Neutrophils Aggravate Acute Liver Injury During Obstructive Cholestasis in Bile Duct–Ligated Mice

Jaspreet S. Gujral,[1,2] Anwar Farhood,[3] Mary Lynn Bajt,[1,2] and Hartmut Jaeschke[1,2]

Obstruction of the common bile duct in a variety of clinical settings leads to cholestatic liver injury. An important aspect of this injury is hepatic inflammation, with neutrophils as the prominent cell type involved. However, the pathophysiologic role of the infiltrating neutrophils during cholestatic liver injury remains unclear. Therefore, we tested the hypothesis that neutrophils contribute to the overall pathophysiology by using bile duct–ligated (BDL) wild-type animals and mice deficient in the β_2 integrin CD18. In wild-type animals, neutrophils were activated systemically as indicated by the increased expression of Mac-1 (CD11b/CD18) and L-selectin shedding 3 days after BDL. Histologic evaluation (48 ± 10% necrosis) and plasma transaminase levels showed severe liver injury. Compared with sham-operated controls (< 10 neutrophils per 20 high-power fields), large numbers of neutrophils were present in livers of BDL mice (425 ± 64). About 60% of these neutrophils had extravasated into the parenchyma. In addition, a substantial number of extravasated neutrophils were found in the portal tract. In contrast, Mac-1 was not up-regulated and plasma transaminase activities and the area of necrosis (21 ± 9%) were significantly reduced in CD18-deficient animals. These mice had overall 62% less neutrophils in the liver. In particular, extravasation from sinusoids and portal venules (PV) was reduced by 91% and 47%, respectively. Immunohistochemical staining for chlorotyrosine, a marker of neutrophil-derived oxidant stress, was observed in the parenchyma of BDL wild-type but not CD18-deficient mice. In conclusion, neutrophils aggravated acute cholestatic liver injury after BDL. This inflammatory injury involves CD18-dependent extravasation of neutrophils from sinusoids and reactive oxygen formation. (HEPATOLOGY 2003;38:355-363.)

The common bile duct can become obstructed in a variety of clinical settings, including sclerosing cholangitis, cholangiocarcinoma, gallstone impaction and extrinsic compression by tumors or enlarged lymph nodes.[1] Complete biliary obstruction causes cholestatic injury to the liver, including hepatocellular necrosis and apoptosis, bile duct epithelial cell proliferation, stellate cell activation, and, eventually, liver fibrosis.[2,3] A well-established experimental animal model of extrahepatic cholestasis is bile duct ligation (BDL) in rodents.[3]

Hepatic inflammation is an important feature of cholestatic liver disease in both humans[4] and experimental animals.[5-7] During the early phase after BDL (<7 days), Kupffer cells are activated as indicated by increased phagocytic activity[8] and increased formation of cytokines[9] and platelet activating factor.[10] In addition, systemic neutrophil priming for reactive oxygen formation has been observed after BDL.[11] These activated neutrophils also accumulate in the liver.[5-8] The formation of neutrophil-chemoattractant CXC chemokines is thought to be involved in hepatic neutrophil recruitment.[7] Moreover, the increased expression of cell adhesion molecules such as intercellular cell adhesion molecule-1 (ICAM-1) after extrahepatic cholestasis[12] can facilitate extravasation of neutrophils as a prerequisite for potential cytotoxicity by these leukocytes.[13] However, despite the extensive evidence for neutrophil activation during obstructive cholestasis in experimental animals and in humans, it is unclear if these phagocytes actually contribute to the in-

Abbreviations: BDL, bile duct ligation; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ALT, alanine transaminase; FACS, flow-activated cell sorting; PBS phosphate-buffered saline; BSA, bovine serum albumin; PV, portal venule; HE, hematoxylin-eosin; HO-1, heme oxygenase-1.

From the [1]Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, AR; [2]Liver Research Institute, University of Arizona, Tucson, AZ; and [3]Department of Pathology, University of Texas Health Science Center, Houston, TX.

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Address reprint requests to: Hartmut Jaeschke, Ph.D., Liver Research Institute, University of Arizona College of Medicine, 1501 N. Campbell Ave., Room 6309, Tucson, AZ 85724. E-mail: Jaeschke@email.arizona.edu; fax: 520-626-5975.

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jury process or if these cells are recruited into the liver to remove cell debris.[14] This question is of fundamental importance for the mechanism of cholestatic liver injury *in vivo*. In addition, if neutrophils aggravate cholestatic liver injury, they may contribute to the development of hepatic fibrosis.[15] If this were the case, neutrophils could be a novel target for therapeutic interventions in obstructive cholestasis and hepatic fibrogenesis.

