cases in the Kanazawa series and 61.8% of 411 cases in the Asan series), and the relatively higher frequency of this type in the Kanazawa series probably reflected the relatively low number of IPNB cases (Table 3; $P < 0.01$). Among the bile duct type of ICC, the majority of cases were of a well- to moderately-differentiated adenocarcinoma in both institutions. The proportion of bile ductular type was similar in the Kanazawa series (10%) and Asan series (10%), and similarly high in group A and low in group B in both institutions. Variants of ICC were similarly rare in group A and group B of both institutions.

Relation of subtypes of ICC with respect to the etiology of non-biliary CALD

While the majority of ICC cases at Kanazawa University were related to HCV infections, those of the Asan Medical Center were related to HBV infections. There was no correlation between the subtypes and the etiology of CALD, particularly hepatitis type C and type B-related liver diseases (data not shown).

Incidence of ICC in the autopsy series

A total of 36 cases of ICC were found in a total of 2853 autopsied cases at the Departments of Pathology, Kanazawa University Graduate School of Medicine from 1988 to 2008. During this period, a total of 294 cases of HCC, 18 cases of combined HC-CC cases, and 36 cases of ICC were autopsied. Among the ICC, 11 cases were associated with long-standing biliary diseases such as hepatolithiasis (30.6%), 17 cases were found in almost normal livers or livers showing non-specific reactive changes (47.2%), and eight cases (22.2%) were associated with non-biliary CALD related to hepatitis B (two cases) or C (five cases) viral infections or of unknown etiology (one case).

DISCUSSION

The findings of this study can be summarized as follows. (i) Of the of autopsied ICC cases 22.2% were associated with non-biliary CALD (group A); (ii) A whole pathological spectrum of ICC found in almost normal livers or livers showing NSRH (group B) was also found in group A; (iii) Bile duct ICC, particularly well- to moderately-differentiated types, were common to both group A and group B in the Kanazawa University cases and Asan Medical Center cases; (iv) Bile ductular ICC occurred relatively frequently in group A in both institutions; (v) IPNB was unlikely to develop in group A, but relatively common in group B, particularly in the Asan Medical Center cases.

To compare the pathological spectrum of ICC arising in groups A and B, a simplified histological classification of ICC was applied in this study. That is, peripheral ICC was categorized as bile duct adenocarcinoma, bile ductular adenocarcinoma, and variants. The bile duct type was a conventional ICC, and was further classified into well-, moderately- and poorly-differentiated types. While perihilar ICC showing IPNB was examined in this study, perihilar ICC showing periductal spread with or without mass formation was not because it can be difficult to differentiate this type from hilar CC or extrahepatic CC showing periductal spread with or without mass formation.

While ICC usually arises in an apparently normal liver, it is also known to arise in non-biliary CALD, particularly liver cirrhosis,[8,14,25,26] and chronic HCV and HBV infection has recently been suggested to be involved in the pathogenesis of ICC. A survey of ICC in our routine autopsy series showed that ICC were found in about 1% of cases (36 of 2853 autopsy cases), and that the incidence of ICC arising in non-biliary CALD was 22.2%. Microscopic pathological findings in non-cancerous parts of the liver in 348 ICC cases from 645 Japanese medical institutions over a period of 2 years (from 1 January 2002 to 31 December 2003) registered by the Japan Liver Cancer Study Group,[27] showed that normal liver, chronic hepatitis/liver fibrosis, and liver cirrhosis were found in 68.4% (238 cases), 20.7% (72 cases) and 10.9% (38 cases) of patients, respectively. In addition, HBsAg and HCVAb were found in 6.2% and 19.1% of 696 ICC cases examined.[26] Taken together, these findings suggest non-biliary CALD, particularly liver cirrhosis, is a background setting for the development of ICC, and chronic hepatitis B and C viral infections are significantly involved in the carcinogenesis of ICC in Japan.

There have been very few pathological studies on ICC arising in non-biliary CALD. As for the clinicopathological features of HCV-related ICC, Yamamoto *et al.* reported that macroscopically, HCV-related ICC were firm and gray-to-tan with nodular-type characterisitics.[5] Particularly, minute nodular ICC appears to be related to hepatitis viral infection and could be detected at an early stage, similar to HCC, by following up cases of chronic hepatitis or cirrhosis.

Histopathological examination revealed that tumor cells were composed of microtubular structures with a fibrous stroma. Tumors were diagnosed as well-differentiated or moderately-differentiated adenocarinoma,[5] and some cases showed poorly-differentiated or anaplastic adenocarcinoma.[25,26]

It was found in this study that the entire spectrum of ICC encountered in group B was also found in group A, and that the majority of ICC were of the bile duct type in both groups. Interestingly, most of them were well- to moderately-differentiated adenocarcinomas, raising the

possibility that the cholangiocarcinogenesis operative in group B may be also involved in group A. As mentioned above, the incidence of ICC was higher in non-biliary CALD according to our survey of ICC in the autopsy series and a survey by the Japan Primary Cancer Study.[27] Taken together, this raises the possibility that the level of risk of the cholangiocarcinogenesis speculated to occur in group B[4,8] is increased in group A. Increased proliferation of bile ductules or progenitor cells in non-biliary CALD may be involved in this high risk of cholangiocarcinogenesis. While the poorly-differentiated type was infrequent in both groups, it was more common in group A than group B. Some environmental factors in non-biliary CALD may be related to this relatively frequent occurrence of poorly differentiated ICC. While the majority of cases in Japan were related to HCV infections, those in Seoul were related to HBV infections. There was no correlation between the subtypes and the etiology of CALD, particularly type C and type B-related chronic liver disease.

It was found that in the ICC of group A, the proportion of bile ductular type was rather high (22.6%)

in comparison with group B (8.4%; $P < 0.001$). This was true for the Kanazawa University cases (17.6%) and also the Asan Medical Center cases (25%). As for the background of bile ductular ICC as a whole, 12 of 47 cases were associated with group A (25.5%). Komuta *et al.* reported that nine of 30 cases (30%) of cholangiolocellular carcinoma, similar to bile ductular ICC, were associated with chronic hepatitis stage 3 or 4,[20] an incidence comparable to that of this study. Therefore, it seems plausible that the incidence of ductular ICC was relatively high in group A and this may have some cholangiocarcinogenetic significance. Bile ductular carcinoma, which is also called cholangiolocellular carcinoma,[16] is known to express phenotypes characterizing bile ductules or hepatic progenitor cells such as NCAM or EpCAM,[18–20] suggesting this type of ICC to have involved hepatic progenitor cells or stem cells during its carcinogenesis or progression. Interestingly, carcinoma cells resembling ductal plate malformation were also found to be intermingled in the ductular type of ICC. Ductal plate malformation is frequently found in Caroli's disease and congenital hepatic fibrosis and is reported to reflect abnormalities in the remodeling of hepatic progenitor cells leading to the ductal formation,[17,21] supporting the suggestion above.

It was found in this study that bile ductular ICC was more likely to develop in group A than group B in both institutions. Some pathological conditions inherent in non-biliary CALD may be involved in the development of bile ductular ICC. Bile ductules or canals of Hering, where hepatic progenitor cells are located, are increased in number in non-biliary CALD.[20] So, it seems conceivable that these proliferated bile ductules in group A led to the alteration of hepatic progenitor cells followed by the increased risk of bile ductular ICC. Of course,

further studies, including gene-expression profiling of the liver and bile ductular ICC, are needed to resolve this issue.

IPNB is characterized by intraductal papillary proliferation of neoplastic biliary lining epithelial cells,[22,23] and is not infrequently associated with mucin overproduction. This tumor is relatively common in Asian countries, and associated with hepatolithiasis in Taiwan and Japan.[22,23] It was found in this study that IPNB was rather frequent in group B (22.5%) in comparison with group A (3.8%; $P < 0.001$), suggesting that IPNB was unlikely to develop in group A. The proportion of IPNB in ICC was high in Seoul in comparison with Kanazawa. That is, about one fourth of ICC belonged to IPNB in group B in Seoul, while IPNB was rare in Kanazawa. In this study, the cases of ICC associated with hepatolithiasis or PSC were excluded. The relatively high rate of IPNB in Seoul might have been related to an environmental factor which is rare or absent in Kanazawa. Some types of biliary tract diseases, such as clonorchiasis, might be related to the development of IPNB in East Asia.[8,28–30] Further clinical and epidemiological surveys on past or present *Clonorchis sinensis* infections and the eating of raw freshwater fish seems necessary to solve this problem.

Based on the huge number of ICC cases in Asia, it was found that the whole pathological spectrum of ICC found in apparently normal livers or NSRH (group B), was also found in non-biliary CALD (group A), and the bile duct type was frequent in both group A and group B, suggesting that the cholangiocarcinogenesis in group B is also involved in group A, and the level of risk is increased in group A. Interestingly, bile ductular ICC was likely to develop in group A, suggesting that carcinogenesis of this type, possibly involving the hepatic progenitor cells, is more specifically related to chronic advanced liver disease. In contrast, IPNB was unlikely to develop in group A. While one fourth of the ICC in Seoul belonged to IPNB, this type was rare in Kanazawa, suggesting an environmental factor in Seoul, which is absent or rare in Kanazawa, to be involved in the development of IPNB.

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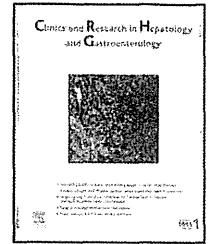
MINI REVIEW

The role of the pathologist in diagnosing and grading biliary diseases

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Summary Pathological features of primary biliary cirrhosis (PBC) are reviewed. Immune-mediated, non-suppurative cholangitis is the initial lesion and is followed by the gradual and extensive destruction of bile ducts and development of chronic cholestasis. Simultaneously, necro-inflammatory activities of the hepatic parenchyma and limiting plates of milder form develop not infrequently. Eventually, liver fibrosis and cirrhosis develop. A new system applicable to needle liver biopsies in which staging is evaluated using a combination of three factors (fibrosis, cholestasis, and bile duct loss) and necro-inflammatory activities of the bile duct and hepatic parenchyma are graded, is proposed. The clinical and therapeutic evaluation of PBC using this system is warranted.
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Introduction

There are many kinds of biliary diseases affecting various anatomical levels of the biliary tree, and each presents characteristic clinical and pathologic features [1—3]. In this minireview, the roles of pathologists in diagnosing and grading biliary diseases, especially primary biliary cirrhosis (PBC), are described. First, the anatomy of the biliary tree is briefly reviewed.

