LIVER

Cholestasis protects the liver from ischaemic injury and post-ischaemic inflammation in the mouse

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Background and hypothesis: Cholestasis is associated with high morbidity and mortality in patients undergoing major liver surgery, but the mechanisms responsible remain elusive. Increased ischaemic liver injury and inflammation may contribute to the poor outcome.

Methods: Common bile duct ligation (biliary obstruction with hyperbilirubinaemia) or selective ligation of the left hepatic duct (biliary obstruction without hyperbilirubinaemia) was performed in C57BL/6 mice before 1 h of hepatic ischaemia and 1, 4 or 24 h of reperfusion. Infection with the intracellular hepatic pathogen *Listeria monocytogenes* for 12 and 48 h was used to study ischaemia-independent hepatic inflammation.

Results: Cholestatic mice showed considerable protection from ischaemic liver injury as determined by transaminase release, histological liver injury and neutrophil infiltration. In cholestatic mice, reduced injury correlated with a failure to activate nuclear factor κ B (NF κ B) and tumour necrosis factor α (TNF α) mRNA synthesis, two key mediators of post-ischaemic liver inflammation. After selective bile duct ligation, both the ligated and the non-ligated lobes showed blocked activation of NF κ B as well as reduced induction of TNF α mRNA synthesis and neutrophil infiltration. By contrast, infection with *L. monocytogenes* showed comparable activation of NF κ B and hepatic recruitment of neutrophils 12 h after infection.

Conclusion: Cholestasis does not increase but rather dramatically protects the liver from ischaemic injury and inflammation. This effect is mediated by a systemic factor, but not bilirubin, and is associated with a preserved capacity to trigger an inflammatory response to other stimuli such as a bacterial pathogen.

Although liver surgery is currently performed safely and mortality is near zero in patients with a normal liver, this procedure is still associated with a major risk of death and complications in the presence of cholestasis. The main reasons for this poor outcome remain elusive, although increased susceptibility to septic complications^{1,2} and an impaired capacity of cholestatic livers to regenerate³ have been described.

Another mechanism that could account for the poor outcome is decreased tolerance to ischaemic liver injury. To our knowledge, the only available study looking at the effect of cholestasis on ischaemic injury used a rat model of short hepatic ischaemia (15 min) followed by only 1 h of reperfusion.⁴ In view of the increased serum transaminase levels in cholestatic animals shortly after reperfusion, it was concluded that cholestasis enhances ischaemic liver injury. As this study did not assess later time points after reperfusion, the influence of cholestasis on post-ischaemic hepatic necrosis and inflammation could not be evaluated.

Periods of hepatic ischaemia followed by reperfusion are characterised by several phases, including the release of reactive oxygen species,⁵ the induction of tumour necrosis factor receptor 1-mediated hepatocellular injury^{6,7} and a nuclear factor κ B (NF κ B)-dependent production of tumour necrosis factor (TNF α).⁸ This cascade mediates inflammation in the post-ischaemic liver, leading to additional injury and necrosis, typically peaking 24 h after reperfusion in the mouse model.

Cholestatic animals are characterised by an increased release of cytokines, increased inflammation and a higher lethality when injected with lipopolysaccharide (LPS) or the Gram-negative pathogen *Escherichia coli*.^{9,10} From this observation it may be expected that cholestasis also induces a more pronounced hepatic inflammation after ischaemia. However,

local expression of the protein kinase B (AKT) in hepatocytes and sphingosine kinase in hepatic stellate cells protects from TNF α /GalN-induced liver injury after bile duct ligation,¹¹ pointing to the possibility that cholestasis might also be protective in some types of liver injury.

Therefore, we designed a series of experiments to evaluate the effect of cholestasis on ischaemic liver injury and inflammation. In addition to a model of common bile duct ligation, selective ligation of the left hepatic duct was used to study the effect of biliary obstruction in the absence of hyperbilirubinaemia. To further explore the ability of the cholestatic liver to respond to other inflammatory stimuli, we infected mice with the Gram-positive intracellular hepatic pathogen *Listeria monocytogenes*.

