, Malmö University Hospital, Lund University, S - 205 02 Malmö, Sweden. E-mail: henrik.thorlacius@med.lu.se.

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the binding of β_1 and β_2 integrins expressed on leukocytes to molecules of the immunoglobulin supergene family expressed on endothelial cells, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).^{8,9,11} The structure of β_2 integrins is characterized by a common β -subunit (CD18), which is linked to 1 of 4 α -subunits (CD11a-d).^{8,9} Accordingly, several different molecules exist that have been shown to mediate leukocyte-endothelial cell interactions, including lymphocyte function antigen (LFA)-1 (CD11a/CD18) and macrophage antigen (Mac)-1 (CD11b/CD18).^{12,13} Of interest, Gujral et al¹⁴ reported that ICAM-1 may play a role in leukocyte recruitment and hepatic injury during obstructive cholestasis. One receptor for ICAM-1 is LFA-1, which has been reported to support leukocyte accumulation in different models of liver inflammation, including alcoholic liver disease,¹⁵ viral hepatitis,¹⁶ endotoxemia,¹⁷ and graft-versus-host disease.¹⁸ The specific role of LFA-1 in obstructive cholestasis, however, is not known at present.

Thus, based on the considerations above, we hypothesized that LFA-1 may be involved in the pathophysiology of cholestatic liver injury. The aim of the present study, therefore, was to analyze the role of LFA-1 in leukocyte recruitment during obstructive cholestasis.

MATERIALS AND METHODS

Animals. Adult male C57BL/6 mice with a body weight of 25–27 g were used. Throughout the experiments, animals were housed 1 per cage on a 12/12 hour light-dark cycle and had free access to standard pellet food and tap water. Animals were anesthetized by intraperitoneal administration of 0.075 mg/g body weight ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 0.025 mg/g body weight xylazine (Janssen Pharmaceutica, Beerse, Belgium). Test substances and fluorescent dyes were administered intravenously via retro-orbital injection. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC) and were approved by the local ethics committee at Lund University.

Experimental protocol. To induce obstructive cholestasis, 15 animals underwent bile duct ligation (BDL). BDL was performed under ketamine/xylazine anesthesia via a midline laparotomy. Using a stereoscopic microscope, the common bile duct was isolated and ligated carefully with a 6-0 silk suture. The laparotomy was then closed by a 5-0 running

suture, and the animals were allowed to recover from anesthesia and operation for 12 hours. Sham-operated animals received phosphate-buffered saline (PBS) intravenously and underwent an identical laparotomy and liver manipulation without BDL (sham, $n = 5$). To delineate the role of LFA-1 in leukocyte recruitment during obstructive cholestasis, mice ($n = 6$) were pretreated 15 minutes prior to BDL with an anti-LFA-1 antibody (anti-LFA-1; M17/4.4.11.9, rat immunoglobulin G [IgG]; Novartis Pharma AG, Preclinical Research, Basel, Switzerland), and other mice ($n = 5$) with an isotype-matched control antibody (control IgG; R3-34, rat IgG; BD Biosciences Pharmingen, San Jose, Calif). Bile duct-ligated animals ($n = 4$), which received PBS only, served as positive controls (PBS).

Intravital fluorescence microscopy. Analysis of the hepatic microcirculation was performed by means of intravital fluorescence microscopy 12 hours after BDL. For this purpose, mice were re-anesthetized, and a transverse subcostal incision was performed. The ligamentous attachments from the liver to the diaphragm and the abdominal wall were gently transected. Subsequently, the mice were positioned on their left side, and the left liver lobe was carefully exteriorized onto an adjustable stage for the microscopic analysis. An equilibration period of 5 minutes was allowed before starting the microscopic observation. For intravital fluorescence microscopy, we used a modified Olympus microscope (BX50WI; Olympus Optical, Hamburg, Germany) equipped with different water immersion lenses ($\times 40$ NA 0.75; $\times 63$ NA 0.9).

The microscopic images were recorded by a charge-coupled device video camera (FK 6990 Cohu; Pieper, Schwerte, Germany) and transferred to CD-ROM for offline evaluation. Blood perfusion within individual microvessels was studied after intravenous injection of 0.1 ml 5% fluorescein isothiocyanate (FITC)-labeled dextran (molecular weight of 150000; Sigma, St Louis, Mo). In vivo labeling of leukocytes with intravenous injection of 0.1 ml of 0.1% rhodamine 6G (Sigma) enabled quantitative analysis of the flow behavior of the leukocytes in both sinusoids and postsinusoidal venules.

A total of 5 postsinusoidal venules with connecting sinusoids were evaluated in each animal. Microcirculatory analysis included determination of sinusoidal perfusion by measuring the number of nonperfused sinusoids given as a percentage of the total number of sinusoids observed. Within sinusoids and postsinusoidal venules, leukocyte adhesion was measured by counting the number of cells that adhered along the endothelium without

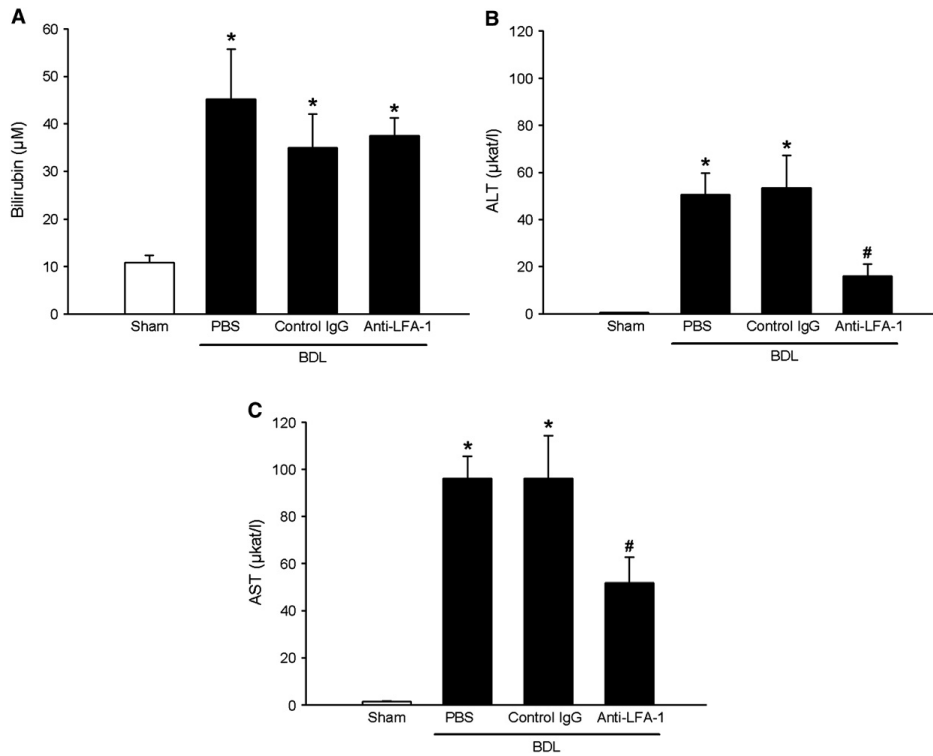


Fig 1. Bilirubin and liver enzymes 12 hours after BDL. Animals were pretreated intravenously with PBS, an isotype-matched control antibody (control IgG), or an antibody against LFA-1 (anti-LFA-1) 15 minutes prior to BDL. Sham-operated mice received only PBS. Levels of bilirubin (A), alanine aminotransferase (B) and aspartate aminotransferase (C) were determined spectrophotometrically. *BDL*, Bile duct ligation; *PBS*, phosphate-buffered saline; *IgG*, immunoglobulin G; *LFA-1*, lymphocyte function antigen-1. Mean values \pm SEM. * $P < .05$ vs sham; # $P < .05$ vs control IgG.

moving during the observation period of 20 seconds; the value is expressed as cells per 10 high-power fields (cells/10 HPFs) or cells per millimeter squared (cells/mm²), respectively. After intravital fluorescence microscopy, animals were killed; blood was drawn from the inferior vena cava for analysis of the serum concentrations of bilirubin and for serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using standard spectrophotometric procedures. Moreover, systemic leukocyte counts, including polymorphonuclear leukocytes (PMNL) and monomorphonuclear leukocytes (MNL), were determined with a hematocytometer.