The biliary tree is composed of intrahepatic bile ducts, right and left hepatic ducts, and the extrahepatic bil-iary system. While there is no sharp delineation of the various segments, the intrahepatic bile ducts are classi-fiable into large and small intrahepatic bile ducts [1,4]. The former consist of the first to third branches of the right and left hepatic ducts, and are accompanied by peribiliary glands. They are lined by a tall columnar epithe-lium on a collagenous duct wall. The latter are classified into septal and interlobular bile ducts. The septal ducts (>100 μm in diameter) are lined by tall columnar cells and are surrounded by the duct wall. In contrast, the interlobular bile ducts are lined by a low columnar and cuboidal epithelium. The interlobular bile ducts are con-nected to bile canaliculi by bile ductules and canals of Hering.

Each segment of the biliary tree has unique anatomical and physiologic characteristics. The bile ducts are lined by biliary epithelial cells (BECs) or cholanigocytes. BECs play

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a number of roles in the biliary system; participating in bile acid reabsorption and drug metabolism, contributing to 30—40% of total bile secretion and mediating immune responses [4].

Clinical and immunological features of PBC

PBC is an autoimmune liver disease which predominantly affects middle-aged to elderly women [5]. Serologically, the most helpful diagnostic test is the demonstration of antimitochondrial antibodies (AMAs), which are found in more than 95% of patients, and serum IgM levels are usually elevated. The main features are usually pruritus and lethargy with skin pigmentation and eventually cholestatic jaundice, although icterus may not develop for years after the initial symptoms. PBC is not infrequently associated with extrahepatic autoimmune diseases such as Sjögren syndrome and chronic thyroiditis. PBC patients may remain asymptomatic for some years and experience a normal life expectancy. Liver function tests show a normal or mildly elevated bilirubin level at early stages, accompanied by a striking and disproportionate elevation of serum alkaline phosphatase. Its natural history is presumed to be about 20 years. Death is usually due to hepatocellular failure, with bleeding from oesophageal varices in approximately 30% of cases.

Following antigen cloning, the major autoantigens which AMAs recognize are now identified as components of the 2-oxo-acid dehydrogenase complex (2-OADC) which is located on the mammalian inner mitochondrial membranes [6]. The occurrence of ANA of the centromere type and autoantibodies against gp210 is also characteristic of PBC [7].

PBC may be a multifactorial disease related to genetic and environmental factors [8]. Genetic factors are suggested by a familial predisposition and an increased prevalence of autoantibodies in relatives of PBC patients. Invernizzi et al. reported that DRB1*08 and DRB1*02 were significantly associated with PBC [9]. The association of PBC with environmental factors has been suggested, and the formation of AMAs during the pathogenesis of PBC has been assumed to be triggered by these environmental factors. The formation of granuloma formation is part of the early bile-duct damage in PBC. Harada et al. have identified several indigenous bacteria, among them *Propionibacterium acnes* (*P. acnes*) as a major clone, in granulomas of PBC, suggesting that *P. acnes* and other enteric bacteria are involved in the pathogenesis [10]. Halogenated xenobiotics actively metabolized in the liver can also modify self-molecules, rendering them immunogenic and resulting in the development of PBC [11].

PBC is characterized by a breakdown in self-tolerance of T and B cells to the conserved mitochondrial self antigen PDC-E2, and then the occurrence of autoreactive T and B cells against PDC-E2 [12]. The hypothesis of molecular mimicry implies that foreign pathogens with homology to self-protein or modified self-protein can break the tolerance.

Autoreactive T cells, recognizing the PDC-E2 component, may be involved in the pathogenesis of bile duct injuries in PBC. In PBC, CD4+ T cells have been shown to recognize amino acids 163—176 of PDC-E2 and there is a corresponding marked increase of CD4+ CD28—T cells in PBC livers [12]. Furthermore, CD8+ cytotoxic T lymphocytes that recognize components of amino acids 159—167 of PDC-E2 have an effector role in the bile duct injury.

Pathology of PBC

The interlobular bile ducts are affected at an early stage in PBC [2]. Parenchymal necro-inflammatory changes involving lobular and periportal areas are not infrequent, though they are usually mild [13]. In time, the cholestatic and necro-inflammatory processes progress and are followed by a progressive fibrosis and eventually cirrhosis.

Bile duct damages

The early lesion of PBC is characterized by injuries to interlobular bile ducts, which eventually undergo destruction. These bile-duct lesions are called chronic non-suppurative destructive cholangitis (CNSDC) or florid duct lesions [14,15]. Epithelial cells of the bile ducts are variably swollen or show an eosinophilic shrunken appearance with pyknotic nuclei. The key pathologic process is immune-mediated damage to bile ducts with cellular senescence of BECs undergoing poor regeneration and eventual apoptotic loss [16—19]. The majority of lymphocytes in the portal tracts are CD4+ (Th1 subset) and CD8+ T cells. Plasma cells and eosinophils can be conspicuous in the early stages. Aggregates of epithelioid cells and a few epithelioid cells are characteristically seen in the vicinity of the bile ducts in the early stages.

Serial sections disclose the segmental disappearance of interlobular bile ducts [2,20]. In contrast, the septal and large intrahepatic ducts, although they may show some inflammation in their walls, are preserved even at an advanced stage of the disease.

Parenchymal and interaface changes

In the early stages, the bile-duct injury and associated inflammation remain confined within the portal tract boundaries. In the parenchyma, single hepatocellular necrosis, Kupffer-cell hyperplasia and sinusoidal infiltration with scattered lymphocytes and pigment-laden macrophages are found, though their degree is usually mild [21]. Subsequently, an extension of the necro-inflammatory process to the periportal parenchyma may eventually develop. This may take either or both of two forms; chronic cholestatic (biliary interface activity) and necro-inflammatory changes (lymphocytic interface activity) [21,22].

Biliary interface activity
Biliary interface activity becomes the main feature as the duct loss progress. In common with other cholestatic disorders, atypical bile ductular proliferation may be a striking feature [1,23]. Another form is cholate stasis showing hydropic hepatocytic swelling and the deposition of copper or copper-associated protein granules, and this may result from the "toxic" effect of hydrophobic bile acids. Mallory bodies and intracellular or canalicular bile pigment dominate the interface at an advanced stage.

Lymphocytic interface activity

Lymphocytic interface activity resembling autoimmune hepatitis (AIH) is involved. In some cases, this lesion seems to reflect an extension to adjacent hepatocytes of the same immunological process in CNSDC, while in other cases, this activity may be apparently independent of CNSDC. Lymphocytic interface activity is present in a substantial number of cases, usually in a milder form. In less than 10% of cases, it may even dominate with variable lobular activities and be associated with clinical features of AIH, so-called synchronous PBC-AIH overlap syndrome [24–26].

Progression of bile duct and hepatic lesions

Histologically, interlobular bile ducts are selectively affected, presenting characteristic findings such as CNSDC, and those affected eventually disappear from the liver. This pathology may evolve over many years without any obvious morphological cholestasis or bilirubinostasis, and this period of evolution is gradually associated with characteristic changes at the interface between the portal tracts and parenchyma — periportal cholate stasis and biliary interface activity and fibrosis. At the same time, hepatitic activities of hepatic lobules and periportal areas are not infrequent, though they are usually mild. Biliary type fibrosis is dense, scar-like in the deeper portions of the septa, but oedematous at the periphery. The second hepatitic activities may also be involved in the progression of PBC, and some cases of PBC with rather prominent hepatitic activities and interface activities may present some features of post-necrotic cirrhosis. Chronic cholestatic and hepatitis activities in various combinations may be responsible for progressive hepatocellular damage and fibrosis in a majority of cases, and liver cirrhosis and hepatic failure may eventually develop [22,23].

Histopathologic diagnosis of PBC

At early and relatively early stages of PBC

Pathologic lesions strongly suggestive of PBC
A florid duct lesion or CNSDC of the interlobular bile duct, is pathognomonic of PBC [15]. Epithelioid cell granulomas around damaged bile ducts, well-formed granulomas or loosely arranged epithelioid cells, are also a characteristic finding of PBC [26]. However, these pathological features strongly suggestive of PBC are distributed heterogeneously within the liver, and might not be sampled by liver biopsy needles.

Bile duct loss or ductopenia which follows chronic destructive cholangitis, is also a characteristic finding of PBC. This ductopenic lesion may develop and progress in the early stages and the more extensive in advanced stages [27]. The presence of arteries unaccompanied by ducts is a useful, yet rough, marker of bile-duct loss [28]. Immunostaining of a biliary cytokeratin such as K19 or K7 is very useful for the detection or identification of bile ducts in portal tracts.

Pathologic lesions suggestive of PBC
Mild chronic cholangitis (lymphocytic or pleomorphic) and variable biliary epithelial damage are frequently found and suggestive of PBC [29], but not diagnostic [30]. Elevated levels of IgM and predominant infiltration by IgM+ plasma cells in portal tracts are typical of PBC [31]. Epithelioid granulomas in the hepatic lobules suggest a diagnosis of PBC. Atypical ductular reactions and the focal deposition of copper or copper-binding proteins are also found in PBC.

Pathological changes raising the possibility of PBC
Small cell changes of hepatocytes in zone 1 and 2 provide us a hint of chronic cholestatic liver disease, particularly PBC [32]. Vague nodularity of the hepatic parenchyma in similar zones is also found in PBC [33]. Prominent eosinophilic infiltration in portal tracts is evident in some cases [34].

Diagnosis of PBC at relatively advanced and advanced stages of PBC

At advanced stages, characteristic features suggestive of PBC are rare, but prominent chronic cholestasis, extensive fibrosis and regenerative nodules are found. PBC should be suspected based on the following features [2,14]:

- a virtual absence of interlobular bile ducts;
- focal lymphocytic aggregates seem to replace the missing bile duct(s);
- peripheral cholate stasis or cholestasis with Mallory bodies and extensive copper deposition;
- a biliary pattern of fibrosis;
- partial or focal preservation of the normal architecture in otherwise established cirrhosis.