Neutrophils enhance tissue injury after hepatic ischemia-reperfusion,[16] endotoxemia,[17] sepsis,[18] alcoholic hepatitis,[19] and remote organ injury.[20] Neutrophils accumulate in sinusoids and postsinusoidal venules.[21,22] After receiving a chemotactic signal from parenchymal cells, neutrophils transmigrate and adhere to hepatocytes.[23] This transmigration process requires β_2 integrins (CD11/CD18) on neutrophils and the transcriptional upregulation of ICAM-1[24] and vascular cell adhesion molecule-1 (VCAM-1)[25] on endothelial cells. Moreover, adherence of neutrophils to hepatocytes involves CD18/ICAM-1 interactions.[26] Adherent neutrophils cause cell injury mainly through formation of reactive oxygen species[27] and release of proteases.[28] Thus, blocking β_2 integrins on neutrophils effectively attenuates the neutrophil-induced oxidant stress and injury.[17,29-31]

Activated neutrophils generate superoxide through the enzyme NADPH (nicotinamide adenine dinucleotide phosphate) oxidase. Superoxide dismutates to hydrogen peroxide and oxygen.[32] Myeloperoxidase released by neutrophils uses hydrogen peroxide and chloride to generate hypochlorous acid, which is a potent oxidant.[32] In addition, it is a chlorinating agent leading to the formation of chlorotyrosine protein adducts.[33,34] These adducts can be detected in tissue by immunohistochemical methods.[35] Previously, we verified that immunostaining for chlorotyrosine protein adducts is a useful marker of neutrophil-induced oxidant stress and cytotoxicity in the liver *in vivo*.[36]

The aim of this study was to determine if neutrophils contribute to liver injury in an experimental model of obstructive cholestasis. We investigated neutrophil activation and cholestatic liver injury in wild-type mice and in animals deficient in CD18 adhesion molecule expression.[37] The cytotoxic activity of liver neutrophils was monitored by staining for chlorotyrosine protein adducts.

Materials and Methods

Animals and Experimental Protocol. Wild-type (C57BL/6J) and CD18-deficient mice were purchased from Jackson Laboratories (Bar Harbor, ME). The expression of CD18 on leukocytes is reduced dramatically but not completely eliminated.[37] All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals*. Before surgery, the mice were anesthetized by intramuscular injection of a cocktail of anesthetics, which included ketamine (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA; 225 mg/kg), xylazine (Rompun; Bayer Corp., Shawnee Mission, KS; 11.4 mg/kg), and acepromazine (Acepromazine maleate; VEDCO, St. Joseph, MO; 2.3 mg/kg). A midline laparotomy was performed, and the common bile duct was ligated twice with a 4-0 silk suture and then cut between the ligatures. After surgery, the animals were intraperitoneally injected with a combination of the antibiotics imipenem and cilastatin (Primaxin; Merck and Co., West Point, PA; 25 mg/kg) and allowed to recover from the anesthesia. Sham-operated animals served as controls. The animals were killed 3 days after surgery by cervical dislocation and exsanguination under pentobarbital anesthesia. Blood and liver samples were collected at this time. Whole blood was used for flow cytometry, and plasma was used for determination of alanine transaminase (ALT) activities and total bilirubin concentrations. Liver samples were fixed in phosphate-buffered formalin, and then embedded in paraffin. Sections of these livers were used for immunohistochemistry and histology.

Flow-Activated Cell Sorting. Mac-1 and L-selectin expressions on peripheral blood neutrophils were determined using flow-activated cell sorting (FACS) analysis, as described in detail.[38] Briefly, 100 μL of whole blood was washed twice with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (PBS/BSA). Cells were suspended in 100 μL of PBS/BSA containing 1 μg of fluorescein isothiocyanate–conjugated RB6-8C5 (anti-Gr1) and, either phycoerythrin-conjugated M1/70 (anti-CD11b) or phycoerythrin-conjugated Mel-14 (anti-L-selectin) antibodies (Pharmingen, San Diego, CA). After incubation for 30 minutes on ice, cells were pelleted by centrifugation and washed twice with PBS. Cells were then lysed with 1 mL Immuno-Lyse (Coulter, Hialeah, FL) for 2 minutes at 37°C and fixed with 250 μL of Coulter Clone fixative. Cells again were pelleted by centrifugation, washed twice with PBS, and resuspended in PBS. Two-color analysis of antibody binding to cells was conducted by flow cytometry using a FACSCalibur Flow Cytometer (Becton Dickinson Biosciences, San Jose, CA). Neutrophils were gated by the forward and light angle scatter, and Gr-1 fluorescein isothiocyanate fluorescence. The intensity of phycoerythrin fluorescence was determined on 10,000 gated events in each sample and expressed as mean fluorescence intensity. Nonspecific fluorescence was determined on cells incubated with isotype and cytochrome-matched control antibodies.

