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Periductal interleukin-17 production in association with biliary innate immunity contributes to the pathogenesis of cholangiopathy in primary biliary cirrhosis

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Summary

An innate immune response to bacterial components is speculated to be involved in the pathogenesis of primary biliary cirrhosis (PBC). Recently, CD4-positive T helper type 17 (Th17) cells, characterized by the secretion of interleukin (IL)-17, have been implicated in the pathogenesis of autoimmune diseases. Human Th17 cells are generated from Th0 cells by IL-6 and IL-1β and maintained by IL-23. In this study, the role of IL-17 in PBC and its association with biliary innate immunity were examined. Using cultured human biliary epithelial cells (BECs), the expression of Th17-related cytokines and chemokines and changes therein on treatment with pathogen-associated molecular patterns (PAMPs) and IL-17 were examined. Immunohistochemistry for IL-17 and Th17-related cytokines was performed using tissue samples of human liver. Consequently, the expression of IL-6, IL-1β, IL-23p19 and IL-23/IL-12p40 mRNAs, and their up-regulation by PAMPs, were found in BECs. Moreover, BECs possessed IL-17-receptors and stimulation with IL-17 induced production of IL-6, IL-1β, IL-23p19 and chemokines. Several IL-17-positive cells had infiltrated damaged bile ducts and the expression of IL-6 and IL-1β was enhanced in the bile ducts of PBC patients. In conclusion, IL-17-positive cells are associated with the chronic inflammation of bile ducts in PBC which is associated causally with the biliary innate immune responses to PAMPs.

Keywords: biliary epithelial cells, innate immunity, interleukin 17, primary biliary cirrhosis, Th17

Introduction

The aetiopathogenesis of primary biliary cirrhosis (PBC) remains speculative. However, the high prevalence of vaginal and urinary tract infections, the presence of bacterial and viral components in bile and liver and the cross-reaction of human and bacterial pyruvate dehydrogenase complex-E2 (PDC-E2) with anti-mitochondrial antibody (AMA) and autoreactive T cells in PBC patients suggest that the presence of bacterial components, including bacterial infections and innate immune responses to bacterial components, are involved in the pathogenesis of PBC, particularly the damage to bile ducts [1,2]. We have reported previously that human biliary epithelial cells (BECs) possess an innate immune system consisting of Toll-like receptors (TLRs), which recognize various pathogen-associated molecular patterns (PAMPs), suggesting biliary innate immunity to be associated closely with PBC [3–5].

CD4-positive helper T cells are essential regulators of immune responses and inflammatory diseases. Immunoreactivity to intra- and extracellular antigens is regulated mainly by two different types of memory CD4-positive T helper type cells, namely Th1 and Th2 cells, which are distinguished principally by their production of different cytokines as well as their ability to induce either cellular (Th1) or humoral (Th2) immune reactions. Recently, a third pathogenic type, Th17 cells, and its association with the chronic inflammation of autoimmune diseases, have been noted [6–8]. Human Th17 cells are characterized by the production of interleukin (IL)-17 (IL-17A and IL-17F) and differentiate from naive T cells (Th0). Studies in mice have identified transforming growth factor (TGF)-β and IL-6 as the critical cytokines driving the differentiation of naive T cells into Th17 cells [9], while a subsequent study indicated that IL-6 and IL-1β, but not TGF-β1, are required for the differentiation in human cells [10]. Recently, the

differentiation of human Th17 cells has been reported to require the activity of TGF-β [11–13], but the importance of TGF-β is still controversial. IL-23 is required for maintaining or stabilizing the cellular functions and for the survival, but not the differentiation, of Th17 cells [6]. Recent studies have identified the subset of T cells producing IL-17 (Th17) as playing a predominant role in the pathogenesis of experimental autoimmune encephalomyelitis [6] and autoimmune arthritis [7,8]. Moreover, Elson *et al.* reported that enteric bacteria-reactive CD4-positive Th17 cells expanded in number in colitic mice and that the relative expression of the IL-17 mRNA transcript in colonic lesions was increased greatly [14]. Leppkes *et al.* reported that the transcriptional factor of Th17 cells, retinoic acid receptor-related organ receptors (ROR) gamma, controls IL-17A and IL-17F production, and these cytokines have a crucial pathogenic role in chronic intestinal inflammation [15]. Because the receptor for IL-17 (IL-17R), a heterodimer of IL-17RA and IL-17RC, is expressed by many cells, IL-17 has the ability to induce the production of several cytokines including IL-6, IL-8 and IL-1, and chemokines such as CXCL (CXC-chemokine ligand)-1 (GRO-α), CXCL2, CXCL3, CXCL6(GCP-2), CXCL8(IL-8), CCL2(MCP-1) and CCL (CC-chemokine ligand)-20 from various cells, including epithelial and vascular endothelial cells [16–20]. These cytokines and chemokines are associated with a continuous (chronic) inflammation and the activation of nuclear factor-κB (NF-κB) and C-Jun N-terminal kinases (JNK) [19]. Although details of the signalling mechanism of the IL-17 pathway remain elusive, Act1 (transcription factor NF-κB activator 1) has been demonstrated recently to be an essential adaptor protein in IL-17 receptor signalling in autoimmune and inflammatory diseases [21].

In chronic hepatitis, particularly chronic viral hepatitis C (CVH-C), the non-suppurative damage of interlobular bile ducts known as hepatitis-associated bile duct damage, or hepatitic duct lesions, is not infrequent [22,23]. However, the difference in the histogenesis of cholangiopathies between PBC and CVH-C remains unclear.

In this study, we found a marked intrahepatic distribution of IL-17-positive cells and speculated that, in the presence of PAMPs, biliary epithelial cells are sufficient sources of IL-6, IL-1β and IL-23 for the generation and stabilization of Th17 cells at sites of periductal antigen-presenting cells such as dendritic cells.

Materials and methods

Cultured human BECs

Three cultured cell lines of human BECs (BEC1–BEC3) were used in this study. BEC1 and BEC2 were newly established from the explanted livers of PBC patients according to methods reported previously [24,25]. BEC3 was established from background liver showing a normal histology far from metastatic foci in surgically resected liver with a metastatic liver tumour. Informed consent for human research was obtained from all patients prior to surgery. This study was approved by the Kanazawa University Ethics Committee. The cultured BECs were incubated with a culture medium composed of Dulbecco's modified Eagle medium (DMEM)/F-12 (Invitrogen, Tokyo, Japan), 5% newborn calf serum (Invitrogen), 0·18 mM adenine (Sigma, St Louis, MO, USA), hydrocortisone (0·4 μg/ml), cholera toxin (10 ng/ml), triiodo-thyronine (1·3 μg/l), insulin transferrin selenium-positive (ITS$^+$) (Becton Dickinson, Franklin Lakes, NJ, USA), 25 mM sodium bicarbonate (Sigma), 1% antibiotics antimycotic, 20 ng/ml of human epidermal growth factor (Invitrogen) and 10 ng/ml of human hepatocyte growth factor (Invitrogen). The cells were grown as monolayers in a 5% CO_2-humidified incubator at 37°C. These BECs had been confirmed to be biliary epithelial cells by the expression of biliary-type cytokeratins (CK7 and CK19) and a marker of polarity (cystic fibrosis transmembrane conductance regulator, CFTR) [26]. All the cultured BECs were used between passages 4 and 9.

Patients and tissue preparations

Liver tissue specimens were used from eight patients with PBC (all positive for AMA by immunofluorescence at least once during follow-up; average age, 57 years; all female; histological stages I/II = 3/5) and, as controls, from nine patients with CVH-C (average age, 58 years; six male/three female; histological stages F1/F2 = 4/5) and five normal livers (average age, 62 years; three male/two female, non-cancerous hepatic regions showing no histological abnormalities from surgically resected livers for metastatic liver tumour). These specimens were selected from registered files in our laboratory, which contained at least three interlobular bile ducts including damaged bile ducts, and were obtained prior to treatments. The pathological diagnoses were based on established criteria including clinical and laboratory data and confirmed by histological review by an independent observer. More than 10 4-μm-thick sections were prepared from each paraffin-embedded block; several were stained with haematoxylin and eosin (H&E) and Gomori's reticulum stain for histological diagnosis and the others were used for immunohistochemistry. In addition to fixed specimens, fresh surgical specimens were available in three cases each of PBC and CVH-C, and used for immunohistochemistry.

Isolation of RNA and real-time polymerase chain reaction (PCR)

For the evaluation of mRNAs of IL-17(A), IL-6, IL-1β, IL-23 (IL-23 p19 and IL-23/IL-12 p40), caspase 1 (IL-1β converting enzyme), chemokines (CXCL1, CXCL2, CXCL3, CXCL6,

Table 1. Primer sequences used in this study.

Target gene	Forward	Reverse
IL-17(A)	5′-TGTCCACCATGTGGCCTAAGAG-3′	5′-GTCCGAAATGAGGCTGTCTTTGA-3′
IL-6	5′-AGTGAGGAACAAGCCAGAGC-3′	5′-AAAGCTGCGCAGAATGAGAT-3′
IL-1β	5′-CCAGGGACAGGATATGGAGCA-3′	5′-TTCAACACGCAGGACAGGTACAG-3′
IL-23 p19	5′-TACTGGGCCTCAGCCAACTC-3′	5′-TACAGCCACAAAGGCCTGGA-3′
IL-23/IL-12 p40	5′-GGAGCGAATGGGCATCTGT-3′	5′-TGGGTCTATTCCGTTGTGTCTTTA-3′
CXCL1	5′-GAAAGCTTGCCTCAATCCTG-3′	5′-CACCAGTGAGCTTCCTCCTC-3′
CXCL2	5′-CTTCTATTTATTTATTTATTTATTTATTTGTTTGTTTT-3′	5′-GAACTAACTTGGGTTTGACCTAAA-3′
CXCL3	5′-TGAAAAAGAGAACAGCAGCTTTCT-3′	5′-AGGACTGAGCTATGTTTGATGAAACA-3′
CXCL6	5′-GCTCCAAGGTGGAAGTGGTA-3′	5′-AGAAAACTGCTCCGCTGAAG-3′
CXCL8	5′-ACACTGCGCCAACACAGAAATTA-3′	5′-TTTGCTTGAAGTTTCACTGGCATC-3′
CCL2	5′-CTGAATTTTGTTTGTTGATGTGAAA-3′	5′-GCAATTTCCCCAAGTCTCTG-3′
CCL20	5′-GCGCAAATCCAAAACAGACT-3′	5′-CAAGTCCAGTGAGGCACAAA-3′
IL-17RA	5′-CCAGATCCCAGCTTTGAGAG-3′	5′-AAATGCCCGCCACATAGTAG-3′
IL-17RC	5′-CTATGGGACGATGACTTGGGAG-3′	5′-AGCGCAGCGGCAAAGAGTA-3′
Act1	5′-AACAAGGAAGCATGAATTTCAGA-3′	5′-ATTCTTGGGCCAGCTGTAGA-3′
GAPDH	5′-GGCCTCCAAGGAGTAAGACC-3′	5′-AGGGGTCTACATGGCAACTG-3′

IL, interleukin; CXCL, CXC-chemokine ligand; CCL, CC-chemokine ligand; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase.