New staging and grading system of PBC

Progression of PBC eventually leading to cirrhosis has been evaluated histologically using classical staging systems proposed by Scheuer [14] and Ludwig et al. [35]. In Scheuer's staging system, PBC is histologically classified into four stages by using characteristic histologic features: stage 1 is characterized by florid duct lesions or CNSDC, and in stage 2, there is a characteristic proliferation of bile ductules. Stage 3 is characterized by fibrosis or scarring and stage 4, by cirrhosis. Ludwig's system applies the histologic features used for the staging of chronic active hepatitis to the staging of PBC: portal hepatitis (stage 1), periportal interface hepatitis (stage 2), bridging necrosis or bridging fibrosis (stage 3), and cirrhosis (stage 4). Histological changes of PBC are, however, heterogeneous in whole liver and sampling errors occur in needle liver biopsies of PBC. Furthermore, as Scheuer pointed out, stage 1 and/or stage 2 lesions are found in stage 3 and stage 4 [36]. In Ludwig's system, bile ductal lesions or cholestatic changes which are very important features of PBC were not evaluated at all. Furthermore, the grading of necro-inflammatory activities of the bile ducts and hepatocytes to reflect the autommune-mediated pathologenesis of PBC is not reflected in these staging systems.

Since publication of the latest staging method, that of Ludwig et al. in 1978 [35], much progress has been made in clinical areas, particularly in therapeutic fields. There are now a number of treatments for PBC such as ursodeoxycholic acid (UDCA) and also combined UDCA and corticosteroid therapy for overlapping syndrome [24–26].

Table 1 Scoring for staging of primary biliary cirrhosis.

A. Scoring of fibrosis

Score 0	No portal fibrosis, or fibrosis limited to portal tracts
Score 1	Portal fibrosis with periportal fibrosis or incomplete septal fibrosis
Score 2	Bridging fibrosis with variable lobular disarray
Score 3	Liver cirrhosis with regenerative nodules and extensive fibrosis

B. Scoring of bile duct loss

Score 0	No bile duct loss
Score 1	Bile duct loss in < 1/3 of portal tracts
Score 2	Bile duct loss in 1/3—2/3 of portal tracts
Score 3	Bile duct loss in > 2/3 of portal tracts

C. Scoring of deposition of copper granules or orcein-positive grai

Score 0	No deposition of granules
Score 1	Deposition of granules in several periportal hepatocytes in <1/3 of portal tracts
Score 2	Deposition of granules in variable periportal hepatocytes in 1/3—2/3 of portal tracts
Score 3	Deposition of granules in many hepatocytes in >2/3 of portal tracts

We recently proposed a new histologic staging and grading system for comprehensive analysis of the histological progression of PBC (staging), and also of the immune-mediated necro-inflammatory activities of small bile ducts (chronic cholangitis) and of hepatocytes (interface and lobular hepatitis) [37,38].

New system for histologic staging and grading of necroinflammatory activities

Staging

Three items (fibrosis, bile duct loss and cholestasis) are chosen for staging. Chronic cholesasis is evaluated by deposition of copper or copper-binding protein by orcein stain [39]. These three items are scored as follows (Table 1A,B,C). For fibrosis (F), a score of 0 means that there is almost no fibrosis or the fibrosis is confined to the portal tracts. A score of 1 means that the fibrosis extends beyond the portal area occasionally with incomplete fibrous septa, a score of 2, that there is bridging fibrosis with variable lobular distortion, and a score of 3, cirrhosis. For bile duct loss (B), a score of 0 means interlobular bile ducts were present in all portal tracts. A score of 1 and 2 means that bile duct loss is evident in less than 1/3 and in 1/3 to 2/3 of portal tracts, respectively. A score of 3 indicates that bile ducts were absent in more than 2/3 of portal tracts. For chronic cholestasis (C), a score of 0 means no deposition of orcein-poistive granules in periportal hepatocytes. A score of 1 indicates deposition in less than one third of periportal hepatocytes of at least one portal tract, and a score of 3, deposition in more than two thirds of periportal hepatocytes along all portal tracts or fibrous septa. A score of 2 is that between 1 and 3. Then, a total score of the three items was obtained: a total score of 0 is stage 1 (no or minimum progression), 1—3 is

Table 2 Staging by summing for scores of three and two items.

	Sum of score	
Stage	3 items	2 items
Stage 1 (no progression)	0	0
Stage 2 (mild progression)	1—3	1—2
Stage 3 (moderate progression)	4—6	3—4
Stage 4 (advanced progression)	7—9	5—6

Three items; fibrosis, bile duct loss and deposition of copper granules on orcein-positive granules. Two items; fibrosis and bile duct loss.

Table 3 Grading of activities of cholangitis and hepatitis of primary biliary cirrhosis.

A. Activities of cholangitis (CA)

CA 0 (no activities)	No cholangitis, but mild duct epithelial damage may be present
CA 1 (mild activities)	One evident chronic cholangitis in the specimen, with or without other duct injuries in less than 1/3 of portal tracts or fibrous septa
CA 2 (moderate activities)	Chronic cholangitis in between CA1 and CA3
CA 3 (marked activities)	At least one CNSDC in the specimen, with or without other duct injuries in 2/3 of portal tracts of fibrous septa

B. Activities of hepatitis (HA)

HA 0 (no activities)	No interface hepatitis, and no or minimum lobular hepatitis
HA 1 (mild activities)	Interface hepatitis affecting 10 continuous hepatocytes in less than 1/3 of portal tracts, and mild to moderate lobular hepatitis
HA 2 (moderate activities)	Interface hepatitis affecting 10 continuous hepatocytes in more than 2/3 of portal tracts, and mild to moderate lobular hepatitis
HA 3 (marked activities)	Interface hepatitis affecting 20 continuous hepatocytes in over half portal tracts, and moderate lobular hepatitis, or bridging or zonal necrosis

CNSDC: chronic non suppurative destructive cholangitis.

stage 2 (mild progression), 4—6 is stage 3 (moderate progression), and 7—9 is stage 4 (advanced progression) (Table 2A). If orcein staining is not available, the sum of the scores for fibrosis and bile duct loss is also applicable as shown in Table 2B.

Grading of necroinflammatory activities

As mentioned above, chronic cholangitis and chronic active hepatitis-like changes are two representative necroinflammatory activities of PBC, which are evaluated by using scoring systems (Table 3).

Chronic cholangitis activities (CA)

Chronic cholangitis is categorized into four grades. Grade 0 means absent or ambiguous bile duct damage. Mild biliary epithelial damage can also be found in grade 0. In grade 3, at least one damaged bile duct showing CNSDC or florid duct lesion [14,15] is found. Damaged bile ducts with periductal epithelioid granulomas are also included in CNSDC. In grade 1, one damaged bile duct showing evident chronic cholangitis entirely surrounded by mild to moderate, duct-oriented lymphoplasmacytes is found, and this type of cholangitis is also occasionally encountered in chronic viral hepatitis. Interlobular bile ducts which are surrounded by small number of lymphoplamsacytes or adjacent to lymphoid cell infiltration in the portal tract are not regarded as evident chronic cholangitis. In grade 2, more than two bile ducts with evident chronic cholangitis are present, and bile ducts in one to two thirds of portal tracts are variably damaged.

Hepatitis activities (HA)

Interface hepatitis and lobular hepatitis are evaluated in combination and are categorized into four grades. Grade 0 means no interface hepatitis. Grade 1 and 2 mean the presence of interface hepatitis affecting about 10 continous hepatocytes at the interface of one portal tract or fibrous septa and of more than two portal tracts or fibrous septa, respectively. Grade 3 means the presence of interface hepatitis affecting more than 20 continous hepatocytes at the limiting plate of many portal tracts or fibrous septa. While no or minimum lobular hepatitis is found in grade 0, mild to moderate lobular hepatitis is found in grade 1 or 2, and moderate lobular hepatitis in grade 3. Occasional zonal necrosis and bridging necrosis is regarded as grade 3.

Conclusions and perspectives

The diagnosis of PBC is usually made by a constellation of clinical, serological and pathological findings. Pathological changes valuable for the diagnosis of PBC differ according to the phase or stage of the disease, and a combination of several pathological lesions may be needed for an exact diagnosis. A new system applicable to needle liver biopsies in which many of the shortcomings of classical staging systems are overcome, has been proposed and is recommended for the clinical and therapeutic evaluation of PBC patients from a histological standpoint.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Interaction Between Toll-Like Receptors and Natural Killer Cells in the Destruction of Bile Ducts in Primary Biliary Cirrhosis

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Primary biliary cirrhosis (PBC) is characterized by chronic nonsuppurative destructive cholangitis (CNSDC) associated with destruction of small bile ducts. Although there have been significant advances in the dissection of the adaptive immune response against the mitochondrial autoantigens, there are increasing data that suggest a contribution of innate immune mechanisms in inducing chronic biliary pathology. We have taken advantage of our ability to isolate subpopulations of liver mononuclear cells (LMC) and examined herein the role of Toll-like receptors (TLRs), their ligands, and natural killer (NK) cells in modulating cytotoxic activity against biliary epithelial cells (BECs). In particular, we demonstrate that Toll-like receptor 4 ligand (TLR4-L)-stimulated NK cells destroy autologous BECs in the presence of interferon alpha (IFN-α) synthesized by TLR 3 ligand (TLR3-L)-stimulated monocytes (Mo). Indeed, IFN-α production by hepatic Mo is significantly increased in patients with PBC compared to disease controls. There were also marked increases in the cytotoxic activity of hepatic NK cells from PBC patients compared to NK cells from controls but only when the NK cells were prepared following ligation of both TLR3-L- and TLR4-L-stimulated LMC. These functional data are supported by the immunohistochemical observation of an increased presence of CD56-positive NK cells scattered around destroyed small bile ducts more frequently in liver tissues from PBC patients than controls. *Conclusion:* These data highlight critical differences in the varied roles of Mo and NK cells following TLR3-L and TLR4-L stimulation. (HEPATOLOGY 2011;53:1270-1281)

