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Platelet C-Type Lectin-Like Receptor 2 Reduces Cholestatic Liver Injury in Mice

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Cholestatic liver injury leads to liver dysfunction. The available evidence suggests that platelets can either promote or reduce liver injury and fibrosis. This study focused on the functions of the C-type lectin-like receptor 2 (CLEC-2), a new special platelet receptor that binds with podoplanin-activating platelets. The role of CLEC-2 and podoplanin in cholestatic liver injury was investigated. Mice were injected intraperitoneally with weekly doses of anti-CLEC-2 antibody (2A2B10) to achieve effective CLEC-2 inhibition in their platelets. Next, left and middle hepatic bile duct ligation (BDL) procedures were performed, and mice were euthanized 1 week later (2A2B10-BDL group). In addition, mice were prepared for control groups, and relevant histological and laboratory variables were compared among these groups. The inhibition of CLEC-2 resulted in increasing hepatocellular necrosis, hepatic inflammation, and liver fibrosis. In addition, podoplanin was strongly expressed in hepatic sinusoidal endothelial cells in BDL-treated mice. Moreover, in 2A2B10-BDL mice, total plasma bile acid levels were significantly increased. In summary, podoplanin is expressed on hepatic sinusoidal endothelial cells upon BDL. Platelets bind with podoplanin via CLEC-2 and become activated. As a result, the total bile acid pool is decreased. Therefore, the CLEC-2–podoplanin interaction promotes liver protection and inhibits liver fibrosis after cholestatic liver injury. (*Am J Pathol* 2020, 190: 1833–1842; <https://doi.org/10.1016/j.ajpath.2020.05.009>)

Cholestatic liver injury due to bile duct obstruction occurs as a result of clinical conditions such as gallstone impaction, biliary atresia, and tumor compression.¹ Bile duct obstruction increases the rate of postoperative complications, including bacteremia, sepsis, gastrointestinal bleeding, immunologic dysfunction, and mortality.^{2–5} In general, cholestatic liver injury involves interplay between chemokines, toxic bile acids, inflammatory cells, endotoxemia, reactive oxygen species, and mitochondrial permeability transition changes.^{6–8} Cholestasis results in the accumulation of hydrophobic bile acid, and hepatic inflammation (a consequence of the initial injury) causes release of several types of cytokines and chemokines.^{9–12} However, the pathologic and molecular process is complex, and the details are still unknown.

Platelets have been shown to both reduce cholestatic liver damage and promote liver injury in different studies. Some reports showed that protease-activated receptors (PARs) promote liver fibrosis.^{13,14} However, platelets have been

shown to minimize cholestatic liver damage, promote liver repair, and inhibit liver fibrosis under some experimental liver injury settings. For example, Joshi et al¹⁵ reported that PAR-4, a key thrombin receptor on mouse platelets, exerts hepatoprotective effects during chronic cholestasis. We instead focused on another platelet receptor, the C-type lectin receptor (CLEC-2), which is a newly discovered platelet receptor for the platelet-activating snake venom rhodocytin.¹⁶ CLEC-2 plays important roles during thrombosis/hemostasis, tumor metastasis, and lymphangiogenesis based on the following findings: thrombus formation was inhibited in CLEC-2–deficient mice¹⁷; podoplanin (an endogenous ligand for CLEC-2 on the surface of tumor cells) facilitates tumor metastasis by inducing platelet aggregation^{18,19}; and lymphatic vessel pattern formation is absent in podoplanin-deficient mice.²⁰ Moreover, CLEC-2

Disclosures: None declared.

expression has been shown to be correlated with liver regeneration after partial hepatectomy in mice.²¹

Thus, the roles of CLEC-2 in thrombosis/hemostasis, tumor metastasis, and lymphangiogenesis have been investigated in detail, but its function in cholestatic liver injury remains unknown. The current study investigated the role of CLEC-2 and podoplanin under cholestatic liver injury conditions using bile duct ligation (BDL)-treated CLEC-2-inhibited mice.

Materials and Methods

Animals

Male mice (9-week-old C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in a clean, temperature-controlled caged environment with 12-hour light–dark cycles. The mice were given free access to regular laboratory chow diet and water. All animals were treated humanely in compliance with the NIH's *Guide for the Care and Use of Laboratory Animals*.²² The Committee of Laboratory Animal Experimentation at University of Yamanashi approved this study.

Treatment and Operative Procedure

Anti-mouse CLEC-2 monoclonal antibody (2A2B10) was prepared at the Cell Engineering Corporation (Osaka, Japan). The procedures for 2A2B10 injections have been detailed elsewhere.²³ Briefly, mice were injected intraperitoneally with 2A2B10 (200 µg) weekly to effectively inhibit CLEC-2 on platelets.