Enzyme-linked immunosorbent assay (ELISA). The right liver lobe was weighed, washed, and homogenized in PBS containing 1% penicillin and streptomycin and amphotericin B (100 U/ml)

and then kept cool in cold, serum-free Dulbecco's modified Eagle medium. After centrifugation, supernatants were collected and stored in -20°C until analysis of CXC chemokines, including macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC). The analysis was performed with double antibody ELISA (Quantikine ELISA kits; R&D Systems Europe Ltd., Abingdon, UK) using recombinant murine KC and MIP-2 as standards. The minimal detectable protein concentrations were less than 0.5 pg/ml.

Flow cytometry. For analysis of surface expression of LFA-1 on neutrophils, blood was collected via cardiac puncture into heparinized syringes at the end of the experiment. Erythrocytes were lysed using red blood cell lysing buffer (Sigma-Aldrich, Stockholm, Sweden), and the leukocytes were recovered after centrifugation. Cells were incubated

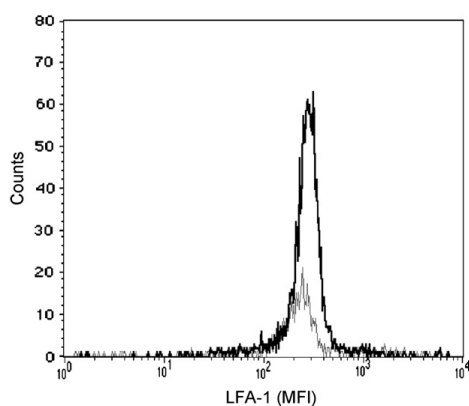


Fig 2. Surface expression of LFA-1 on neutrophils after BDL compared to sham-operated mice. Cells were stained with antimouse Gr-1 to identify and gate the neutrophil population and anti-CD11a/LFA-1 to quantify LFA-1 expression (mean fluorescence intensity [MFI]) on neutrophils collected from BDL- (black) or sham-operated (gray) animals. BDL, Bile duct ligation; LFA-1, lymphocyte function antigen-1.

with anti-CD16/CD32 to block Fc γ III/II receptors and reduce nonspecific labeling for 5 minutes. The cells were stained at 4°C for 30 minutes simultaneously with phycoerythrin-conjugated anti-Gr-1 (clone RB6-8C5) and FITC-conjugated anti-CD11a/LFA-1 (clone M17/4) monoclonal antibodies (mAbs) (all purchased from BD Biosciences Pharmingen). Flow-cytometric analysis was performed according to standard settings on a flow cytometer (FACSsort; Becton Dickinson, Mountain View, Calif). A viable gate was used to exclude dead and fragmented cells.

Histology. Tissue samples were taken from the left liver lobe and fixed in 4% formaldehyde phosphate buffer over night. Dehydrated, paraffin-embedded, 6- μ m sections were stained with hematoxylin and eosin and analyzed under light microscopy. For histologic quantification of hepatocellular injury (“biliary infarcts”), 5 random HPFs were evaluated by use of a semiautomatic image analysis system (NIS-Elements Advanced Research; Nikon, Kanagawa, Japan), and findings are given as percentage of liver surface as described previously.¹⁹

Statistics. All data are presented as mean values \pm standard error of the mean (SEM). Statistical evaluations were performed using Kruskal-Wallis 1-way analysis of variance on ranks followed by multiple comparisons versus control group (Dunn’s method)

(SigmaStat; Jandel, San Rafael, Calif). Statistical significance was accepted for a value of $P < .05$.

RESULTS

Hepatocellular damage. In all experimental groups, ligation of the common bile duct resulted in the development of obstructive cholestasis after 12 hours with increased serum concentrations of bilirubin ranging between 35 and 45 μ M compared with sham-operated controls (\sim 11 μ M; $P < .05$; Fig 1, A). We also found that BDL was associated with severe hepatocellular damage, as indicated by a marked increase of ALT and AST activities in PBS-treated (50.6 ± 9.2 and 96.2 ± 9.2 μ kat/L, respectively) and control IgG animals (53.4 ± 13.9 and 96.1 ± 18.1 μ kat/L, respectively) compared to sham levels of ALT and AST (0.49 ± 0.08 and 1.33 ± 0.15 μ kat/L, respectively) (Fig 1, B, C). Surface expression of CD11a increased on circulating neutrophils after BDL (Fig 2).

Therefore, we hypothesized that LFA-1 may constitute a potential target in cholestatic liver damage. Pretreatment with the anti-LFA-1 antibody decreased serum ALT activity to 16.0 ± 5.0 μ kat/L and AST levels to 51.7 ± 10.9 μ kat/L ($P < .05$; Fig 1, B, C), corresponding to a 70% and 46% reduction, respectively. Hematoxylin and eosin-stained liver sections of sham-operated controls exhibited a normal hepatic architecture (Fig 3, A), whereas marked destruction of the liver tissue with broad areas of biliary infarcts and massive infiltration of leukocytes was observed in cholestatic mice (Fig 3, B). Administration of the anti-LFA-1 antibody markedly decreased this cholestatic liver damage (Fig 3, C). In fact, inhibition of LFA-1 reduced BDL-induced biliary infarcts by 62% ($P < .05$; Fig 3, D).

Hepatic leukocyte recruitment and sinusoidal perfusion. Analysis of the hepatic microcirculation by intravital fluorescence microscopy showed that numbers of rolling and adherent leukocytes in postsinusoidal venules of sham-operated controls were 2.6 ± 0.7 cells/min and 144.2 ± 21.8 cells/ mm^2 , respectively (Fig 4, A, B). BDL triggered recruitment of leukocytes in the hepatic microcirculation with a greater than 5-fold increase in the numbers of rolling and adherent leukocytes in PBS- and control IgG-treated mice ($P < .05$; Fig 4, A, B). Administration of the anti-LFA-1 antibody did not decrease the number of rolling leukocytes but did significantly decrease the number of adherent leukocytes in postsinusoidal venules to levels comparable with those of sham-operated controls (Fig 4, A, B). Thus, immunoneutralization of LFA-1