CXCL8, CCL2 and CCL20), IL-17 receptor (IL-17RA and IL-17RC) and Act1 in BECs, total RNA was isolated from cultured cells using the RNeasy Total RNA System (Qiagen, Hilden, Germany), following the manufacturer's instructions. Then, 1 μg of total RNA was reverse-transcribed with an oligodeoxythymidylic acid (oligo)-(dT) primer and ReverTra Ace (Toyobo, Osaka, Japan) to synthesize a cDNA template for PCR. With regard to caspase 1, IL-17RA, IL-17RC and Act1, conventional PCR was performed in order to examine the presence of these molecules. The reaction profile consisted of initial denaturation at 94°C for 3 min followed by 28–35 cycles with 30 s of denaturation at 94°C, 30 s of annealing of primers at 55°C and 60 s of extension at 72°C. After the PCR, 5 μl aliquots of the products were subjected to 1·5% agarose gel electrophoresis. As for the other molecules, to carry out relative quantification, real-time quantitative PCR was performed for measurements according to a standard protocol using the SYBR Green PCR Master Mix and ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Tokyo, Japan), and relative gene expression was calculated using the comparative cycle threshold method. The primers used in this study are listed in Table 1.

Treatment with PAMPs and IL-17

Cultured BECs were stimulated with Pam3CSK4 (TLR-1/2 ligand, 100 ng/ml; Invivogen, San Diego, CA, USA), polyinosinic–polycytidylic acid [poly(I:C), a synthetic analogue of viral dsRNA, TLR-3 ligand, 25 μg/ml; Invivogen], lipopolysaccharide (LPS) (ultrapure grade, TLR-4 ligand, 1 μg/ml; Invivogen), or recombinant human IL-17 (1000 U/ml; PeproTech, London, UK), for 3 h and used for the preparation of samples for PCR analysis.

Immunohistochemistry

The deparaffinized and rehydrated sections for studying IL-17, IL-6, IL-1β, IL-23 p19 and caspase 1 were microwaved in 10 mM citrate buffer for 20 min in a microwave oven. Following the blocking of endogenous peroxidase, these sections were incubated at 4°C overnight with antibodies against IL-17 (goat polyclonal immunoglobulin (Ig)G, 1 μg/ml; R&D, Minneapolis, MN, USA) or caspase 1 (mouse monoclonal, 2·5 μg/ml; Imgenex, San Diego, CA, USA), and then at room temperature for 1 h with anti-goat immunoglobulins conjugated to a peroxidase-labelled dextran polymer (Simple Staining Kit; Nichirei, Tokyo, Japan) or anti-mouse immunoglobulins conjugated to a peroxidase labelled-dextran polymer (Envision System; Dako, Tokyo, Japan), respectively. As for IL-6, IL-1β and IL-23 p19, the sections were incubated for 15 min with anti-IL-6 antibody (rat monoclonal IgG1κ, 10 μg/ml; Abcam, Cambridge, UK), anti-IL-1β antibody (mouse monoclonal IgG2b, 5 μg/ml; Abcam) or IL-23 p19 antibody (mouse monoclonal IgG1κ, 10 μg/ml; BioLegend, San Diego, CA, USA), and then the catalysed signal amplification (CSA) system (Dako) was used. After a benzidine reaction, sections were counterstained lightly with haematoxylin. As a negative control, normal mouse IgG1κ or IgG2b or goat IgG was used as the primary antibody.

Among IL-17-induced chemokines, antibodies against CCL2 and CXCL6 were commercially available for immunohistochemistry using fresh sections. After fixation of frozen 5 μm sections in cold acetone for 10 min and the blocking of endogenous peroxidase, these sections were incubated with human CCL2 antibody (mouse monoclonal; 10 μg/ml; R&D Systems Inc.) or human CXCL6 antibody (goat polyclonal; 4 μg/ml; Santa Cruz, Santa Cruz, CA, USA) at room temperature for 60 min.

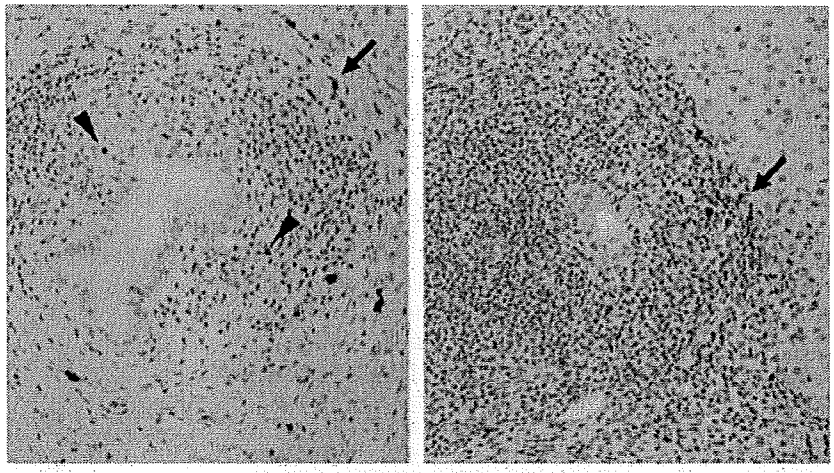

Fig. 1. Immunohistochemistry for interleukin (IL)-17 using tissue sections of primary biliary cirrhosis (PBC) and chronic viral hepatitis C (CVH-C). Several IL-17-positive mononuclear cells are scattered in the interface area of inflamed portal tracts in PBC and CVH-C (arrows). Moreover, IL-17-positive cells are found around chronic non-suppurative destructive cholangitis (CNSDC) in PBC (arrowheads), but not around interlobular bile ducts showing hepatitis-associated bile duct injury in CVH-C.

Histological examination

For the semi-quantitative evaluation of the immunohistochemistry, three to five representative portal tracts containing interlobular bile ducts including damaged bile ducts were chosen in each section for assessment. A total of 37 bile ducts in PBC and 44 in CVH-C were evaluated. IL-17-positive infiltrating mononuclear cells were counted around bile ducts in a high-power field (×400). For IL-6, IL-1β and IL-23 p19, immunoreactivity in bile ducts was graded semiquantitatively as follows: 0, absence of expression; 1, low constitutive expression; 2, intermediate expression; and 3, high expression. The final score indices of PBC and CVH-C were defined as the mean of individual cases.

Statistical analysis

Data were analysed using the paired *t*-test and Mann–Whitney *U*-test; $P < 0.05$ was considered statistically significant.

Results

Intrahepatic distribution of IL-17-positive cells in liver tissue

Representative images of the immunohistochemistry with anti-IL-17 antibody are shown in Fig. 1. IL-17-positive infiltrating cells were scattered mainly within the inflammed portal area of PBC and CVH-C, especially in interface areas. No epithelial and mesenchymal elements other than infiltrating cells expressed IL-17 in livers. Moreover, in PBC, IL-17-positive infiltrating mononuclear cells were accumulated around the damaged interlobular bile ducts such as chronic non-suppurative destructive cholangitis (CNSDC). In CVH-C, several interlobular bile ducts showing hepatitis-associated bile duct damage were found, but IL-17-positive cells were not accumulated around these bile ducts, including the undamaged ones. In normal livers, IL-17-positive mononuclear cells were very few or absent around interlobular bile ducts. Semi-quantitative evaluation revealed that number of IL-17-positive mononuclear cells around bile ducts to be significantly greater in PBC (5·7 ± 0·5) [mean ± standard error of the mean (s.e.m.)] than in CVH-C (1·3 ± 0·3) ($P < 0.05$).

Expression of IL-6, IL-1β, IL-23 and caspase 1 mRNAs and their regulation by PAMPs in cultured BECs

Real-time PCR analysis revealed that LPS, Pam3CSK4 and poly(I:C) enhanced the mRNA expression of both Th17-inducible cytokines, IL-6 and IL-1β in cultured BECs, with the increases being statistically significant (Fig. 2). Regarding the Th17-maintaining cytokine (IL-23), the expression of IL-23 p19 mRNA was up-regulated by stimulation with all these PAMPs, but that of the IL-23/IL-12 p40 component was induced by LPS and Pam3CSK4, but not poly(I:C) (Fig. 2). Cultured BECs constantly expressed caspase 1 mRNA, which is necessary for the production of activated IL-1β (Fig. 3).

Expression of IL-6, IL-1β, IL-23 p19 and caspase 1 in interlobular bile ducts

Immunohistochemistry revealed that the expression of IL-1β, IL-6 and IL23p19 were basically restricted in biliary epithelial cells and several infiltrating inflammatory cells. Hepatocytes, excluding several periportal hepatocytes, were negative for these cytokines. IL-23 p19 and caspase 1 were expressed constantly in biliary epithelial cells lining all intrahepatic bile ducts, irrespective of anatomical levels of diseased livers and normal livers (Fig. 4). However, the expression of IL-6 and IL-1β was prominent in the

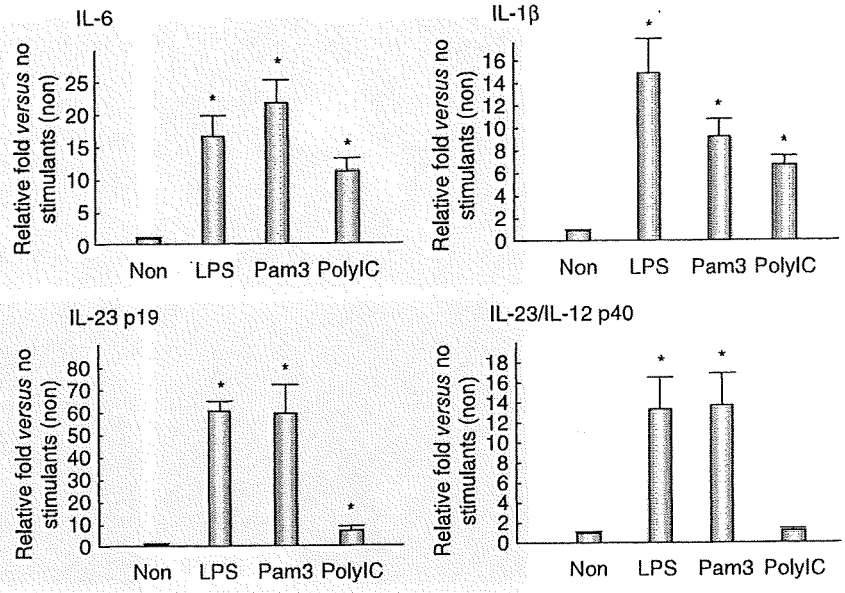

Fig. 2. Quantitative analysis of T helper 17 (Th17)-inducible cytokines [interleukin (IL)-6 and IL-1β] and Th17-maintaining cytokines (IL-23 p19 and IL-23/IL-12 p40) in cultured human biliary epithelial cells (BECs). Expression of IL-6 mRNA was up-regulated 16·6 ± 4·3 [mean ± standard error of the mean (s.e.m.)]-fold by stimulation with lipopolysaccharide (LPS) [Toll-like receptor (TLR)-4 ligand], 21·7 ± 2·5-fold with Pam3CSK4 (TLR-1/2 ligand) and 11·3 ± 1·5-fold with poly(I:C) (TLR-3 ligand). The relative fold-increase in the expression of IL-1β caused by LPS, Pam3CSK4 and poly(I:C), compared with no stimulant (non), was 14·9 ± 4·1, 9·2 ± 1·6 and 6·7 ± 0·6, respectively; that of IL-23 p19 was 60·7 ± 3·0, 60·0 ± 15·7 and 7·3 ± 0·6, and that of IL-23/IL-12 p40 was 13·4 ± 3·3, 13·7 ± 3·6 and 1·2 ± 0·3, respectively. LPS and Pam3CSK4 up-regulated the production of all these cytokines significantly ($P < 0·05$). Poly(I:C) also up-regulated the production of IL-6, IL-1β and IL-23 p19 ($P < 0·05$), but not IL-23/IL-12 p40. Duplicate experiments were performed using three lines of cultured biliary epithelial cells (BECs) (BEC1–BEC3) and data are shown as the mean ± s.e.m.