BDLs were next performed in the mice to create a left and middle hepatic BDL model (Figure 1A). The procedures for left and middle hepatic BDLs have been described in detail elsewhere.²⁴ Briefly, mice were anesthetized with continuous vaporized 2% isoflurane. After an upper midline laparotomy (20 mm), the xiphoid was retracted superiorly with a clamp, and the middle lobe was gently held superiorly to expose the murine porta hepatis. The hepatic bile duct proximal to the point where the left and middle hepatic ducts merge was ligated. The cystic duct was then ligated and the gallbladder removed. After the procedures, the mice were kept on a warming pad in their cage under an infrared lamp until fully awake and active. Then, 1 week later, they were euthanized. At the preliminary experiment stage, the mice receiving 2A2B10 injections and BDL were mostly dead 2 weeks later. Therefore, the cholestatic liver injury was evaluated 1 week later in the current study.

This study comprised 4 experimental groups (Figure 1B). The control group mice ($n = 5$) were animals receiving control rat IgG (Molecular Innovations, Novi, MI) injections and simple laparotomy. The 2A2B10 group mice ($n = 5$) received 2A2B10 injections and simple laparotomy. The BDL group mice ($n = 12$) received control rat IgG injections and left and middle hepatic BDLs. The 2A2B10-BDL group mice ($n = 12$) received 2A2B10 injections and left and middle hepatic BDLs.

Sample Collection

Blood was collected from the vena cava into heparin tubes during euthanasia, and plasma was isolated. Liver tissues were fixed in 10% formalin and embedded into paraffin blocks, and the remaining tissues were frozen and stored at -80°C . The left, middle, and right lobes were stored separately. Some liver sections were stained with hematoxylin and eosin. All evaluations such as immunostaining and PCR were performed by using left and middle lobe samples. Pathologists (S.F. and H.A.) evaluated the liver pathology in a blind manner, observing at least five fields from each animal in each group.

TUNEL Staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed with an available kit (Merck Millipore, Darmstadt, Germany), according to the manufacturer's instructions. Hepatocyte apoptosis around the periportal areas and Glisson's sheath was quantitated by counting the average number of TUNEL-positive cells in five fields of liver tissue.

Immunohistochemistry

Paraffin-embedded liver sections (3-µm thick) were stained with primary antibodies using Dako Antibody Dilution Solution (Agilent Technologies, Santa Clara, CA). The primary antibodies used in these experiments included rabbit polyclonal anti- α -smooth muscle actin (α -SMA; Abcam, Cambridge, MA; 1:200, 60 minutes, room temperature), rabbit polyclonal anti-myeloperoxidase (Mpo; Abcam; 1:50, 60 minutes, room temperature), rat monoclonal anti-podoplanin antibody (PDPN; OriGene Technologies, Hereford, Germany; 1:800, overnight, 4°C), mouse monoclonal anti-hepatic sinusoidal endothelial cells antibody (SE-1; Novus Biologicals, Littleton, CO; 1:200, overnight, 4°C), rabbit polyclonal anti-CD68 antibody (CD68; Abcam; 1:500, overnight, 4°C), and rabbit polyclonal anti-CD41 antibody (CD41; Abcam; 1:100, overnight, 4°C). The VECTASTAIN ABC Elite Kit (Vector Laboratories, Burlingame, CA) and the Dako Liquid DAB+ Substrate Chromogen System (Agilent Technologies) were used for visualization. In addition, the slides were counterstained with filtered Mayer's hematoxylin (MilliporeSigma, Burlington, MA) for 2 minutes.

Quantitative analyses were performed by using ImageJ software version 1.51 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij/>). For α -SMA and Mpo, five fields of liver tissue were randomly selected to calculate the percentage of positively stained areas.

Immunofluorescence Staining

Paraffin-embedded liver sections (3-µm thick) were deparaffinized and rehydrated. The sections were then incubated with epitope retrieval solution (Agilent Technologies) for 20 minutes at 98°C and stained with hamster monoclonal anti-podoplanin

antibody (gp36; Abcam; 1:100, overnight, 4°C), mouse monoclonal antihepatic sinusoidal endothelial cells antibody (SE-1; Novus Biologicals; 1:200, overnight, 4°C), or rabbit polyclonal anti-CD68 antibody (Abcam; 1:500, overnight, 4°C). The sections were washed and incubated for 1 hour with Alexa Fluor 488–conjugated anti-hamster (Thermo Fisher Scientific, Waltham, MA), Alexa Fluor 568–conjugated anti-mouse (Thermo Fisher Scientific), or Alexa Fluor 568–conjugated anti-rabbit (Thermo Fisher Scientific) secondary antibodies. Nuclei were stained with TO-PRO-3 (Thermo Fisher Scientific). Finally, the slides were mounted by using SlowFade Gold Antifade Reagent (Thermo Fisher Scientific). The images were captured by using an FSX100 laser confocal microscope (Olympus, Tokyo, Japan).

