ORIGINAL ARTICLE

Interferon γ accelerates NF- κ B activation of biliary epithelial cells induced by Toll-like receptor and ligand interaction

K Harada, K Isse, Y Nakanuma

J Clin Pathol 2006;59:184–190. doi: 10.1136/jcp.2004.023507

See end of article for authors' affiliations

Correspondence to:
Dr Y Nakanuma,
Department of Human
Pathology, Kanazawa
University Graduate
School of Medicine,
Kanazawa 920-8640,
Japan; pbcpsc@kenroku.
kanazawa-u.ac.jp

Accepted for publication
3 May 2005

Background: The Toll-like receptor (TLR) family recognises pathogen associated molecular patterns (PAMPs) and plays a pivotal role in the innate immune response. Biliary epithelial cells (BECs) lining the intrahepatic bile ducts are potentially exposed to bacterial components in bile, and murine BECs possess TLRs that recognise PAMPs, resulting in nuclear factor κ B (NF- κ B) activation.

Aims: To examine the presence of TLRs in human BECs and the influence of cytokines and PAMPs on TLR expression and NF- κ B activation.

Methods: The expression of TLR2–5, MD-2, MyD88, and IRAK1 was examined in human liver tissue and cultured BECs by immunohistochemistry or reverse transcription polymerase chain reaction. The influence of PAMPs (peptidoglycan and lipopolysaccharide) in cultured cells preincubated with interferon γ (IFN γ) was evaluated by NF- κ B activation.

Results: TLR2–5, MyD88, and IRAK-1 proteins were detectable in BECs of the intrahepatic biliary tree in human liver tissue. TLR2–5, MD-2, MyD88, and IRAK-1 mRNA was demonstrated in human cultured BECs. The expression of these TLRs was upregulated by IFN γ , and TLR2 was upregulated by tumour necrosis factor α . Interleukins 4 and 6 failed to induce TLR upregulation. Interestingly, preincubation with IFN γ synergistically increased the upregulation of NF- κ B induced by PAMPs in cultured BECs.

Conclusion: These results suggest that the TLR family is present in human biliary cells and participates in the innate immunity of the intrahepatic biliary tree. Disordered regulation of TLRs after intracellular signalling by cytokines and PAMPs may be involved in immune mediated biliary diseases.

The intrahepatic biliary tree is a conduit through which bile passes to the extrahepatic bile duct.¹ There have been several reports that pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), lipoteichoic acids, and bacterial DNA fragments are detectable in bile,^{2–8} suggesting that the biliary epithelium may be exposed to these PAMPs. Biliary epithelial cells (BECs) produce various proinflammatory cytokines including interleukin 6 (IL-6) and tumour necrosis factor α (TNF α) when treated with LPS and cytokines.^{5–10} Moreover, the T cell derived cytokine milieu around bile ducts is associated with the aberrant expression of immune regulating molecules in affected bile ducts and the pathogenesis of inflammatory biliary diseases, such as primary biliary cirrhosis (PBC).^{9–12}

“Recently, we demonstrated that Toll-like receptor molecules are involved in biliary innate immunity using cultured murine biliary epithelial cells”

The Toll-like receptor (TLR) family plays a crucial role in the innate recognition of PAMPs.¹³ To date, 10 TLRs (TLR1–10) have been identified. TLR2 is responsible for the recognition of peptidoglycan (PGN) and lipoteichoic acid, whereas TLR4 recognises LPS.^{6–8} These responses to PAMPs transduce intracellular signals, leading to nuclear factor κ B (NF- κ B) translocation, which results in the synthesis of inflammatory cytokines such as TNF α .^{5–7, 8, 14–15} Epithelial cells lining the mucosal surface of the gastrointestinal tract^{16–17} and urogenital tract¹⁸ and immunocompetent cells have been shown to possess innate immunity involving TLR systems.

Recently, we demonstrated that Toll-like receptor molecules are involved in biliary innate immunity using cultured murine BECs.⁵ In our present study, we examined the expression of TLRs in human biliary epithelium, the

responsiveness to PAMPs, and the influence of cytokines using cultured human BECs, assuming that the TLR system of the biliary tree is involved in innate immunity and cytokine mediated inflammatory reactions in the intrahepatic biliary tree.