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The cholangitis of primary biliary cirrhosis (PBC) has been called an orchestrated immune attack, including involvement of autoantibodies, CD4+, and CD8+ T cells.[1,2] This concept has led to the thesis that a multilineage response against the immunodominant autoantigen PDC-E2 is an essential component of disease pathogenesis.[3] It is unclear whether the natural history of PBC is "entirely" secondary to adaptive autoimmune responses; epidemiologic analysis has suggested a role of transient exposure

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to environmental agents in the etiology of PBC.[4] The data presented herein suggest that innate immune mechanisms contribute to the pathology characteristic of PBC by either accelerating disease or by specific chronic destruction of small bile duct epithelial cells.[5] Indeed, one paradox in PBC has been the relative lack of a therapeutic response to the various immunosuppressive drugs that have been administered to PBC patients, despite the observation that PBC is a model autoimmune disease.[6] A more detailed analysis of the effector mechanisms involved in the pathogenesis of human PBC has led us to suggest that in addition to the documented adaptive autoimmune responses there is also a direct role of innate immune responses in the biliary pathology of PBC.[2,5,7-9]

The studies described herein take advantage of our ability to culture primary human biliary epithelial cells (BEC) *in vitro* as well as to isolate subpopulations of liver infiltrating mononuclear cells.[8,10,11] Although there are significant numbers of natural killer (NK) cells present around small bile ducts, especially during the early stages of PBC,[12] we note that there are NK cells present throughout the disease course. Importantly, we focused on these NK cells and report herein that such NK cells are highly cytotoxic for autologous BEC following ligation of the Toll-like receptor 4 (TLR4) expressed by NK cells in the presence of interferon-α (IFN-α). Furthermore, this function of NK cells is dependent on the activation of monocytes (Mo) by way of TLR3. We submit that activation of Mo and their crosstalk with NK cells contribute to the pathology of PBC. The data supporting this view are the basis of the present report.

Patients and Methods

Subjects and Protocol. A total of 22 explanted liver tissues constitute the present study. Eight of these 22 liver tissues were from patients with PBC, three from patients with hepatitis B virus infection, eight with hepatitis C virus infection, and three with alcoholic liver disease. The term control diseases in this report refers to patients with diseases other than PBC. All patients had endstage liver cirrhosis without detectable signs of other acute liver injury from an unrelated cause. The diagnosis of PBC was based on established criteria[2] and sera from each of these patients had readily detectable high titers of antimitochondrial antibodies.[2] The immunohistochemical studies reported herein were performed on fresh tissue samples from wedge biopsies of 47 patients including 11 normal controls with metastatic liver disease, 14 patients with PBC, 16

with hepatitis C, and six with primary sclerosing cholangitis (PSC). All of the tissues from patients used herein for immunohistological studies were classified as early stage without detectable signs of cirrhosis. Samples were obtained and studied after informed consent of the donor and all experimental protocols were approved by the Research Ethics Committee of Kyushu University and the University of California at Davis. The isolation, verification of purity, and the specific protocols used are described below.

Isolation of Intrahepatic BECs and Liver-Infiltrating Mononuclear Cells (LMCs). The liver mononuclear cell populations were isolated as described in detail by our laboratory.[7] Briefly, liver specimens were first digested with 1 mg/mL of collagenase type I. Cells from the digested tissue were purified using a Ficoll-hypaque gradient to obtain LMC.[9] The LMC were allowed to adhere by incubating the cells overnight in tissue culture plates and an enriched population of adherent cells harvested. This adherent cell population was maintained in tissue culture until the cells reached full confluence, usually by day 14, and the nonadherent cell population aspirated, washed, and cryopreserved in media containing 7.5% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen.

BECs were separated from adherent cells using CD326 (EpCAM) conjugated MicroBeads (Miltenyi Biotec) specific for epithelial cells. Cells were then resuspended in media consisting of a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal calf serum (FCS), epithelial growth factor (10 ng/mL), cholera toxin (10 ng/mL), hydrocortisone (0.4 μg/mL), triiodothyronine (1.3 μg/L), transferrin (5 μg/mL), insulin (5 μg/mL), adenine (24.3 μg/mL), and 10 ng/mL hepatocyte growth factor (R&D systems, Minneapolis, MN) and cultured.[7] The purity of the cells was verified by immunohistochemical examination of an aliquot of these cells for the expression of cytokeratins 7 and 19 using appropriate antibodies (Dako, Glostrup, Denmark) and only cultures that were >90% positive for these cytokeratins and >95% viable (as determined by trypan blue) were used for the studies reported herein. The cultures used in the studies herein were between four to six passages to exclude the possibility for potential loss of phenotype after prolonged *in vitro* culture.

Isolation of T Cells, Mo, NK Cells, Myeloid Dendritic Cells (mDC), Plasmacytoid DC (pDC), and Natural Killer T (NKT) Cells. As reported,[8] the T cells used for the studies were isolated from LMC using a Pan T cell isolation kit II (Miltenyi Biotec).[8]

Similarly the highly enriched population of Mo and NK cells used were purified using Mo and NK cell isolation kits, respectively (Miltenyi Biotec).[8] The purity of the CD3+ T cells, Mo, and NK cells used were >90% as determined by flow cytometric analysis of an aliquot from each isolation. In efforts to ensure the purity of the cell population being studied, the population of T cells, Mo, or NK cells were each harvested separately. In addition, the same assay was performed following depletion of each of the three cell lineages from LMCs in efforts to confirm that the data obtained were indeed the function of the lineage being studied. The mDCs (BDCA-1+), pDC (BDCA-2+), and NKT cells were isolated using the mDC, pDC, and NKT cell isolation kits (Miltenyi Biotec), respectively, which included two magnetic separation steps. The purity of BDCA-1+ mDCs and the CD3+ CD56+ NKT cells were each >80% as determined by flow cytometric analysis of an aliquot of the cell preparation used for the study. An enriched population of mDC and NKT cells were harvested separately and, once again, the same assay was performed following depletion of the specific cell population in efforts to confirm that the function identified was due to the specific cell lineage being studied.

Cytotoxicity Assay Against Autologous BEC. The cytotoxic activity of LMC was assessed using an 8-hour ^{51}Cr release assay using autologous BEC as target cells.[7] Briefly, the detached BECs were labeled with 2 μCi/mL ^{51}Cr (Amersham) overnight, washed 3× in media and 5 × 10^3 ^{51}Cr-labeled cells dispensed into individual wells of a 96-well round-bottom plate. The nonstimulated, the interleukin (IL)-2, or TLR-activated LMCs were added to triplicate wells at an effector to target cell ratio of 20:1 in a total volume of 200 μL of complete RPMI medium. The IL-2-stimulated effector LMCs used for the assay were stimulated for 3 days with IL-2 (100 units/mL) and the TLR-activated LMC comprised of a series of cell cultures incubated with a single or mixture of TLR ligands each at a predetermined optimal concentration of 2-10 μg/mL of the appropriate TLR-L prior to their addition to the target cells. The TLR ligands used included TLR2 ligand (lipoteichoic acid, LTA: TLR2-L), TLR3 ligand (polyinosine-polycytidylic acid, poly (I:C): TLR3-L), TLR4 ligand (lipopolysaccharide, LPS: TLR4-L), TLR5 ligand (Flagellin: TLR5-L), TLR7/8 ligand (CL097: TLR7/8-L), TLR9 ligand type A (ODN2216, CpG type A: TLR9-LA), and TLR9 ligand type B(ODN2006, CpG type B: TLR9-LB). The combination of TLR ligands used for activation of LMC included (1) TLR2-L + the ligands for either TLR3, 4, 5, 7/8, 9-LA, or 9-LB; (2) TLR3-L +

the ligands for either TLR4, 5, 7/8, 9-LA, or 9-LB; (3) TLR4-L + ligands for either TLR5, 7/8, 9-LA, or 9-LB; (4) the TLR5-L + the ligands for either 7/8, 9-LA, or 9-LB; (5) TLR7/8-L + the ligands of either 9-LA to TLR9-LB; (6) TLR9-LA + TLR9-LB. The TLR ligands were purchased from Invitrogen (San Diego, CA). Controls consisted of triplicate wells containing target cells cultured in media alone and target cells that were incubated with 10% Triton X-100 to determine spontaneous and maximal ^{51}Cr release, respectively. Following incubation of the cocultures of the effector with target cells for 8 hours, 100 μL of supernatant fluid was collected from each well and counted and the percentage of specific ^{51}Cr release calculated as (cpm of experimental release − cpm of spontaneous release) / (cpm of maximal release − cpm of spontaneous release) × 100). Experiments using the combination of TLR3-L and TLR4-L were performed on aliquots of samples at least three times from each of the patients. As further controls, polymyxin B and chloroquine were used as specific inhibitors of LPS and poly I:C, respectively, for assays involving TLR4 and TLR3-induced activation. Although polymyxin B was added at the time of TLR4 activation, chloroquine was added 2 hours prior to the activation of the TLR3 pathway for the cytotoxicity assay.

Hepatic Mo, T cells, and NK cells were isolated from LMC following in vitro activation with TLR3-L and TLR4-L for 3 days. Subsequently, highly enriched populations of Mo, T cells, NK cells, and LMC depleted of Mo, T cells, and NK cells were assessed for their cytotoxic activity against autologous BEC at an effector-to-target cell ratio of 5:1. Thence enriched populations of NK cells and LMC were stimulated with several combinations of TLR3-L and TLR4-L in the presence of a variety of supernatant fluids prepared as described above. The combinations included (1) activation of the appropriate cell cultures with TLR3-L and TLR4-L in the presence of supernatant of unfractionated LMC; (2) the activation of the appropriate cell cultures with TLR3-L in the presence of supernatant of TLR4-L-activated LMC; (3) activation of the appropriate cell cultures with TLR4-L in the presence of supernatant of TLR3-L-activated LMC; and (4) activation of the appropriate cell cultures with supernatants of TLR3-L and TLR4-L-stimulated LMC. The stimulated NK and LMC were then assessed for cytotoxicity against autologous BEC. Finally, unfractionated LMC and highly enriched populations of mDC, Mo, NKT, or LMC depleted of mDC, Mo. or NKT cells were cultured at 1 × 10^5/200 μL in 96-well plates for 48 hours in the presence of either TLR3-L or supernatant fluids obtained from cultures of NK

Table 1. Primer Sequences Used for Real-Time Polymerase Chain Reaction Analyses

	PCR Product Size	Forward Primer	Reverse Primer
NKG 2D	200	GTCTCAAAATGCCAGCCTTC	TCGAGGCATAGAGTGCACAG
NKp46	258	ATGGGGCTGTTGAATACCAG	TCTCTCCCGAGATCACTTCG
CD94	279	CCACGGAGTAACATCCCATC	GAAGCTGCAGTGAACCATGA
NKG2A	174	TCCATGGGTGACAATGAATG	CTGCAAATGCAAACGCTTTA
FasL	257	TCTACCAGCCAGATGCACAC	CAAGATTGACCCCGGAAGTA
TRAIL	143	GGCAACTCCGTCAGCTCGTTA	GGTCCCAGTTATGTGAGCTGCTA
Granzyme B	257	TCCCTGTGAAAAGACCCATC	TTCGCACTTTCGATCTTCCT

cells stimulated with TLR4-L. The cultures were then assessed for cytotoxicity against autologous BEC.