Biochemical Measurements

Plasma total bilirubin (T-Bil), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured to assess hepatic parenchymal damage using FUJI DRI-CHEM analyzers (Fujifilm Co., Tokyo, Japan). Enzyme-linked immunosorbent assay kits (Abcam) were used to measure plasma serotonin levels and Total Bile Acid Assay Kits (Cell Biolabs, Inc., San Diego, CA) for plasma and urinary total bile acid levels.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from left liver lobes by using the RNeasy Mini Kit (Qiagen, Germantown, MD). RNA concentrations were measured in a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific). Total RNA (1 µg) was reverse transcribed by using random primers and the high-capacity cDNA archive kit (Thermo Fisher Scientific). Quantitative RT-PCR (Thermo Fisher Scientific) was performed for the following transcripts: *Tgfb1* (Mm01178820_m1), *Col1a1* (Mm00801666_g1), *Timp1* (Mm01341361_m1), *Epcam* (Mm00493214_m1), *Tnfa* (Mm99999068_m1), *Il6* (Mm00446190_m1), podoplanin (*Pdpn*) (Mm01348912_g1), *OSTα* (*Slc51a*) (Mm00521530_m1), *OSTβ* (*Slc51b*) (Mm01175040_m1), CyclinD1 (*Ccnd1*) (Mm00432359_m1), *Cyp7a1* (Mm00484150_m1), FXR (*Nr1h4*) (Mm00436425_m1), and *Gapdh* (4352339E-127039). The ΔC_t values were determined for all genes relative to the control *Gapdh* transcript. The $\Delta\Delta C_t$ s were calculated by using treated group means relative to the strain-matched control group means.

Statistical Analysis

Prism 5 (GraphPad Software, San Diego, CA) was used to perform statistical analyses. Statistical significance was evaluated by using the *t*-test and one-way analysis of variance within each time point followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

Figure 1 Schematic representation of this study and representative image of euthanasia. **A** and **B**: Schematic representation of left and middle hepatic bile duct ligation (BDL) (**A**) and this study (**B**). **C**: Representative image of surface appearance at the time of euthanasia in control group (left panel) and BDL group (right panel). $n = 5$ control-sham and 2A2B10-sham (**B**); $n = 12$ control-BDL and 2A2B10-BDL (**B**). 2A2B10, anti-C-type lectin-like receptor 2 antibody; CL, caudate lobe; GB, gall bladder; LL, left lobe; LMBDL, left and middle hepatic bile duct ligation; ML, middle lobe; RL, right lobe.

Results

Hepatocellular Necrosis and Apoptosis Are Increased in BDL-Treated CLEC-2–Inhibited Mice

To evaluate the effect of CLEC-2 during cholestasis, left and middle hepatic BDLs were performed in normal mice and in CLEC-2–inhibited mice. After BDL, common bile duct dilation, left and middle lobe edema, and dull-yellowish appearances were observed (Figure 1C). However, the general liver appearances of BDL and 2A2B10-BDL mice were similar (data not shown).

The body weights of 2A2B10-BDL mice were significantly reduced, and their liver weight/body weight ratios were significantly increased compared with those of BDL mice (Figure 2, A and B). The 2A2B10-BDL mice had

Figure 2 Quantitative analysis and representative photomicrographs of liver injury phenotypes. **A:** Body weight difference before the time of operation and euthanasia. **B:** Relative liver weight (%) to body weight. Blood total bilirubin (T-Bil) (**C**), aspartate aminotransferase (AST) (**D**), and alanine aminotransferase (ALT) (**E**) levels, as measured by using an enzymatic assay. The lines inside the box plot represent the median size, and **plus signs** indicate the average size. **F: Left column:** Images of hematoxylin and eosin (H&E) staining. **Right column:** Images of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. **Arrow** indicates necrotic region; **arrow head**, apoptotic cell. **G:** Quantitative analysis of necrotic area in left liver sections are expressed as percentage of positive staining area relative to total area in five random fields at 100 $\times$ magnification. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus bile duct ligation (BDL). Scale bar: 200 μ m (**F**, left column); 50 μ m (**F**, right column). Original magnification: $\times 100$ (**F**, left column); $\times 400$ (**F**, right column). 2A2B10, anti-C-type lectin-like receptor 2 antibody.

significantly higher plasma T-bil, AST, and ALT levels than the BDL mice (Figure 2, C–E). Also, in agreement with the observed AST and ALT levels, the multifocal acute hepatocellular necrotic areas were more extensive in 2A2B10-BDL mice (Figure 2F) than in the BDL mice. Moreover, the extent of necrotic areas (quantitative assessments) was significantly increased in 2A2B10-BDL mice compared with BDL mice (Figure 2G). These data show that CLEC-2 inhibition resulted in increased hepatocellular necrosis. Moreover, TUNEL staining was performed to evaluate apoptosis. The number of the apoptotic hepatocytes was significantly higher ($P < 0.05$) in 2A2B10-BDL mice (26.6 ± 6.8 per field) than in the BDL mice (5.5 ± 2.2 per field) (Figure 2F).