MATERIALS AND METHODS

Liver tissue specimens

Liver specimens from eight histologically normal livers (mean age, 58 years; male/female, three/five), 23 cases of PBC (stage I/II/III/IV, 10/eight/one/four; mean age, 60 years; male/female, two/21), 11 cases of extrahepatic biliary obstruction (EBO; mean age, 68 years; male/female, seven/four), and 26 cases of hepatitis C virus related liver cirrhosis (HCV-LC; mean age, 65 years; male/female, 16/10) were obtained from the liver disease file of our laboratory. Formalin fixed, paraffin wax embedded sections were prepared for immunohistochemistry.

Among the fresh human liver tissues investigated, samples from four normal livers (non-cancerous parts obtained from surgically resected livers of metastatic liver tumour), four HCV-LCs, and five PBCs (explanted livers) were used for reverse transcription polymerase chain reaction (RT-PCR). Frozen sections were prepared for microdissection from one normal liver, two PBCs, and one HCV-LC. Informed consent was obtained from all patients before surgery.

Abbreviations: BEC, biliary epithelial cell; EBO, extrahepatic biliary obstruction; HCV-LC, hepatitis C virus related liver cirrhosis; ICC, intrahepatic cholangiocarcinoma; IFN γ , interferon γ ; IFN γ R, interferon γ receptor; IL, interleukin; IL-4/5/6R, interleukin 4/5/6 receptor; LPS, lipopolysaccharide; NF- κ B, nuclear factor κ B; PAMP, pathogen associated molecular pattern; PBC, primary biliary cirrhosis; PCR, polymerase chain reaction; PGN, peptidoglycan; RT, reverse transcription; Th1, T helper type 1; TLR, Toll-like receptor; TNF α , tumour necrosis factor α ; TNFR, tumour necrosis factor receptor

Table 1 Primer sequences for reverse transcription polymerase chain reaction

	Forward	Reverse	Product size	Cycle number	Annealing temperature
TLR2	5'-GCCAAAGTCTTGATTGATGG-3'	5'-TTGAAGTCTCCAGCTCCTG-3'	347 bp	28	55°C
TLR3	5'-CCATTCCAGCCTCTTCGTAA-3'	5'-GGATGTTGGTATGGGTCTCG-3'	505 bp	25	55°C
TLR4	5'-TGGATACGTTTCCTTATAAG-3'	5'-GAAATGGAGGCACCCCTTC-3'	507 bp	28	55°C
TLR5	5'-GGAACCAAGCTCCTAGTCCT-3'	5'-GATGGCATCCTGGATATTGG-3'	575 bp	28	55°C
MD-2	5'-GTCCACCCTGTTTTCTTCAT-3'	5'-GGGCTCCAGAAATAGCTTC-3'	404 bp	25	55°C
MyD88	5'-CCAACCTTCAGCAGTGACAA-3'	5'-GTGTGTATGCTGGTGCCTGT-3'	398 bp	25	55°C
IRAK-1	5'-GAGTGGACTGCAGTGAAGCA-3'	5'-CTACCACCCCAAAGCTGAAG-3'	499 bp	25	55°C
IL-4R	5'-CAGAGAGCCTGTCCTGGAC-3'	5'-CACAGTGGTGGCTCAGAGA-3'	407 bp	28	65°C
IL-5R	5'-CTCCACAAAGGCTTTTCAGC-3'	5'-CTCCCCAGTGTGCTTTTGT-3'	308 bp	40	55°C
IL-6R	5'-TGGACACTCACACGGACACT-3'	5'-GTGGGAGGTGGAGAGAAGAGAGA-3'	600 bp	28	65°C
IFN γ R	5'-GGCAGCATCGCTTAAACTC-3'	5'-CACCTGCTACCTAGGAACC-3'	601 bp	22	60°C
TNFR1	5'-GAGAGGCCATAGCTGTCTGG-3'	5'-GTTTCTGAAGCCGTGAAGG-3'	303 bp	35	60°C
TNFR2	5'-GGATGAAGCCAGTTAACCA-3'	5'-TGCTCTGTCTTCATGGGTGA-3'	500 bp	30	60°C
GAPDH	5'-GGCCTCCAAGGAGTAAGACC-3'	5'-AGGGGTCTACATGGCAACTG-3'	147 bp	18	60°C

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN γ R, interferon γ receptor; IL-4/5/6R, interleukin 4/5/6 receptor; TLR, Toll-like receptor; TNFR, tumour necrosis factor receptor.

