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RESEARCH PAPER

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

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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-GP1bα 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-GP1bα 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 homoeostatic 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)

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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 pretreated i.v. 2 h prior to BDL with an anti-GP1bα 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 pg ml^{-1}.

Statistics

All data are presented as mean values ± 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-GP1bα 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-GP1bα 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-GP-1bα 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 ± 0.03 U g^{-1} in the liver (Figure 2, $P<0.05$ vs sham, $n=7$–10). Platelet depletion by anti-GP1bα decreased MPO levels to 0.10 ± 0.01 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-GP-1bα 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-GP-1bα 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 HPF, 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).

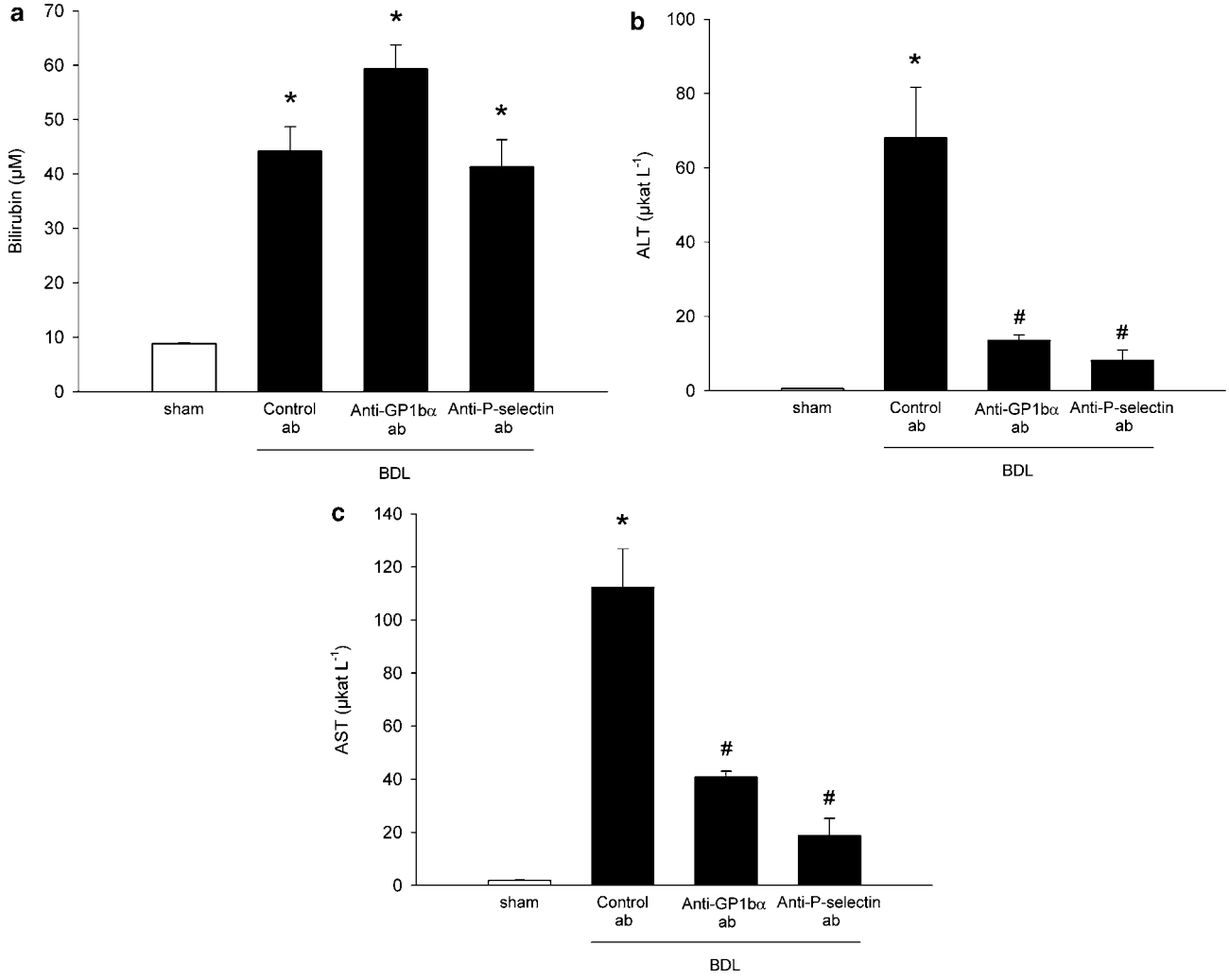

Figure 1 Bilirubin and liver enzymes 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1bα (anti-GP1bα ab) or against P-selectin (anti-P-selectin ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. The levels of (**a**) bilirubin (**b**) alanine aminotransferase (ALT) and (**c**) aspartate aminotransferase (AST) were determined spectrophotometrically. Data represent means ± 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 ± 34.6	1.7 ± 0.3