Hepatic Inflammation and Liver Fibrosis Increase in BDL-Treated CLEC-2-Inhibited Mice

Hepatic inflammation and liver fibrosis were next examined. Peribiliary collagen deposition, as indicated by Sirius Red

staining, was increased in livers of 2A2B10-BDL mice compared with those of BDL mice (Figure 3A). The presence of fibrosis from one portal area to another one (bridging fibrosis) was also observed in 2A2B10-BDL mice. The peribiliary expression of α -SMA (a marker of activated hepatic stellate cells and portal fibroblasts) was increased in livers of 2A2B10-BDL mice compared with those of BDL mice (Figure 3A). Expression of Mpo (most abundantly expressed in neutrophil granulocytes) was also increased in livers of 2A2B10-BDL mice (Figure 3A). In addition, accumulation of neutrophils in not only the peribiliary zone but also between the centrilobular and periportal zones (zone 2) was observed. In agreement with the liver histology, the extent of staining (quantitative assessments) after Sirius Red, α -SMA, and Mpo was significantly increased in 2A2B10-BDL mice compared with BDL mice (Figure 3, B–D).

The expression of profibrogenic and proinflammatory factors was next examined by using real-time PCR. Hepatic expression of profibrogenic *Tgfb1*, *Colla1*, *Timp1*, and

Figure 3 **A:** Representative photomicrographs of the left liver sections in control, bile duct ligation (BDL), and anti-C-type lectin-like receptor antibody (2A2B10)-BDL groups. Images of Sirius red (**left column**), α -smooth muscle actin (α -SMA) (**middle column**), and myeloperoxidase (Mpo) (**right column**). **B** and **C:** Quantitative analysis of immunohistochemical staining in the BDL and 2A2B10-BDL groups. Quantitative analysis of staining for Sirius Red (**B**) and α -SMA (**C**) in left liver sections is expressed as percentage of positive staining area relative to total area in five random fields at 200 $\times$ magnification. **D:** Quantitative analysis of Mpo-positive cells is expressed as an average number of staining cells in five random fields at 200 $\times$ magnification. The lines in the box plot represent the median size, and **plus signs** indicate the average size. *** P < 0.001 versus BDL. Scale bars = 100 μ m (**A**). Original magnification, $\times$ 200 (**A**).

Epcam mRNAs was increased significantly in livers of 2A2B10-BDL mice compared with expression in livers of control and BDL mice (Figure 4, A–D). Hepatic expression of proinflammatory genes *Tnfa* and *Il6* was also increased significantly in livers of 2A2B10-BDL mice compared with those of BDL mice (Figure 4, E and F). These data show that CLEC-2 inhibition results in increased hepatic inflammation and liver fibrosis.

Podoplanin Is More Highly Expressed in Sinusoidal Endothelial Cells in BDL-Treated Mice than in Control Mice

Expression of the CLEC-2 ligand podoplanin in the liver was examined. After immunohistochemical staining, the expression of podoplanin was scarce in control mice. Conversely, expression of podoplanin was detected easily on the hepatic sinusoid in BDL mice (Figure 5A). Consistent with this finding, the quantitative assessment of podoplanin staining was significantly higher in BDL mice than in control mice (Figure 5B), and podoplanin (*Pdpn*) mRNA was also significantly increased in BDL mice and 2A2B10-BDL mice (Figure 5C). To further elucidate the location of podoplanin expression, immunohistochemical staining was performed with various antibodies in the liver sections. These immunohistochemical staining results suggest that the podoplanin-

positive cells were sinusoidal endothelial cells (Figure 5D). Moreover, the immunofluorescence staining revealed that podoplanin was expressed on a portion of the sinusoidal endothelial cells but not on macrophages such as Kupffer cells (Figure 5E). These data suggest that podoplanin expression was dramatically increased on sinusoidal endothelial cells upon BDL.

Platelets Are Decreased on Hepatic Sinusoid in BDL-Treated CLEC-2–Inhibited Mice Compared with BDL-Treated Mice

Platelet accumulation was examined by using immunohistochemical staining of CD41. As a result, a large number of platelets accumulated on the hepatic sinusoid in the BDL mice; however, platelets were decreased in 2A2B10-BDL mice compared with BDL mice (Figure 6). This result might suggest that activated platelets are associated with liver protection of cholestatic liver injury.

Total Plasma Bile Acid Levels Are Increased in CLEC-2–Inhibited Mice

The total plasma bile acid levels were significantly increased in 2A2B10-BDL mice compared with levels in BDL mice (Figure 7A). In contrast, urinary bile acid levels were

Figure 4 Quantitative analysis of liver injury markers performed by quantitative RT-PCR. Fibrosis markers: *Tgfb1* (A), *Col1a1* (B), *Timp1* (C), and *Epcam* (D). Markers of inflammation: *Tnfa* (E) and *Il6* (F). The lines inside the box plot represent the median size, and **plus signs** indicate the average size. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control; † $P < 0.05$, †† $P < 0.01$ versus bile duct ligation (BDL). 2A2B10, anti-C-type lectin-like receptor 2 antibody.

significantly decreased in 2A2B10-BDL mice compared with BDL mice (Figure 7B). Taken together, these results suggest that CLEC-2 plays a role in modifying the bile acid pool.