MATERIALS AND METHODS

Animals

Specific pathogen-free C57BL/6 mice (Harlan Laboratories, Zeist, The Netherlands) were used for all experiments. Animals were fed on a standard laboratory diet with water and food ad libitum and were kept under controlled environmental conditions. Procedures were performed under isoflurane anaesthesia. Three mice were injected intraperitoneally with LPS (5 mg/kg, *E. coli* serotype O127:B8, Sigma-Aldrich, Steinheim, Germany), and liver tissue and blood were procured after 1 h to serve as positive controls. All animal experiments were approved by the veterinary office of the canton of Zurich and were carried out according to institutional guidelines.

Abbreviations: CBDL, common bile duct ligation; EMSA, electrophoretic mobility shift assay; LPS, lipopolysaccharide; NF κ B, nuclear factor κ B; SBDL, selective bile duct ligation; TNF α , tumour necrosis factor α

Ligation of the common or the left bile duct

- *Common bile duct ligation (CBDL)*: After mid-line laparotomy, the common bile duct was mobilised and dissected above the pancreas between two 8-0 nylon sutures.
- *Selective bile duct ligation (SBDL)*: Selective ligation of the left hepatic duct was performed by placing two 9-0 nylon sutures around the left bile duct just above the bifurcation.¹²
- *Sham procedure (control)*: In control animals, laparotomy and mobilisation of the bile duct were performed as in the two other groups but without ligation.
- Cholecystectomy was performed in all the three groups. Bile duct ligations or sham laparotomies were performed 5 days¹³ before ischaemia or infection with *L. monocytogenes*.

Partial hepatic ischaemia

Partial hepatic ischaemia with 60 min of ischaemia and 1, 4 or 24 h of reperfusion was used as described previously.^{6, 14} The abdomen was opened through the initial mid-line incision. Inflow occlusion to the median and left liver lobes was performed by placing a microvascular clamp (Aesculap, San Francisco, California, USA). Reperfusion was initiated by removing the clamp. At the indicated reperfusion times, blood samples were taken from the inferior vena cava, the liver was flushed with saline and either fixed in 4% buffered formaldehyde or immediately shock frozen in liquid nitrogen.

Serum aspartate aminotransferase and total bilirubin

Serum levels of aspartate aminotransferase and total bilirubin were measured using a serum multiple analyzer (Ektachem DTSCII, Johnson & Johnson, Rochester, New Brunswick, USA).

Histological examination of liver sections

Formalin-fixed tissues were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin using standard techniques. To quantify the necrotic liver surface, 10 random high power fields ($\times 100$) per liver were assessed by using a semiautomatic image analysis system (Saisam V.4.2.2, Microvision Instruments, Evry Cedex, France) and expressed as a percentage of the liver surface. Neutrophils were visualised by chloroacetate esterase staining of sections embedded in paraffin wax using standard techniques.

NF κ B staining was performed on these sections after antigen retrieval in a TRIS-based buffer (Ventana Medical Systems, Illkirch, France) by boiling in a microwave oven for 30 min. Slides were incubated with a rabbit polyclonal antibody to the p65 subunit of NF κ B (1:200, Lab Vision, Fremont, California, USA catalogue number RB-1638-P01). The staining was performed in an automatic slide staining system (Discovery XT System; Ventana Medical Systems) using the iVIEW DAB-Kit (Ventana Medical Systems) with appropriate positive and negative controls.

Electrophoretic mobility shift assays for NF κ B

Oligonucleotides used in electrophoretic mobility shift assays (EMSAs; NF κ B consensus, top strand: 5'-GAT CAG TTG AGG GGA CTT TCC CAG GC-3'; NF κ B consensus, bottom strand: 5'-GAT CGC CTG GGA AAG TCC CCT CAA CT) were designed to have 5'-GATC overhangs when annealed, allowing radioactive labelling by fill-in reactions, as described previously.¹⁵ A total of 5 μ g of protein from nuclear extracts prepared from mouse livers using the NE-PER extraction kit (Pierce, Perbio Science, Lausanne, Switzerland) were used for individual DNA-binding reactions. The binding reactions were performed and the EMSA gels were run and processed as described previously.¹⁵ In supershift experiments, 1 μ g of the NF κ B p65 antibody (C-20;