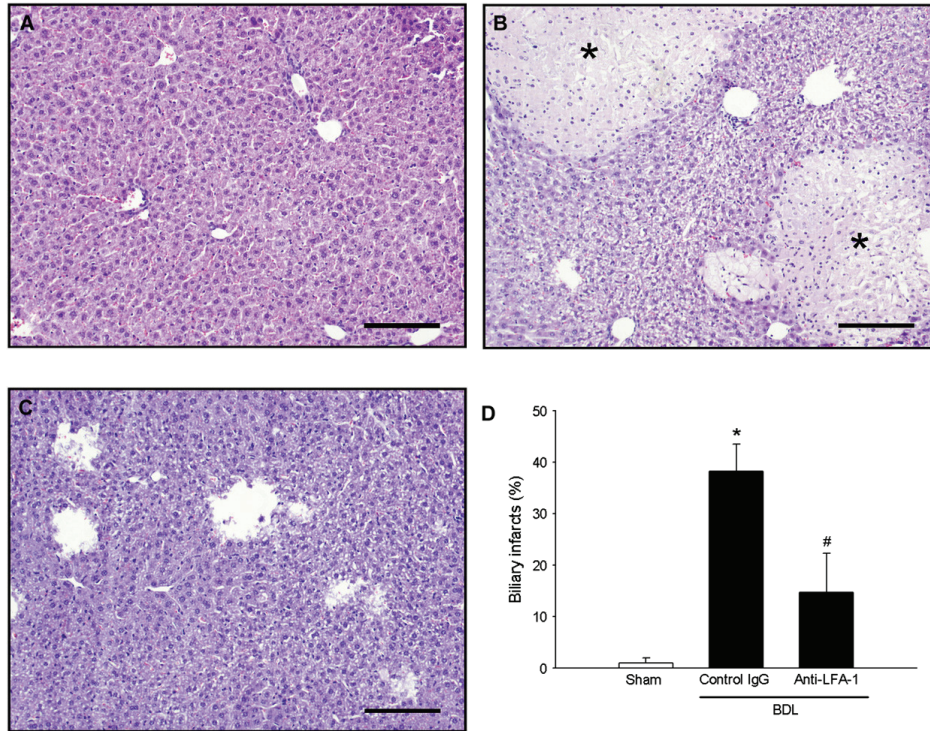


Fig 3. Hematoxylin and eosin-stained cross-sections of liver tissue harvested 12 hours after sham operation (A) or BDL (B and C). Sham-operated mice exhibited a normal hepatic architecture (A), whereas BDL in control antibody-treated animals resulted in severe destruction of the liver, with large areas of clusters of injured hepatocytes (biliary infarcts) (B, asterisks) and massive infiltration of the liver tissue with leukocytes. In contrast, pretreatment with the anti-LFA-1 antibody markedly decreased the cholestatic liver damage (C). Morphometric quantification of the percentage of biliary infarcts in the liver (D). BDL, Bile duct ligation; LFA-1, lymphocyte function antigen-1; IgG, immunoglobulin G. Mean values \pm SEM. * $P < .05$ vs sham; # $P < .05$ vs control IgG. Scale bars: 200 μ m.

decreased BDL-induced leukocyte adhesion in postsinusoidal venules by more than 90% ($P < .05$). In addition, leukocyte adhesion in sinusoids was increased in all bile duct-ligated animals ($P < .05$; Fig 4, C).

Interestingly, there were no marked differences between PBS, control IgG, and anti-LFA-1 mice in the levels of sinusoidal leukocyte adhesion (Fig 4, C). Moreover, analysis of systemic leukocyte counts demonstrated that all experimental groups presented with comparable numbers of PMNLs and MNLs in the systemic circulation (Table). In addition to the recruitment of leukocytes, BDL was accompanied with a perfusion failure of the hepatic microcirculation, as indicated by an increased number of nonperfused sinusoids in PBS (28% \pm

4%) and control IgG mice (28% \pm 2%) compared with sham-operated controls (5% \pm 1%) ($P < .05$; Fig 5). Notably, injection of the anti-LFA-1 antibody reduced this perfusion failure to 17% \pm 1% in BDL mice ($P < .05$ vs PBS and control IgG).

Hepatic formation of CXC chemokines. Leukocyte extravasation into the liver parenchyma has been reported to be directed by secreted CXC chemokines. Analysis of CXC chemokines in isolated liver tissue showed that release of MIP-2 and KC was low but detectable in the sham-operated group (Fig 6, A, B). Of interest, BDL resulted in a marked increase of hepatic MIP-2 and KC concentrations without significant differences among PBS-, control IgG-, and anti-LFA-1-treated animals (Fig 6, A, B).

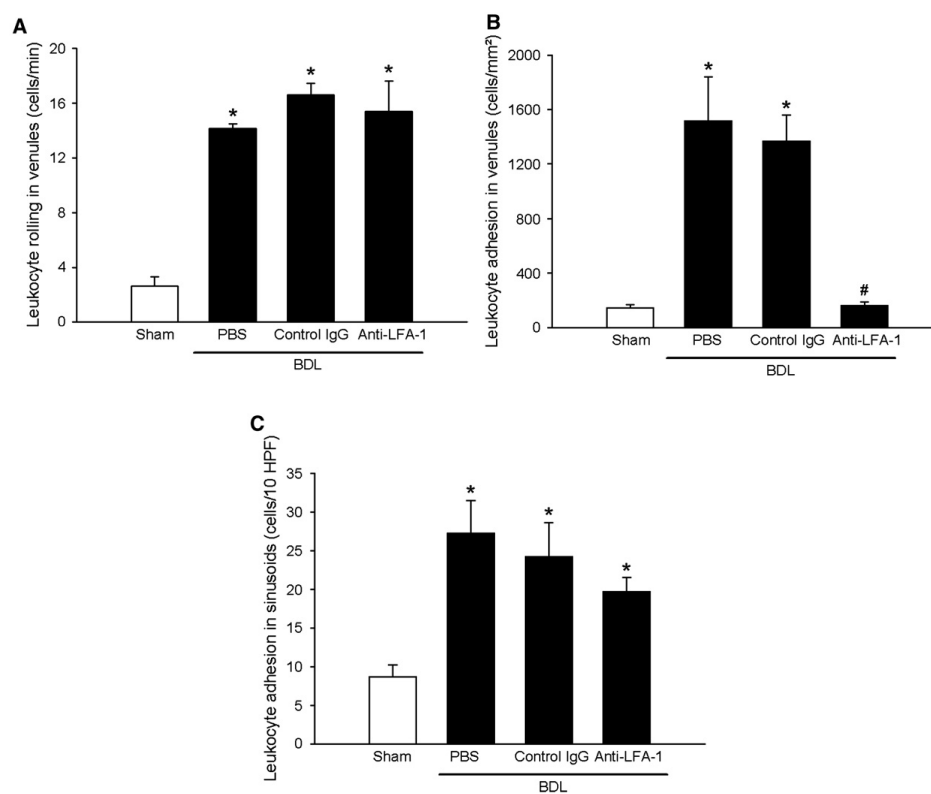


Fig 4. Rolling (A) and adherent (B) leukocytes in postsinusoidal venules and leukocyte adhesion in sinusoids (C), as assessed by intravital fluorescence microscopy 12 hours after BDL. Animals were pretreated intravenously with PBS, an isotype-matched control antibody (control IgG), or an antibody against LFA-1 (anti-LFA-1) 15 minutes prior to BDL. Sham-operated mice received only PBS. *BDL*, Bile duct ligation; *PBS*, phosphate-buffered saline; *IgG*, immunoglobulin G; *LFA-1*, lymphocyte function antigen-1. Mean values \pm SEM. * $P < .05$ vs sham; # $P < .05$ vs control IgG.