Discussion

The coagulation cascade has been shown to participate in cholestatic liver injury. Experimental evidence supports a role for PARs, including the thrombin receptor PAR-1, in

promoting liver fibrosis.^{13,14} PAR-1 deficiency reduces hepatic collagen deposition in models of carbon tetrachloride, BDL, and alpha-naphthylisothiocyanate-induced liver fibrosis.^{25–28} However, emerging evidence suggests that under some experimental settings of liver injury, platelets

Figure 5 Representative photomicrographs and quantitative analysis of podoplanin expression in the left liver sections. **A:** Immunohistochemical staining for podoplanin in the control (left panel) and bile duct ligation (BDL) groups (right panel). **B:** Quantitative analysis of staining for podoplanin in left liver sections is expressed as the percentage of positive staining area relative to total area in five random fields at 200× magnification. **C:** Quantitative analysis of podoplanin (*Pdpn*) performed by quantitative RT-PCR. The lines inside the box plot represent the median size, and **plus signs** indicate the average size. **D:** Immunohistochemical staining for podoplanin (top panel), hepatic sinusoidal endothelial cells (SE-1) cells (middle panel), and CD68 (bottom panel) in the BDL group. **E:** Immunofluorescence staining for podoplanin (red), hepatic SE-1 cells (green, left panel), CD68 (green, right panel), and TO-PRO-3 (blue) in the BDL group. * $P < 0.05$, ** $P < 0.01$ versus control. Scale bars: 100 μm (A); 50 μm (D). Original magnification: $\times 200$ (A); $\times 400$ (D and E).

Figure 6 Representative photomicrographs in the left liver sections. **A:** Images of CD41 staining in the bile duct ligation (BDL) (left panel) and anti-C-type lectin-like receptor 2 antibody (2A2B10)-BDL (right panel) groups. **B:** Quantitative analysis of positive CD41 cells is expressed as an average number of staining cells in five random fields at 400 $\times$ magnification. *** P < 0.001 versus BDL. Scale bars = 50 μ m. Original magnification, $\times$ 400.

can promote liver repair and inhibit liver fibrosis. Kodama et al²⁹ showed that thrombocytopenia exacerbates liver fibrosis, and Joshi et al¹⁵ reported that PAR-4 exerts hepatoprotective effects during chronic cholestasis. However, the contribution of platelets in experimental settings of liver damage and fibrosis seems to be context dependent. The available evidence suggests that platelets can either promote or reduce liver injury and fibrosis.

CLEC-2, a new platelet receptor different from the PARs, reportedly induces platelet activation through the tyrosine kinase-dependent pathway by binding to podoplanin, an endogenous ligand. The CLEC-2-podoplanin interaction is associated not only with thrombosis/hemostasis but also with tumor metastasis, lymphangiogenesis, and tissue regeneration.^{17,20,21,30,31} To the best of our knowledge, no studies have found a correlation between CLEC-2 and cholestatic liver injury. The current study found that CLEC-2 inhibition results in increased hepatocellular necrosis, apoptosis, hepatic inflammation, and liver fibrosis. Some reports showed that hepatocellular apoptosis and necrosis consequently aggravated hepatic inflammatory responses in cholestatic liver injury.^{12,32} Hepatocellular apoptosis and necrosis are important inflammatory stimulus, indicating that the association between apoptosis and inflammation causes further liver injury.

In the current study, mice were injected weekly with 2A2B10, an anti-CLEC-2 monoclonal antibody. Importantly, the 2A2B10 injection does not cause a decrease in levels of other major platelet adhesion molecules.²²

Figure 7 Quantitative analysis of plasma total bile acid (**A**) and urinary bile acid levels (**B**). The lines inside the box plot represent the median size, and plus signs indicate the average size. ** P < 0.01, *** P < 0.001 versus control; † P < 0.05 versus bile duct ligation (BDL). 2A2B10, anti-C-type lectin-like receptor 2 antibody.

Furthermore, 2A2B10 injections induced platelet CLEC-2 deficiency in the current study (Supplemental Figure S1A). Moreover, it was revealed that 2A2B10 almost selectively inhibited rhodocytin-induced platelet aggregation (Supplemental Figure S1B). Rhodocytin is reportedly a CLEC-2 ligand and mediates platelet activation through multiple pathways.¹⁶ The current findings show that the roles of CLEC-2 and PARs differ. It may be that the PAR pathway leads to a coagulation cascade that promotes liver fibrosis, whereas the CLEC-2 pathway protects the liver, although both PARs and CLEC-2 activate platelets.