Santa Cruz Biotechnology, Santa Cruz, California, USA) was added to the binding reactions.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from 50 mg of liver tissue using TRIzol reagent (Invitrogen, Paisley, UK) and 5 μ g of RNA was reverse transcribed using ThermoScript RT-PCR System (Invitrogen). Quantitative real-time polymerase chain reaction amplification and data analysis were performed using an ABI Prism 7000 Sequence Detector System (PE Applied Biosystems, Rotkreuz, Switzerland). TaqMan gene expression assay (PE Applied Biosystems) for TNF α (assay ID Mm 00443258_m) was used to quantify mRNA expression after normalisation to the house-keeping gene 18S (TaqMan ribosomal RNA control reagents, PE Applied Biosystems). Samples were calculated as

$$2^{-((\Delta C_t \text{ after ischaemia/reperfusion}) - (\Delta C_t \text{ before ischaemia/reperfusion}))}$$

where ΔC_t represents the differences in C_t values of the TNF α transcript and the endogenous control 18S RNA. The results shown represent fold induction after ischaemia compared with baseline levels in control, CBDL and SBDL mice before ischaemia.

Myeloperoxidase activity in liver tissue

Measurement of myeloperoxidase activity in liver tissue was performed as described previously¹⁶ with some modifications.^{17, 18} In brief, 50 mg of liver tissue were homogenised on ice in 500 μ l of 50 mM potassium phosphate (KPO₄) buffer, pH 7.4, with a hand tissue grinder, followed by centrifugation at 15 000 *g* for 15 min at 4°C. The pellet was washed in the same buffer and centrifuged for 5 min at 15 000 *g*, followed by resuspension in 10 volumes of 50 mM KPO₄ buffer, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide (Fluka Chemie Buchs, Switzerland) and incubation at 60°C for 2 h. After sonication, samples were subjected to three freeze-thaw cycles and again sonicated for 10 s followed by centrifugation at 15 000 *g* at 4°C for 15 min. The supernatant (100 μ l) was mixed with 100 μ l of *o*-diansidine dihydrochloride (10 mg/ml KPO₄ buffer; Sigma-Aldrich), 0.3% hydrogen peroxide (H₂O₂) was added and the absorbance was measured at 405 nm for 1 min to determine the Δ OD/min/mg protein.

Culture and infection with *L. monocytogenes*

L. monocytogenes (NF-L512, strain 10403S containing the actA-gfpuv-plcB transcriptional fusion in a single copy on the bacterial chromosome) was obtained from N Freitag (Seattle, Washington, USA) and cultured in brain heart infusion broth (Oxoid, Basingstoke, Hampshire, UK). Overnight cultures were plated on brain heart infusion agar plates (Oxoid) to determine bacterial titres. For intravenous injection, the original culture was diluted in phosphate buffered saline to inject 2×10^6 (12-h groups) or 1×10^5 colony-forming units (48-h groups) in 200 μ l.

Livers of control and CBDL mice were removed 12 or 48 h after infection. To determine bacterial titres, left liver lobes of infected animals (48 h) were homogenised, and organ suspensions were plated out in serial 10-fold dilutions on brain heart infusion agar plates. Whole livers (12 h) or the right and median lobes (48 h) were fixed in 4% buffered formaldehyde and processed for histological analysis.

Data presentation and statistical analysis

Data are presented as mean (standard deviation, SD). Statistical comparison of two groups at various time points was carried out by two-sided Student's *t* test for independent samples (SPSS Inc., Chicago, Illinois, USA). Comparison of >2 groups was performed by analysis of variance and Newman-Keuls retrospective analysis. Values of $p < 0.05$ were considered to be significant.

RESULTS

Does cholestasis increase ischaemic injury to the liver?

To assess the effect of obstructive jaundice on ischaemic liver injury, a histological quantification of liver necrosis was

performed after 1 h of ischaemia and 1, 4 and 24 h of reperfusion. No necrosis could be detected histologically at 1 h of reperfusion. However, 4 and 24 h of reperfusion showed a significant reduction of necrosis in cholestatic animals

Figure 1 Common bile duct ligation (CBDL) protects against hepatic ischaemia reperfusion injury. Liver injury was measured by (A) histological evaluation of parenchymal liver injury (haematoxylin and eosin (H&E) staining) at 4 and 24 h of reperfusion and (B) aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels at baseline and after 1 h of ischaemia followed by 1, 4 and 24 h of reperfusion. (C) Myeloperoxidase (MPO) activity in liver tissue before ischaemia and 24 h after reperfusion. (D) H&E stainings ($\times 100$, upper left, control; upper right, CBDL) and stainings of neutrophils in injured areas with chloroacetate esterase ($\times 400$, lower left, control; lower right, CBDL) at 24 h of reperfusion. White bars, control; black bars, CBDL. Mean and (SD) are shown for 6–8 animals per group. *Indicates significance ($p < 0.05$).