damaged bile ducts in PBC, but faint or lacking in CVH-C and normal livers (Fig. 4). As shown in Fig. 5, the semi-quantitative evaluation also showed the expression of IL-6 and IL-1β to be significant in PBC, compared with CVH-C ($P < 0·05$).

Effect of IL-17 on biliary epithelial cells and chemokine expression in bile ducts

Conventional reverse transcription (RT)–PCR revealed that cultured BECs possess receptors for IL-17 (IL-17RA and IL-17RC) and also Act1 (an essential adaptor protein in IL-17 receptor signalling) (Fig. 3). Therefore, biliary epithelial cells could be affected by the periductal cytokine milieu associated with IL-17; consequently, we examined the effect of IL-17 in cultured BECs. The expression of IL-6, IL-1β and IL-23 p19, but not IL-23/IL-12 p40, was up-regulated by IL-17 (Fig. 6). Moreover, expression of all the IL-17-induced chemokines, CXCL1, CXCL2, CXCL3, CXCL6, CXCL8, CCL2 and CCL20, was also up-regulated by IL-17 (Fig. 6). The expression of CCL2 and CXCL6 was examined in intrahepatic bile ducts using fresh liver sections. The damaged bile ducts expressed CCL2 and CXCL6 in PBC, but not or only faintly in CVH-C (Fig. 7).

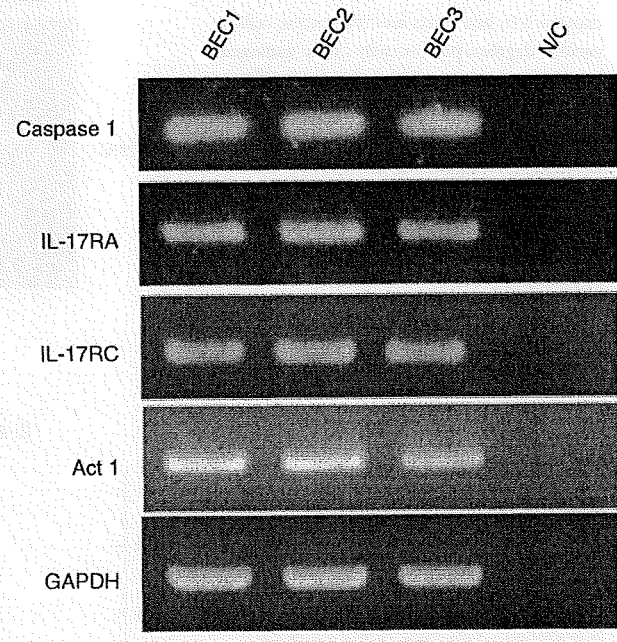

Fig. 3. Gel images of reverse transcription–polymerase chain reaction for caspase 1, interleukin (IL)-17RA, IL-17RC, Act1 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) [internal control for reverse transcription–polymerase chain reaction (RT–PCR)] using three cultured human biliary epithelial cell lines (BECs) (BEC1–BEC3). The BECs constantly expressed all these molecules, including GAPDH. N/C, negative control without cDNA.

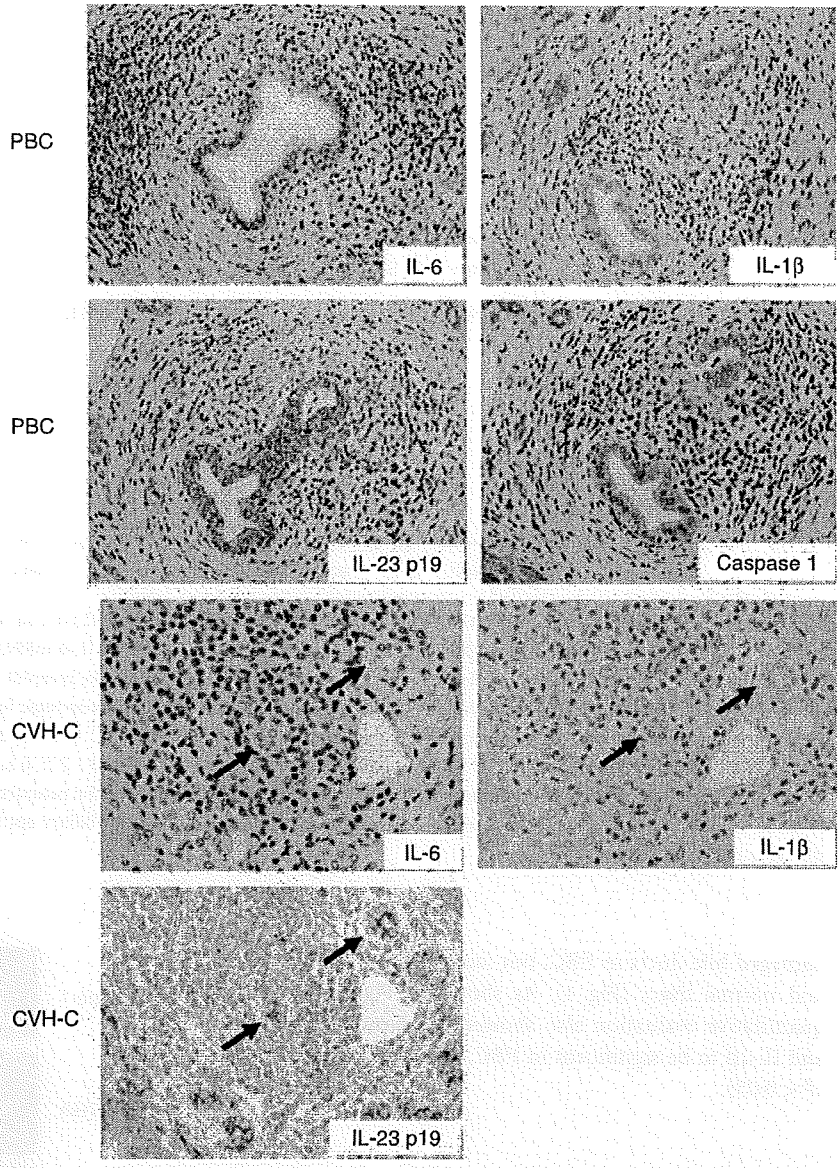

Fig. 4. Immunohistochemistry for T helper type 17 (Th17)-inducible cytokines [interleukin (IL)-6 and IL-1β], a component of IL-23 (IL-23 p19), and caspase 1. IL-6 and IL-1β are expressed in bile ducts and infiltrating cells to various degrees; prominently in the damaged bile ducts of primary biliary cirrhosis (PBC), but faintly or not at all in interlobular bile ducts of chronic viral hepatitis C (CVH-C). Caspase 1, which cleaves IL-1β for its secretion, is also expressed in the damaged bile ducts of PBC. In contrast, IL-23 p19 is constantly expressed in interlobular bile ducts, irrespective of PBC and CVH-C.

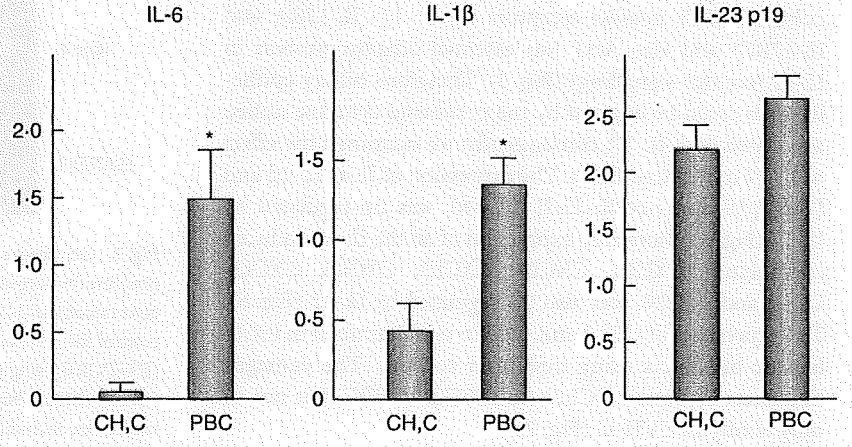

Fig. 5. Semiquantitative evaluation for the expression of interleukin (IL)-6, IL-1β and IL-23 p19 in interlobular bile ducts including damaged bile ducts. The scores for IL-6 were 0·02 ± 0·02 (mean ± standard error of the mean) and 1·5 ± 0·28 in chronic viral hepatitis C (CVH-C) and primary biliary cirrhosis (PBC), respectively; those for IL-1β were 0·48 ± 0·10 and 1·34 ± 0·12; and those for IL-23 p19 were 2·2 ± 0·2 and 2·6 ± 0·08, respectively. Statistical analysis revealed the expression of IL-6 and IL-1β to be significantly up-regulated in PBC, compared with CVH-C ($P < 0·05$).