In efforts to study the influence of IFN-α, an additional cytotoxicity assay was performed in which highly enriched populations of NK cells were stimulated with TLR4-L in the presence or absence of recombinant IFN-α. In parallel, the supernatant fluids from TLR3-L-stimulated Mo in the presence or absence of anti-IFN-α antibody (Abcam) were studied. Similarly, in nested experiments, anti-TNF-related apoptosis inducing ligand (TRAIL) monoclonal antibody (mAb) (R&D Systems, final concentration: 1 μg/mL), anti Fas-L mAb (R&D Systems, final concentration: 1 μg/mL), or Granzyme B inhibitor (BioVision, final concentration: 10 μM) were used in the same cytotoxicity assay in attempts to identify the effector molecules involved. Importantly, each of these experiments was performed on samples from all PBC patients and control liver disease patients at least three times.

IL-12, IL-15, IL-18, and IFN-α Production from Mo. In efforts to identify the nature of the cytokines that were involved in promoting NK cell effector function, supernatants from the TLR3-L-stimulated hepatic Mo cultured for 3 days were analyzed for levels of IL-12, IL-15, IL-18, and IFN-α. These cytokines were selected based on previously published data that reported their involvement in NK cell functional activity.[13] Assays were performed using a sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems), using a combination of unlabeled and biotin- or enzyme-coupled monoclonal antibody to each cytokine. Data reported herein represent results obtained from each of the experiments performed on samples from all patients at least three times.

Isolation and Quantitation of Messenger RNA (mRNA) for Select Markers. Aliquots of NK cells from PBC patients and disease controls were cultured in media alone (unstimulated) or cultured in the presence of TLR4-L, IFN-α, or the combination of TLR4-L and IFN-α for 24 hours. Total RNA was isolated from the cultured NK cells using RNAeasy columns (Qiagen, Valencia, CA) and quantitative analyses car-

ried out utilizing a real-time polymerase chain reaction (PCR) assay using SYBR Green PCR Master Mix (Invitrogen) and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Tokyo, Japan). The relative levels of NKG2D and NKp46 (activating receptors), CD94 and NKG2A (inhibitory receptors), and FasL, TRAIL, and Granzyme B (effector function markers) were determined using the primers noted in Table 1. Data are expressed as the fold-change in levels of mRNA versus unstimulated NK cells.

Immunohistochemical Staining of Human Liver Specimens for CD56 Expression. Deparaffinized and rehydrated sections and frozen sections of liver tissues from 11 normal controls with a diagnosis of metastatic liver disease, 14 patients with PBC, 16 with hepatitis C, and six with PSC were used for the detection of CD56-expressing cells using standard immunostaining. Endogenous peroxidase was blocked using normal goat serum diluted 1:10 (Vector Laboratories, Burlingame, CA) for 20 minutes; CD56 was diluted 1:100 (Dako) and immunostaining was performed on coded sections and the data interpreted by a "blinded" pathologist.

Statistical Analysis. All experiments were performed in triplicate and data points shown are the mean values of results of these triplicates. Comparisons between the points for certain datasets are expressed as mean ± standard deviation (SD), and the significance of differences was determined by Student's t test. All analyses were two-tailed and P-values <0.05 were considered significant. Statistical analyses were performed using Intercooled Stata 8.0 (StataCorp, College Station, TX).

Results

Autologous BEC Killing Assay by LMC. As noted in Fig. 1A and as expected, LMC when cocultured with autologous BEC demonstrated no detectable cytotoxicity (0.5 ± 4.3%). However, following incubation of LMCs with IL-2 (100 μl/mL) a marked increase in cytotoxic activity against autologous BEC was observed (48.3 ± 9.7%). It is well known that innate immune effector cells can be activated *in vitro*
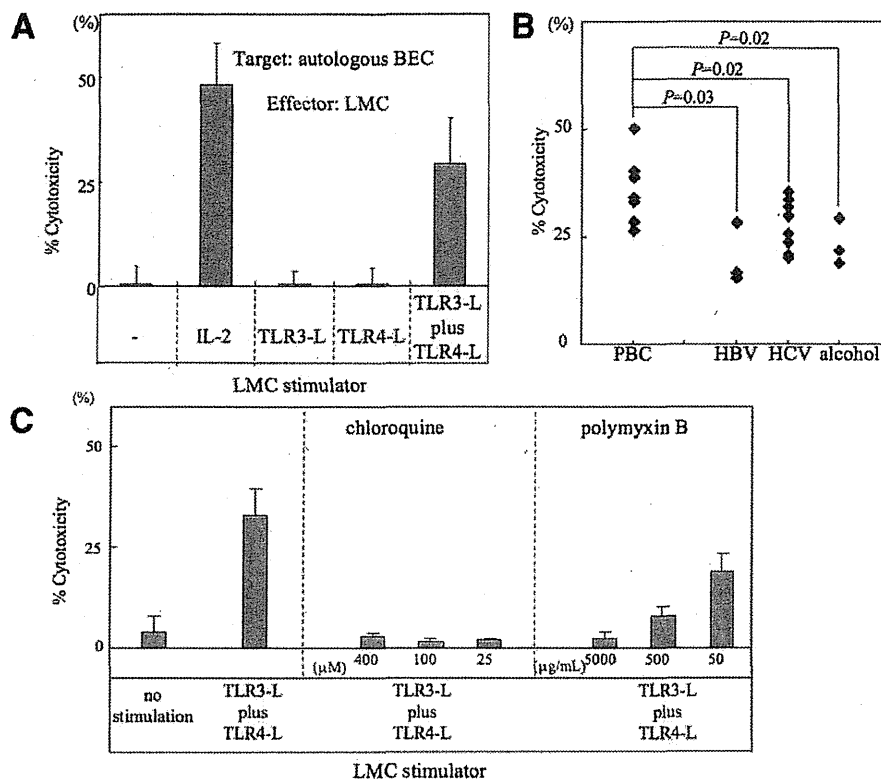
Fig. 1. (A) *In vitro* activation requirements of LMC for cytotoxicity against BEC. LMC isolated from eight patients with PBC and 14 control patients were cultured *in vitro* with either IL-2, TLR3-L alone, TLR4-L alone, or a mixture of TLR3-L+TLR4-L for 3 days and then washed and assayed for cytotoxicity against autologous BEC using the standard ^{51}Cr release assay. LMC cultured in media alone served as a negative control. The assay was performed in triplicate for each activation agent and expressed as mean ± SD. Representative data from one PBC patient are shown. (B) The net cytotoxicity for LMC against BEC was performed. There were statistical differences in the degree of net cytotoxicity induced by TLR3-L and TLR4-L activation of LMC in cells from PBC when compared to other control liver diseases. (C) The use of inhibitors of the TLR3 and TLR4 signaling pathways on the cytotoxicity of activated LMC against autologous BEC. LMC from eight PBC patients and 14 control patients were activated *in vitro* with TLR3-L+TLR4-L in the presence of various concentrations of either chloroquine (TLR3 pathway inhibitor) or polymyxin B (TLR4 pathway inhibitor) and tested for cytotoxicity against autologous BEC. The left panel shows the control cytotoxicity data of LMC cultured in media alone or following activation with TLR3-L and TLR4-L. The middle and right panels reflect data obtained on aliquots of the same LMC activated using TLR3-L and TLR4-L but cultured in the presence of chloroquine or polymyxin B, respectively. Each culture was performed in triplicate and the data shown are mean ± SD. The data shown are from one PBC patient but are representative.

by way of a number of TLR pathways besides IL-2. Thus, we studied a variety of TLR ligands either individually or in various combinations as outlined in Materials and Methods. First, whereas LMC did not demonstrate any detectable cytotoxicity against autologous BEC following ligation of any single TLR ligand (for example, the CTL activity following TLR3-L ligation was 0.5 ± 3.1% and following TLR4 ligation was 0.6 ± 3.9%) (Fig. 1A; Supporting Fig. 1A), use of the combination of TLR3-L and TLR4-L led to significant cytotoxicity against autologous BEC (CTL activity; 29.3 ± 11.1%). Importantly, LMC did not induce significant cytotoxicity against autologous BEC using any other combination of TLR ligands (Supporting Fig. 1B). To exclude the possibility that the cytotoxicity noted using the combination of TLR3-L+TLR4-L was not due to the direct effect of the

TLR ligands on BEC instead of LMC, we cocultured BEC with TLR3-L and TLR4-L in a similar cytotoxic assay described above. However, no detectable cytotoxic activity was found (data not shown).