Figure 2 Selective bile duct ligation (SBDL) of the left bile duct reduces ischaemic liver injury and neutrophil infiltration despite the absence of hyperbilirubinaemia. (A) Model of common bile duct ligation (CBDL) and SBDL of the left lobe. Grey, biliary obstruction; diagonal black lines; ischaemia/reperfusion. RL: right lobe, ML: median lobe, LL: left lobe, CL: caudate lobe. (B) Representative haematoxylin and eosin sections ($\times 200$) of portal fields from sham livers (left), CBDL livers (middle) and the left lobe of SBDL (right) showing comparable bile duct proliferation in CBDL and SBDL livers. (C) Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels after 1 h of ischaemia and 24 h of reperfusion in mice subjected to sham laparotomy or SBDL before ischaemia. IR: 1 h ischaemia/24 h reperfusion. Values indicate mean (SD) ($n=6$ per group). (D) Myeloperoxidase (MPO) activity at 24 h of reperfusion. Values represent the mean (SD) of the same animals as in (C). Control, sham laparotomy; SBDL non-lig; non-ligated (median) lobe of SBDL mice; SBDL lig, ligated (left) lobe of SBDL mice. *Indicates significance ($p<0.05$).

(fig 1A,D). Although higher at 1 h, transaminase levels were significantly lower at 4 and 24 h in cholestatic animals (fig 1B). These findings also correlated with a near-absence of neutrophil infiltration in the ischaemic lobes of cholestatic animals at 24 h of reperfusion as determined by myeloperoxidase activity (fig 1C) and chloroacetate esterase staining (fig 1D). Taken together, these results suggest decreased, rather than increased, ischaemic injury in cholestatic mice.

Does cholestasis reduce liver injury and inflammation due to systemic or local factors?

The reduction in ischaemic injury and neutrophil infiltration in CBDL mice could be mediated by local or systemic factors. An appealing candidate is bilirubin, as its serum levels are strongly increased after CBDL. Bilirubin functions as a potent

intracellular antioxidant in neurones¹⁹ and has protective properties against cold ischaemic injury of the liver.²⁰ SBDL (fig 2A) leads to histological changes in the affected lobe, which are comparable to those observed after CBDL (fig 2B), yet does not lead to an increase in serum bilirubin levels (serum bilirubin levels for sham and SBDL: all animals below the limit of detection (0.1 mg/dl), CBDL: 15 (1.4) mg/dl). To test whether bilirubin is responsible for reduced liver injury and inflammation, we subjected SBDL mice to 1 h of ischaemia and 24 h of reperfusion. In comparison with control animals, SBDL mice showed significantly lower levels of transaminase (fig 2C), and neutrophil infiltration was reduced in both the non-ligated and ligated lobes (fig 2D). These findings indicate that the observed protection from liver injury and inflammation is due to a factor that is systemically released from the ligated lobe but is not related to hyperbilirubinaemia.

Figure 3 Common or selective bile duct ligation (CBDL or SBDL, respectively) inhibits the activation of nuclear factor κ B (NF κ B) in response to hepatic ischaemia. (A) DNA-binding activity of NF κ B assessed by electrophoretic mobility shift assay (EMSA). Nuclear protein extracts (5 μ g) from livers after sham laparotomy, CBDL with or without 1 h of ischaemia and 1 h of reperfusion, or mice treated with a single intraperitoneal injection of lipopolysaccharide (LPS; 5 mg/kg) were subjected to EMSA. The figure shows one of two assays from different animals with comparable results. (B) Densitometric analysis of both assays ($n=5-6$ per group). Values indicate mean (SD) of the densitometric analysis. *Indicates significance (sham v control + 1 h ischaemia/1 h reperfusion, $p<0.05$). (D) Same as (B), but from livers after SBDL, with or without 1 h of ischaemia and 1 h of reperfusion. (C) Densitometric analysis of (D) and a second gel including four animals per group. SBDL non-lig, non-ligated (median) lobe of SBDL mice; SBDL lig, ligated (left) lobe of SBDL mice; 1/1, 1 h ischaemia/1 h reperfusion. Values are mean (SD). *Indicates significance ($p<0.05$).