Some studies have implicated platelet-derived serotonin as a mediator capable of suppressing cholestatic liver injury and biliary fibrosis.^{33,34} About 95% of the circulating serotonin is stored in platelets. Jang et al³³ found that serotonin ameliorates liver injury and reduces the bile acid pool through adaptation of renal transporters during cholestasis. In that study, it was also shown that serotonin reduces the renal bile transporters OST α and OST β , which elevate plasma bile salt levels. In the current study, plasma levels of serotonin were increased in BDL mice, whereas they remained constant in 2A2B10-BDL mice (Supplemental Figure S2A). Moreover, total bile acid was significantly increased in 2A2B10-BDL mice, and urinary bile acid was significantly decreased in 2A2B10-BDL mice. Renal transporters OST α (*Slc51a*) and OST β (*Slc51b*) were also significantly increased in 2A2B10-BDL mice (Supplemental Figure S2, B and C). These results suggest that BDL mice platelets are activated and release serotonin via a CLEC-2 pathway, whereas 2A2B10-BDL mice platelets remain inactive and do not release serotonin because of the inhibition of CLEC-2. Furthermore, released serotonin via a CLEC-2 pathway reduces bile transporters OST α and OST β in the kidney and up-regulates the urinary bile acid. Therefore, serotonin protects cholestatic liver injury

Figure 8 Schematic representation of the proposed role of podoplanin and C-type lectin-like receptor 2 (CLEC-2) in cholestasis. Platelets bind podoplanin expressed on liver sinusoidal endothelial cells via CLEC-2. Platelets activate to reduce cholestatic liver injury. SYK, spleen associated tyrosine kinase.

in mice by modulating bile salt homeostasis. However, it is noteworthy that the liver injury related to serotonin/bile acid has been assessed only by a single group and has not yet been confirmed by others.³⁵ Thus, although platelets have been proposed to affect cholestatic injury through other mechanisms, caution is required. Further studies are warranted to confirm this theory.

We showed that CLEC-2 is involved in injury reduction, either by actual reduction of injury or by a role in repair. Hepatocyte proliferation markers were examined by using immunohistochemical staining and quantitative RT-PCR, and no differences were found between BDL-mice and 2A2B10-BDL mice (Supplemental Figure S3). These results suggest that the mechanisms of liver regeneration after partial hepatectomy and of cholestatic liver injury are different.

An intriguing finding of this study is the fact that expression of the CLEC-2 ligand podoplanin was very low in control mice, whereas expression on hepatic sinusoidal endothelial cells in BDL mice was high. This finding suggests that podoplanin expression on sinusoidal endothelial cells is associated with conditions under BDL. It was previously reported that podoplanin is also expressed on hepatic sinusoidal endothelial cells in mice after partial hepatectomy.²¹ We conclude that different interventions such as bile duct obstruction or partial hepatectomy may trigger expression of podoplanin, although the detailed mechanism for this action is unclear. Hitchcock et al³⁶ reported that podoplanin is expressed on monocytes and Kupffer cells in the liver after salmonella infection. The podoplanin expression sites may be situation dependent.

The current study has certain limitations. Colonna et al³⁷ reported on the molecular characterization of two novel C-type lectin-like receptors, one of which is selectively expressed in human dendritic cells. Alternatively, Chaipan et al³⁸ also reported that DC-SIGN and CLEC-2 mediate HIV type 1 capture by platelets. Thus, although dendritic cell infiltration into the liver in this model occurred, its involvement would be small. The expression of CLEC-2 has been reported to be almost specific of platelets, and 2A2B10 injections induced platelet-specific CLEC-2 deficiency without affecting CLEC-2 levels on leukocytes.^{22,39}

Conclusions

This report is the first regarding the role of the new platelet receptor CLEC-2 during cholestatic liver injury. We showed that platelet activation via CLEC-2 minimizes cholestatic liver injury (Figure 8). Furthermore, podoplanin was expressed on liver sinusoidal endothelial cells after BDL. Subsequently, platelets were found to be bound with podoplanin on sinusoidal endothelial cells via CLEC-2. The activated platelets via CLEC-2 combined with podoplanin to reduce the bile acid pool, protect the hepatocyte, and inhibit liver fibrosis. On the basis of these results, CLEC-2 and podoplanin interactions can reduce the extent of liver damage during cholestasis.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.05.009>.

References

- Hofmann AF: Cholestatic liver disease: pathophysiology and therapeutic options. *Liver* 2002, 22 Suppl 2:14–19
- Armstrong CP, Dixon JM, Duffy SW, Elton RA, Davies GC: Wound healing in obstructive jaundice. *Br J Surg* 1984, 71:267–270
- Dixon JM, Armstrong CP, Duffy SW, Elton RA, Davies GC: Upper gastrointestinal bleeding. A significant complication after surgery for relief of obstructive jaundice. *Ann Surg* 1984, 199:271–275
- Dixon JM, Armstrong CP, Duffy SW, Davies GC: Factors affecting morbidity and mortality after surgery for obstructive jaundice: a review of 373 patients. *Gut* 1983, 24:845–852
- Jiang WG, Puntis MC: Immune dysfunction in patients with obstructive jaundice, mediators and implications for treatments. *HPB Surg* 1997, 10:129–142