Studies were then carried out to evaluate the differences if any in the cytotoxicity of BEC following TLR3-L and TLR4-L stimulation of LMC from PBC as compared with LMC isolated from other disease controls. The net cytotoxicity of LMCs from PBC patients (n = 8) against BEC was 36.4 ± 7.5. In the case of LMCs from HBV (n = 3), HCV (n = 8), and alcohol-related cirrhosis (n = 3) controls, the net cytotoxicity was 20.2 ± 7.1, 27.7 ± 5.9, and 23.4 ± 5.5, respectively, as shown in Fig. 1B. There were statistical differences in the degree of net cytotoxicity induced by TLR3-L+TLR4-L activation of LMC in cells from PBC when compared to similarly activated LMCs
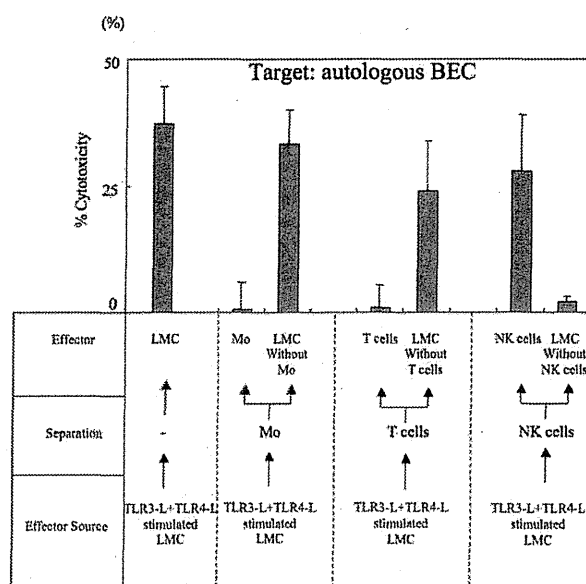
Fig. 2. Identification of the cell lineage within LMC that mediate cytotoxicity against autologous BEC. Cultures of LMC were activated in vitro with TLR3-L and TLR4-L and then aliquots assayed for cytotoxicity against autologous BEC (control) or used to isolate or deplete specific cell lineages. Thus, LMC were either enriched for Mo or depleted of Mo, enriched for T cells, or depleted of T cells and enriched for NK cells or depleted of NK cells and each of these tested for cytotoxicity against autologous BEC. Results of mean cytotoxicity (mean ± SD) of data obtained on one PBC patient are displayed.

from other control liver diseases (PBC versus HBV-related cirrhosis: $P = 0.03$, PBC versus HCV-related cirrhosis: $P = 0.02$, PBC versus alcohol-related cirrhosis: $P = 0.02$). Subsequently, in efforts to confirm that the activation by TLR4-L (LPS) and TLR3-L (poly I:C) was indeed induced by way of the respective TLR pathways, use was made of pretreatment of the activation agents with previously defined optimum concentrations of polymyxin B for LPS and chloroquine for poly I:C. As shown in Fig. 1C, polymyxin B inhibited CTL activity in a dose-dependent manner and chloroquine inhibited CTL activity even at the lowest concentration used.

NK Cells Are Cytotoxic for Autologous BEC in the Presence of TLR3-L+TLR4-L-Stimulated LMC.

The ability of cells to induce cytotoxic activity against autologous BEC following the ligation of TLR3-L+TLR4-L was next examined. Cultures of LMC, stimulated with TLR3-L+TLR4-L, were used to either isolate enriched populations of Mo, T cells, NK cells, or isolate cultures depleted of each of these cell lineages. These enriched and depleted cell cultures were assessed for their cytotoxicity against autologous BEC. Unfractionated TLR3-L+TLR-4-activated LMC were used

for purposes of a positive control. As shown in Fig. 2, whereas Mo did not demonstrate any significant cytotoxicity against autologous BEC (CTL activity; 0.6 ± 5.4%), LMC depleted of Mo demonstrated significant cytotoxicity against autologous BEC (CTL activity; 33.2 ± 6.8%). Similarly, whereas T cells did not demonstrate significant cytotoxicity against autologous BEC (CTL activity; 0.8 ± 4.5%), LMC depleted of T cells had significant cytotoxicity against autologous BEC (CTL activity; 24.0 ± 10.0%). On the other hand, whereas NK cells demonstrated significant cytotoxicity against BEC (CTL activity; 28.0 ± 11.0%), LMC depleted of NK cells did not show significant cytotoxicity against autologous BEC (CTL activity; 2.0 ± 1.1%). These data indicate that it is the NK cell lineage following TLR3-L and TLR4-L stimulation that is responsible for significant cytotoxic activity against autologous BEC. Representative data from one PBC patient is shown in Fig. 2.

TLR4-L-Stimulated NK Cells with Supernatants from TLR3-L-Stimulated LMC Are Cytotoxic for Autologous BEC.

In efforts to identify the potential mechanisms by which activation of TLR3-L+TLR4-L in cultures of LMC generate cytotoxic activity of NK cells against autologous BEC, data obtained in preliminary studies showed that the activation of enriched population of NK cells with TLR3-L+TLR4-L did not lead to significant cytotoxicity against autologous BEC (Fig. 3A). These data indicate that the generation of cytotoxic activity against autologous BEC was likely due to the presence of a second population of cells. Experiments were thus carried out to clarify the relationship of NK cells, LMC, TLR3-L, and TLR4-L. We prepared supernatant fluids from LMC cultured in the presence of the appropriate ligands for either TLR3, TLR4, or TLR3+TLR4. As shown in Fig. 3A, NK cells only demonstrated cytotoxicity against autologous BEC when cultured in the presence of TLR4-L and supernatant fluids prepared from TLR3-L-activated LMC (CTL activity; 26.3 ± 11.0%), but not when cultured in the presence of TLR3-L and supernatant fluids prepared from LMC with TLR4-L (CTL activity; 0.2 ± 2.1%). The NK cells, in addition, did not kill autologous BEC in the presence of supernatant from TLR3-L and TLR4-L-stimulated LMC (CTL activity; 0.8 ± 2.8%) as shown in Fig. 3A. These data indicate that NK cells cytotoxicity against autologous BEC requires not only the activation of TLR4-L but also cytokines that are synthesized by LMC upon TLR3-L activation.
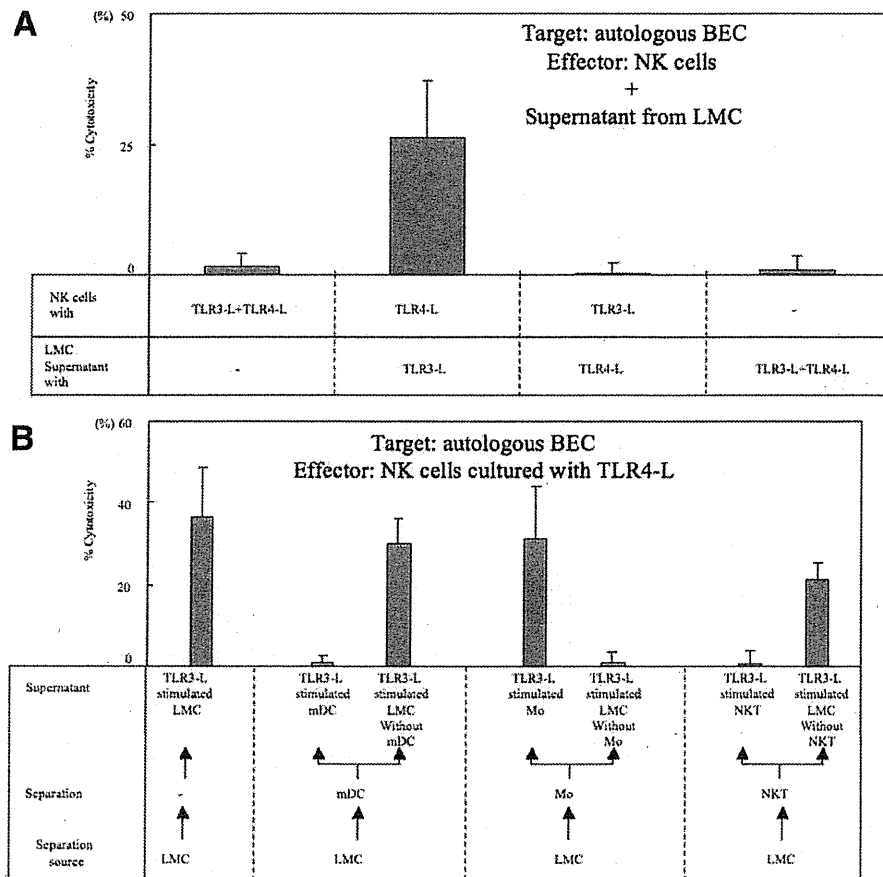
Fig. 3. (A) The activation requirements of NK cells in mediating cytotoxicity against autologous BEC. Highly enriched population of NK cells were cultured in vitro in the presence of (1) TLR3-L+TLR4-L; (2) TLR4-L and supernatant fluid from LMC cultured in the presence of TLR3-L; (3) TLR3-L and supernatant fluid from LMC cultured in the presence of TLR4-L; and (4) supernatant fluids from LMC cultured in the presence of TLR3-L+TLR4-L. Cultures were performed in triplicate and the mean ± SD of the net percent cytotoxicity calculated. The data shown are from one PBC patient and are representative. (B) Identification of the cell lineage that is the source of the factor required to mediate cytotoxicity of autologous BEC by TLR4-L-activated NK cells. A pool of a highly enriched population of NK cells was cultured with TLR4-L in the presence of supernatant fluids from (1) unfractionated LMC cultured with TLR3-L (control); (2) highly enriched populations of mDC or LMC depleted of mDC-stimulated with TLR3-L; (3) highly enriched population of Mo or LMC depleted of Mo-stimulated with TLR3-L; and (4) highly enriched population of NKT cells to LMC depleted of NKT cells stimulated with TLR3-L. These cultures were tested for cytotoxicity against autologous BEC. Each culture was performed in triplicate and the data shown reflect mean ± SD of net percent cytotoxicity of the triplicate cultures. The data shown are from one PBC patient and are representative.