Is post-ischaemic activation of NF κ B altered in cholestasis?

NF κ B, a transcriptional activator of genes encoding inflammatory cytokines, mediates the inflammatory response to warm hepatic ischaemia.^{8, 21, 22} We tested the hypothesis that the markedly decreased inflammation found in cholestatic animals may be related to an altered activation of NF κ B in response to ischaemia. In control animals, 1 h of ischaemia followed by 1 h of reperfusion significantly increased NF κ B DNA-binding activity, as assessed by EMSAs. Consistent with a previous study,²³ cholestatic animals already showed an increased NF κ B-binding activity at baseline. In contrast with control animals, NF κ B-binding activity did not increase any further in cholestatic animals after ischaemia compared with baseline (fig 3A,B). This is not due to maximum activation of the NF κ B pathway during cholestasis, as nuclear extracts from livers of LPS-treated mice showed a several-fold stronger NF κ B-binding activity, indicating that further activation was possible (fig 3A).

To test whether local or systemic factors are responsible for the blocked activation of NF κ B, we again performed 1 h of ischaemia followed by 1 h of reperfusion 5 days after SBDL. In this model, bilirubin levels were normal (fig 2C) and no activation of NF κ B was found in the non-ligated lobe before ischaemia (fig 3C). Activation of NF κ B in response to ischaemia was blocked in the ligated (not shown) and also in the non-ligated lobe (fig 3C, D). Paralleling the finding with respect to post-ischaemic inflammation, the inhibited activation of NF κ B is due to the release of a systemic factor, but not bilirubin.

Does the reduced activation of NF κ B lead to diminished up regulation of TNF α ?

Activation of NF κ B⁸ and subsequent transcription and release of TNF α are crucial events in the evolution of reperfusion injury,^{6, 4} and are particularly important for inflammation in the post-ischaemic liver.⁸

To assess whether the impaired induction of NF κ B-binding activity leads to reduced transcription of TNF α ,^{25, 26} we quantified TNF α mRNA levels by real time polymerase chain reaction. After 1 h of ischaemia and 1 h of reperfusion, expression levels of mRNA for TNF α increased about fivefold in control animals. This initial induction was less in CBDL mice and the non-ligated lobes of SBDL mice (about threefold) and even absent in the ligated lobes of SBDL animals. In line with the activation of NF κ B, transcripts for TNF α further increased in the control group but they returned to baseline in CBDL mice as well as in both the ligated and the non-ligated lobes of SBDL mice at 4 h of reperfusion (fig 4). Thus, the inhibited activation of NF κ B resulted in a blocked induction of TNF α .

Does cholestasis also impair NF κ B activation and hepatic inflammation in a non-ischaemic model of liver inflammation?

To test whether activation of NF κ B and hepatic inflammation are also reduced in a non-ischaemic model of liver inflammation, control and CBDL mice were infected with *L. monocytogenes*. Infection with this Gram-positive intracellular pathogen is a

Figure 4 Common or selective bile duct ligation (CBDL or SBDL, respectively) inhibits the induction of tumour necrosis factor α (TNF α) in response to ischaemia. The induction of TNF α mRNA in postschaemic livers was determined by real time polymerase chain reaction at baseline and after 1, 4 and 24 h of reperfusion. Results indicate the fold induction by ischaemia from the respective baseline condition. Mean (SD) are shown for 5–6 animals per group. ■, control; ▲, CBDL; ◇, SBDL, non-ligated side; △, SBDL, ligated side. *Indicates a significant difference between control and the ligated side of SBDL ($p < 0.05$); †indicates a significant difference between control and all three other groups ($p < 0.05$).

well-established model to study liver inflammation.^{27–28} *L. monocytogenes* mainly infects the spleen and the liver. In the liver, the pathogen enters hepatocytes through binding of the hepatocyte growth factor receptor via the listerial surface protein internalin B. Infected hepatocytes activate NF κ B,^{29–30} followed by rapid neutrophil infiltration²⁷ and formation of microabscesses.

At 12 h after infection, immunohistochemical staining for NF κ B showed nuclear translocation in hepatocytes at sites of listerial infection in both control and cholestatic mice (fig 5A), clusters of infiltrating neutrophils were observed in non-cholestatic as well as in cholestatic livers (fig 5A), and the numbers of microabscesses were comparable (fig 5B).