Hepatic Neutrophil Sequestration and Mononuclear Cell Infiltration. To assess neutrophil accumulation in the livers, tissue sections were stained for chloroacetate esterase present on neutrophils, using a Naphthol-ASD Chloroacetate Esterase Kit (Sigma, St. Louis, MO). The number of neutrophils present in the sinusoids and extravasated into the parenchymal tissue were counted in 20 high-power fields. The sum of sinusoidal and extravasated neutrophils was expressed as the total neutrophil sequestration in the liver.[24] The number of neutrophils present in 20 portal venules (PV) and the extravasated ones in 20 portal tracts of similar size also were counted. Sections of formalin-fixed paraffin-embedded liver samples were stained with hematoxylin-eosin (HE) for evaluation of mononuclear cell infiltration. The numbers of mononuclear cells present in the tissue were counted in 20 high-power fields. All cell counts were performed in a blinded fashion.

Immunohistochemistry. Protein adducts of chlorotyrosine were detected by immunohistochemistry using a rabbit anti-chlorotyrosine polyclonal antibody.[36] Liver sections were deparaffinized and rehydrated, and then incubated sequentially with the Immunopure Peroxidase Suppressor (Pierce) and the DAKO protein block (Dako Corp., Carpinteria, CA) for 30 minutes each. This was followed by incubation with the primary antibody for 1 hour at room temperature. Color was developed with a horseradish peroxidase–based labeled streptavidin biotin (LSAB) kit (Dako) and diamino benzidine chromogen (Dako) according to the manufacturer's instructions.

Plasma Bilirubin and Liver Injury. As an indicator of the extent of cholestasis, bilirubin levels were measured in the plasma using the Total Bilirubin kit (Sigma). ALT activities were determined in the plasma using the DG 159-UV kit (Sigma). HE-stained liver sections were used for evaluation of liver cell injury. The percentage of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared with the entire histologic section.[16,24,25,39]

Western Blotting. Heme oxygenase-1 (HO-1) induction in the liver was evaluated by Western Blot analysis, as described.[40] Briefly, frozen liver samples were homogenized in 25 mmol/L HEPES buffer (pH 7.5) containing 5 mmol/L ethylenediaminetetraacetic acid, 2 mmol/L dithiothreitol, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), and 1 μg/mL of each of the protease inhibitors pepstatin, leupeptin, and aprotinin. The homogenates were centrifuged at 14,000*g* at 4°C for 20 minutes. The supernatants (50 μg per well) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 4% to 20% Tris-glycine gels (Invitrogen, Carlsbad, CA). The gels were transferred onto polyvinylidine difluoride membranes (PVDF; Immobilon-P; Millipore, Bedford, MA). The membranes were then blocked with 5% milk overnight at 4°C. This was followed by incubation with the primary antibody for 2 hours at room temperature. A mouse anti–HO-1 monoclonal immunoglobulin G (Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada) was used as the primary antibody (1:2,000). The membranes were washed and then incubated with the secondary antibody (1:5,000), anti-mouse immunoglobulin G–HRP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hour at room temperature. Enhanced chemiluminescence (Amersham Pharmacia Biotechnology, Inc., Piscataway, NJ) was used to visualize the proteins. Densitometry analysis was performed on the blots with a Bio-Rad GS700 Imaging Densitometer (Bio-Rad, Hercules, CA) and Quantity One 4.1.1 software (Bio-Rad).

Statistical Analysis. Data are given as means ± SE. Comparisons between multiple groups were performed with 1-way ANOVA followed by Bonferroni *t* test. If the data were not normally distributed, the Kruskal-Wallis Test (nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test was performed. $P < .05$ was considered significant.

Results

In pilot experiments, we determined that BDL causes significant liver injury in C57BL/6J mice after 3 days, with all animals surviving. Therefore, we used this time point to determine systemic neutrophil activation by flow cytometry. The increased expression of the β_2 integrin Mac-1 (CD11b/CD18) and the shedding of L-selectin were evaluated on peripheral blood neutrophils of wild-type and CD18-deficient mice. Mac-1 expression in wild-type animals increased to 270 ± 44% of sham-operated controls 3 days after BDL (Fig. 1). L-selectin expression was reduced by 65% compared with baseline. Sham-operated CD18-deficient mice expressed 58% less Mac-1 compared with wild-type animals (Fig. 1). These results are consistent with the fact that this gene is not completely eliminated in these mice.[37] After BDL, Mac-1 expression did not significantly increase in CD18-deficient animals (Fig. 1). However, L-selectin shedding was still observed to a similar degree as in wild-type animals (−63%).

Liver injury after BDL was assessed by measuring ALT activities in plasma and by evaluation of hepatocellular necrosis in liver sections stained with HE. Sham-operated animals in both groups had low levels of ALT in plasma and no necrosis in the liver (Fig. 2). After BDL, the wild-type animals showed a fortyfold increase in the plasma ALT activities compared with sham-operated controls (Fig. 2A) and 49% necrosis in the liver (Fig. 2B). On the