Table. Systemic leukocyte counts

| | <i>MNL</i> | <i>PMNL</i> | <i>Total</i> |
|-------------------|---------------|---------------|---------------|
| Sham | 1.6 \pm 0.1 | 0.4 \pm 0.0 | 2.0 \pm 0.1 |
| PBS + BDL | 1.7 \pm 0.2 | 0.5 \pm 0.1 | 2.2 \pm 0.3 |
| Control IgG + BDL | 1.2 \pm 0.2 | 0.3 \pm 0.0 | 1.5 \pm 0.2 |
| Anti-LFA-1 + BDL | 1.4 \pm 0.1 | 0.4 \pm 0.0 | 1.8 \pm 0.2 |

MNL, Mononuclear leukocytes; *PMNL*, polymorphonuclear leukocytes; *PBS*, phosphate-buffered saline; *BDL*, bile duct ligation; *IgG*, immunoglobulin G; *LFA*, lymphocyte function antigen-1. Systemic leukocyte counts ($\times 10^3$ cells per microliter of blood) of sham-operated (Sham) or bile duct-ligated (BDL) animals, which were treated with PBS, control antibody (Control IgG), or anti-LFA-1 (Anti-LFA-1) antibody. Leukocytes were counted in a hemacytometer and defined as MNLs or PMNLs. Mean values \pm SEM.

DISCUSSION

Obstructive cholestasis is associated with hepatocellular damage, and first-line treatment is usually endoscopic or operative decompression. Such

intervention can be insufficient or delayed, and novel approaches to ameliorate cholestatic liver injury are warranted. This study demonstrates that LFA-1 mediates cholestasis-induced leukocyte

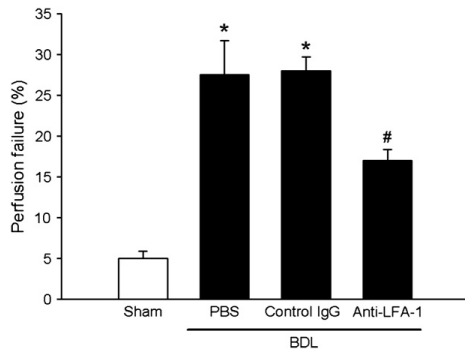


Fig 5. Perfusion failure of liver sinusoids as assessed by intravital fluorescence microscopy 12 hours after BDL. Animals were pretreated intravenously with PBS, an isotype control antibody (control IgG), or an antibody against LFA-1 (anti-LFA-1) 15 minutes prior to BDL. Sham-operated mice received only PBS. *BDL*, Bile duct ligation; *PBS*, phosphate-buffered saline; *IgG*, immunoglobulin G; *LFA-1*, lymphocyte function antigen-1. Mean values \pm SEM. * $P < .05$ vs sham; # $P < .05$ vs control IgG.

adhesion in the liver microcirculation and that inhibition of LFA-1 function prevents hepatic accumulation of leukocytes, cholestatic liver injury, and sinusoidal perfusion failure. Thus, these findings suggest that LFA-1 may represent a useful target as protection against liver damage in condition with obstructive cholestasis.

Recruitment of leukocytes is considered to be a rate-limiting step in BDL-induced liver injury.^{7,14} Leukocyte accumulation is a multistep process supported by sequential interactions between specific groups of adhesion molecules expressed on circulating leukocytes and the microvascular endothelium. The mechanistic roles of these specific adhesion molecules in cholestasis-induced leukocyte recruitment, however, remain elusive. In this study, we observed that the surface expression of LFA-1 on circulating leukocytes was increased in BDL mice, which led us to hypothesize that LFA-1 might be involved in mediating cholestatic liver injury. We used the technique of intravital fluorescence microscopy, which enables the visualization and quantification in vivo of the different stages of leukocyte-endothelial cell interactions (ie, leukocyte rolling and adhesion). By using this method, we found that inhibition of LFA-1 function decreased BDL-provoked venular attachment of leukocytes by more than 90%, suggesting that LFA-1 is a dominant adhesion molecule in supporting firm leukocyte adhesion in the liver during obstructive cholestasis.

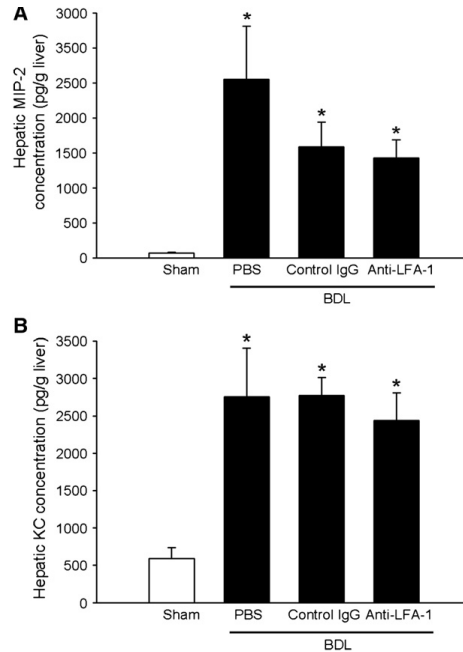


Fig 6. Hepatic levels of macrophage inflammatory protein-2 (MIP-2) (A) and cytokine-induced neutrophil chemoattractant (KC) (B) 12 hours after BDL. Animals were pretreated intravenously with PBS, an isotype control antibody (control IgG), or an antibody against LFA-1 (anti-LFA-1) 15 minutes prior to BDL. Sham-operated mice received only PBS. *BDL*, Bile duct ligation; *PBS*, phosphate-buffered saline; *IgG*, immunoglobulin G; *LFA-1*, lymphocyte function antigen-1. Mean values \pm SEM. * $P < .05$ vs sham.

Thus, we have demonstrated a critical role of LFA-1 in mediating firm adhesion of leukocytes onto postsinusoidal venules in cholestatic liver injury. This finding is in line with a previous study¹⁴ showing that ICAM-1, which is the most important receptor for LFA-1 on endothelial cells, plays an important role for BDL-induced leukocyte recruitment and liver damage. Moreover, immunoneutralization of LFA-1 had no effect on leukocyte rolling in postsinusoidal venules, which is not surprising because leukocyte rolling in the liver has been demonstrated to be predominately mediated by P-selectin.^{10,20,21} One hallmark of cholestatic liver injury is decrease in microvascular perfusion.

In this study, BDL caused widespread failure of sinusoidal perfusion, which improved in animals pretreated with the anti-LFA-1 antibody. The

relation between LFA-1–mediated leukocyte recruitment and sinusoidal perfusion is not exactly clear at present, but it may be related to the tissue protection exerted by blocking accumulation of leukocytes as observed in this study. For example, extravascular edema formation and endothelial cell swelling associated with tissue injury appear to be major determinants in causing microvascular compression and decreased organ perfusion.²² We found that administration of the anti-LFA-1 antibody decreased serum activities of ALT and AST by 46% to 70%, respectively, and improved the hepatic microscopical structure in BDL mice, suggesting that targeting LFA-1 protects the liver against severe hepatocellular damage in obstructive cholestasis. Considering the key role of leukocytes in cholestatic liver injury^{7,14} and our finding that inhibition of LFA-1 concomitantly decreased leukocyte accumulation and hepatocyte injury, we conclude that a mechanistic link exists between LFA-1–dependent leukocyte recruitment and cholestatic liver damage.

It is well-known that obstructive cholestasis induces a complex inflammatory reaction in the liver after only a few hours.⁴ This reaction involves rapid production and release of cytokines and CXC chemokines by Kupffer cells and hepatocytes; accumulating bile acids may initiate this cascade reaction.⁴ In this experimental model of obstructive cholestasis, we found that BDL mice had increased hepatic levels of MIP-2 and KC, which are murine homologues of human growth-related oncogene chemokines.^{23,24} Because these CXC chemokines attract neutrophils in particular,²⁴⁻²⁶ they are considered to be important mediators of several pathologic processes, including septic lung injury,²⁷ glomerulonephritis,²⁸ bacterial meningitis,²⁹ and endotoxemic liver injury.³⁰ Indeed, it was reported recently that MIP-2 and KC regulate in particular endotoxin-induced transmigration and extravascular tissue accumulation of leukocytes in the liver without affecting intravascular leukocyte-endothelial cell interactions. In contrast, we found that inhibition of LFA-1 had no effect on CXC chemokine production in cholestatic livers, suggesting that the decrease in leukocyte infiltration in BDL mice is not related to changes in CXC chemokine production after immunoneutralization of LFA-1.