At 48 h after infection, numerous hepatocytes of cholestatic mice showed a positive nuclear signal for NF κ B, whereas no more positive nuclei were detected in control animals (fig 5C). In line with this observation, cholestatic mice showed a diffuse infiltration of neutrophils, whereas only a few remaining abscesses were found in control animals (fig 5C). Despite this massive inflammation, the titres of *L. monocytogenes* were >200 times in cholestatic livers as in controls, indicating an inefficient clearance of the pathogen (fig 5D).

Figure 5 Activation of nuclear factor κ B (NF κ B) and liver inflammation in response to infection with *Listeria monocytogenes*, which were not impaired in cholestatic mice. Infection with *L. monocytogenes*, staining for the p65 subunit of NF κ B and determination of listerial titres were performed as described in the text. (A) Clusters of positively staining hepatocytes in areas of infection are observed in control and common bile duct ligation (CBDL) mice (upper left, control; upper right, CBDL) 12 h after injecting *L. monocytogenes*. Haematoxylin and eosin (H&E) staining showing typical microabscesses in both groups ($\times 200$, lower left, control; lower right, CBDL). (B) The number of microabscesses 12 h after infection was counted in 10 random high power fields (HPF) ($\times 100$). (C) Staining for NF κ B showing numerous positive hepatocytes in cholestatic but not in control mice (upper left, control; upper right, CBDL), which is associated with a diffuse neutrophil infiltration (H&E, $\times 63$; lower left, control; lower right, CBDL), 48 h after injecting *L. monocytogenes*. (D) Titres of *L. monocytogenes* 48 h after infection. Values indicate mean (SD; $n = 4$ per group). *Indicates significance ($p < 0.05$).

DISCUSSION

This study was designed to investigate the effect of biliary obstruction on hepatic ischaemia. Cholestatic animals showed reduced liver injury, impaired NF κ B activation, inhibited TNF α transcription and reduced hepatic inflammation in response to ischaemia. Experiments with SBDL suggested that the absence of inflammation is related to a systemically released factor independent of hyperbilirubinaemia. In contrast with ischaemia, infection with *L. monocytogenes* in cholestatic mice resulted in activation of NF κ B and severe inflammation, showing preserved initiation of the inflammatory pathway in response to a different type of injury.

The finding that cholestasis protects against ischaemic liver injury was unexpected in the light of a previous study showing increased early release of transaminases in cholestatic animals.⁴ In our study, we showed that the initial increase in transaminase levels does not correlate with increased liver injury at later time points. The higher transaminase levels at 1 h of reperfusion may be due to increased damage of sinusoidal endothelial cells⁴ without subsequent consequences. Sinusoidal cell damage is a predominant feature of cold ischaemic injury, as it is observed during the preservation of liver grafts. By contrast, normothermic ischaemic injury predominantly causes damage to hepatocytes (for a review see Selzner *et al*⁷). Another plausible mechanism is a “wash-out” of transaminases immediately after reperfusion from cells that are already damaged by the cholestatic liver injury itself. However, in view of the dramatic protection of cholestatic livers at later time points, we did not investigate this isolated finding further.

An interesting observation in our study was the near-absent infiltration of neutrophils in post-ischaemic cholestatic livers at 24 h of reperfusion, the reperfusion time point where neutrophil infiltration reaches its pinnacle.^{8, 17} This prompted us to focus on two key mediators of the inflammatory cascade involved in hepatic ischaemia, NF κ B and TNF α . Activation of the transcription factor NF κ B^{8, 21} and the subsequent production and release of TNF α ²⁴ plays a pivotal part in the inflammation triggered by ischaemic liver injury. In line with a reduced infiltration of neutrophils in cholestatic livers, NF κ B-binding activity did not increase in response to ischaemia in cholestatic animals. A possible explanation for this observation could be a maximal activation of the NF κ B pathway by bile duct ligation. Therefore, we included LPS-treated mice, which showed a further extensive activation of NF κ B, as well as a stronger induction and release of TNF α , which is consistent with results of a previous study.²¹ In addition, ligation of the left hepatic lobe was sufficient to block the activation of NF κ B in the post-ischaemic liver. The failure to activate NF κ B was also observed in the non-ligated median lobe. This lobe did not express increased nuclear translocation of NF κ B before ischaemia, indicating that activation of NF κ B is not required for the observed protection. In line with this finding, the induction of TNF α and infiltration of neutrophils were inhibited in the post-ischaemic livers of CBDL mice, as well as in the ligated and the non-ligated lobes of SBDL mice. This shows that a systemically released factor mediates the protection from post-ischaemic inflammation in cholestatic livers.