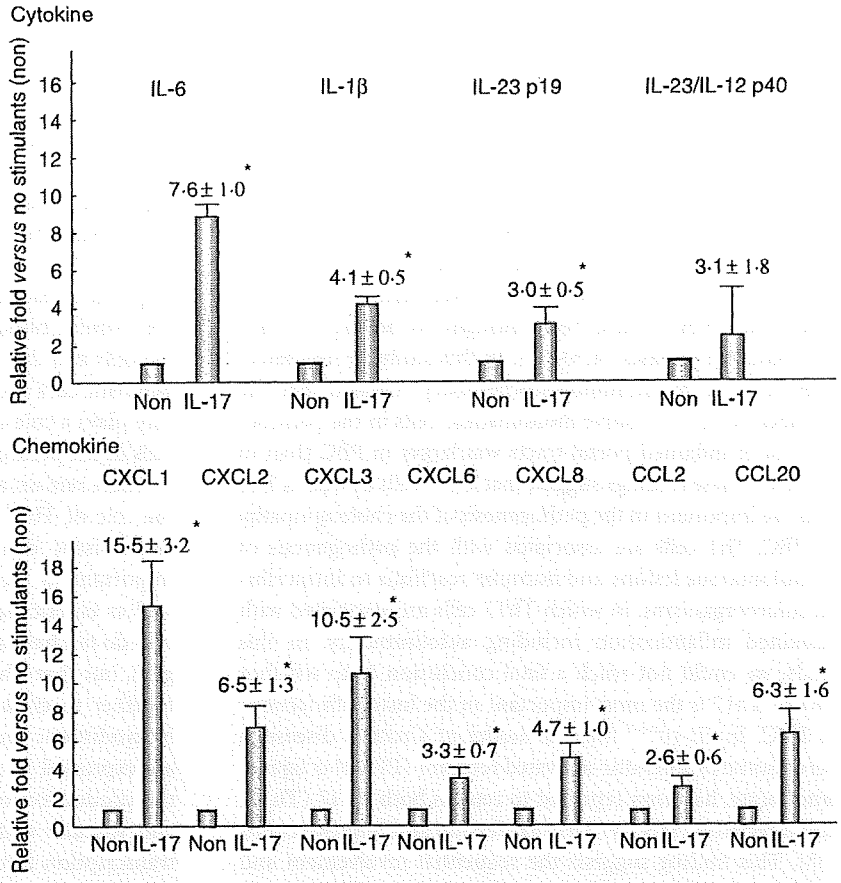

Fig. 6. The effect of interleukin (IL)-17 on the production of cytokines and chemokines in cultured human biliary epithelial cells (BECs). Real-time polymerase chain reaction revealed that the expression of Th17-inducible cytokines (IL-6 and IL-1β), Th17-maintaining cytokines (IL-23 p19 and IL-23/IL-12 p40) and IL-17-induced chemokines [epithelial-derived neutophil activating protein (CXCL)-1, CXCL2, CXCL3, CXCL6, CXCL8, monocyte chemoattractant protein (CCL)-2 and CCL20] is basically up-regulated by stimulation with IL-17 and there are significant differences except for IL-23/IL-12 p40 between no stimulants (non) and IL-17 ($P < 0{\cdot}05$). Duplicate experiments were performed using three lines of BECs, and data are shown as the mean ± standard error of the mean.

Discussion

Th17 cells are part of the mucosal host defence system and their major role seems to be protection against infections sustained by extracellular bacteria. Moreover, since the discovery that under certain conditions, Th17 cells can also be involved in the pathogenesis of chronic inflammatory disorders including models of some autoimmune diseases, there has been intense interest in the relative contributions of Th17 and Th1/Th2 cells to the pathogenesis of these diseases. Th17 cells are characterized by the production of primarily IL-17(A), known formerly as CTLA8. Lan *et al.* have already examined the IL-17-positive cells in PBC and concluded no differerence in intraportal IL-17-positive cells between PBC and chronic active hepatitis including CVH-C, non-alcoholic steatohepatitis and autoimmune hepatitis [27]. However, in the present study we noted interlobular bile ducts showing bile duct damage and cholangitis, including CNSDC, which is characteristic histology of PBC, and evaluated IL-17-positive mononuclear cells around these bile ducts in PBC. Our immunohistochemical examination revealed that IL-17-positive infiltrating mononuclear cells were present mainly at the interface of inflammed portal tracts in cases of PBC and CVH-C, and also in PBC, accumulated around the damaged interlobular bile ducts including CNSDC. In CVH-C, hepatitis-associated bile duct damage is seen frequently, but there is no periductal accumulation of IL-17-positive infiltrating cells. These findings suggest that Th17 cells are associated with interface hepatitis in chronic liver diseases and that the Th17-related peribiliary

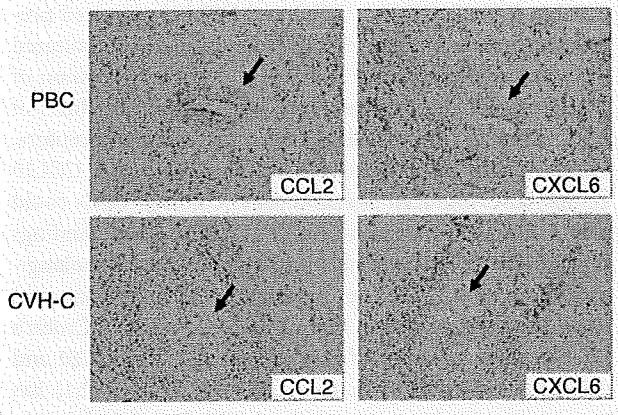

Fig. 7. Immunohistochemistry for monocyte chemoattractant protein (CCL)-2 and epithelial-derived neutophil activating protein (CXCL)-6 using frozen sections of primary biliary cirrhosis (PBC) and chronic viral hepatitis C (CVH-C). In PBC, damaged bile ducts show aberrant expression of CCL2 and CXCL6, but interlobular bile ducts in CVH-C lack expression of these chemokines.

cytokine milieu is enhanced in PBC and implicated in the histogenesis of the sustained cholangitis of PBC. Although some epithelial cells such as synovial cells in rheumatoid arthritis patients and glandular cells in salivary glands have been reported to be a source of IL-17 [28,29], no epithelial elements within livers expressed IL-17 and cultured BECs also lacked IL-17 mRNA (data not shown).

So far, studies on the Th1/Th2 balance in liver diseases have been based mainly on the profile of cytokines using peripheral blood lymphocytes or liver tissues, and both PBC and CVH-C have been thought to belong to Th1-predominant diseases [4,30–32]. In this study, *in situ* detection of IL-17 by immunohistochemistry revealed that the number of IL-17-positive mononuclear cells in the periductal area of inflamed portal tracts was larger in PBC than in CVH-C. These findings suggest that Th17 cells as well as Th1 cells are important in the pathogenesis of the cholangiopathy of PBC. Th1 cells are associated with the pathogenesis of granulomatous lesions and immune reactions to intracellular microorganisms, in which Th17 cells are associated with sustained inflammation including autoimmunity. In this study, we could not reach a final conclusion as to whether Th1 or Th17 is the most important in the histopathogenesis of PBC. In IL-10$^{(-/-)}$ mice, a model of Crohn's disease, a peroxisome proliferator-activated receptor (PPAR) α ligand, fenofibrate, has been reported to repress both IL-17 (Th17) and interferon (IFN)-γ (Th1) expression and improve colitis [33]. This finding suggests the sequential involvement and different functions of Th17 and Th1 cells rather than an exclusive role for these Th cells during the development of inflammatory and autoimmune diseases. Therefore, we speculate that also in PBC, both Th1 and Th17 cytokine milieus are important in the pathogenesis of cholangiopathy.

As Th17-inducible cytokines, TGF-β and IL-6 were identified in mice. However, a later study demonstrated that, in humans, IL-1β but not TGF-β is needed together with IL-6 to induce Th17 cells. IL-23 was also identified initially as a Th17-inducible cytokine, but is now recognized as a functional cytokine of Th17 cells. In contrast, IL-27, IL-12p35 and IFN-gamma play roles in the regulation of Th1 cell differentiation and also act as anti-Th17 differentiation cytokines [34]. Recently, a role of innate immunity via TLR signalling in the differentiation of Th17 cells has been described in inflammatory bowel diseases and multiple sclerosis; TLR signalling in B cells and T cells is responsible for the induction of Th17 [35,36] and TLR signalling in T cells is responsible for the induction of Th17, but not Th1 cells [37]. The present study demonstrated that bacterial PAMPs (LPS and Pam3CSK4) induced the production of Th17-inducible cytokines (IL-6 and IL-1β) and a Th17-maintaining cytokine (IL-23) in cultured BECs. Because the expression of IL-1β accompanied that of caspase 1 in cultured BECs, IL-1β could be released into the culture medium in a functional cleaved form. As for anti-Th17 differentiation cytokines, cultured BECs did not express mRNAs of IL27 nor IFN-gamma, irrespective of the presence of PAMPs. Although IL-12 p35 mRNA was constantly detected, significant changes by the treatments with bacterial PAMPs (LPS and Pam3CSK4) were not found (data not shown). Immunohistochemistry also revealed that interlobular bile ducts, in particular, damaged bile ducts in PBC, expressed IL-6 and IL-1β. IL-23 p19 and caspase 1 were expressed constitutively in intrahepatic bile ducts including interlobular bile ducts of normal livers as well as PBC and CVH-C. These results suggest that the biliary innate immune response to bacterial components involves the production of Th17-inducible and -maintaining cytokines in biliary epithelial cells and the differentiation into Th17 cells of periductal dendritic cells and macrophages; that is, biliary innate immunity plays a role in the induction and maintenance of Th17 cells in the periductal area in cases of PBC.

There remain a number of unanswered questions, such as the role of Th17 cells in host defence and how Th17 cells cause tissue damage. It seems likely that Th17 cells are important in the host response to commensal bacteria as well as enteric pathogens. An alternative view is that Th17 cells do not have a specific role in host defence against pathogens, but that Th17 and regulatory T cells (T_{reg}) cells work together to elicit or restrain tissue inflammation. Because heterodimeric receptors for IL-17 (IL-17RA and IL-17RC) are expressed in many cells, IL-17 has the ability to induce the production of cytokines and chemokines by epithelial and vascular endothelial cells. Recently, the presence of a transcription factor, Act1, has reported to be imperative in IL-17 receptor signalling in autoimmune and inflammatory diseases [20,21]. This study has demonstrated that biliary epithelial cells also constantly possess IL-17RA, IL-17RC and Act1, and produced Th1-inducible cytokines (IL-6 and IL-1β) on treatment with IL-17, suggesting that periductal IL-17 itself could enhance the induction of the Th17-dominant milieu. Moreover, we have confirmed that the production of all IL-17-induced chemokines (CXCL1, CXCL2, CXCL3, CXCL6, CXCL8, CCL2 and CCL20), reported previously in several cells [16–20], were expressed in cultured BECs stimulated with IL-17 and also that the expression of CCL2 and CXCL6 was enhanced in damaged bile ducts of PBC. Because Th17 cells possess chemokine receptors, CCR2, CCR4 and CCR6 [10,38,39], CCL2 and CCL20 among IL-17-induced chemokines in cultured BECs could play a role in the attraction of Th17 cells. These findings suggest that IL-17 takes part in the chronicity of cholangiopathy in PBC via the production of cytokines and chemokines. Moreover, in IL-10$^{(-/-)}$ mice, a model of Crohn's disease, the expression of chemokines (CCL2, CCL20 and CXCL10) as well as of IL-17 (Th17) and IFN-γ (Th1) has been reported to be attenuated by the PPAR α ligand fenofibrate [33], supporting that CCL2 and CCL20 are important in the pathogenesis of Th17-related chronic inflammation. Therefore, we speculate that these chemokines are important in sustaining the chronic inflammation accompanying several immune cells including effecter cells and dendritic

cells. Further study is needed to clarify the mechanism of Th17 cell-mediated bile duct injury in PBC.