6. Roggin KK, Kim JC, Kurkchubasche AG, Papa EF, Vezeridis AM, Tracy TF: Macrophage phenotype during cholestatic injury and repair: the persistent inflammatory response. *J Pediatr Surg* 2001, 36: 220–228
7. Houdijk AP, van Lambalgen AA, Thijs LG, van Leeuwen PA: Gut endotoxin restriction improves postoperative hemodynamics in the bile duct-ligated rat. *Shock* 1998, 9:282–288
8. Gores GJ, Miyoshi H, Botla R, Aguilar HI, Bronk SF: Induction of the mitochondrial permeability transition as a mechanism of liver injury during cholestasis: a potential role for mitochondrial proteases. *Biochim Biophys Acta* 1998, 1366:167–175
9. Koeppl TA, Trauner M, Baas JC, Thies JC, Schlosser SF, Post S, Gebhard MM, Herfarth C, Boyer JL, Otto G: Extrahepatic biliary obstruction impairs microvascular perfusion and increases leukocyte adhesion in rat liver. *Hepatology* 1997, 26:1085–1091
10. Lin M, Rippe RA, Niemelä O, Brittenham G, Tsukamoto H: Role of iron in NF-kappa B activation and cytokine gene expression by rat hepatic macrophages. *Am J Physiol* 1997, 272: G1355–G1364
11. Saito JM, Maher JJ: Bile duct ligation in rats induces biliary expression of cytokine-induced neutrophil chemoattractant. *Gastroenterology* 2000, 118:1157–1168
12. Wen YA, Liu D, Zhou QY, Huang SF, Luo P, Xiang Y, Sun S, Luo D, Dong YF, Zhang LP: Biliary intervention aggravates cholestatic liver injury, and induces hepatic inflammation, proliferation and fibrogenesis in BDL mice. *Exp Toxicol Pathol* 2011, 63:277–284
13. Anstee QM, Dhar A, Thursz MR: The role of hypercoagulability in liver fibrogenesis. *Clin Res Hepatol Gastroenterol* 2011, 35: 526–533
14. Tripodi A, Anstee QM, Sogaard KK, Primignani M, Valla DC: Hypercoagulability in cirrhosis: causes and consequences. *J Thromb Haemost* 2011, 9:1713–1723
15. Joshi N, Kopec AK, O'Brien KM, Towery KL, Cline-Fedewa H, Williams KJ, Copple BL, Flick MJ, Luyendyk JP: Coagulation-driven platelet activation reduces cholestatic liver injury and fibrosis in mice. *J Thromb Haemost* 2015, 13:57–71
16. Suzuki-Inoue K, Fuller GLJ, Garcia A, Eble JA, Pöhlmann S, Inoue O, Gartner TK, Hughan SC, Pearce AC, Laing GD, Theakston RDG, Schweighoffer E, Zitzmann N, Morita T, Tybulewicz VLJ, Ozaki Y, Watson SP: A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. *Blood* 2006, 107:542–549
17. May F, Hagedorn I, Pleines I, Bender M, Vögtle T, Eble J, Elvers M, Nieswandt B: CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis. *Blood* 2009, 114:3464–3472
18. Suzuki-Inoue K, Kato Y, Inoue O, Kaneko MK, Mishima K, Yatomi Y, Yamazaki Y, Narimatsu H, Ozaki Y: Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. *J Biol Chem* 2007, 282: 25993–26001
19. Christou CM, Pearce AC, Watson AA, Mistry AR, Pollitt AY, Fenton-May AE, Johnson LA, Jackson DG, Watson SP, O'Callaghan CA: Renal cells activate the platelet receptor CLEC-2 through podoplanin. *Biochem J* 2008, 411:133–140
20. Schacht V, Ramirez MI, Hong YK, Hirakawa S, Feng D, Harvey N, Williams M, Dvorak AM, Dvorak HF, Oliver G, Detmar M: T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *EMBO J* 2003, 22: 3546–3556
21. Kono H, Fujii H, Suzuki-Inoue K, Inoue O, Furuya S, Hirayama K, Akazawa Y, Nakata Y, Sun C, Tsukiji N, Shirai T, Ozaki Y: The platelet-activating receptor C-type lectin receptor-2 plays an essential role in liver regeneration after partial hepatectomy in mice. *J Thromb Haemost* 2017, 15:998–1008
22. Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council: Guide for the Care and Use of Laboratory Animals. Eighth Edition. Washington, DC, National Academies Press, 2011
23. Shirai T, Inoue O, Tamura S, Tsukiji N, Sasaki T, Endo H, Satoh K, Osada M, Sato-Uchida H, Fujii H, Ozaki Y, Suzuki-Inoue K: C-type lectin-like receptor 2 promotes hematogenous tumor metastasis and prothrombotic state in tumor-bearing mice. *J Thromb Haemost* 2017, 15: 513–525
24. Aoki H, Aoki M, Yang J, Katsuta E, Mukhopadhyay P, Ramanathan R, Woelfel IA, Wang X, Spiegel S, Zhou H, Takabe K: Murine model of long-term obstructive jaundice. *J Surg Res* 2016, 206:118–125