Supernatant from TLR3-L-Stimulated Mo Induces NK Cell Cytotoxicity. We next carried out studies in efforts to identify the cell lineage that was the source of the cytokine(s) in the supernatant fluids from TLR3-L-activated unfractionated LMC that induced TLR4-L-stimulated NK cell cytotoxicity against autologous BEC. Highly enriched populations of mDC, Mo, NKT cells, and the corresponding population of LMCs depleted of mDC, Mo, and NKT cells were stimulated with TLR3-L and the supernatant harvested; insufficient quantities were available to study the pDC fraction. NK cells were cultured with TLR4-L in the presence or absence of each of these supernatant fluids and analyzed for cytotoxicity against autolo-gous BEC as described in Materials and Methods. As noted in Fig. 3B, whereas TLR4-L-stimulated NK cells cultured in the presence of supernatant fluids from TLR3-L unfractionated LMC demonstrated significant cytotoxicity; similarly TLR4-L-stimulated NK cells, when cultured with supernatant fluids of TLR3-L, stimulated mDC, and NKT cells did not demonstrate detectable cytotoxicity against autologous BEC. However, the TLR4-L-activated NK cells, cultured in the presence of TLR3-L-activated Mo, readily demonstrated cytotoxicity. The identification of Mo as the source of the cytokine required for TLR4-L-activated NK cells to induce cytotoxicity against autologous BEC was confirmed by results obtained with
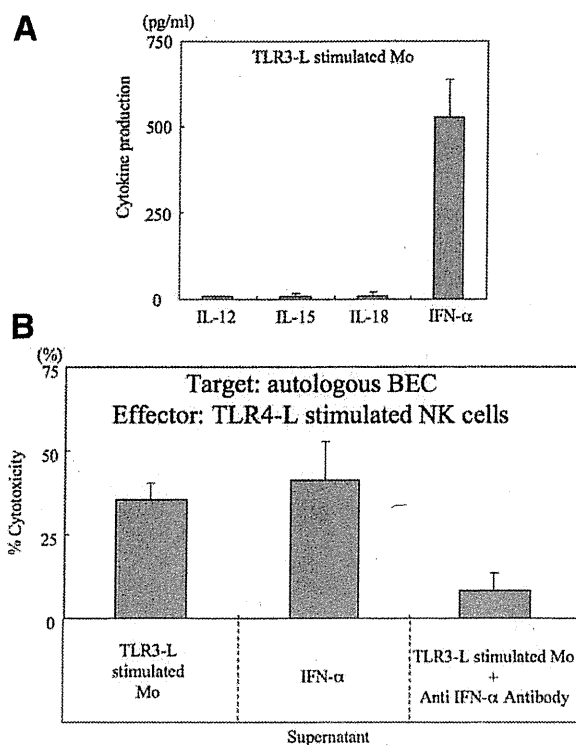
Fig. 4. (A) Analysis of cytokines synthesized by *in vitro* TLR3-L-activated hepatic Mo. Mo from the liver of the 22 patients included in the present study were isolated and cultured *in vitro* and the supernatant fluids analyzed for levels of IL-12, IL-15, IL-18, and IFN-α. Cultures were performed in triplicate and the data displayed represents mean ± SD of values obtained from cultures from one representative patient. Statistical differences between PBC patients and disease controls are described in the text. (B) IFN-α is required by TLR4-L-stimulated NK cells to mediate cytotoxicity against autologous BEC. Aliquots of TLR4-L-stimulated NK cells were cultured in the presence of either supernatant fluids from TLR3-L-stimulated hepatic Mo (control), IFN-α, or supernatant fluids from TLR3-L-stimulated Mo incubated with previously determined optimum concentration of anti-IFN-α monoclonal antibody. Cultures were performed in triplicate and assayed for cytotoxicity against autologous BEC. Data displayed are net percent cytotoxicity and the data shown are from one representative PBC patient.

supernatant fluids from TLR3-L-stimulated LMC depleted of mDC, and NKT cells, respectively.

TLR3-L-Stimulated Mo Produce IFN-α. The nature of the cytokine synthesized by TLR3-L-activated Mo that promoted cytotoxicity in TLR4-L-activated NK cells was studied next. We reasoned that the cytokine responsible for this activity was most likely IL-12, IL-15, IL-18, or IFN-α, which have previously been shown to generally activate NK cells. As seen in Fig. 4A, whereas TLR3-L-stimulated Mo produced low but detectable levels of IL-12 (7.9 ± 3.4 pg/mL), IL-15 (9.8 ± 8.0 pg/mL), and IL-18 (10.0 ± 9.6 pg/mL), the major cytokine synthesized was shown to be IFN-α (530.1 ± 106.2 pg/mL). In efforts to confirm that it

was indeed IFN-α that was responsible for inducing TLR4-L-activated NK cell cytotoxicity, aliquots of TLR4-L-activated NK cells were cultured in the presence or absence of various concentrations of either IL-12, IL-18, IL-15, or IFN-α (Fig. 4A). Data derived from such studies demonstrated that whereas TLR4-L-activated NK cells cultured in the presence of IL-12, IL-18, or IL-15 (10-20 pg/mL) had no detectable cytotoxicity (data not shown), TLR4-L-activated NK cells cultured in the presence of recombinant IFN-α (500 pg/mL) readily induced cytotoxicity against autologous BEC (cytotoxicity; 41.2 ± 11.4%) (Fig. 4B). The identity of IFN-α as the cytokine responsible for inducing cytotoxicity in cultures of TLR4-L-activated NK cells was confirmed with the use of anti-IFN-α antibody. Thus, pretreatment of supernatant fluids from TLR3-L-activated Mo with anti-IFN-α reduced the cytotoxicity of TLR-4-stimulated NK cells against autologous BEC (cytotoxicity; 8.5 ± 5.2%). We also examined the relative levels of IFN-α synthesized by TLR3-L-activated Mo from patients with other diseases as compared with Mo from PBC patients in efforts to determine whether there was a qualitative and/or quantitative difference in the synthesis of this cytokine. IFN-α production from TLR3-L-activated Mo from PBC patients (n = 8; 355 ± 132 pg/mL) was significantly higher than similarly activated Mo from HBV-related cirrhosis (n = 3; 175 ± 74 pg/mL: $P < 0.03$), HCV related cirrhosis (n = 8; 175 ± 57 pg/mL: $P < 0.01$), or those from alcohol-related cirrhosis (n = 3; 180 ± 54 pg/mL: $P < 0.03$).

Contribution of Other Molecules to Liver NK Cell Cytotoxicity Against Autologous BEC. Although the above studies identified IFN-α as the cytokine synthesized by TLR3-L-activated Mo, we next attempted to identify the nature of the molecules synthesized by NK cells that were potentially involved in mediating cytotoxicity against autologous BEC. First, we evaluated the expression of activating receptors, inhibitory receptors, and effectors using reverse transcriptase (RT)-PCR methods on mRNA isolated from unstimulated NK cells, TLR4-L-stimulated NK cells, IFN-α-stimulated NK cells, and the combination of TLR4-L and IFN-α-stimulated NK cells. As shown in Fig. 5A, based on the activation signals the cultured cells expressed effector molecules such as FasL, TRAIL, and/or Granzyme B. Among these effector molecules, TRAIL appeared to be the molecule involved in promoting the cytotoxicity of TLR4-L-activated NK cells. Thus, as shown in Fig. 5B, the addition of monoclonal anti-TRAIL antibody but not anti-FasL antibody or anti-Granzyme B significantly reduced the cytotoxicity
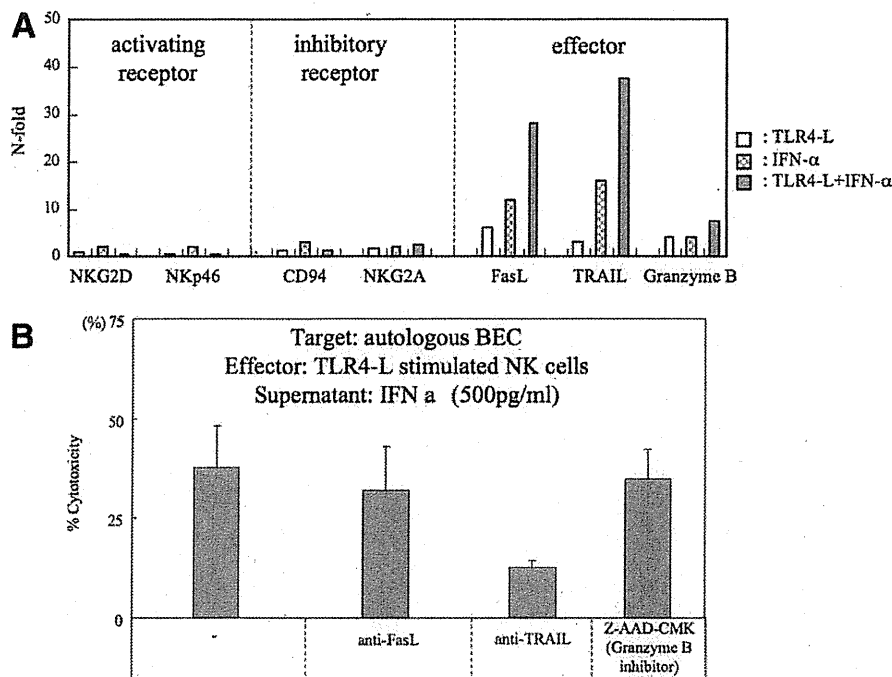
Fig. 5. (A) Expression of activating receptor, inhibitory receptor, and effectors based on TLR4-L, IFN-α, or a combination of TLR4-L and IFN-α stimulation. Activating receptors and inhibitory receptors were not expressed following stimulation from TLR4-L, IFN-α, or their combination. Effectors such as FasL, TRAIL, and Granzyme B were synergistically expressed dependent on the stimulation. Data displayed are from one representative PBC patient. (B) Contribution of TRAIL to liver NK cell cytotoxicity against autologous BEC. Aliquots of NK cells were cultured in the presence of TLR4-L and 500 pg/mL of IFN-α alone (control) or with the addition of predetermined optimum concentrations of anti-FAS-L, anti-TRAIL, or Z-AAD-CMK (inhibitor of Granzyme B) and then assayed for cytotoxicity against autologous BEC. Cultures were performed in triplicate and the data displayed reflect net percent cytotoxicity expressed as mean ± SD of the triplicate cultures. The data shown are from one PBC patient and are representative.

of TLR4-L-activated NK cells. These data indicate that IFN-α from Mo and TLR4-L-activated NK cells induce TRAIL to mediate cytotoxicity against liver BEC.