In conclusion, the present study demonstrated that IL-17-positive cells were accumulated around the damaged bile ducts in PBC and that biliary epithelial cells possessed the ability to produce Th17-inducible cytokines (IL-6 and IL-1β) and Th17-maintaining cytokine (IL-23) as a result of the innate immune response. These results suggest that periductal IL-17-secreting cells facilitate the migration of inflammatory cells including Th17 cells around the bile ducts in PBC, which could further aggravate the chronic cholangitis, and also that the differentiation into Th17 cells is associated closely with biliary innate immunity. It seems likely that periductal Th17 cells propagate and modulate the chronic cholangitis and bile duct damage in PBC, although further studies are necessary to establish the exact roles of Th17 cells in the aetiopathogenesis of cholangiopathy in PBC.

Acknowledgements

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Disclosure

The authors declare no conflicts of interest.

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REVIEW

Lymphocyte recruitment and homing to the liver in primary biliary cirrhosis and primary sclerosing cholangitis

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Abstract The mechanisms operating in lymphocyte recruitment and homing to liver are reviewed. A literature review was performed on primary biliary cirrhosis (PBC), progressive sclerosing cholangitis (PSC), and homing mechanisms; a total of 130 papers were selected for discussion. Available data suggest that in addition to a specific role for CCL25 in PSC, the CC chemokines CCL21 and CCL28 and the CXC chemokines CXCL9 and CXCL10 are involved in the recruitment of T lymphocytes into the portal tract in PBC and PSC. Once entering the liver, lymphocytes localize to bile duct and retain by the combinatorial or sequential action of CXCL12, CXCL16, CX3CL1, and CCL28 and possibly CXCL9 and CXCL10. The relative importance of these chemokines in the recruitment or the retention of lymphocytes around the bile ducts remains unclear. The available data remain limited but underscore the importance of recruitment and homing.

Keywords Lymphocyte homing · Mucosal addressin cellular adhesion molecule-1/MAdCAM-1 · Chemokines · Primary biliary cirrhosis · Primary sclerosing cholangitis

Introduction

Primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are chronic cholestatic liver diseases characterized by progressive destruction of bile ducts. Whereas PBC affects the small intrahepatic bile ducts, PSC involves the intra- and/or extrahepatic bile ducts. PBC is an organ-specific autoimmune disease with female predominance, genetic predisposition, high titer serum anti-mitochondrial autoantibodies, disease-specific antinuclear autoantibodies, frequent association with other autoimmune diseases, and strong indications that the destruction of small bile ducts is autoimmune-mediated [1]. PSC shares many of these features including *p*-ANCA autoantibodies in up to 80% of patients, genetic predisposition, and association with other autoimmune diseases. In particular, there is a link between inflammatory bowel disease (IBD), with >70% of PSC patients having IBD, most frequently ulcerative colitis, at some point during their lives. Nonetheless, PSC differs from classical autoimmune diseases in male rather than female predominance and is poorly responsive to immunosuppressive treatment. In addition, there is only circumstantial evidence that bile duct destruction in PSC is an immune-mediated response to an autoantigen [2, 3].

Both PBC and PSC are characterized by massive infiltration of T cells into the portal tract, the infiltrate consisting predominantly of T cells [4-7]. For reviews and discussion on the immunobiology of PBC, including generic mechanisms of autoimmunity, we refer to several recent and key publications [8-22].

In PBC liver compared to peripheral blood, there is a 100-fold enrichment of CD4+ T cells and a 10-fold enrichment of CD8+ T cells specific for the major PBC autoantigen, strongly suggesting that both populations participate in the bile duct injury seen in PBC [1]. In keeping with a major role for cellular immune mechanisms in PBC, there is type-1 cytokine predominance in serum and liver of PBC patients [23, 24]. Very little is known

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about the contribution of T cells to the lesions of PSC and the cytokine milieu in PSC liver, except that liver-infiltrating lymphocytes (LIL) from PSC patients produce significantly increased amounts of TNFα and IL-1β but decreased levels of IFNγ [7].

According to the general paradigm, leukocyte extravasation is a complex multistep process that begins with "tethering" and rolling of blood-born leukocytes, i.e., reversible and transient interactions mediated mainly by selectins. This slows down the immune cells sufficiently to allow them to interact with chemokines presented by endothelial cells. These can then increase the affinity and avidity of leukocyte integrins for their endothelial adhesion molecule receptors, thereby allowing firm adhesion to occur. The adhesion molecules involved in firm attachment frequently belong to the immunoglobulin superfamily, which includes intercellular adhesion molecules (ICAM)-1–5, vascular cell adhesion molecule 1 (VCAM-1), and mucosal addressin cellular adhesion molecule 1 (MAdCAM-1). Firm adhesion is a prerequisite for subsequent transendothelial migration into the tissue. Once lymphocytes have entered a specific tissue, they follow sequential and combinatorial chemotactic gradients to their final destination.

Chemokines play an essential role at every step of this adhesion cascade and subsequent migration. They do so by increasing the affinity and avidity of integrins for their adhesion molecules, providing chemotactic signals for transendothelial migration and the subsequent migration to specific compartments within a tissue and by triggering integrin activation required for the binding to adhesion and cell-matrix molecules involved in tissue retention. They are divided into four subfamilies (CC, CXC, C, and CX3C, with CC and CXC chemokines representing the largest group) based on the arrangement of the first two of four conserved cysteine residues. They can be further subdivided functionally into homeostatic and inflammatory chemokines (see Table 1 for the nomenclature and receptor usage of inflammatory cytokines). Inflammatory chemokines are induced or upregulated during inflammation and mediate leukocyte recruitment; homeostatic chemokines are constitutively expressed and regulate navigation of leukocyte precursors during hematopoiesis and of mature lymphocytes in secondary lymphoid tissue [25]. Note, however, that some chemokines (e.g., CCL21) do not neatly fit into one of these categories.

There is increasing evidence that chemokines do not establish soluble chemokine gradients at the luminal endothelial surface but instead are immobilized via attachment to specific glycosaminoglycans [26, 27]. Therefore, the production of specific glycosaminoglycans by endothelial cells may represent mechanisms to provide tissue-specific recruitment signals [26, 27]. In addition, although endothelial cells can produce many chemokines, themselves, they are also able to take up, transcytose and then present chemokines produced by surrounding cells [27, 28].

Chemokines exert their effects through binding to seven-membrane-spanning G-protein coupled receptors expressed on leukocyte and lymphocyte subsets. Frequently, a certain chemokine can bind to several different receptors; conversely, a receptor can be shared by several chemokines. There is extensive cross-talk between chemokines on numerous levels, e.g., through downregulation of shared receptors [29], enhancing the surface density of unrelated receptors [30] or through blocking of signaling events downstream from receptor activation [31].

Naïve lymphocytes recirculate between blood and peripheral lymph nodes. During this stage, they express L-selectin and CCR7, which allow them to enter lymph nodes which express the L-selectin ligand peripheral lymph node addressin (PNad) and the CCR7 ligands, CCL19 and CCL21. Once lymphocytes encounter their cognate antigen, they generally downregulate CCR7 and L-selectin expression and instead acquire a different set of adhesion receptors that allow them to be recruited to sites of inflammation and/or to "home," i.e., to preferentially return to the tissue in which they were originally activated. Recruitment and homing to certain tissues has been shown to involve the recognition of specific "addressins," i.e., tissue-specific ligands expressed by vascular endothelial cells, by homing receptors expressed on the lymphocyte. A further level of specificity is provided by chemokine receptor pairings. For example, homing to the gut is mediated by α4β7 integrin on lymphocytes binding to mucosal MAdCAM-1. The chemokine CCL25, which is capable of activating α4β7, is expressed preferentially in the small intestine, where it interacts with lymphocytes expressing its cognate receptor CCR9. Skin homing involves interactions between cutaneous lymphocyte antigen with E-selectin and CCL17 with CCR4. No specific addressin has been identified for the liver.

A unique feature of the liver is its dual blood supply, with arterial blood entering via the hepatic arteries and venous blood coming from the gut via the portal veins. Hepatic arterioles and portal venules transport arterial and venous blood into the sinusoids, which then take it through the lobule to the central vein. Lymphocytes can enter the liver at several sites, the most important probably being the portal vessels and the sinusoids. Portal veins constitutively express low levels of ICAM-1 and occasionally ICAM-2 and VCAM-1 [32-35]. E-selectin is generally not detectable [32, 36], whereas P-selectin may be weakly expressed on some portal vein endothelia [32]. Lymphocyte extravasation at this site is thought to proceed according to the general paradigm.

Table 1 Chemokines, their official and common names, and their receptors

Official nomenclature	Common name	Abbreviation	Receptor
CCL2	Monocyte chemoattractant protein-1	MCP-1	CCR2, CCR4, CCR9
CCL3	Macrophage inflammatory protein-1α	MIP-1α	CCR1, CCR5
CCL4	Macrophage inflammatory protein-1β	MIP-1β	CCR5, CCR9
CCL5	Regulated upon activation normal T l		CCR1, CCR3, CCR5, CCR9
CCL20	Macrophage inflammatory protein-3α, Liver and activation regulated chemokine	MIP-3α,	
LARC	CCR6		
CCL21	Secondary lymphoid chemokine	SLC	CCR7
CCL25	Thymus-expressed chemokine	TECK	CCR9
CCL28			CCR10
CXCL9	Monokine induced by IFNγ	MIG	CXCR3
CXCL10		IP-10	CXCR3
CXCL11	IFN-inducible T cell a chemoattractant	I-TAC	CXCR3
CXCL12	Stromal (cell)-derived factor-1	SDF-1	CXCR4
CXCL16			CXCR6
CX3CL1	Fractalkine		CX3CR1

Sinusoidal endothelium constitutively expresses ICAM-1 [32, 37] and ICAM-2 [32], whereas levels of VCAM-1 and E-selectin are minimal or undetectable [32, 36]. Note, however, that constitutive expression of E-selectin has been reported on cultured hepatic sinusoidal endothelial cells (HSEC) [38]. Lymphocyte recruitment in the sinusoids does not involve the classical tethering and rolling step in cultured human [39] or mice HSEC [40]. Instead, the vast majority of lymphocytes immediately arrest. This is thought to be due to the low to undetectable levels of sinusoidal selectin expression, which seems to be dispensable in the low-shear environment of the sinusoids. There are indications that vascular adhesion protein 1 (VAP-1) plays a particularly important role in lymphocyte extravasation in the sinusoids.