25. Sullivan BP, Weinreb PH, Violette SM, Luyendyk JP: The coagulation system contributes to alphaVbeta6 integrin expression and liver fibrosis induced by cholestasis. *Am J Pathol* 2010, 177: 2837–2849
26. Fiorucci S, Antonelli E, Distrutti E, Severino B, Fiorentina R, Baldoni M, Caliendo G, Santagada V, Morelli A, Cirino G: PAR1 antagonism protects against experimental liver fibrosis. Role of proteinase receptors in stellate cell activation. *Hepatology* 2004, 39:365–375
27. Rullier A, Gillibert-Duplantier J, Costet P, Cubel G, Haurie V, Petitbois C, Taras D, Dugot-Senard N, Deleris G, Bioulac-Sage P, Rosenbaum J: Protease-activated receptor 1 knockout reduces experimentally induced liver fibrosis. *Am J Physiol Gastrointest Liver Physiol* 2008, 294:G226–G235
28. Kallis YN, Scotton CJ, Mackinnon AC, Goldin RD, Wright NA, Iredale JP, Chambers RC, Forbes SJ: Proteinase activated receptor 1 mediated fibrosis in a mouse model of liver injury: a role for bone marrow derived macrophages. *PLoS One* 2014, 9:e86241
29. Kodama T, Takehara T, Hikita H, Shimizu S, Li W, Miyagi T, Hosui A, Tatsumi T, Ishida H, Tadokoro S, Ido A, Tsubouchi H, Hayashi N: Thrombocytopenia exacerbates cholestasis-induced liver fibrosis in mice. *Gastroenterology* 2010, 138:2487–2498. e24987
30. Suzuki-Inoue K, Inoue O, Ding G, Nishimura S, Hokamura K, Eto K, Kashiwagi H, Tomiyama Y, Yatomi Y, Umemura K, Shin Y, Hirashima M, Ozaki Y: Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. *J Biol Chem* 2010, 285: 24494–24507
31. Kato Y, Kaneko MK, Kunita A, Ito H, Kameyama A, Ogasawara S, Matsuura N, Hasegawa Y, Suzuki-Inoue K, Inoue O, Ozaki Y, Narimatsu H: Molecular analysis of the pathophysiological binding of the platelet aggregation-inducing factor podoplanin to the C-type lectin-like receptor CLEC-2. *Cancer Sci* 2008, 99:54–61
32. Galle PR, Hofmann WJ, Walczak H, Schaller H, Otto G, Stremmel W, Krammer PH, Runkel L: Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. *J Exp Med* 1995, 182:1223–1230
33. Jang JH, Rickenbacher A, Humar B, Weber A, Raptis DA, Lehmann K, Stieger B, Moritz W, Soll C, Georgiev P, Fischer D, Laczkó E, Graf R, Clavien PA: Serotonin protects mouse liver from cholestatic injury by decreasing bile salt pool after bile duct ligation. *Hepatology* 2012, 56:209–218
34. Lesurtel M, Graf R, Aleil B, Walther DJ, Tian Y, Jochum W, Gachet C, Bader M, Clavien PA: Platelet-derived serotonin mediates liver regeneration. *Science* 2006, 312:104–107
35. Kyritsis K, Chen L, O'Brien A, Francis H, Hein TW, Venter J, Wu N, Ceci L, Zhou T, Zawieja D, Gashev AA, Meng F, Invernizzi P, Fabris L, Wu C, Skill NJ, Saxena R, Liangpunsakul S, Alpini G, Glaser SS: Modulation of the tryptophan hydroxylase 1/monoamine oxidase-A/5-hydroxy tryptamine/5-hydroxytryptamine receptor 2A/2B/2C axis regulates biliary proliferation and liver fibrosis during cholestasis. *Hepatology* 2020, 71:990–1008
36. Hitchcock JR, Cook CN, Bobat S, Ross EA, Flores-Langarica A, Lowe KL, Khan M, Dominguez-Medina CC, Lax S, Carvalho-Gaspar M, Hubscher S, Rainger GE, Cobbald M, Buckley CD,

- Mitchell TJ, Mitchell A, Jones ND, Van Rooijen N, Kirchhofer D, Henderson IR, Adams DH, Watson SP, Cunningham AF: Inflammation drives thrombosis after Salmonella infection via CLEC-2 on platelets. *J Clin Invest* 2015, 125:4429–4446
37. Colonna M, Samaridis J, Angman L: Molecular characterization of two novel C-type lectin-like receptors, one of which is selectively expressed in human dendritic cells. *Eur J Immunol* 2000, 30: 697–704
 38. Chaipan C, Soilleux EJ, Simpson P, Hofmann H, Gramberg T, Marzi A, Geier M, Stewart EA, Eisemann J, Steinkasserer A, Suzuki-Inoue K, Fuller GL, Pearce AC, Watson SP, Hoxie JA, Baribaud F, Pöhlmann S: DC-SIGN and CLEC-2 mediate human immunodeficiency virus type 1 capture by platelets. *J Virol* 2006, 80:8951–8960
 39. Suzuki-Inoue K, Inoue O, Ozaki Y: Novel platelet activation receptor CLEC-2: from discovery to prospects. *J Thromb Haemost* 2011, 9 Suppl 1:44–55