Laser capture microscopy and RNA amplification

Approximately 100 cells lining bile ducts were microdissected from the frozen sections using Microdissection System PixCellTM II (Arcturus, Mountain View, California, USA). Total RNA was extracted and amplified using the PicoPure RNA isolation kit and RiboAmpTM RNA amplification kit (Arcturus), respectively.

Immunohistochemistry

Dewaxed sections heat treated in citrate buffer were incubated overnight with polyclonal rabbit antibodies against human TLR2–5 and intracellular adaptor molecules (MyD88 and IRAK-1) (1 μ g/ml, Santa Cruz Biotechnology, Santa Cruz, California, USA), and then with the ENVISON system (Dako, Tokyo, Japan) for one hour. After reacting with benzidine, sections were counterstained with haematoxylin. As a negative control, normal rabbit IgG (1 μ g/ml) was used as the primary antibody; this always resulted in negative staining.

For the semiquantitative evaluation of TLR4, five representative septal or interlobular bile ducts¹ were chosen in each case (40 bile ducts in normal livers, 115 in PBC, 55 in

EBO, and 130 in HCV-LC) for assessment, and were evaluated as cytoplasmic or cytoplasmic and membranous (luminal, lateral, and/or basal) expression.

RT-PCR analysis

The expression of TLR2–5, MD-2 (coreceptor of TLR4),^{15–17} MyD88, and IRAK-1 mRNA in liver tissues, microdissected bile ducts, and cultured cells was detected by means of RT-PCR. In cultured cells, mRNA encoding IL-4, IL-5, IL-6, interferon γ (IFN γ), and TNF α receptors (IL-4R, IL-5R, IL-6R, IFN γ R, and TNFR1/2, respectively) was also examined. Total RNA from liver tissues and cultured cells, and amplified RNA from microdissected bile ducts, were used for reverse transcription. PCR was performed using DNA polymerase and specific primers (table 1); the PCR consisted of denaturation at 94°C (one minute), annealing (one minute), and extension at 72°C (two minutes) (table 1 shows the annealing temperatures and cycle numbers). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard.

For quantitative analysis, real time PCR was also performed according to a standard protocol using SYBR green and the ABI 7700 sequence detection system (ABI, Tokyo, Japan). Primers were newly designed to meet specific criteria using Primer Express Software (ABI).

Cultured cells

Two human intrahepatic cholangiocarcinoma (ICC) cell lines (CCKS1^{19–20} and HuCCT1²¹), and three human intrahepatic bile duct BEC cell lines (HIBEC1–3) were investigated. HIBEC1–3 are derived from explanted livers of one patient with HCV-LC²² and two patients with PBC.⁵

Stimulators

Human IL-4, IL-6, IFN γ , and TNF α were purchased from PeproTech (London, UK). PGN (InvivoGen, San Diego, California, USA) and Ultra Pure LPS (InvivoGen) were used as ligands for TLR2 and TLR4, respectively. Cultured cells were treated with the cytokines (1000 U/ml) for three hours and then analysed by RT-PCR.

Estimation of NF- κ B activation

Cultured cells were first treated with IFN γ for 36 hours and then continuously with PGN (1 μ g/ml) or LPS (1 μ g/ml) for two hours, after which NF- κ B activation was measured by the DNA binding capacity of NF- κ B using the TransAMTM NF- κ B kit (Active Motif, Carlsbad, California, USA).^{5–23} Treatment with IFN γ , PGN, or LPS alone was also examined.

Figure 1 Gel image of reverse transcription polymerase chain reaction for TLR2–5, MD-2, MyD88, IRAK-1, and GAPDH (positive control) mRNA in whole liver tissue from normal liver (lanes 1–4), PBC (lanes 5–9), and HCV-LC (lanes 10–13), and in microdissected bile ducts of normal liver (lane 14), PBC (lanes 15 and 16), and HCV-LC (lane 17). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCV-LC, hepatitis C virus related liver cirrhosis; PBC, primary biliary cirrhosis; TLR, Toll-like receptor.