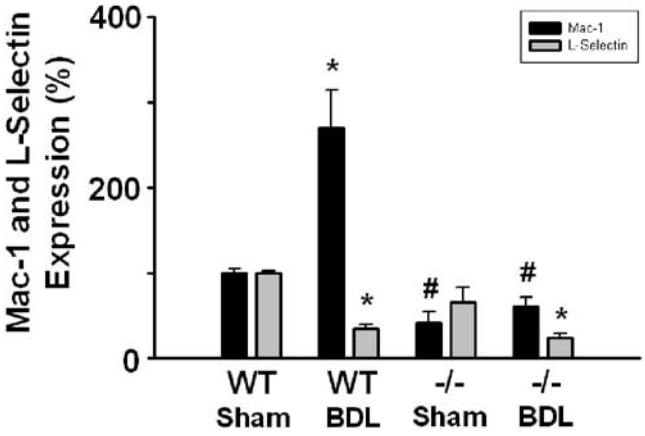

Fig. 1. Systemic neutrophil activation 3 days after BDL in mice. Wild-type (WT) animals or CD18-deficient (−/−) mice were sham-operated or subjected to BDL. The animals were killed after 3 days. Blood was collected and neutrophils were analyzed for Mac-1 (CD11b/CD18) and L-selectin expression by flow cytometry. Baseline expression of Mac-1 and L-selectin in sham-operated wild-type animals was set as 100%. Data represent means ± SE of n = 5 animals per group. $^{*}P < .05$ compared with Sham; $^{\#}P < .05$ compared with WT.

other hand, in CD18-deficient mice, the plasma ALT and liver cell necrosis after BDL were reduced by 80% and 70%, respectively, compared with the wild-type animals. To compare the extent of cholestasis between groups, plasma bilirubin levels were measured. The sham-operated wild-type and CD18-deficient mice had a plasma bilirubin level of 0.6 ± 0.1 mg/100 mL. After BDL for 3 days, these levels increased to 10.4 ± 2.0 mg/100 mL in the wild-type mice ($P < .05$). Similar levels were observed in BDL CD18-deficient animals (8.1 ± 0. 8 mg/100 mL).

To assess neutrophil infiltration, liver sections were stained for chloroacetate esterase, a marker for neutrophils.[16,17,24,25] The number of neutrophils present in sinusoids or extravasated into the parenchymal tissue were quantified by counting them in 20 randomly selected high-power fields (×400). Only about 10 neutrophils per 20 high-power fields were observed in sham-operated animals of both wild-type and CD18-deficient animals (Fig. 3C). No neutrophils were seen extravasated into the parenchyma. On the other hand, after BDL, the total number of neutrophils in the liver increased more than fortyfold in wild-type animals (Fig. 3A and C). Approximately 60% of these neutrophils were located in the parenchyma, *i.e.,* had extravasated. In contrast, the increase in the number of hepatic neutrophils in CD18-deficient mice after BDL was significantly lower (sixteenfold increase over baseline) (Fig. 3B and C). More importantly, only 15% of these neutrophils had extravasated. Thus, overall, the number of neutrophils located in the parenchyma was reduced by 91% in CD18-deficient mice compared with wild-type animals. The extent of mononuclear cell infiltration into the tissues also was evaluated by counting the number of mononuclear cells in 20 high-power fields. No mononuclear cells were seen in the sham-operated wild-type and CD18-deficient controls. However, after BDL for 3 days, a mild mononuclear cell infiltration was observed in livers of wild-type mice (10 ± 2 cells/20 high-power fields). C18-deficient animals had a similar infiltrate (12 ± 1 cells/20 high-power fields).

To evaluate if the extravasated neutrophils were cytotoxically active, we immunostained liver sections for chlorotyrosine protein adducts.[36] HE stained sections of sham-operated control sections showed a normal architecture with no necrosis (Fig. 4A). No positive staining for chlorotyrosine protein adducts could be seen in these livers (Fig. 4B). On the other hand, after BDL, liver sections