In summary, LFA-1 mediates firm leukocyte adhesion in the liver microvasculature in obstructive cholestasis. Blockade of LFA-1–dependent leukocyte adhesion attenuates cholestatic liver injury and sinusoidal perfusion failure. Taken together, our data suggest that targeting LFA-1 may help to

protect against pathologic inflammation and liver damage in cholestatic liver diseases.

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IV

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**Rho-kinase inhibitor attenuates cholestasis-induced
CXC chemokine formation, leukocyte recruitment and
hepatocellular damage in the liver**

M.W. Laschke, M.D.,^{1,2,*} S. Dold, M.D.,^{1,2} B. Jeppsson, M.D., Ph.D.,¹ M.K.

Schilling, M.D.,³ M.D. Menger, M.D.,² and H. Thorlacius, M.D., Ph.D.¹

¹*Department of Surgery, Malmö University Hospital, Malmö, Lund University, Sweden,*

²*Institute for Clinical & Experimental Surgery, University of Saarland, Homburg/Saar,
Germany,*

³*Department of General, Visceral, Vascular and Pediatric Surgery, University of
Saarland, Homburg/Saar, Germany*

Running title: Rho-kinase signaling in cholestasis

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***Corresponding author / reprint requests:**

Dr. med. Matthias W. Laschke, M.D.

Institute for Clinical & Experimental Surgery

University of Saarland

D-66421 Homburg/Saar

Germany

phone: +49 6841 162 6554

fax: +49 6841 162 6553

e-mail: matthias.laschke@uniklinik-saarland.de

Abstract

Background. In the present experimental study, we analyzed the role of Rho-kinase during obstructive cholestasis by studying the effect of the Rho-kinase inhibitor Y-27632 on hepatic CXC chemokine formation, leukocyte recruitment and hepatocellular damage.

Materials and methods. C57BL/6 mice underwent bile duct ligation (BDL) to induce obstructive cholestasis. Mice were pretreated with Y-27632 (1 and 10 mg/kg) or the vehicle PBS. Sham-operated animals served as controls. After 12 hours, hepatic accumulation of leukocytes and sinusoidal perfusion were determined using intravital fluorescence microscopy. Hepatocellular damage was monitored by measuring serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). CXC chemokines in the liver were analyzed by ELISA.

Results. Administration of 10 mg/kg of Y-27632 protected against cholestasis-induced hepatocellular damage indicated by a more than 87% reduction of ALT and AST in BDL mice. Moreover, this Rho-kinase inhibitor significantly decreased BDL-induced production of CXC chemokines by 83% and leukocyte recruitment by 60%. Finally, treatment with Y-27632 restored sinusoidal perfusion in cholestatic animals.

Conclusions. Our findings indicate that the Rho-kinase signaling pathway plays a key role in the pathophysiology of cholestatic liver injury. Thus, targeting Rho-kinase activity may represent a new therapeutic approach in the treatment of inflammation and liver injury in cholestatic liver diseases.

Key Words: Cholestasis, Rho-kinase, bile duct ligation, leukocytes, CXC chemokines, Y-27632, intravital fluorescence microscopy

Introduction

Cholestasis triggers immediate liver injury characterized by hepatocellular necrosis, microvascular perfusion failure, which over-time may result in liver fibrosis. During the last years, it has become evident that cholestatic liver injury results from hepatic inflammation, which is initiated by the accumulation of hydrophobic bile acids in the liver tissue [1,2]. This, in turn, leads to Kupffer cell activation with increased production and release of pro-inflammatory mediators [3,4] and subsequent leukocyte recruitment to the liver [5-8]. In this process, it has been shown that CXC chemokines, i.e. macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC) are important regulators of leukocyte extravasation in the liver [9]. However, the intracellular signaling pathways underlying this inflammatory reaction are not well understood.

Interestingly, Zhou et al. could demonstrate that expression of Rho-kinase is strongly upregulated in the liver during chronic cholestasis, which may be of importance for regulating hepatic vascular resistance [10]. The small G-protein Rho and its downstream effector Rho-kinase act as important molecular switches regulating a variety of essential cellular functions, including actin cytoskeleton organization, cell adhesion, migration and proliferation [11-14]. Moreover, the Rho/Rho-kinase pathway has been implicated in several inflammatory processes, such as chemokine expression and leukocyte-endothelial cell interactions [15,16]. Accordingly, inhibition of Rho-kinase has been shown to be effective against liver injury in models of ischemia-reperfusion [17] and sepsis [16,18] as well as carbon tetrachloride-induced hepatic fibrosis [19] and cell death [20]. However, the potential role of Rho-kinase in cholestasis-induced inflammation in the liver remains elusive.

Based on these findings, we hypothesized that the Rho-kinase signalling pathway may also play a crucial role in hepatic inflammation associated with obstructive

cholestasis. Therefore, the aim of our present *in vivo* study was to analyze the effect of Y-27632, a Rho-kinase inhibitor, on hepatic CXC chemokine formation, leukocyte recruitment and hepatocellular damage under cholestatic conditions. For this purpose, Y-27632-treated mice underwent bile duct ligation (BDL), which represents a well established experimental approach to induce obstructive cholestasis in rodents [6-8].

Materials and Methods

Animals

Adult male C57BL/6 mice with a body weight of 25-27g were used for the study. The animals were housed one per cage on a 12-12 hour light-dark cycle and had free access to standard pellet food and tap water. The mice 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. Fluorescent dyes were administered intravenously (i.v.) via retroorbital injection. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, USA), and were approved by the local ethics committee at Lund University.

Experimental protocol

BDL to induce obstructive cholestasis was performed under ketamine/xylazine anesthesia via a midline laparotomy. Using a surgical microscope, the common bile duct was prepared and carefully ligated with a 7-0 Prolene suture. Finally, the laparotomy was closed again by a 5-0 running suture and the animals were allowed to recover from anesthesia and surgery for 12 hours before the livers were analyzed. Sham operated animals underwent an identical laparotomy and liver manipulation without BDL (sham; n=7). To analyze the role of Rho-kinase in hepatic inflammation associated with obstructive cholestasis, mice were pretreated 15 min before BDL with 1 mg/kg (n=6) and 10 mg/kg (n=7) of the Rho-kinase inhibitor Y-27632. Bile duct ligated animals, which received PBS only, served as positive controls (PBS; n=5).