Adhesion molecules in PBC and PSC

VAP-1

Unlike most other adhesion molecules, VAP-1 does not belong to the selectin, integrin, or immunoglobulin super families but is a syaloglycoprotein and copper-containing semicarbazide-sensitive amine oxidase that exists in both membrane-bound and soluble forms [41]. It mediates sialic-acid-dependent adhesion of lymphocytes to peripheral lymph node high endothelial venules (HEV) [42] and promotes shear-dependent lymphocyte adhesion to, and transmigration across, hepatic sinusoidal endothelium in vitro [39, 43, 44]. Both its adhesive function and enzyme activity contribute to these processes [38, 39, 45]. In addition, VAP-1 has been shown to activate hepatic endothelial cells, resulting in the upregulation of E-selectin, P-selectin, ICAM-1 transcription and translation, and enhanced production of CXCL8 (IL-8), thereby increasing lymphocyte adhesion [46, 47]. The lymphocyte ligand of VAP-1 has not been identified. From available data, it also remains unclear at which step of the adhesion cascade VAP-1 comes into play. Studies with isolated hepatic sinusoidal endothelial cells suggest that it mediates firm adhesion but does not affect lymphocyte rolling [47], whereas the results obtained with liver sections point to a role in the early tethering interactions [43, 44]. Recent data obtained with VAP-1 expressing rabbit heart endothelial cells suggest that VAP-1 functions after the initial tethering step [45]. VAP-1 can use primary amine groups on the surface of lymphocytes as a substrate. During the reaction, a transient covalent bond is formed between the endothelial VAP-1 and the lymphocyte. This comes into play after the initial tethering and reduces the rolling velocity, thereby providing time for the chemokine-mediated integrin activation that is a prerequisite for firm adhesion.

In humans, VAP-1 is constitutively expressed in endothelial cells of several organs and tissues, with the greatest abundance found in high endothelial venules of peripheral lymph nodes. Hepatic vascular and sinusoidal endothelium, but not bile ducts or hepatocytes, also express low constitutive levels of this protein [43, 44]. Inflammation or inflammatory mediators can induce VAP-1 in a tissue-specific manner [48, 49]. However, data from a very limited number of patients with acute liver allograft rejection or PBC

suggest that inflammation does not enhance VAP-1 expression in portal and sinusoidal endothelium or induce it in other cell types since both the pattern and intensity of VAP-1 immunoreactivity were the same in normal and diseased livers [43] (see also Table 2). Consistent with these observations, cultured HSEC were found to constitutively produce low levels of VAP-1, which were not enhanced by incubation with TNFα, IL-1β, IFNγ, bile acids, or LPS [39]. All of these agents (with the exception of bile acids which were not tested) can enhance VAP-1 expression in certain other tissues in an organ- and cell type-specific manner [49].

Of particular note, there are indications that VAP-1 in human peripheral lymph nodes has marked lymphocyte subset selectivity, mediating shear-resistant adhesion of CD8+ T cells and NK cells but binding CD4+ T cells poorly [42, 50]. Whether VAP-1 on hepatic endothelium is equally subset-specific has not been investigated. Furthermore, in mice, VAP-1 selectively mediates the hepatic recruitment of Th2 cells, whereas Th1 cells used α4β1 integrin [51]. However, in vitro generated Th1 and Th2 cells, as used in this study, frequently do not adequately reproduce the behavior of their in vivo counterparts [52, 53]. In view of the importance of Th1 and CD8+ T cells in PBC and possibly PSC, it would be interesting to establish the relevance of these findings and also their pertinence to human liver.

ICAM-1 and VCAM-1

In normal human liver, there is constitutive, though weak expression of ICAM-1 on sinusoidal endothelium, still weaker or even undetectable levels have been reported on portal endothelium [32-34, 37], and no staining has been detected on bile ducts. Immunoreactivity for VCAM-1, if at all detectable, is generally confined to the portal endothelium in normal liver [32-34, 54]. Note, however, that in primary culture, both human biliary epithelial cells (BEC) and HSEC constitutively express low levels of ICAM-1, whereas VCAM-1 is not always detectable [39, 55]. As is seen in numerous other endothelial and epithelial cell types, expression of ICAM-1 is enhanced and that of VCAM-1 induced or upregulated by incubation with inflammatory cytokines (TNFα or IL-1β) or IFNγ.

In PBC and PSC, ICAM-1 and VCAM-1 are induced or upregulated on portal and sinusoidal endothelium [34, 35, 37, 56] (see also Table 2). This upregulation of ICAM-1 and VCAM-1 is not only applied to PBC and PSC but is also seen in other inflammatory liver diseases, including acute and chronic rejection and viral hepatitis [32, 33, 57], although there are some indications that the pattern is somewhat disease-specific [58]. However, upregulation of ICAM-1 expression on bile ducts seems to be quite specific for PBC, PSC, and other liver diseases in which bile ducts are the major targets of immune-mediated destruction [32, 34, 35, 59, 60]. VCAM-1 is only very occasionally detected on bile ducts of PBC and PSC patients [34, 35]. Note, however, that immunoreactivity for ICAM-1 on bile ducts and ductules is detected in some but not all patients with PSC and PBC [34, 35, 37, 61]. Both its presence and its distribution seem to depend on the disease stage since it is seen almost exclusively in more advanced disease [35]. This suggests that ICAM-1 induction or upregulation is a consequence rather than a cause of bile duct inflammation. Lymphocytes expressing the ICAM-1 ligand, LFA-1, are seen only occasionally in normal liver, and their frequency is greatly increased in various liver diseases [33, 34, 58]. Unlike hepatocellular liver diseases, LFA-1+ T cells are found mostly around damaged bile ducts in the vicinity of ICAM-1-expressing BEC [37, 61]. This indicates that upregulation of biliary ICAM-1 can result in enhanced lymphocyte recruitment to bile ducts.

MAdCAM-1 and the aberrant homing of mucosal lymphocytes to PSC liver

There is a strong link between PSC and IBD, and certain molecules involved in lymphocyte recruitment are shared between the gut and the liver. This is thought to be part of an enterohepatic lymphocyte recirculation that allows for immune surveillance across both liver and gut, which encounter some of the same antigens due to their connection via the portal circulation. This prompted Grant et al. [62] to formulate the hypothesis that PSC is mediated by long-lived memory T cells that were originally activated in the gut and are recruited to the liver due to aberrant inflammation-induced expression of adhesion molecules

Table 2 Changes in adhesion molecule expression in PBC and PSC

Adhesion molecule	Portal vein			Sinusoidal EC			Bile ducts		
	normal	PBC	PSC	Normal	PBC	PSC	Normal	PBC	PSC
ICAM-1	−/(+)	++	++	+	++	++	−	−/+	−/+
VCAM-1	−/(+)	++	++		++	++	−	−/(+)	−/(+)
VAP-1	+	+	+	+	+	+	−	−	−
MAdCAM-1	−	+	+	−	+	+	−	−	−

"−/(+)" negative or occasionally weakly positive. "−/+" negative in some, positive in other patients

and chemokines that are usually restricted to the gut. When these cells become activated in the liver by cross-reactive self-antigens in the liver or by gut antigens that have entered the liver via the portal vein, the inflammatory response may become exaggerated and, if prolonged, result in chronic inflammation.

Several lines of evidence support this hypothesis. Under normal conditions, the mucosal vessels of the gut are essentially the only site of MAdCAM-1 expression in humans [63]. However, this adhesion molecule, while not detected in normal liver, can be expressed by hepatic endothelium of patients with chronic inflammatory liver disease [64, 65]. Discussions of the role that aberrant homing of mucosal lymphocytes plays in the pathogenesis of the hepatic complications of IBD underscores the importance of MAdCAM-1 expression in inflammatory liver diseases "associated with IBD" [62, 66, 67]. However, this statement is somewhat incomplete. In one of the only two existing studies on the expression of this adhesion molecule in human liver diseases, Hillan et al. [65] detected MAdCAM-1 expression in association with portal tract inflammation, whether due to hepatitis B or C, PBC, or PSC. In particular, portal vessels and lymphoid aggregates stained positive, and this is similar to the results reported by Grant et al. [64], who detected some additional staining of the sinusoids. However, the proportions of specimens positive for MAdCAM-1 differed between the studies. Grant et al. [64] reported that the majority of specimens from PSC and autoimmune hepatitis (AIH) patients (88% and 70%), but only 64% of PBC and 14% of chronic rejection specimens, showed immunoreactivity. In contrast, 71% of needle biopsy samples from PBC patients, but only 20% of specimens of PSC patients, were positive for MAdCAM-1 in the investigation by Hillan et al. [65]. However, in that study, all explanted liver samples from PBC and PSC patients demonstrated immunoreactivity for this adhesion molecule. This may simply reflect the focal nature of MAdCAM-1 expression, limiting the detection in small biopsy samples. It could indicate, however, that MAdCAM-1 is increasingly upregulated with disease progression and that could suggest that expression of this adhesion molecule is a consequence rather than a cause of inflammation. In order to clarify the true extent of MAdCAM-1 expression and its relation to the disease stages in PSC and PBC, further investigations are clearly needed.

In a study of nine PSC patients, the MAdCAM-1 ligand, $\alpha4\beta7$ integrin, was expressed on LIL from seven with 10% and 50% of infiltrating lymphocytes in portal tracts staining positive for $\alpha4\beta7$ expression compared to <10% of lymphocytes in PBC samples [5]. Grant et al. [64], themselves, reported that only ~10% of LIL in PSC expressed $\alpha4\beta7$ compared to ~40% of PBL from the same patients. However, PSC liver contains a higher proportion of $\alpha E\beta7$+ T cells compared to peripheral blood, and $\alpha4\beta7$ T cells have been reported to differentiate within tissue into $\alpha E\beta7$+ cells. Unfortunately, LIL from PBC or AIH patients were not analyzed by flow cytometry, even though immunohistochemistry reveals that a proportion of intrahepatic lymphocytes in all liver diseases studied were positive for $\alpha4\beta7$. Further studies are called for in order to resolve the question whether $\alpha4\beta7$ expression characterizes mainly LIL in PSC or pertains to essentially all liver diseases.