Figure 2 Immunohistochemistry for TLR2–5, MyD88, and IRAK-1 in normal liver (A–C, E–H) and PBC (D). (A) TLR2 was expressed in the cytoplasm of septal bile ducts (arrow) and bile ductules (arrowhead). Hepatocytes (HC) are faintly positive. (B) TLR3 was expressed in large bile ducts (arrow), endothelial cells (arrowhead), and hepatocytes. (C) TLR4 was expressed in the cytoplasm of interlobular bile ducts (large arrow), endothelial cells (arrowhead), and Kupffer cells (small arrows). Hepatocytes were faintly positive or negative. (D) Interlobular bile ducts (arrow) in PBC expressed TLR4 in the lateral and luminal surfaces in addition to the cytoplasm. (E) TLR5 was expressed in the cytoplasm of septal bile ducts (arrow) and hepatocytes. (F) MyD88 was expressed in interlobular bile ducts (arrow) and endothelial cells (arrowhead). Hepatocytes were faintly positive or negative. (G) IRAK-1 was expressed in interlobular bile ducts (arrow), endothelial cells (arrowhead), and hepatocytes. (H) A semiserial section adjacent to that shown in (F). The positive signals were eliminated when the slide was incubated with normal rabbit IgG (negative control). PBC, primary biliary cirrhosis; TLR, Toll-like receptor.

Statistical analysis

The paired *t* test was used and significance was set at $p < 0.05$.

RESULTS

TLR expression in human liver tissue

Detection of TLR mRNA

Amplification of TLR2–5, MD-2, MyD88, and IRAK-1 mRNA was detected in all liver tissue and microdissected bile duct samples by RT-PCR (fig 1).

Expression of TLR protein

In normal liver, TLR2–5, MyD88, and IRAK-1 were immunohistochemically expressed diffusely in the cytoplasm of the intrahepatic large bile ducts, septal bile ducts, interlobular bile ducts, bile ductules, and intrahepatic peribiliary glands¹ (fig 2). Hepatocytes expressed TLR3, TLR5, and IRAK-1. Endothelial cells in portal tracts expressed TLR3, TLR4,

MyD88, and IRAK-1. Several Kupffer cells were positive for TLR4 and MyD88.

In the pathological livers, TLR2–5, MyD88, and IRAK-1 were diffusely expressed in the cytoplasm of bile ducts, as seen in normal liver. Moreover, TLR4 was also expressed on the luminal, lateral, and basal surfaces of bile ducts in variable combinations in pathological livers (fig 2D). Membranous (luminal, lateral, and/or basal) and cytoplasmic expression of TLR4 was found in 15 of 40 bile ducts in normal liver, 92 of 115 in PBC, 44 of 55 in EBO, and 108 of 130 in HCV-LC, with relative increased membranous staining in pathological livers (fig 3). TLR3 and MyD88 positive inflammatory cells were found scattered in the portal tracts.

Baseline expression of TLRs and cytokine receptors in cultured BEC

RT-PCR revealed that TLR2–5, MD-2, MyD88, and IRAK-1 mRNA was amplified in the two ICC and three HIBEC

Figure 3 Pattern of TLR4 expression in bile ducts. Five representative septal or interlobular bile ducts were chosen in each case for the assessment. Membranous (luminal, lateral, and/or basal) and cytoplasmic expression was found in 15 of 40 bile ducts in normal liver, 92 of 115 bile ducts in PBC, 44 of 55 bile ducts in EBO, and 108 of 130 bile ducts in HCV-LC. Membranous and cytoplasmic expression was significantly increased in pathological livers, compared with normal liver (* $p < 0.05$). EBO, extrahepatic biliary obstruction; HCV-LC, hepatitis C virus related liver cirrhosis; PBC, primary biliary cirrhosis; TLR, Toll-like receptor.

cultured cell lines without stimulation (fig 4). IL-4R, IL-6R, IFN γ R, and TNFR2 mRNA was also detected in all cells. TNFR1 was detected in both ICC cell lines, but not in the HIBEC cell lines. IL-5R was found in none of the cell lines. Therefore, IL-4, IL-6, IFN γ , and TNF α were chosen as cytokine stimulators for the studies that followed.

Cytokine induced TLRs and MD-2 expression

We investigated the effect of IL-4, IL-6, IFN γ , and TNF α treatment on the expression of TLR2–5 and MD-2 mRNA in cultured cells. As shown in the representative gel images (fig 5A), treatment with IFN γ upregulated the expression of TLR2–5 in all cells. In addition, TNF α upregulated the expression of TLR2. None of the cell lines showed upregulation of TLRs by IL-4 or IL-6. The expression of MD-2 was not affected by cytokine treatment.