Intravital fluorescence microscopy

Twelve hours after BDL, analysis of the hepatic microcirculation and hepatocellular apoptosis was performed by means of intravital fluorescence microscopy. For this purpose, the laparotomy of the anesthetized mice was opened again. Moreover, a transverse subcostal incision was performed and the ligamentous attachments from the liver to the diaphragm and the abdominal wall were gently released. Subsequently, the mice were positioned on their left side and the left liver lobe was carefully exteriorized onto an adjustable stage for the microscopic analysis. An equilibration period of 5 min was allowed before starting the microscopical observation. For intravital fluorescence microscopy, we used a modified Olympus microscope (BX50WI, Olympus Optical Co. GmbH, Hamburg, Germany) equipped with different water immersion lenses (x40 NA 0.75; x63 NA 0.9). The microscopic images were recorded by a charge-coupled device video camera (FK 6990 Cohu, Pieper GmbH, Schwerte, Germany) and transferred to CD-ROM for off-line evaluation. Blood perfusion within individual microvessels was studied after i.v. injection of 0.1ml 5% fluorescein isothiocyanate (FITC)-labeled dextran 150,000 (contrast enhancement by intravascular staining of plasma; Sigma Chemical Co., St. Louis, MO, USA). In vivo labeling of leukocytes with 0.1% rhodamine-6G (0.1ml i.v., Sigma Chemical Co.) enabled quantitative analysis of leukocyte flow behaviour in both sinusoids and postsinusoidal venules. Five postsinusoidal venules with connecting sinusoids were evaluated in each animal. Microcirculatory analysis included determination of sinusoidal perfusion by measuring the number of non-perfused sinusoids given as a percentage of the total number of sinusoids observed. Within sinusoids and postsinusoidal venules, leukocyte adhesion was measured by counting the number of cells that adhered along the endothelium without moving during the observation period of 20 s and is expressed as cells/mm² or cells/10 HPF (high power fields), respectively. Rolling leukocytes were defined as cells moving with a velocity less than two-fifths of the centerline velocity within postsinusoidal venules, and are given as

cells/min passing a reference point within the microvessel.

After intravital fluorescence microscopy, blood was drawn from the inferior vena cava for analysis of the serum levels of bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as serological indicators of hepatocellular injury, using standard spectrophotometric procedures. Moreover, systemic leukocyte counts, including polymorphonuclear leukocytes (PMNL) and monomorphonuclear leukocytes (MNL), were determined with a hematocytometer.

Enzyme-linked immunosorbent assay (ELISA)

To quantify hepatic levels of CXC chemokines an ELISA technique was used. For this purpose, 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 (DMEM). After centrifugation, supernatants were collected and stored in -20°C until analysis of CXC chemokines, including macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC), by the use of double antibody Quantikine ELISA kits (R&D Systems) using recombinant murine KC and MIP-2 as standards. The minimal detectable protein concentrations were less than 0.5 pg/ml.

Histology

After the intravital microscopic analysis, tissue samples were taken from the left liver lobe 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. For histological quantification of hepatocellular injury ('biliary infarcts'), five random high-power fields were evaluated by use of a semiautomatic image analysis system (NIS-Elements Advanced Research, Nikon Corporation, Kanagawa, Japan) and

findings are given as a percentage of liver surface as described previously [21].

Statistics

All data are presented as mean values \pm SEM. Statistical evaluations were performed using Kruskal-Wallis one way analysis of variance on ranks followed by the appropriate post-hoc test (Bonferroni) compensating for multiple comparisons (SigmaStat; Jandel Corporation, San Rafael, CA, USA).

Results

Liver injury

BDL significantly increased serum levels of bilirubin (~30 μM) when compared to sham-operated controls (~12 μM ; $P=0.002$ vs. PBS, $P<0.001$ vs. Y-27632 1mg/kg, $P=0.001$ vs. Y-27632 10mg/kg) (Fig. 1A), suggesting that BDL is an appropriate approach to induce obstructive cholestasis under experimental conditions. Notably, serum levels of bilirubin were similar in all BDL groups, indicating that the different treatment protocols did not affect the level of cholestasis in BDL mice. Cholestasis resulted in severe hepatocellular damage, as indicated by a massive release of liver enzymes. Thus, serum levels of ALT and AST were 0.5 ± 0.0 and 1.2 ± 0.1 $\mu\text{kat/l}$ in sham mice and 55.7 ± 11.3 ($P<0.001$ vs. sham) and 90.1 ± 18.3 $\mu\text{kat/l}$ ($P=0.001$ vs. sham) in BDL animals, respectively (Figs. 1B and C). Notably, it was found that administration of 10 mg/kg of the Rho-kinase inhibitor Y-27632 almost completely abolished the increase of liver enzymes, reducing ALT and AST levels down to 4.4 ± 1.8 ($P<0.001$ vs. PBS) and 11.4 ± 4.0 $\mu\text{kat/l}$ ($P=0.002$ vs. PBS), respectively, in cholestatic animals (Figs. 1B and C). Thus, this inhibition of Rho-kinase signaling decreased liver enzyme release by more than 87% in BDL mice. Histological examination of isolated liver tissues revealed that sham-operated animals exhibited a normal hepatic architecture (Fig. 2A). In contrast, BDL caused a severe destruction of the liver microarchitecture in PBS-treated mice with broad areas of necrosis (Fig. 2B). In fact, the percentage of biliary infarcts in BDL mice receiving PBS was $28.1 \pm 5.9\%$ of the liver surface. This cholestasis-induced tissue necrosis was significantly reduced to $8.1 \pm 4.8\%$ ($P=0.031$) in BDL animals receiving 10mg/kg of the Rho-kinase inhibitor Y-27632 (Figure 2C). Thus, inhibition of Rho-kinase reduced cholestasis-induced biliary infarcts by 71%.

Leukocyte recruitment and sinusoidal perfusion

Intravital fluorescence microscopy allows for the detailed investigation of the different steps in the recruitment process of leukocytes into the liver. In sham-operated control animals, we could detect only minor leukocyte rolling (4.3 ± 1.4 cells/min) and adhesion (161.9 ± 65.9 cells/mm²) in postsinusoidal venules (Figs. 3A and B). In contrast, BDL significantly increased leukocyte rolling and adhesion in PBS-treated mice. Thus, the numbers of rolling and adherent leukocytes were 19.4 ± 2.5 cells/min ($P < 0.001$ vs. sham) and 1267.3 ± 178.0 cells/mm² ($P < 0.001$ vs. sham), respectively, in cholestatic mice (Figs. 3A and B). Of interest, treatment with the Rho-kinase inhibitor Y-27632 (10 mg/kg) reduced BDL-induced leukocyte rolling and adhesion by 54% ($P = 0.001$ vs. PBS) and 60% ($P = 0.002$ vs. PBS), respectively (Figs. 3A and B). Comparable results were found for leukocyte trapping in liver sinusoids. Numbers of stationary leukocytes in sinusoids were found to be significantly ($P < 0.001$) increased in BDL animals when compared to sham-operated controls (Fig. 3C). This leukocytic response to obstructive cholestasis was also inhibited by treatment with 10 mg/kg of Y-27632 (Fig. 3C). Analysis of systemic leukocyte counts demonstrated that all experimental groups presented with comparable numbers of PMNLs and MNLs (Table 1). In addition to the recruitment of leukocytes, BDL was accompanied with a profound perfusion failure of the hepatic microcirculation, as indicated by significantly increased numbers of non-perfused sinusoids in PBS-treated mice ($30.0 \pm 3.7\%$ vs. $5.3 \pm 0.7\%$ in sham operated animals; $P < 0.001$) (Fig. 4). Interestingly, it was observed that application of 10mg/kg of Y-27632 could restore microvascular perfusion by reducing the percentage of non-perfused sinusoids down to $11.2 \pm 0.8\%$ ($P < 0.001$ vs. PBS) in cholestatic animals (Fig. 4).

CXC Chemokines

CXC chemokines are important regulators of leukocyte trafficking in the liver [9]. Analysis of CXC chemokines in isolated liver tissue showed that release of MIP-2 and

KC was low but detectable in sham operated mice (Figs. 5A and B). In contrast, it was observed that BDL resulted in a massive increase of hepatic CXC chemokines levels in PBS-treated animals, i.e. MIP-2 increased by 18-fold ($P=0.009$) and KC increased by 10-fold ($P=0.006$) when compared to sham operated mice (Figs. 5A and B). We found that pretreatment with 10mg/kg of the Rho-kinase inhibitor Y-27632 reduced BDL-induced formation of MIP-2 by 83% ($P=0.024$ vs. PBS) and KC by 44% (Figs. 5A and B).