Control ab + BDL	331 ± 28.5	1.5 ± 0.2
Anti-GP1 ab + BDL	40 ± 5.9*	1.2 ± 0.2
Anti-P-selectin ab + BDL	330 ± 22.9	1.7 ± 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-GP1bα ab or an anti-P-selectin ab prior to induction of BDL. Animals not exposed to BDL served as sham. Data are means ± 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 ± 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 ± 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 ± 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-1bα 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).

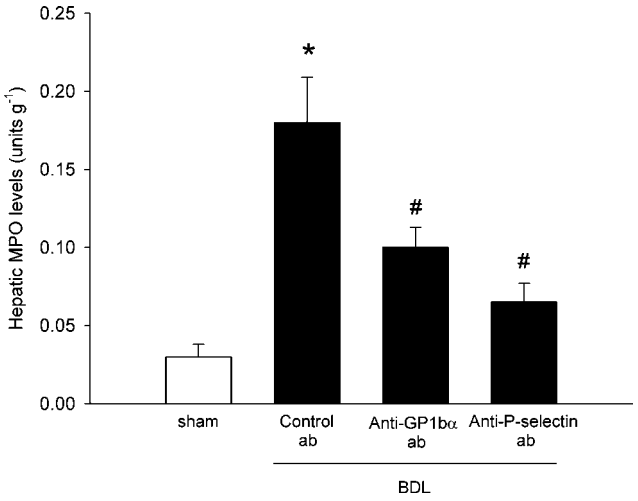

Figure 2 Hepatic levels of myeloperoxidase (MPO) 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1bα (anti-GP1bα ab) or against P-selectin (anti-P-selectin ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. Data represent means ± 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-1bα 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).

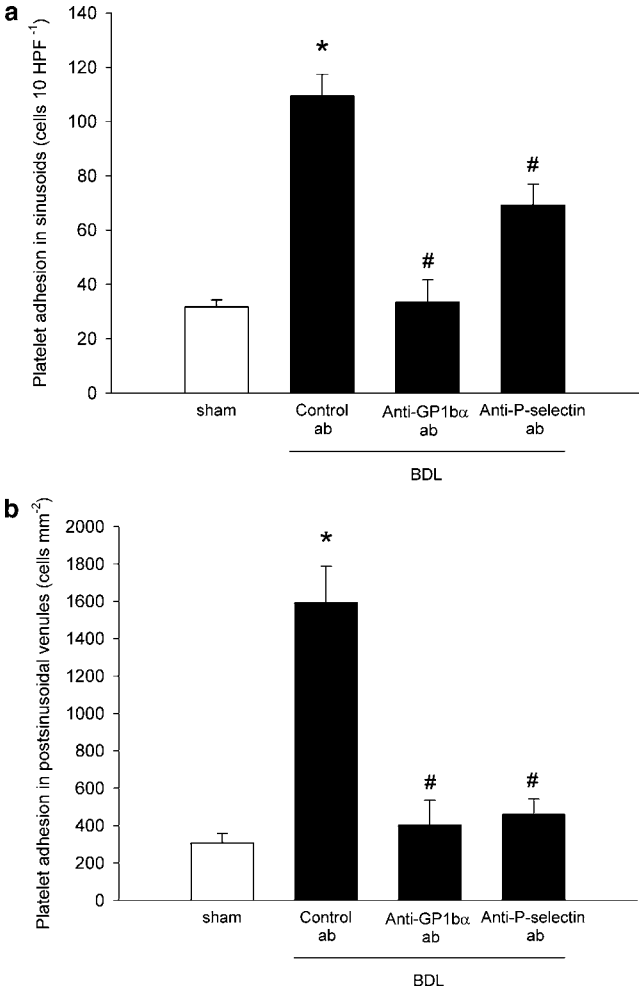

Figure 3 Platelet adhesion in (**a**) sinusoids and (**b**) postsinusoidal venules 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1bα (anti-GP1bα ab) or against P-selectin (anti-P-selectin ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. Data represent means ± s.e.mean ($n = 7$–8). *$P < 0.05$ vs sham and $^{\#}P < 0.05$ vs Control ab + BDL.