The upregulation of TLR2–5 and MD-2 mRNA expression after treatment with IFN γ and TNF α was measured quantitatively using real time PCR. In all cultured cells, IFN γ significantly upregulated TLR2–5. The average increase in expression (shown as fold increase relative to unstimulated cells) was 3.5, 2.3, 2.0, and 2.3, respectively (fig 5B). TNF α also significantly upregulated TLR2 approximately fourfold (fig 5B).

Influence of preincubation with IFN γ on NF- κ B activation by LPS and PGN treatment

As shown in fig 6, treatment with IFN γ , PGN, or LPS alone increased NF- κ B activation 1.4, 2.3, and 2.6 fold in HuCCT1 cells and 1.8, 2.8, and 3.3 fold in HIBEC1 cells, respectively, compared with untreated cells. Preincubation with IFN γ

Figure 4 Reverse transcription polymerase chain reaction analysis of TLR2–5, MD-2, MyD88, IRAK-1, and cytokine receptors in cultured cholangiocarcinoma cells (HuCCT1 and CCKS1) and biliary epithelial cells (HIBEC1–3). TLR2–5, MD-2, MyD88, and IRAK-1 mRNA was detected in all cells. IL-4R, IL-6R, IFN γ R, and TNFR2 were expressed by all cells. TNFR1 was detected in cholangiocarcinoma cells only. None of the cells expressed IL-5R. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN γ R, interferon γ receptor; IL-4/5/6R, interleukin 4/5/6 receptor; TLR, Toll-like receptor; TNFR, tumour necrosis factor receptor.

followed by LPS treatment increased NF- κ B activation 5.8 fold (HuCCT1) and 6.8 fold (HIBEC1), and IFN γ preincubation followed by PGN treatment increased NF- κ B activation 5.9 fold (HuCCT1) and 11.0 fold (HIBEC1). This increased NF- κ B activation seen after sequential treatment with IFN γ and LPS, or IFN γ and PGN, was higher than that found by simply summing the values, which would be a 4.0 fold and a 5.1 fold increase in HuCCT1 and HIBEC1 cells, respectively, for IFN γ plus LPS, and a 3.7 fold and a 4.6 fold increase in HuCCT1 and HIBEC1 cells, respectively, for IFN γ plus PGN. Therefore, this effect was regarded as synergistic rather than additional.

DISCUSSION

We found diffuse immunohistochemical staining for TLR2–5, MyD88, and IRAK-1 in the intrahepatic biliary tree of normal human liver, irrespective of anatomical level. This was confirmed by the detection of the corresponding mRNA species by RT-PCR in the microdissected bile ducts and untreated cultured BECs. MD-2 mRNA was also detected in

Figure 5 Effects of cytokines on TLR2–5, and MD-2 mRNA expression. Cultured cholangiocarcinoma cells (HuCCT1 and CCKS1) and biliary epithelial cells (HIBEC1–3) were treated with phosphate buffered saline alone or with IL-4, IL-6, IFN γ , or TNF α . (A) Representative reverse transcription polymerase chain reaction (RT-PCR) gel images of (A) HuCCT1 and (B) HIBEC1. Treatment with IFN γ upregulated the expression of all TLRs except for MD-2. TNF- α also upregulated TLR2. Real time RT-PCR analysis of the effects of (C) IFN γ and (D) TNF α on TLR2–5 and MD-2 mRNA expression. Quantitative analysis revealed that the mean (SD) fold increase in TLR2, TLR3, TLR4, TLR5, and MD-2 mRNA expression after IFN γ treatment was 3.5 (1.1), 2.3 (0.7), 2.0 (0.6), 2.3 (0.8), and 1.1 (0.7), respectively; these results were significant except for MD-2. After TNF α treatment, the corresponding figures were 3.9 (1.3), 1.4 (0.2), 1.0 (0.1), 1.1 (0.3), and 1.1 (0.2); the results were significant for TLR2 only. Results are shown as mRNA expression relative to that without cytokine treatment. Bars indicate mean $\pm$ SD. * $p < 0.05$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN γ , interferon γ ; interleukin; PBS, phosphate buffered saline; TLR, Toll-like receptor; TNFR, tumour necrosis factor receptor.

cultured cells. However, the MD-2 protein could not be analysed, because a suitable antibody is not available. Thus, BECs expressing TLRs and related molecules should be regarded as active participants in biliary innate immunity.