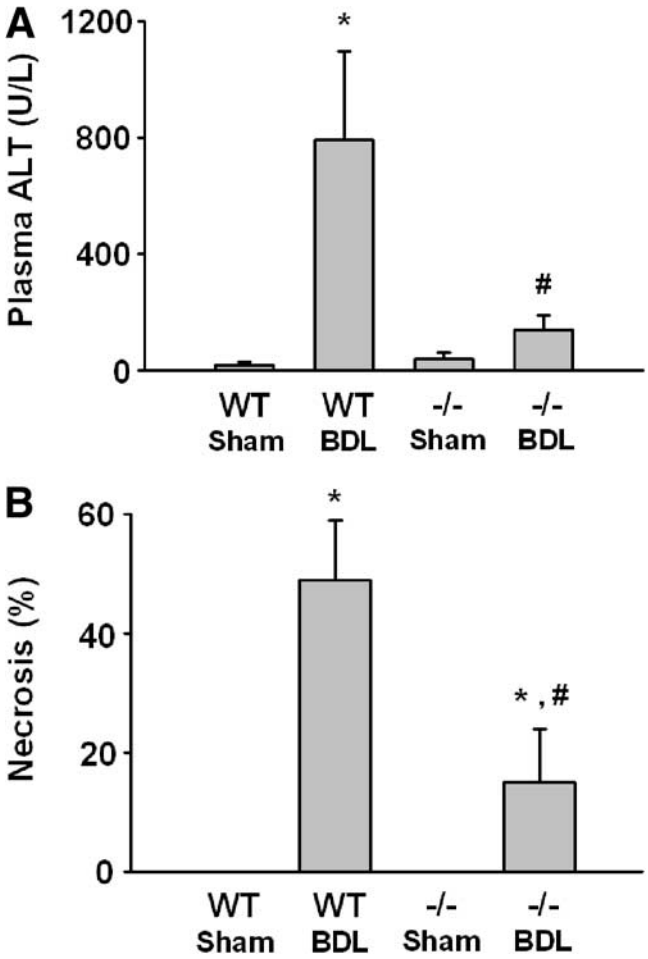

Fig. 2. Hepatocellular injury 3 days after BDL in mice. Wild-type (WT) animals or CD18-deficient (−/−) mice were sham-operated or subjected to BDL. (A) The animals were sacrificed after 3 days. ALT activities were determined in the plasma. Formalin-fixed liver sections were stained with HE. (B) Necrosis was estimated in histological sections and expressed as percentage of the total area. Data represent means ± SE of n = 5 animals per group. $^{*}P < .05$ compared with Sham; $^{\#}P < .05$ compared with WT.

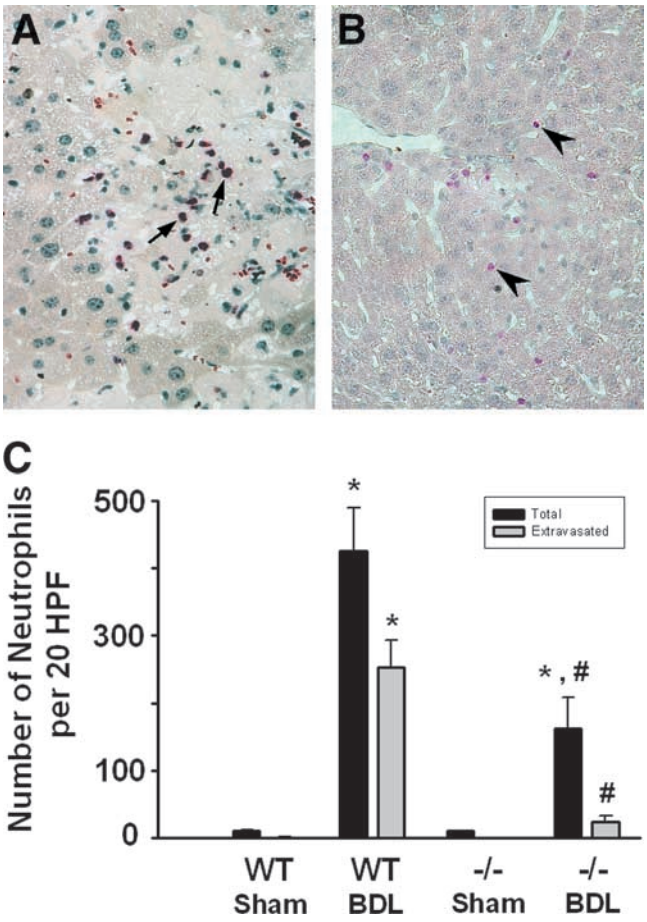

Fig. 3. Accumulation of neutrophils in the liver after BDL in mice. Wild-type (WT) animals or CD18-deficient mice (−/−) were sham-operated or subjected to BDL. The animals were killed after 3 days. Formalin-fixed liver sections were stained for chloroacetate esterase to assess hepatic neutrophil accumulation. Neutrophils were identified by positive staining and morphology. Livers from (A) wild-type mice showed a number of neutrophils extravasated (**arrows**) into the parenchymal tissue, especially around necrotic foci. In contrast, the number of extravasated neutrophils in the livers from (B) CD18-deficient mice was reduced drastically. Most of the neutrophils sequestered in these livers remained in the sinusoids (**arrowheads**). The number of neutrophils present in sinusoids and extravasated into the parenchymal tissue were counted in 20 high-power fields. (C) The data are expressed as the total number of neutrophils in liver sections and the number of extravasated neutrophils. Data represent means ± SE of n = 5 animals per group. $^{*}P < .05$ compared with sham; $^{\#}P < .05$ compared with WT. (Original magnification ×400.)

from wild-type animals showed confluent areas of necrotic hepatocytes especially in midzonal areas (Fig. 4C). These areas were infiltrated with neutrophils. Moreover, extensive positive staining for chlorotyrosine protein adducts could be observed in these areas (Fig. 4D). In contrast, the livers from BDL CD18-deficient mice showed less severe hepatocellular injury (Fig. 4E). The confluent areas of necrotic hepatocytes were less frequent together with a lower number of extravasated neutrophils. Little positive staining for chlorotyrosine protein adducts could be seen in these livers (Fig. 4F).