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 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

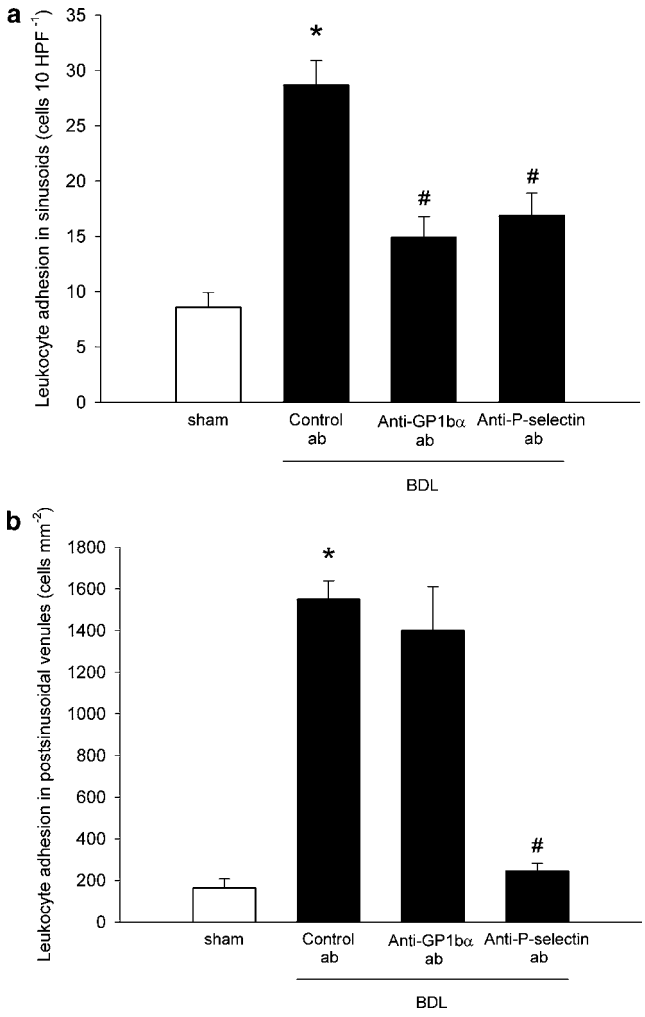

Figure 4 Leukocyte adhesion in (**a**) sinusoids and (**b**) postsinusoidal venules 12 h ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1bα (anti-GP1bα ab) or against P-selectin (anti-P ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. Data represent means ± 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.

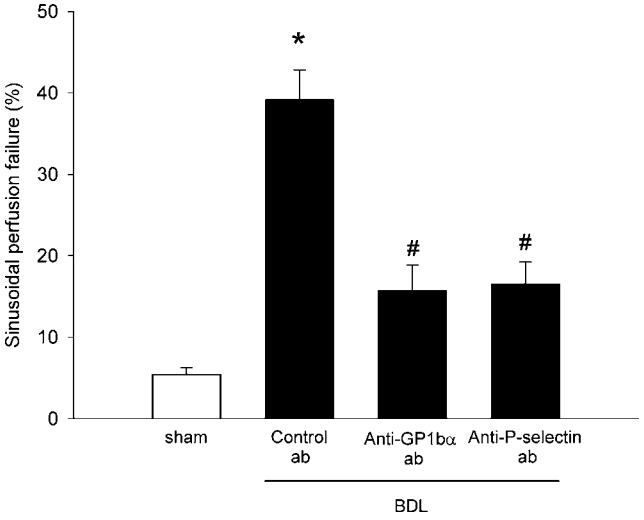

Figure 5 Sinusoidal perfusion failure 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1bα (anti-GP1bα ab) or against P-selectin (anti-P-selectin ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. Data represent means ± 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