Discussion

Endoscopic and surgical decompression are the main approaches to relieve obstructive cholestasis but may not be sufficient to prevent hepatic injury and septic complications. Therefore, pathophysiologic studies are required to define mechanisms behind cholestasis-provoked liver damage. In the present study, we analyzed for the first time the role of the Rho-kinase signaling pathway in cholestasis-induced hepatic inflammation. We found that inhibition of Rho-kinase markedly reduced CXC chemokine formation, leukocyte recruitment, perfusion failure and hepatocellular damage in cholestatic mice. Based on our new findings, we suggest that Rho-kinase plays a key role in the pathophysiology of cholestatic liver injury. Thus, targeting the Rho-kinase signaling pathway may represent a novel therapeutic strategy in the management of inflammation and liver damage associated with obstructive jaundice.

Although Rho-kinase-mediated signaling has mainly been described in conjunction with several actomyosin-based cellular processes, such as cell migration, cytokinesis and contraction [22], it has become increasingly evident during the last years that the Rho-kinase signalling pathway is also involved in several pathological conditions within different organ systems. For example, two recent studies reported that Rho-kinase activity is increased during cholestasis and that Rho-kinase plays an important role in regulating portal pressure in rats with secondary biliary cirrhosis [10,23]. Our study extends on these findings by showing for the first time that inhibition of Rho-kinase signalling dose-dependently protects against cholestasis-induced hepatic injury. In fact, we found, herein, that administration of 10mg/kg of the Rho-kinase inhibitor Y-27632 decreased BDL-provoked increases in the serum levels of ALT and AST by more than 87% as well as the percentage of biliary infarcts by 71%, suggesting that Rho-kinase signalling constitutes a prominent role in cholestasis-induced hepatocellular damage.

The activity of Rho-kinase may be blocked by several compounds. For our study, we used the pyridine derivative Y-27632, which inhibits the kinase activity of both Rho-kinase-1 and -2 by competing with ATP for binding to their catalytic sites [24]. By now, Y-27632 is a well established pharmacological agent to analyze the involvement of Rho-kinase in the pathogenesis of various diseases [25]. Our present findings add now cholestatic liver injury to the list of diseases in which Rho-kinase appears to regulate tissue damage, including ischemia-reperfusion damage of the liver [17] and heart [26], septic liver injury [16,18], cerebral ischemia [27,28] and pulmonary hypertension [29].

Accumulating data in the literature suggest that hepatic accumulation of leukocytes constitutes a rate-limiting step in liver damage associated with bile flow obstruction, which is based on the hepatoprotective effect exerted by targeting of specific adhesion molecules regulating leukocyte-endothelium interactions in the liver [6,7]. Accumulation of activated leukocytes in the liver tissue results in the release of large amounts of reactive oxygen species, which has been described as a major pathophysiologic mechanism in acute cholestatic liver injury [6]. In the present study, we found that treatment with the Rho-kinase inhibitor Y-27632 significantly decreased leukocyte rolling and adhesion in postsinusoidal venules as well as leukocyte trapping in liver sinusoids of cholestatic animals. In fact, inhibition of Rho-kinase reduced BDL-induced leukocyte adhesion by 60%, which correlates very well with the 87% reduction in the serum activities of ALT and AST observed in cholestatic mice pretreated with the Rho-kinase inhibitor Y-27632. The cholestatic inflammatory reaction is characterized by activation of Kupffer cells, resulting in an increased production and release of pro-inflammatory mediators [3,4]. Considering that secretion of CXC chemokines regulates leukocyte trafficking into the inflamed liver [9], it is interesting to note that BDL caused a clear-cut increase in the formation of MIP-2 and KC, which is in line with previous observations [8]. Herein, we could also demonstrate that inhibition of Rho-kinase effectively reduces

this cholestasis-associated release of CXC chemokines. Thus, this role of Rho-kinase in controlling MIP-2 and KC production in cholestatic livers may help explain the inhibitory effect of Y27632 on BDL-induced leukocyte accumulation observed in the present study. In this context, it should be mentioned that beside MIP-2 and KC, BDL also up-regulates the expression of IFN-inducible protein-10 (IP-10) and monocyte chemoattractant protein-1 (MCP-1) in the liver [30,31]. Accordingly, inhibition of Rho-kinase may also affect their expression during obstructive cholestasis. However, the functional role of these chemokines in cholestatic liver injury remains to be determined.

Microcirculatory changes are characteristic of cholestatic liver damage. Acute cholestasis is characterized by a marked increase in the number of non-perfused sinusoids, which may play a role in the pathophysiology of cholestasis-provoked hepatic damage. Indeed, we observed that BDL significantly increased the number of non-perfused sinusoids. Interestingly, administration of the Rho-kinase inhibitor Y-27632 significantly improved the microvascular perfusion in BDL mice. Since leukocytes trapped in the sinusoids may interfere with the blood flow in the narrow sinusoids, it may be suggested that the reduced sinusoidal accumulation of leukocytes may partially explain the restored microvascular perfusion in the livers of cholestatic animals. In this context, it is interesting that recent reports have shown that Rho-kinase is not only up-regulated in chronic bile flow obstruction but also that Rho-kinase controls contraction of microvessels in the liver and accordingly portal pressure in secondary biliary cirrhosis [10,23], which may be a complementary or alternative explanation for the improved sinusoidal perfusion observed in cholestatic mice treated with the Rho-kinase inhibitor. In the study by Anegawa et al. [23], it was shown that Rho-kinase activation in chronic cholestasis causes defective endothelial nitric oxide function and subsequently increased intrahepatic vascular resistance. Whether alterations in endothelial nitric oxide synthase may play a role in acute cholestatic liver injury is currently under investigation

in our laboratory. Thus, inhibition of the Rho-kinase signaling pathway may improve hepatic blood perfusion in two distinctly different ways. On the one hand inhibition of Rho-kinase attenuates leukocyte-dependent obstruction and on the other hand normalizes intrahepatic vascular resistance, which may be of benefit for patients with cholestatic liver disease.

In summary, the present study demonstrates for the first time that the Rho-kinase signaling pathway plays a key role in the pathophysiology of acute cholestatic liver injury. In fact, inhibition of Rho-kinase by Y-27632 attenuated CXC chemokine formation, leukocyte recruitment, hepatocellular damage and microvascular perfusion failure in the liver. Our novel findings suggest that targeting Rho-kinase activity may represent a new therapeutic approach in the treatment of inflammation and liver damage in cholestatic liver diseases.

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Figure legends

Figure 1: Bilirubin and liver enzymes 12 hours after ligation of the common bile duct. Mice were pretreated i.v. with PBS or Y-27632 (1 and 10 mg/kg) 15 min before bile duct ligation (BDL). Sham-operated mice served as controls. Levels of bilirubin (A), alanine aminotransferase (ALT) (B) and aspartate aminotransferase (AST) (C) were determined spectrophotometrically. Means \pm SEM. ^aP=0.002 vs. sham; ^bP<0.001 vs. sham; ^cP=0.001 vs. sham; ^dP<0.001 vs. sham; ^eP=0.011 vs. sham; ^fP<0.001 vs. PBS; ^gP=0.001 vs. sham; ^hP=0.003 vs. sham; ⁱP=0.002 vs. PBS.