NK Cells Around BEC in the Liver. Finally, we investigated the relative levels of NK cells around bile ducts in sections of liver by immunohistochemistry. Comparative analyses of sections of liver from PBC patients and patients with liver diseases other than PBC demonstrated that CD56^{+} NK cells predominantly invaded the portal area only in sections from PBC patients. Thus, whereas the number of CD56^{+} NK cells invading portal areas was determined to be 8 ± 4.4 cells per small bile duct from PBC patients, those for sections of liver from patients with hepatitis C gave values of 2.7 ± 2.1 CD56+NK cells per small bile duct ($P < 0.01$), those from PSC gave values of 1.1 ± 1.2 CD56+NK cells ($P < 0.01$), and those from normal liver gave a value of 0.8 ± 1.0 CD56+NK cells ($P < 0.01$). Representative histochemical images are displayed in Fig. 6.

Discussion

Studies of the mechanisms of a variety of autoimmune diseases, including PBC, have predominantly focused on the contributory role of adaptive T and B cell responses in the pathogenesis of disease.[14-16] It is thus generally assumed that the major effector mechanisms that induce tissue pathology are those mediated by autoantigen-specific CD8^{+} T cells and autoantigen-specific antibodies that directly and/or indirectly contribute to tissue pathology. Interestingly, the institution of immunosuppressive agents that predominantly target pathways involved in the activation and effector mechanisms employed by cells of the adaptive immune system have so far failed to result in clear therapeutic benefit in patients with PBC. This therapeutic failure of inhibiting adaptive immunity in patients with chronic autoimmune diseases such as PBC has prompted a need for the reevaluation of this line of thinking. Thus, it is reasonable to consider that alternate immune effector mechanisms are functioning and contributing to the pathogenesis of human PBC.

We submit that the involvement of innate immune effector mechanisms in any chronic disease including
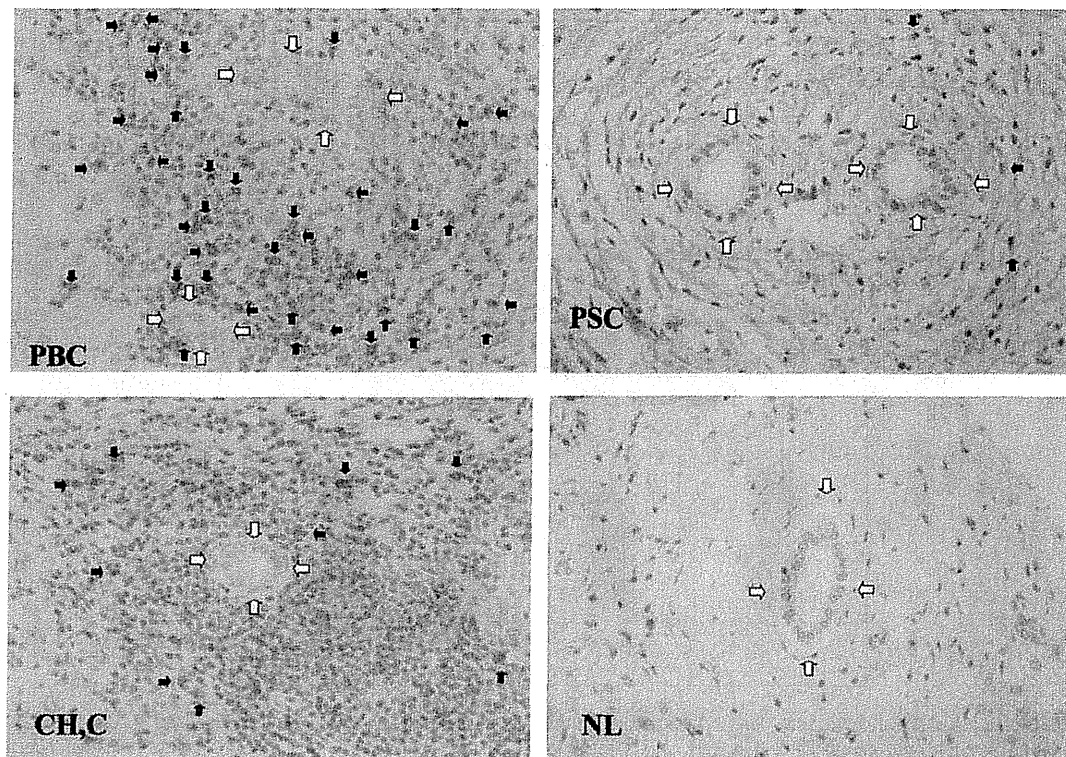
Fig. 6. Immunohistochemistry of CD56-positive cells in liver. Mononuclear cells expressing CD56 are seen in the biliary epithelial layer and periductal tissue. In PBC, CD56$^+$ cells are seen within the biliary epithelium (white arrow) and also at high density around the bile ducts (black arrow). In PSC and hepatitis C, CD56$^+$ cells are scattered, and in normal liver CD56$^+$ cells are rare around the bile ducts. Statistical differences between PBC patients and controls are described in the text.

autoimmune diseases such as PBC needs to be considered and evaluated. Thus, whereas it is easy to visualize a role for innate immune involvement in the initial stages of the disease process followed by the emergence of adaptive immune responses, it is clear that destruction of tissues during the chronic stages must require removal of dying cells and products of lytic cells. The removal of such unwanted tissues in addition to autophagy must involve the function of innate immune mechanisms. It naturally follows that the activated state of the innate immune system must result in proinflammatory cascades contributing to the pathology of the autoimmune disease. It should also be noted that the adaptive immune system has been shown to affect the character and magnitude of innate inflammatory responses.[17]

One of the major cell lineages of the innate immune system that is known to mediate target cell destruction are cells of the NK cell lineage.[18] Our previous findings of a high frequency of NK cells within cellular infiltrates around small bile duct cells of the liver in PBC patients[12] prompted us to examine the potential role this cell lineage plays in the pathogenesis of human PBC. Data presented

herein demonstrate that NK cells from TLR3-L and TLR4-L-stimulated LMC kill autologous BEC, especially in PBC patients when compared to other control liver diseases; there have been descriptions of crosstalk between NK cells and other innate immune populations by way of TLRs.[13,19] One explanation for this observation is the finding of high levels of IFN-α in the sera of patients with PBC as compared with sera from patients with other liver diseases and otherwise control individuals. Thus, IFN-α is known to activate NK cell and contributes to enhance NK cell mediated cytotoxicity (Supporting Fig. 2 highlights these pathways).

The data herein also demonstrate that CD56-expressing NK cells upon ligation of TLR4 in the presence of IFN-α activates NK cells[20] and induces TRAIL.[21] The function of NK cells appears to vary depending on the disease process.[22] For example, the phenotypes of NK cells in patients with inflammatory bowel disease are different from those from normal intestinal mucosa.[23] NK cell activation receptor NKp46-positive NK cells have been shown to recognize and destroy beta cells in type I diabetes.[24] However, as previously shown, NKp46 is not induced on NK cells by

TLR4-L in the presence of IFN-α. Hence, it is our working hypothesis that the function of local resident NK cells in CNSDC is distinct from that noted in patients with PBC as exemplified by the unique expression of TRAIL in the latter but not the former.

There was no detectable cytotoxic effect when BECs were cultured with either TLR3-L or TLR4-L alone in our assay. Up-regulation of NK cell activating ligands has been reported in several liver subpopulations, including BEC, and has been implicated in liver injury.[25] It is not clear whether NK cell-activating ligands are also up-regulated on BEC in PBC and involved in the increased sensitivity to NK cell killing. Studies are in progress to define the relative sensitivity of BEC to NK cell cytotoxicity.

NK cells are cytotoxic for autologous BEC in the presence of TRAIL. The fact that human cholangiocytes constitutively express death receptor 5, which is the natural receptor for TRAIL, coupled with the finding of elevated levels of TRAIL expression and apoptosis in cholangiocytes of PBC patients,[26] suggests that TRAIL/Death receptor 5-mediated apoptosis may be the major pathway involved in the pathogenesis of chronic cholestatic disease.

Our data indicate that there are two requirements for NK cell-mediated cytotoxicity. One requirement depends on the source of TLR4 ligation and the other is the source of IFN-α, reportedly elevated in CNSDC.[27] Indeed, IFN-α appears to be derived from a monocytoid lineage, potentially plasmacytoid dendritic cells (pDC)[28]; in our study we were not able to address this issue because of insufficient quantities of pDC in this experimental protocol. This is an issue that should be examined in the future. An additional remaining question is the identification of the source of the ligands for TLR3 and TLR4 that activate Mo and NK cells, respectively. Thus, whereas exogenous sources of ligands for TLR3 and 4 were used herein, it will be important to identify the natural ligands that are functional in patients with PBC because such data will be of major clinical importance. In this regard, it is of interest to note that there is a correlation of urinary tract infections and PBC[29,30]; this could be the candidate source for TLR-L.

Sera from patients with autoimmune diseases often reflect the presence of elevated levels of inflammatory cytokines, including type 1 and 2 interferons (IFN), TNF-α, and IL-12.[31-33] IFN is induced by both a TLR-dependent and independent pathway in systemic autoimmunity.[34] Additionally, activation and proliferation of both autoantigen specific and nonspecific CD8 T cell responses are characterized by

the expression of CD38 and Ki-67 expression.[35] Previous work has demonstrated that pDC is a major source of type 1 IFN in response to ligation of TLR7.[36] In this regard, the characteristics of pDC that contribute to their pathogenic role include the observation that TRAIL-expressing pDC induces death of CD4 T cells that express TRAIL-associated death receptors.[37] In addition, pDC inhibit T cell proliferation through an indoleamine oxidase (IDO)-dependent pathway[38] and, finally, pDC rapidly migrate to the site of autoimmune mediated injury and/or infection and attract CD4+ T cells to the site.[39] We should note that in this study we did not evaluate IFN production from pDC in the presence of TLR7/8-L (CL097), but we did note the absence of cytolytic activity of LMC incubated with TLR4-L and TLR7/8-L (CL097).

Finally, it has also been demonstrated that CX3CL1 is expressed by BEC from patients with PBC and appears involved in the recruitment of intrahepatic lymphocytes into bile ducts.[8,40] This interaction promotes NK cell activation.[41] In conclusion, therefore, there is a complex but nonetheless well-defined relationship between liver mononuclear cell subpopulations and the biliary cell pathology of PBC. These interactions provide several steps that can potentially be modulated to reduce inflammation and will be the focus of further studies.

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