Our recent study showed that cultured murine BECs express TLR2–5 and MD-2 constitutively, and respond to PAMPs by the activation of NF- κ B and the production of TNF α .⁵ We found in our present study that cultured human

Figure 6 Effect of IFN γ (interferon γ) on peptidoglycan (PGN) and lipopolysaccharide (LPS) induced nuclear factor κ B (NF- κ B) activation. HuCCT1 and HIBEC1 cells were exposed to IFN γ , and then PGN or LPS. Results are shown as relative activation of NF- κ B, compared with that without treatment. IFN γ by itself increased NF- κ B activation 1.4 and 1.8 fold in HuCCT1 and HIBEC1 cells, respectively. PGN or LPS alone increased NF- κ B activation 2.3 or 2.6 fold in HuCCT1 cells, and 2.8 and 3.3 fold in HIBEC1 cells, respectively. However, preincubation with IFN γ followed by PGN or LPS increased NF- κ B activation 5.9 and 5.8 fold in HuCCT1 cells and 11.0 and 6.8 fold in HIBEC1 cells, respectively. The increase in NF- κ B activation attributable to IFN γ pretreatment followed by LPS or PGN was synergistic, compared with the responses to IFN γ , LPS, or PGN alone. DNA–NF- κ B binding activity was effectively competed for by the wild-type consensus oligonucleotide, but not mutated oligonucleotide (data not shown).

BECs also induce NF- κ B activation in response to stimulation with PGN (a TLR2 ligand) and LPS (a TLR4 ligand). Constitutive expression of intracellular signalling molecules (MyD88 and IRAK-1) in BECs also supports the hypothesis that the activation of TLRs is followed by the activation of associated intracellular downstream signalling molecules, the activation of NF- κ B, and the production of inflammatory mediators.

T cell derived inflammatory cytokines participate in the regulation of TLR expression in several cell types.^{17, 24–26} Our study showed that IFN γ (a T helper type 1 (Th1) cytokine) upregulated the expression of TLR2–5 mRNA in cultured BECs. TNF α also upregulated the expression of TLR2. Interactions between TLRs and Th1 cytokines are involved in the pathogenesis of inflammatory bowel diseases.^{16, 17} Therefore, it is possible that this is also the case in some biliary diseases, such as PBC, which has a Th1 predominant peribiliary cytokine milieu. However, we found no differences between the expression of TLR in the biliary tree between normal and diseased bile ducts, except for TLR4, suggesting that the upregulation of these molecules is not reflected in protein concentrations in vivo. Interestingly, the bile ducts of pathological livers showed both membranous and cytoplasmic staining for TLR4, suggesting that the expression pattern may reflect the upregulation of TLRs in BECs.²⁷

“Our study showed that the T helper type 1 cytokine interferon γ upregulated TLR2–5 mRNA and accelerated the upregulation of pathogen associated molecular pattern induced NF- κ B activation in biliary epithelial cells”

We examined the effect of cytokines on PAMP induced NF- κ B activation in cultured BECs. This effect may reflect the combined influence of the periductal inflammatory micro-environment and the constituents of the bile on the development and persistence of immune mediated biliary diseases. It was found that the increase in NF- κ B activation by sequential treatment with IFN γ and LPS or IFN γ and PGN was higher than simply summing the effects of IFN γ and LPS alone, or IFN γ and PGN alone, suggesting a synergistic effect. Such synergy in intracellular signalling is a known property of the TLR system.²⁸

Although several bacterial and viral species have been reported to be associated with the pathogenesis of biliary diseases, including PBC and hepatolithiasis,^{29–33} the exact roles of BEC in the development of these biliary diseases is still unclear. Our study showed that the Th1-type cytokine IFN γ upregulated TLR2–5 mRNA and accelerated the upregulation of PAMP induced NF- κ B activation in BECs. TNF α , which is also produced by Th1 cells, upregulated TLR2. In situ BECs, which constitute the inflamed bile ducts, are

shown to produce TNF α .⁹ This suggests that a Th1 dominant peribiliary milieu leads to the increased susceptibility to PAMPs and the production of biologically active materials, including cytokines from BECs.^{2, 4, 29} Li suggested that well coordinated innate immunity signalling enables human cells and tissues to respond efficiently to various substances.³⁴ However, aberrant regulation of these pathways causes various diseases, such as asthma. Thus, increased susceptibility in BECs caused by a Th1 predominant cytokine milieu may be involved in the pathogenesis of the chronic cholangitis seen in PBC.^{11, 12}