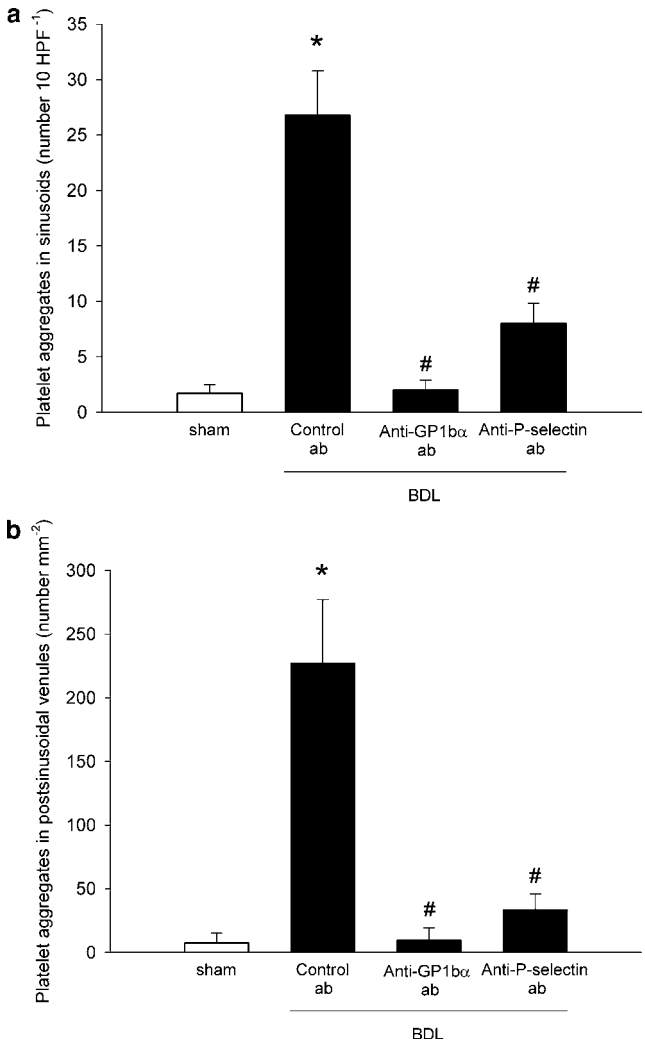

Figure 6 Platelet aggregates in (**a**) sinusoids and (**b**) postsinusoidal venules 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1bα (anti-GP1bα ab) or against P-selectin (anti-P-selectin ab) prior to bile 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 ± s.e.mean ($n = 7$–8). *$P < 0.05$ vs sham and $^{\#}P < 0.05$ vs Control ab + BDL.

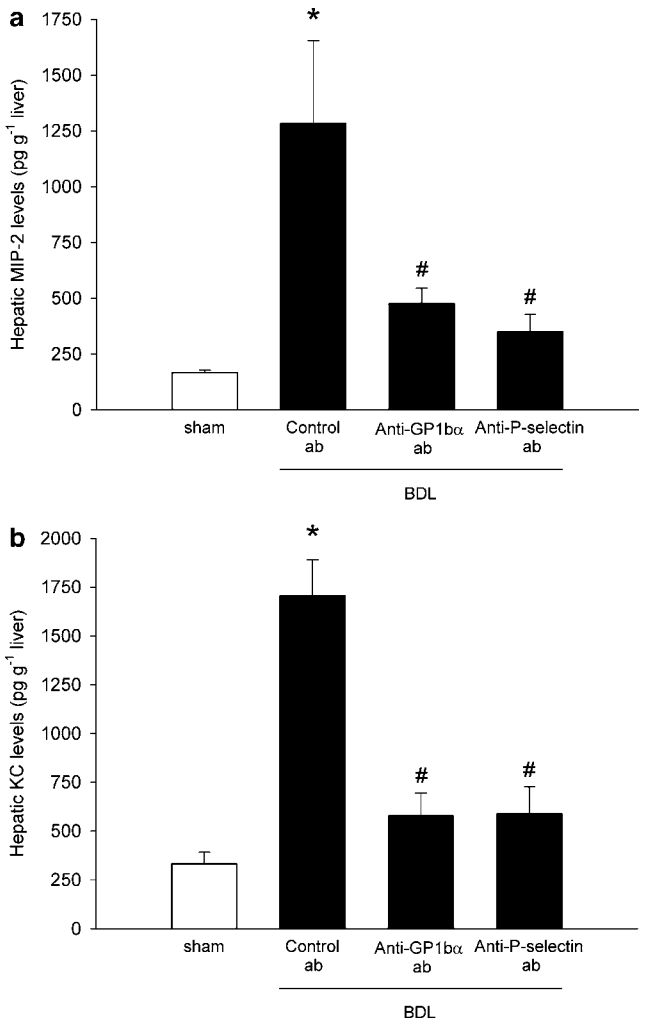

Figure 7 Hepatic levels of (**a**) macrophage inflammatory protein-2 (MIP-2) and (**b**) cytokine-induced neutrophil chemoattractant (KC) 12 h after ligation of the common bile duct. Mice were pretreated i.v. with an iso-type control antibody (Control ab), an antibody against GP1bα (anti-GP1bα ab) or against P-selectin (anti-P-selectin ab) prior to bile duct ligation (BDL). Sham animals received only phosphate-buffered saline. Data represent means ± s.e.mean ($n = 6$–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 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 postsinusoidal 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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