Figure 2: H&E histology of liver tissue harvested 12 hours after sham operation (A) or ligation of the common bile duct (B, C). Sham-operated mice exhibited a normal hepatic architecture (A), whereas BDL in PBS-treated animals resulted in a severe liver damage with broad areas of necrosis (B, asterisks). In contrast, pretreatment with Y-27632 (10 mg/kg) markedly reduced cholestatic liver injury (C). Scale bars: 200 μ m.

Figure 3: Rolling (A) and adherent leukocytes (B) in postsinusoidal venules and leukocyte adhesion in sinusoids (C), as assessed by intravital fluorescence microscopy 12 hours after ligation of the common bile duct. Animals were pretreated i.v. with PBS or Y-27632 (1 and 10 mg/kg) 15 min before bile duct ligation (BDL). Sham operated mice served as controls. ^aP<0.001 vs. sham; ^bP=0.001 vs. sham; ^cP=0.001 vs. PBS; ^dP<0.001 vs. sham; ^eP<0.001 vs. sham; ^fP<0.002 vs. PBS; ^gP<0.001 vs. sham; ^hP<0.001 vs. sham; ⁱP<0.001 vs. PBS.

Figure 4: Perfusion failure of liver sinusoids, as assessed by intravital fluorescence microscopy 12 hours after ligation of the common bile duct. Animals were pretreated i.v. with PBS or Y-27632 (1 and 10 mg/kg) 15 min before bile duct ligation (BDL). Sham

operated mice served as controls. Means \pm SEM. ^aP<0.001 vs. sham; ^bP<0.001 vs. sham; ^cP<0.001 vs. PBS.

Figure 5: Hepatic levels of macrophage inflammatory protein-2 (MIP-2) (A) and cytokine-induced neutrophil chemoattractant (KC) (B) 12 hours after ligation of the common bile duct. Animals were pretreated i.v. with PBS or Y-27632 (1 and 10 mg/kg) 15 min before bile duct ligation (BDL). Sham operated mice served as controls. Means \pm SEM. ^aP=0.009 vs. sham; ^bP=0.010 vs. sham; ^cP=0.024 vs. PBS; ^dP=0.006 vs. sham; ^eP=0.009 vs. sham.

Table 1. Systemic leukocyte counts.

| Systemic leukocyte counts (x10³ cells/μl blood) | | | |
|--|---------------|---------------|---------------|
| | <i>MNL</i> | <i>PMNL</i> | <i>Total</i> |
| Sham | 1.6 \pm 0.1 | 0.4 \pm 0.0 | 2.0 \pm 0.2 |
| BDL + PBS | 1.6 \pm 0.2 | 0.4 \pm 0.1 | 2.0 \pm 0.2 |
| BDL + 1 mg/kg Y-27632 | 1.5 \pm 0.2 | 0.6 \pm 0.1 | 2.1 \pm 0.2 |
| BDL + 10 mg/kg Y-27632 | 1.3 \pm 0.1 | 0.4 \pm 0.1 | 1.6 \pm 0.1 |

Systemic leukocyte counts of sham-operated (Sham) or bile duct-ligated animals, which were treated with PBS (BDL + PBS) or Y-27632 (1 and 10 mg/kg; BDL + Y-27632). Leukocytes were counted in a hemacytometer and defined as mononuclear (MNL) or polymorphonuclear cells (PMNL). Means \pm SEM.

Figure 1

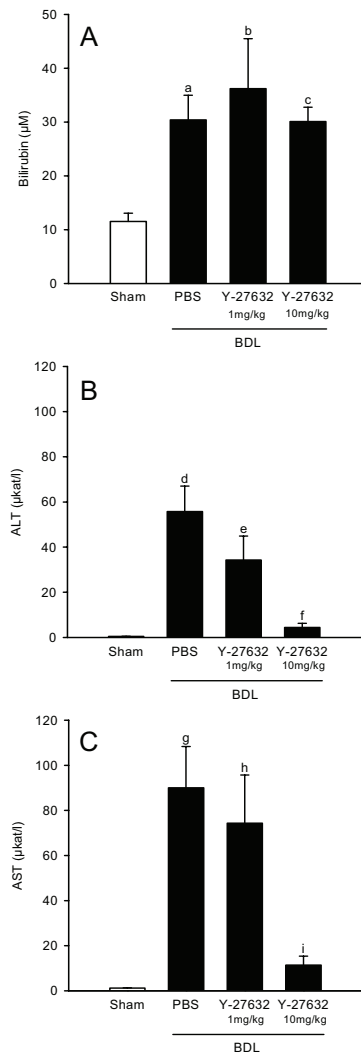


Figure 2

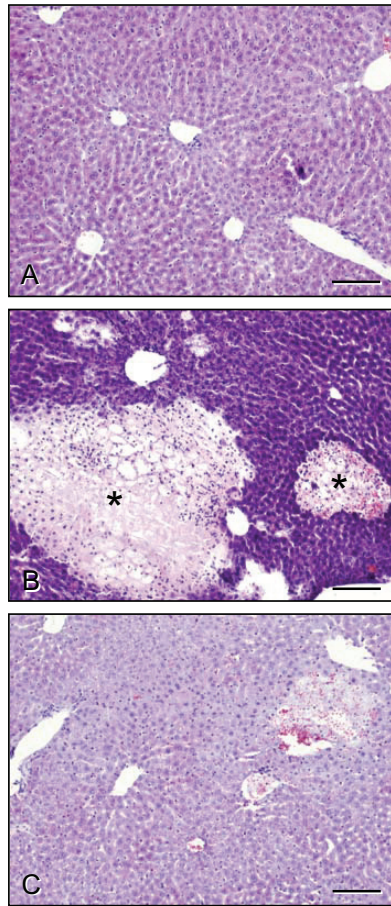


Figure 3

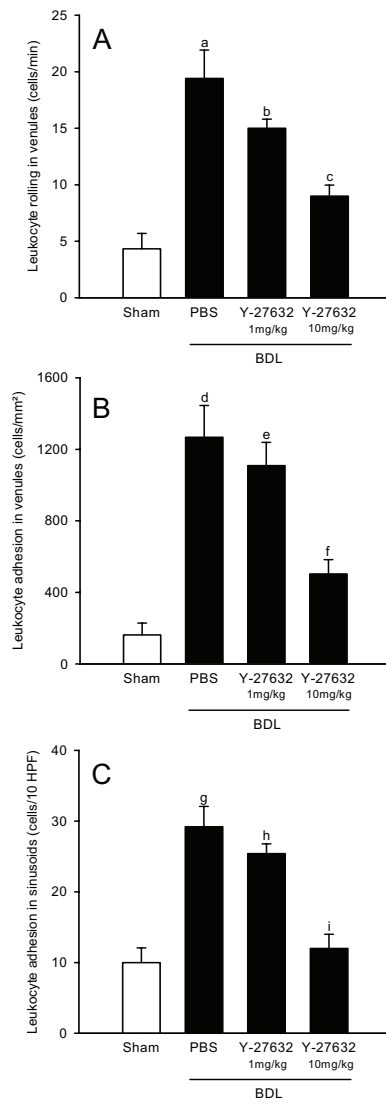


Figure 4

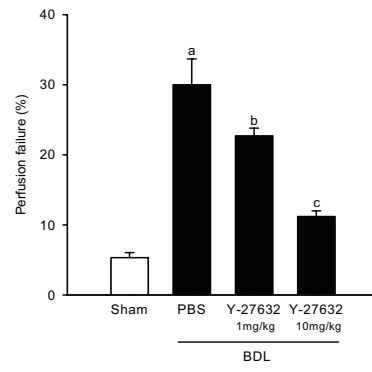


Figure 5