Chemokines in PBC and PSC

Chemokines that can trigger $\alpha4\beta7$-mediated binding to MAdCAM-1

CCL25 Interactions between the gut-specific chemokine CCL25 with its receptor CCR9 results in activation of the $\alpha4\beta7$ integrin, thereby allowing firm adhesion of lymphocytes to MAdCAM-1 [68]. Note, however, that it did not trigger the shape changes associated with motility and transendothelial migration. In addition to the MAdCAM-1–$\alpha4\beta7$ pairing, this provides another level of specificity in the gut homing of lymphocytes. Analysis of CCL25 and CCR9 expression in various liver diseases indicates that CCL25 immunoreactivity, detected mainly on sinusoidal endothelium in areas of interface hepatitis, was highly specific for PSC since it was not seen in PBC and other inflammatory liver diseases [69] (see also Table 3). CCR9 was detected on 20% of LIL in PSC where it was frequently co-expressed with $\alpha4\beta7$ while <2% of LIL were CCR9 positive in other liver diseases. Although these results await independent confirmation, they strongly suggest that the MAdCAM-1/$\alpha4\beta7$/CCL25/CCR9 axis plays a specific role in the pathogenesis of PSC but not in PBC and other inflammatory liver diseases. They also provide further support for the hypothesis that PSC involves aberrant homing of mucosal lymphocytes to the liver. Note, however, that CCR9+ lymphocytes are found almost exclusively in the small intestine, whereas few are detected in the colon [70]. Similarly, CCL25 is specifically expressed in the small intestine and not in the colon. Therefore, one would expect PSC to be associated mainly with Crohn's disease, which affects the entire gut, but not with ulcerative colitis, affecting only the colon. For as yet unknown reasons, the opposite is the case. However, it is interesting that the ulcerative colitis associated with PSC has a unique phenotype in which there is ileal involvement and rectal sparing [71]. An understanding of the factors that induce the expression of CCL25 in liver and intestine may shed some light on this paradox but remains elusive.

Table 3 Changes in chemokine expression in PBC and PSC

Chemokine	Portal vein			Sinusoidal EC			Bile ducts		
	normal	PBC	PSC	normal	PBC	PSC	Normal	PBC	PSC
IP-10	−	+ (?)	ND	−/+	ND	ND	−	+	ND
MIG	−/+	+ (?)	ND	−/+	ND	ND	−	+	ND
ITAC	−	ND	ND	+	ND	ND	ND	ND	ND
CCL21									
CCL25	−	−	−	−	−	++	−	−	−
CCL28	−	+	+	−	−	−	−	++	++
CXCL12 (SDF-1)	−	−	−	−	−	−	+	++	++
CXCL16	+	+	+	+	+	+	+	++	++
CX3CL1 (fractalkine)	+	+	+	−	−	−	(+)	++	(+)

ND not determined; "−" negative; "(+)" faint; "−/+" nondetectable in some, weakly positive in others; "+" clearly detectable; "++" strong staining

CCL21 If aberrant expression of MAdCAM-1/α4β7 integrin and CCL25/CCR9 are specifically involved in the recruitment of lymphocytes to liver diseases associated with IBD, the question then arises as to what, if any, function MAdCAM-1 expression has in PBC and other hepatic diseases. In this context, it is interesting to note that not only CCL25 but also CCL21, CCL28, and CXCL12 are capable of triggering α4β7-mediated adhesion of human PBL to MAdCAM-1 under shear stress in vitro [68, 72, 73]. Therefore, whether these chemokines play a role in the recruitment of T lymphocytes to PBC liver via MAdCAM-1 will depend on the actual number of α4β7-positive lymphocytes that enter the liver. CCL21 is a chemokine usually expressed on HEV in peripheral lymph nodes and Peyer's patches, and its ability to activate the α4β7 integrin-mediated adhesion of lymphocytes to MAdCAM-1 [68, 72] plays an important role in the recruitment of naïve lymphocytes to Peyer's patches. In addition, CCL21 can also trigger the shape changes that precede transendothelial migration and can activate α4β1 integrin-mediated lymphocyte binding to VCAM-1 under fluid flow [68].

A characteristic of PSC, PBC, and a variety not only of other autoimmune disease but also of chronic hepatitis C (CHC) is the presence of lymphocyte aggregates in the target organ. These organize into lymphoid follicles that contain HEV expressing either PNAd or MAdCAM-1 [64]. In mice, ectopic expression of CCL21 results in lymphoid neogenesis [74, 75]; the presence of CCL21 is sufficient to trigger integrin-dependent adhesion of naïve lymphocytes to nonlymphoid tissue and to induce their extravasation [76]. In human autoimmune diseases such as ulcerative colitis or rheumatoid arthritis, the lymphoid neogenesis is associated with expression of CCL21 mRNA in endothelial cells, including the HEV within the lymphoid follicles as well as blood vessels outside these organized lymphoid structures [76].

CCL21 is restricted to a few small lymphatic vessels in normal liver but is expressed in portal tracts in PSC and PBC, particularly in lymphoid aggregates [77]. The strongest immunoreactivity is seen on vascular endothelium of vessels exhibiting the morphology of HEV. Lymphoid aggregates in PBC; PSC and other liver diseases also demonstrated MAdCAM-1 immunoreactivity, and some vessels with HEV morphology within these aggregates are occasionally positive for MAdCAM-1 staining [64, 65]. This suggests a functional role for CCL21 in the recruitment of CCR7+ lymphocytes to the lymphoid aggregates within PBC and PSC portal tracts. CCR7, the receptor for CCL21, was expressed on a significantly higher percentage of PBL (76% vs. 50%) and intrahepatic T cells (20% vs. 9%) from PSC and PBC patients compared to healthy controls [77]. Of note, a high proportion of LIL in PSC were CD45RA+, and not all could be characterized as primed T cells that had reverted to a CD45RA+ phenotype. This suggests that CCL21 plays a role in the recruitment of naïve T cells to the liver, which would be consistent with the observation that murine CCL21 can recruit naïve T lymphocytes to nonlymphoid tissues [76]. Together, these results support the hypothesis that lymphoid neogenesis provides an environment for interactions between immune cells and antigen-presenting cells within the peripheral tissue that represents the antigen source [75] and, therefore, a site where autoreactive naïve T cells may be primed directly at the site of inflammation [76].

CCL28 Like CCL25 and CCL21, CCL28 can trigger α4β7-mediated adhesion of human PBL to MAdCAM-1 as well as α4β1-mediated binding to VCAM-1 under shear stress in vitro [68, 72, 73]. It also induces transwell chemotaxis of PBL and LIL [78]. Furthermore, it can mediate static adhesion of LIL from PSC, PBC, or alcoholic liver disease (ALD) to immobilized MAdCAM-1 via α4β7 and to VCAM-1 via α4β1 [78]. Both of these adhesion molecules are expressed in the portal endothelium in PBC

and PSC liver, whereas bile ducts never express MAdCAM-1 and are only occasionally positive for VCAM-1 in PBC patients [34, 35, 64, 65].

CCL28 is not detected in normal liver, but intense staining is seen in livers of patients with PSC, PBC, or ALD, not only on injured bile ducts but also on portal endothelium and on reactive bile ductules [78]. Primary human cholangiocytes can be induced to express CCL28 mRNA and protein by incubation with IL-1β or LPS. The CCL28 receptor, CCR10, is expressed on almost 17% of liver-infiltrating CD3+ T cells in inflamed liver but only 6% in healthy control liver, with the vast majority of CCR10+ cells being CD4+ T cells. Since MAdCAM-1 is not, and VCAM-1 only occasionally, detected on bile ducts, the main function of CCL28 expression in bile ducts may not be in the extravasation of lymphocytes but in their localization to bile ducts. Since binding to fibronectin is also mediated by α4β1 integrin, CCL28 may also play a role in the attachment of lymphocytes to this matrix molecule and thereby enhance their tissue retention.

Of note, Foxp3 mRNA is preferentially contained in the CCR10+ T cell population, indicating that this T cell subset includes natural regulatory T cells. A decreased frequency of Tregs has been reported from the liver and peripheral blood of PBC patients [79, 80], although increased numbers were found by others [81]. It is possible that this decrease is due to reduced expression of CCR10 or other chemokine receptors that have been implicated in the recruitment of Tregs, such as CCR8 [82] or CCR5 [83]. However, first-degree relatives of PBC patients also have a decreased frequency of Tregs in peripheral blood [79], suggesting that there is a genetic defect in the production of this lymphocyte subset rather than a defect in their homing to the liver.

CXCL12 CXCL12, also called stromal (cell)-derived factor 1 (SDF-1), is a chemokine that induces its effects via interaction with its specific receptor CXCR4. These effects include enhancing both the initial tethering of lymphocytes and their subsequent firm α4β1 integrin-mediated adhesion to VCAM-1 on endothelial cells under fluid flow [68, 84]. In addition, CXCL12 is capable of triggering α4β7-mediated binding of PBL or CD4+ T cells to MAdCAM-1 and fibronectin under shear stress [68, 73]. Furthermore, SDF-1 stimulates the chemotaxis of T cells, NKT cells, and NK cells isolated from the liver of HCV patients and healthy controls [85] and the transendothelial migration of naïve and memory CD4+ and CD8+ T cells [86]. In normal liver, SDF-1 is expressed exclusively on bile ducts in portal tracts [87, 88]. Immunoreactivity largely remains confined to the bile duct but is greatly enhanced not only in PBC and PSC liver [69, 88] but also in the liver of patients with AIH, CHC, or acute or chronic liver allograft rejection [85, 87, 88]. Interlobular and septal bile ducts show the strongest immunoreactivity, but reactive bile ductules are also positive. Both normal and diseased liver contained SDF-1 mRNA, and transcript for this chemokine was detected in laser-captured BEC, but not hepatocytes, from patients and controls [88]. Although the frequency of CXCR4 positivity was equally high in PBL and LIL, the latter exhibited greater intensity of CXCR4 expression, with both CD3+ T cells and CD19+ B cells showing higher expression levels surrounding SDF-1 positive bile ducts [87, 88]. Hepatic gene expression profiles using cDNA array analysis of PBC and PSC liver specimens reveals that CXCR4 is among the most strongly upregulated genes in PBC liver compared to normal liver and even compared to PSC liver [89]. Conversely, expression of the SDF-1 gene is more markedly enhanced in PSC than in PBC. The exclusive expression of SDF-1 on bile ducts in human liver indicates that this chemokine does not have a role in the recruitment of CXCR4+ lymphocytes to liver. However, once lymphocytes have entered the portal tract; SDF-1 functions in attracting them to bile ducts. In addition, SDF-1 may have a role in lymphocyte retention via its ability to augment their adhesion to fibronectin [73, 85, 90]. The biliary basement membrane demonstrates immunoreactive fibronectin in 80% of PBC patients but not in disease and normal control livers [91]. It remains to be determined whether this includes overexpression of the CS1 splice variant of fibronectin, which contains the binding site for VLA-4 (α4β1 integrin). Overexpression of CS1-fibronectin has been implicated in the retention of activated T cells in the synovium of rheumatoid arthritis patients [92]. Note that the interaction between lymphocytes and CS1-fibronectin is usually mediated by α4β1 integrin, but α4β7 integrin may also bind to this ligand [73]. Since SDF-1 can activate both of these integrins, it would be capable of triggering adhesion to fibronectin regardless of the integrin mediating this interaction.

Other chemokines

CXCR3 ligands

As their common names indicate, the CXC chemokines human monokine induced by IFN-γ (MIG, CXCL9), IFNγ-inducible protein of 10 kDa (IP-10, CXCL10), and IFN-inducible T cell α chemoattractant (I-TAC, CXCL11) are induced by IFNγ. They trigger adhesion and transendothelial migration under fluid flow as well as chemotaxis by signaling through a common receptor, CXCR3 [55, 86, 93]. This receptor is expressed selectively on activated effector or memory T cells [94], although more recent data suggest that activation is not an absolute requirement [93].