Another indicator for tissue oxidant stress can be the induction of HO-1, an enzyme that is regulated by the redox-sensitive transcription factor AP-1.[41] Therefore, HO-1 protein expression was evaluated in the livers of

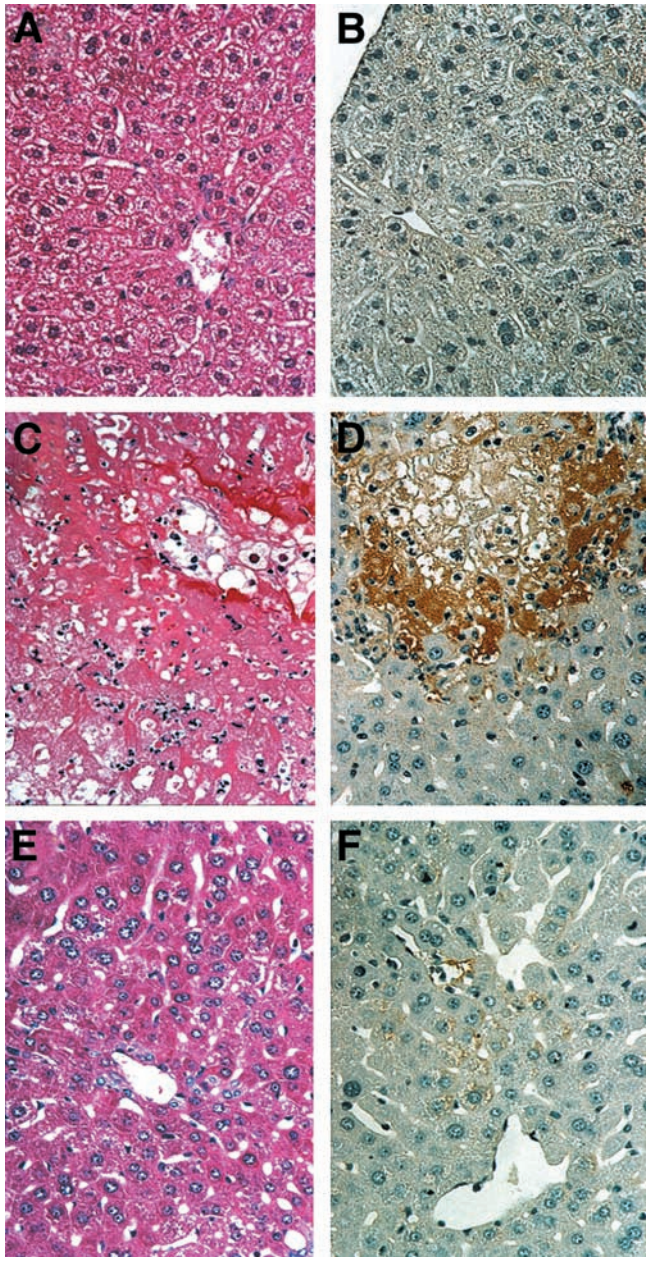

Fig. 4. Liver injury and chlorotyrosine adduct formation after BDL. Wild-type animals or CD18-deficient mice were sham-operated or subjected to BDL. The animals were killed after 3 days. Formalin-fixed and paraffin-embedded liver sections were either stained with HE (A, C, and E) or immunostained for chlorotyrosine protein adducts (B, D, and F). (A) Livers from sham-operated controls had normal architecture and had (B) no positive staining for chlorotyrosine protein adducts. (C) Three days after BDL, the livers from wild-type mice showed severe liver cell injury. (D) This was accompanied by extensive positive staining for chlorotyrosine protein adducts. In contrast, the livers from CD18-deficient mice had (E) reduced liver cell injury and (F) very limited chlorotyrosine adduct staining. (Original magnification ×400.)

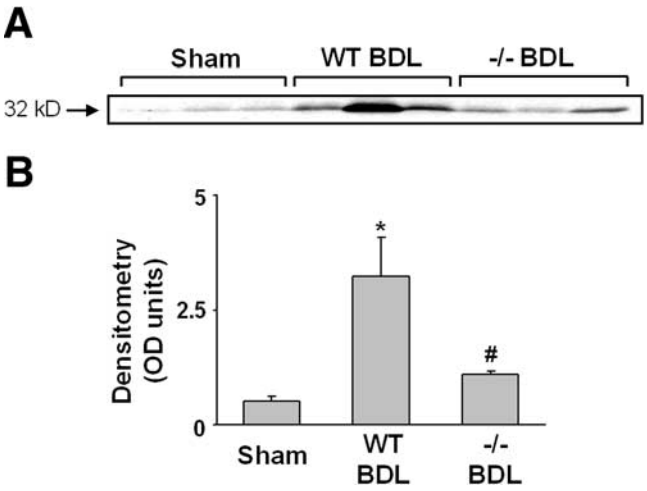

Fig. 5. HO-1 induction in the liver after BDL in mice. Wild-type (WT) animals or CD18-deficient (−/−) mice were sham-operated or subjected to BDL. The animals were killed after 3 days. (A) Liver samples were homogenized and evaluated for HO-1 induction by Western blotting. (B) A densitometry analysis was performed on the blot and the data were expressed as OD units. Data represent means ± SE of n = 3 animals per group. *$P < .05$ compared with sham; #$P < .05$ compared with WT.