In conclusion, our study has provided the first evidence that human intrahepatic bile ducts express TLR2–5, MyD88, and IRAK-1, which form a PAMP recognition system, thus contributing to biliary innate immunity. Moreover, IFN γ synergistically increased the reactivity of BECs to PAMPs. These findings imply that innate immunity involving the TLR system plays a role in the pathophysiology of the intrahepatic biliary tree, and may be linked to the development of acquired immunity.

Authors' affiliations

K Harada, K Isse, Y Nakanuma, Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa 920–8640, Japan

REFERENCE

- 1 Nakanuma Y, Hosono M, Sanzen T, *et al.* Microstructure and development of the normal and pathologic biliary tract in humans, including blood supply. *Microsc Res Tech* 1997;**38**:552–70.
- 2 Hiramatsu K, Harada K, Tsuneyama K, *et al.* Amplification and sequence analysis of partial bacterial 16S ribosomal RNA gene in gallbladder bile from patients with primary biliary cirrhosis. *J Hepatol* 2000;**33**:9–18.
- 3 Osnes T, Sandstad O, Skar V, *et al.* Lipopolysaccharides and beta-glucuronidase activity in choledochal bile in relation to choledocholithiasis. *Digestion* 1997;**58**:437–43.
- 4 Sasatomi K, Noguchi K, Sakisaka S, *et al.* Abnormal accumulation of endotoxin in biliary epithelial cells in primary biliary cirrhosis and primary sclerosing cholangitis. *J Hepatol* 1998;**29**:409–16.
- 5 Harada K, Ohira S, Isse K, *et al.* Lipopolysaccharide activates nuclear factor-kappaB through toll-like receptors and related molecules in cultured biliary epithelial cells. *Lab Invest* 2003;**83**:1657–67.
- 6 Chow JC, Young DW, Golenbock DT, *et al.* Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* 1999;**274**:10689–92.
- 7 Jiang Q, Akashi S, Miyake K, *et al.* Lipopolysaccharide induces physical proximity between CD14 and toll-like receptor 4 (TLR4) prior to nuclear translocation of NF-kappa B. *J Immunol* 2000;**165**:3541–4.
- 8 Kaisho T, Akira S. Toll-like receptors as adjuvant receptors. *Biochim Biophys Acta* 2002;**1589**:1–13.
- 9 Yasoshima M, Kono N, Sugawara H, *et al.* Increased expression of interleukin-6 and tumor necrosis factor-alpha in pathologic biliary epithelial cells: in situ and culture study. *Lab Invest* 1998;**78**:89–100.
- 10 Park J, Gores GJ, Patel T. Lipopolysaccharide induces cholangiocyte proliferation via an interleukin-6-mediated activation of p44/p42 mitogen-activated protein kinase. *Hepatology* 1999;**29**:1037–43.
- 11 Harada K, Van de Water J, Leung PS, *et al.* In situ nucleic acid hybridization of cytokines in primary biliary cirrhosis: predominance of the Th1 subset. *Hepatology* 1997;**25**:791–6.
- 12 Dienes HP, Lohse AW, Gerken G, *et al.* Bile duct epithelia as target cells in primary biliary cirrhosis and primary sclerosing cholangitis. *Virchows Arch* 1997;**431**:119–24.
- 13 Anderson KV. Toll signaling pathways in the innate immune response. *Curr Opin Immunol* 2000;**12**:13–19.
- 14 Akashi S, Saitoh S, Wakabayashi Y, *et al.* Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. *J Exp Med* 2003;**198**:1035–42.
- 15 Shimazu R, Akashi S, Ogata H, *et al.* MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 1999;**189**:1777–82.
- 16 Abreu MT, Vora P, Faure E, *et al.* Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *J Immunol* 2001;**167**:1609–16.
- 17 Abreu MT, Arnold ET, Thomas LS, *et al.* TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. *J Biol Chem* 2002;**277**:20431–7.
- 18 Schilling JD, Mulvey MA, Vincent CD, *et al.* Bacterial invasion augments epithelial cytokine responses to Escherichia coli through a lipopolysaccharide-dependent mechanism. *J Immunol* 2001;**166**:1148–55.