These chemokines are either not demonstrable in normal liver [55, 87, 95-97] or, when detected, are confined to sinusoidal endothelium and occasional portal venules [87, 98-100]. Their upregulation in various inflammatory liver diseases seems to follow chemokine- and disease-specific patterns. In PBC liver samples, IP-10 and MIG were induced in portal areas, including the damaged bile ducts [96]. In chronic liver allograft rejection, which is also characterized by progressive bile duct loss, IP-10 was induced on bile ducts in portal tracts and was upregulated on sinusoids [87]. MIG was not seen on any bile ducts but was increased on sinusoidal endothelium; I-TAC was induced on portal endothelium and some bile ducts but was not detected on sinusoids. This contrasts with the findings in CHC liver, where IP-10 and I-TAC are either found to be upregulated almost exclusively on hepatocytes [97, 99, 101] or exclusively on sinusoids [98, 102], whereas MIG has been reported to be induced or enhanced selectively on sinusoids [97, 98, 100]. A high frequency of CXCR3 lymphocytes, predominantly CD4+ T cells, was detected in PBC portal tracts, including damaged bile ducts [96]. In addition, PBC patients exhibit a significantly higher frequency of peripheral CXCR3+ CD4+ T cells compared to healthy controls. Similar results have been reported from patients with liver allograft rejection [87] and CHC patients [55, 100, 101]. Note that increased CXCR3 expression on infiltrating T cells is not confined to inflammatory diseases of the liver but seems to be a general feature of inflammation [103].

Together, these results suggest that expression of IP-10 in bile ducts may be characteristic of liver diseases where bile ducts are specifically targeted, including PBC and chronic liver allograft rejection; expression on hepatocytes seems to be typical of certain stages of forms of CHC; whereas induction or upregulation on sinusoidal cells is seen in both chronic rejection and CHC, but not in PBC. MIG also seems to participate in the recruitment of T cells to the portal tract in PBC, whereas it is expressed only on sinusoidal endothelium in chronic rejection and chronic hepatitis C. The available data on I-TAC are limited but suggest that this chemokine may be involved in the portal inflammation seen in chronic rejection but may play a role in the recruitment into the liver parenchyma via the sinusoids in CHC.

Plasma levels of IP-10 and MIG were elevated not only in PBC patients [96, 104] but also in their first degree relatives compared to controls [96]. In PBC patients, levels increased with advancing disease stage. Increased serum concentrations of IP-10 are also seen in patients with AIH [104], CHC [102, 105], and chronic hepatitis B [104], although the latter is not a consistent finding [102].

Interestingly, in situ hybridization studies indicate that IP-10 mRNA is expressed in hepatocytes of AIH, PBC, and chronic hepatitis B and C patients but not in portal areas, including the damaged bile ducts of PBC patients [104]. Similarly, others detected IP-10 transcripts mainly in hepatocytes, particularly around areas of focal and piecemeal necrosis in CHC patients [105]. On the other hand, not only a human hepatocyte-derived cell line (Huh-7) [99] but also cultured hepatic BEC [55, 106] and cultured hepatic sinusoidal endothelial cells [55, 98] are capable of producing all three CXCR3 ligands. IFNγ alone is generally sufficient to induce the production of these chemokines, whereas TNFα and IL-1β alone are often ineffective but further augment IFNγ-induced secretion. Relatively high levels of TNFα and IFNγ are present in most inflammatory liver diseases, including PBC, CHC, and liver allograft rejection. Therefore, other microenvironmental factors must contribute to the disease-specific expression patterns of the CXCR3 ligands. These could include HCV infection itself since it has been shown that transfection of HCV proteins or HCV RNA can stimulate the transcription and protein synthesis of chemokines, including IP-10 MIG and I-TAC [99, 107]. In PBC, LIL may play a role in further enhancing the chemokine production of BEC induced by certain other stimuli [106]. In addition, PBC LIL has been reported to exhibit enhanced chemotaxis to IP-10 and other chemokines compared to LIL from patients with viral hepatitis.

CXCL16 Another chemokine that might be involved in the recruitment of lymphocytes to bile ducts is CXCL16, constitutively expressed on bile ducts, hepatocytes, sinusoids, and to a lesser extent, on portal vessels [108]. Expression of this chemokine is upregulated on bile ducts in liver tissue from PBC and PSC, but not CHC, patients, whereas hepatocyte and sinusoid staining is enhanced in CHC. This pattern recaptures the disease-specific distribution of CXCR3 ligands. In matched samples, significantly higher percentages of LIL than PBL were positive for the CXCL16 receptor, CXCR6, in liver donors as wells as patients with end-stage CHC [108]. In cell culture, cholangiocytes demonstrate stronger expression of CXCL16 than hepatocytes and sinusoidal endothelial cells, which were nonetheless also positive. This chemokine was shown to enhance the adhesion of LIL and, to a lesser degree, PBL to primary cholangiocytes under static conditions, by activating α4β1 integrin (VLA-4) and thereby increasing its ability to bind to VCAM-1. This suggests that CXCL16 is important for the retention of lymphocytes on bile duct epithelium either through VCAM-1 or, since VCAM-1 is rarely detectable even in PBC and PSC, possibly through the other α4β1 integrin ligand, fibronectin. There are indications, though, that CXCR6 may play a more general role in the targeting of lymphocytes to, or their localization within, inflamed tissue

since increased numbers of CXCR6 T cells are not only found in inflamed liver but also in inflamed synovium of psoriatic and rheumatoid arthritis [109]. Interestingly, CXCR6 is preferentially expressed on type 1 (IFNγ-expressing) CD4+ and CD8+ T cells, particularly by highly differentiated effector Th1 cells and CD56+ granzyme A-expressing effector CD8+ cells. These are the cell types that are implicated in the destruction of bile ducts in PBC.

Fractalkine Fractalkine (CX3CL1) is an unusual molecule in that it (1) is the first and, to date, only member of the CX3C subclass of chemokines, (2) exists in both membrane-bound and soluble form, and (3) is both an adhesion molecule and a chemokine. It consists of a chemokine domain atop a mucin-like stalk connected to a single transmembrane region and a short intracellular tail. Fractalkine in its membrane-anchored state mediates leukocyte adhesion in an integrin-independent manner under static and flow conditions [110, 111]. There are indications that as in the interactions between other adhesion molecules and their lymphocyte ligands, adhesion to fractalkine requires a chemokine-mediated activation step [30]. In contrast, soluble fractalkine acts as a true chemokine capable of activating integrin ligands of other adhesion molecules and fibronectin [112, 113] and of triggering chemotaxis and transendothelial migration of certain monocyte and lymphocyte subsets [114, 115].

Both adhesion and chemotaxis are mediated by interactions between the chemokine domain of fractalkine with the fractalkine receptor CX3CR1 [115], but only chemotaxis requires signaling through G proteins. The two forms of fractalkine can show antagonistic activities [115-117]. CX3CR1 is expressed on monocytes, particularly CD16+ monocytes [118], but, above all, is a defining feature of cytotoxic effector lymphocytes, as identified by their cell-surface expression of CD57 and CD11b and intracellular expression of granzyme B and perforin [117]. Included in this group are terminally differentiated CD8+ T cells, NK cells, γδ T cells, and a small subgroup of CD4+ T cells. Of note, among CD4+ T cells, expression of CX3CR1 is almost exclusively found on those lacking the costimulatory molecule CD28 [116, 119]; all $CD28^-$ CD4+ T cells are positive for CX3CR1 [119]. In addition, CX3CR1 expression and responsiveness to fractalkine is seen predominantly in Th1 cells [120]. However, since CX3CR1 expression is confined to a small subset of CD4+ T cells, the relevance of these findings remains to be established.

Immunohistochemistry revealed that CX3CL1 is expressed on endothelial cells of small vessels in normal liver and faint staining is also detected on some bile ducts [121]. In liver biopsy samples from PBC patients, both the signal intensity and the number of bile ducts showing immunoreactivity is clearly increased, whereas neither the pattern nor the intensity of staining is altered in PSC, extrahepatic biliary obstruction, or CHC. From these data, it would appear that upregulation of fractalkine on bile ducts is specific to PBC. However, another group showed fractalkine expression in inflammatory foci surrounding regenerating nodules and also in bile-duct-like structures in CHC [122]. Even stronger immunoreactivity was detected in acute hepatitis due to acute HBV infection. In addition to areas of inflammation and necrosis, there was intense staining in regenerating epithelial cells within ductular reactions.

The fractalkine receptor, CX3CR1, was expressed in a few mononuclear cells in the portal tracts of normal livers and expression was somewhat increased in other liver diseases but was significantly higher in PBC patients, where CX3CR1-positive lymphocytes were found predominantly around damaged bile ducts [121]. Of note, CX3CR1 expression is rapidly downregulated on CD4+ T cells after receptor engagement by fractalkine [119]. Consequently, CX3CR1+ CD4+ T cells could not be detected in inflamed colonic tissue from IBD patients, whereas $CD28^-$CD4+ T cells, which all express this receptor, could be demonstrated in inflamed mucosa. Therefore, $CD28^-$ CD4+ T cells may have escaped detection in PBC liver [121]. Note, however, that the proportion of $CD28^-$ CD4+ T lymphocytes is significantly increased among PBL and intrahepatic T lymphocytes of PBC patients compared to healthy controls [123]. In particular, the intra-epithelial lymphocytes of small bile ducts in PBC patients contained a much higher number of $CD28^-$ CD4+ T cells than seen in other liver diseases or normal liver [124]. In contrast to CD28+ CD4+ T cells, $CD28^-$ CD4+ T cells are cytotoxic, express high levels of IFNγ, and are resistant to apoptosis. They are strongly implicated in autoimmunity since their frequency is significantly increased in a variety of autoimmune diseases. The detection of fractalkine on BEC in PBC together with the observation that this chemokine can act as a chemoattractant for the $CD28^-$ subset of CD4+ T cells [117, 119] suggest an important role for fractalkine in the recruitment of these cells to the bile ducts. In addition, it has been shown that fractalkine can also function as a co-stimulatory molecule and thereby increase granule exocytosis and IFNγ production in $CD28^-$ CD4+ T cells [116].

PBC patients exhibit a much higher precursor frequency of CD4+ T cells that do not require co-stimulation for proliferation and IFNγ production in response to the major PBC antigen PDC-E2 163-176 [123]. Furthermore, some of the co-stimulation-independent clones established from these PDC-E2 163-176-specific CD4+ T cells lacked CD28 expression. Nonetheless, this co-stimulatory function of fractalkine may not play an important role in PBC, since BECs, which are the major cell type expressing CX3CL1 in PBC liver, have been shown not to act as antigen-