sham-operated animals and 3 days after BDL (Fig. 5). A very low expression of HO-1 was found in sham-operated wild-type and CD18-deficient animals. After BDL, HO-1 expression was increased sixfold in wild-type animals compared with sham-operated controls (Fig. 5). HO-1 induction in CD18-deficient animals in response to BDL was significantly less than in wild-type BDL animals (Fig. 5).

In addition to neutrophils in sinusoids and parenchyma, we observed a substantial number of neutrophils in the portal tracts after BDL (Fig. 6). No neutrophils were seen in the hepatic portal tracts of sham-operated animals. After BDL, the livers from wild-type animals had 189 ± 42 neutrophils per 20 portal tracts. Neutrophil infiltration in the portal tracts of BDL CD18-deficient animals was significantly reduced in comparison with the wild-type BDL animals (Fig. 6). Little to no staining for chlorotyrosine protein adducts was observed in the vicinity of these extravasated neutrophils (data not shown). Because the neutrophils in the portal tract all migrated from the PV, we quantified the number of neutrophils in the PV. Compared with sham-operated wild-type or CD18-deficient mice (<1 neutrophil/20 PV), the number of adherent neutrophils in these vessels was increased after BDL (wild-type, 11.7 ± 1.4 neutrophils/20 PV; CD18-deficient, 9.4 ± 1.8).

Discussion

The aim of the current study was to investigate a potential role of neutrophils in liver injury induced by obstructive cholestasis in mice. Our data showed systemic neutrophil activation, hepatic neutrophil accumulation with extravasation into the parenchyma, and substantial liver injury 3 days after BDL. In contrast, animals deficient in CD18 expression on neutrophils had fewer neutrophils in the liver. More importantly, neutrophil extravasation was prevented almost completely, and the liver injury was drastically reduced. These data suggest that neutrophils play a critical role in the aggravation of liver injury during obstructive cholestasis.

After BDL, circulating neutrophils express significantly more Mac-1 (CD11b/CD18) and less L-selectin than neutrophils from sham-operated animals. These parameters clearly indicate systemic neutrophil activation after BDL. Portal endotoxemia, which occurs after BDL,[10,42] may be responsible for the formation of inflammatory mediators such as tumor necrosis factor α, platelet activating factor, and CXC chemokines.[7,9,10,42] These mediators are known to be potent activators of neutrophil Mac-1 expression, L-selectin shedding, and priming for reactive oxygen formation.[38,43-45] After systemic activation, neutrophils accumulate in sinusoids without the involvement of cellular adhesion molecules.[46] In contrast, neutrophil adherence in portal or postsinusoidal venules requires adhesion molecules.[47] On the other hand, the transmigration from both sinusoids and venules involves β_2 integrin/ICAM-1 interactions, β_1 integrin/VCAM-1 interactions, or both.[13,23] The drastically reduced number of extravasated neutrophils after BDL in livers of CD18-deficient mice supports the conclusion that the transmigration process through the sinusoidal endothelial cell

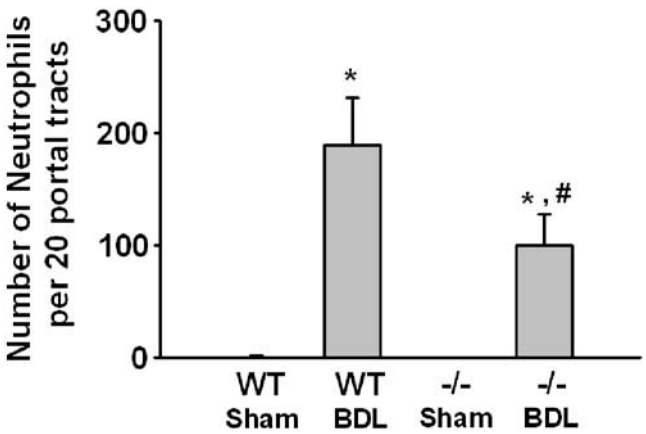

Fig. 6. Neutrophil accumulation in portal tracts 3 days after BDL. Wild-type (WT) animals or CD18-deficient (−/−) mice were sham-operated or subjected to BDL. The animals were killed after 3 days. Formalin-fixed liver sections were stained for chloroacetate esterase to assess the accumulation of neutrophils in the portal tracts. The number of neutrophils present in 20 portal tracts was determined. Data represent means ± SE of n = 5 animals per group. *$P < .05$ compared with sham; #$P < .05$ compared with WT.