Hyperbilirubinaemia is a hallmark of cholestasis. Bilirubin is shown to have potent antioxidative properties in neurones,¹⁹ as well as in cold stored livers *ex vivo* and in a rat transplantation model.²⁰ Bilirubin also inhibits TNF α -induced activation of NF κ B and the expression of adhesion molecules including intracellular adhesion molecule-1 in endothelial cells *in vitro*.³¹ These observations prompted us to assess whether hyperbilirubinaemia is responsible for the inhibition of the inflammatory response. As the application of bilirubin *in vivo* is hampered by

a very short half life,³² we chose a model of local biliary obstruction in the left lobe. This model induces cholestatic changes in the liver comparable to those observed in the CBDL model¹² in the absence of increased systemic levels of bilirubin or bile acids.¹² SBDL of the left lobe was sufficient to inhibit the activation of NF κ B and the induction of TNF α in response to ischaemia in both the ligated and the non-ligated lobes. Thus, bilirubin is neither responsible nor required for the blocked activation of NF κ B in response to ischaemia under conditions of biliary obstruction. However, the blocked activation in the non-ligated lobe indicates that another factor, which is released from the ligated lobe, inhibits the induction of the inflammatory response. These findings, however, do not preclude a potential role for bilirubin as a protective molecule in a different context.

A recent study showed that bile duct ligation protects the liver from TNF α /GalN-induced hepatocellular apoptosis.¹¹ In contrast with the protection from post-ischaemic liver injury and inflammation found in our study, reduced hepatocellular apoptosis in the TNF α /GalN was limited to the ligated lobe, and was found to be mediated by two local factors, AKT and sphingosine kinase.¹¹ In another study it was found that cholestasis markedly reduced the occurrence of arrhythmias after cardiac ischaemia in a rat model. This effect was mediated by endogenous opioids but did not affect infarct size.³³

To better understand the reduced inflammation and activation of NF κ B in CBDL mice after ischaemia, we analysed the ability of cholestatic mice to activate NF κ B in hepatocytes during infection with *L. monocytogenes*. Ischaemia⁸ and infection with *L. monocytogenes*³⁰ led to an inhibitor of NF κ B kinase 2-dependent activation of NF κ B. However, cholestatic mice showed normal activation of NF κ B in infected hepatocytes 12 h after infection, accompanied by a comparable recruitment of neutrophils at sites of infection in cholestatic and control mice. This finding suggests that the activation of the NF κ B pathway after ischaemia is inhibited upstream of inhibitor of NF κ B kinase 2. It also indicates that the recruitment of neutrophils into the cholestatic liver is not inhibited, given that an adequate stimulus is provided.

The inability of cholestatic animals to clear *L. monocytogenes* was associated with considerable activation of NF κ B and the presence of a severe inflammatory response 48 h after infection. The impaired clearance may be due to reduced bactericidal activity of neutrophils in cholestatic animals, as it has been described for *Staphylococcus aureus*.³⁵ In addition, listeria are resistant to bile toxicity as a result of a bile salt hydrolase,³⁵ which is reflected by the accumulation of the germ in the bile duct³⁶ and the gall bladder³⁷ in normal animals. The reduced clearance of *L. monocytogenes* also provides further evidence that cholestasis reduces the resistance to bacterial infections, as has already been shown for *E. coli*¹³ and *S. aureus*.³⁴

We conclude that, in contrast with previous data⁴ and beliefs, cholestasis dramatically reduces post-ischaemic tissue necrosis and inflammation in the liver. The underlying mechanisms for poor tolerance of the cholestatic liver for surgery remain unclear, and future studies may focus on an increased susceptibility for infections with Gram-positive and Gram-negative bacteria. From our data, it is also tempting to conclude that cholestasis should not be considered an obstacle to inflow occlusion preventing blood loss during liver surgery. However, interspecies differences need to be considered and further experimental and clinical studies will be required before this concept can be adopted in clinical practice.

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