Take home messages

- Our study provides the first evidence that human intrahepatic bile ducts express Toll-like receptors (TLRs) 2–5, MyD88, and IRAK-1, which form a pathogen associated molecular pattern (PAMP) recognition system, thus contributing to biliary innate immunity
- Interferon γ (IFN γ) synergistically increased the reactivity of biliary epithelial cells to PAMPs
- Thus, innate immunity involving the TLR system may play a role in the pathophysiology of the intrahepatic biliary tree in immune mediated diseases, such as primary biliary cirrhosis

- 19 **Saito K**, Minato H, Kono N, *et al*. Establishment of the human cholangiocellular carcinoma cell line (CCKS1) [in Japanese]. *Kanzo* 1993;**34**:122–9.
- 20 **Sugawara H**, Yasoshima M, Katayanagi K, *et al*. Relationship between interleukin-6 and proliferation and differentiation in cholangiocarcinoma. *Histopathology* 1998;**33**:145–53.
- 21 **Miyagiwa M**, Ichida T, Takiwa T, *et al*. A new human cholangiocellular carcinoma cell line (HuCC-T1) producing carbohydrate antigen 19/9 in serum-free medium. *In Vitro Cell Dev Biol* 1989;**25**:503–10.
- 22 **Kamihira T**, Shimoda S, Harada K, *et al*. Distinct costimulation dependent and independent autoreactive T-cell clones in primary biliary cirrhosis. *Gastroenterology* 2003;**125**:1379–87.
- 23 **Renard P**, Ernest I, Houbion A, *et al*. Development of a sensitive multi-well colorimetric assay for active NFkappaB. *Nucleic Acids Res* 2001;**29**:E21.
- 24 **Liu S**, Salyapongse AN, Geller DA, *et al*. Hepatocyte toll-like receptor 2 expression in vivo and in vitro: role of cytokines in induction of rat TLR2 gene expression by lipopolysaccharide. *Shock* 2000;**14**:361–5.
- 25 **Matsumura T**, Ito A, Takii T, *et al*. Endotoxin and cytokine regulation of toll-like receptor (TLR) 2 and TLR4 gene expression in murine liver and hepatocytes. *J Interferon Cytokine Res* 2000;**20**:915–21.
- 26 **Faure E**, Thomas L, Xu H, *et al*. Bacterial lipopolysaccharide and IFN-gamma induce Toll-like receptor 2 and Toll-like receptor 4 expression in human endothelial cells: role of NF-kappa B activation. *J Immunol* 2001;**166**:2018–24.
- 27 **Nagai Y**, Akashi S, Nagafuku M, *et al*. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol* 2002;**3**:667–72.
- 28 **Ahonen CL**, Doxsee CL, McGurran SM, *et al*. Combined TLR and CD40 triggering induces potent CD8+ T cell expansion with variable dependence on type I IFN. *J Exp Med* 2004;**199**:775–84.
- 29 **Harada K**, Tsuneyama K, Sudo Y, *et al*. Molecular identification of bacterial 16S ribosomal RNA gene in liver tissue of primary biliary cirrhosis: is *Propionibacterium acnes* involved in granuloma formation? *Hepatology* 2001;**33**:530–6.
- 30 **Selmi C**, Balkwill DL, Invernizzi P, *et al*. Patients with primary biliary cirrhosis react against a ubiquitous xenobiotic-metabolizing bacterium. *Hepatology* 2003;**38**:1250–7.
- 31 **Harada K**, Ozaki S, Kono N, *et al*. Frequent molecular identification of *Campylobacter* but not *Helicobacter* genus in bile and biliary epithelium in hepatolithiasis. *J Pathol* 2001;**193**:218–23.
- 32 **Xu L**, Shen Z, Guo L, *et al*. Does a betaretrovirus infection trigger primary biliary cirrhosis? *Proc Natl Acad Sci U S A* 2003;**100**:8454–9.
- 33 **Mason AL**, Xu L, Guo L, *et al*. Detection of retroviral antibodies in primary biliary cirrhosis and other idiopathic biliary disorders. *Lancet* 1998;**351**:1620–4.
- 34 **Li L**. Regulation of innate immunity signaling and its connection with human diseases. *Curr Drug Targets Inflamm Allergy* 2004;**3**:81–6.