layer is a CD18-dependent process. This conclusion is consistent with previous *in vitro* experiments[48] and with observations in an endotoxemia model of liver injury.[24,25] These data further support the hypothesis that even primed neutrophils accumulated in sinusoids do not cause relevant liver injury without transmigration.[22,49]

Mononuclear cells are another leukocyte cell type expressing β_2 integrins. In agreement with a recent report,[8] we found a mild mononuclear cell infiltrate in sinusoids 3 days after BDL. Compared with neutrophils, these mononuclear cells represented less than 2.5% of the total number of infiltrating leukocytes. In addition, there was no significant difference in the mononuclear cell number in livers of wild-type and CD18-deficient animals. These data do not support the hypothesis that mononuclear cells are involved directly in cholestatic liver injury at 3 days after BDL. However, mononuclear cell infiltrate become more prominent later and may contribute to the injury at that time.[8,42]

Previous detailed analysis of blood leukocyte counts in the CD18-deficient mice showed increased basal levels of neutrophils and mononuclear leukocytes compared with wild-type animals.[37] In spite of higher blood counts, Wilson et al. observed reduced emigration of these neutrophils into the peritoneal cavity in a chemical-induced peritonitis model. This is consistent with inhibition of CD18-dependent extravasation in these mice. Thus, the protective effect in our model is unlikely to be caused by neutropenia or lack of other leukocytes in the CD18-deficient mice. In contrast, the missing CD18-receptor prevented neutrophil extravasation and cytotoxicity in the liver.

The extensive formation of chlorotyrosine protein adducts in wild-type animals after BDL indicates the generation of reactive oxygen species, hydrochlorous acid in particular. This is consistent with data in an endotoxemia model, in which the cytotoxicity of extravasated neutrophils correlated with chlorotyrosine adduct formation.[36] Based on the higher susceptibility of glutathione peroxidase–deficient animals during endotoxemia, it was concluded that the reactive oxygen species generated by adhering neutrophils diffused into hepatocytes and caused an intracellular oxidant stress, which killed the cells.[27] The mechanism of injury did not involve lipid peroxidation.[27] Consistent with these results, it was reported that lipid peroxidation is a late event ($>$1 week after BDL), which is unrelated to the initial injury phase after onset of cholestasis.[5,50] Moreover, our observation that the neutrophil-derived oxidant stress was eliminated in CD18-deficient mice indicates that an adherence-dependent oxidant stress is important for the neutrophil cytotoxicity. The overall beneficial effect of CD18 deficiency is a combination between the reduced number of transmigrated neutrophils and the functional inactivation (reduced superoxide formation) of neutrophils that lack β_2 integrins, especially Mac-1 (CD11b/CD18).[17,29]

Further evidence for an oxidant stress in the liver 3 days after BDL comes from the observation that the stress protein HO-1 was induced. The expression of this enzyme in hepatic parenchymal cells is under the control of the redox-sensitive transcription factor AP-1.[41] Antioxidant treatment can suppress HO-1 expression in the liver.[41] Our finding that BDL induced HO-1 only marginally in CD18-deficient animals suggests that the oxidant stress in the liver was substantially less than in wild-type animals. This further supports the conclusion that the oxidant stress was caused mainly by neutrophils located in the hepatic parenchyma.

The presence of neutrophils in portal tract areas as well as in necrotic areas of the hepatic parenchyma suggests that neutrophils transmigrated from both PV and sinusoids. These findings are different from previous results in endotoxemic animals, where neutrophils transmigrated only from sinusoids.[22] However, the accumulation of bile acids in the portal tract after BDL stimulates the formation of CXC chemokines, which are at least in part responsible for neutrophil recruitment and transmigration in the portal tracts.[7] Because all adhesion molecules such as ICAM-1, VCAM-1, and platetet-endothelial cell adhesion molecule-1 are expressed on venular endothelium in the liver,[13] the formation of a chemotactic gradient of CXC chemokines in the portal tract area can trigger neutrophil transmigration. Interestingly, only the neutrophils, which extravasated from the sinusoids into the parenchyma, are associated with areas of necrosis. This correlated with the formation of chlorotyrosine protein adducts as an indicator of reactive oxygen formation by neutrophils. In contrast, portal tracts do not stain positive for chlorotyrosine protein adducts, and the neutrophils in these areas are not associated with tissue injury. This suggests that only neutrophils that accumulated in and extravasated from sinusoids caused serious additional injury after BDL. The fact that the periportally located neutrophils do not cause tissue injury does not mean that they are irrelevant. In contrast, these cells could interact with stellate cells and promote fibrogenesis.[15,50] Further experiments are necessary to test this hypothesis.

In summary, our data clearly show neutrophil accumulation in sinusoids and PV after BDL with extravasation from both locations. However, only neutrophils, which migrated from sinusoids into the parenchyma, generated reactive oxygen and substantially aggravated tissue injury. Neutrophil transmigration and cytotoxicity was dependent on β_2 integrins (CD18). Thus, neutrophils could be

an important therapeutic target to improve early cholestatic liver injury.

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