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Repair-Related Activation of Hedgehog Signaling Promotes Cholangiocyte Chemokine Production

Alessia Omenetti¹, Wing-Kin Syn¹, Youngmi Jung¹, Heather Francis^{2,3}, Alessandro Porrello⁴, Rafal P. Witek¹, Steve S. Choi¹, Liu Yang¹, Marlyn Mayo⁵, M. Eric Gershwin⁶, Gianfranco Alpini^{2,3,7}, and Anna Mae Diehl¹

Alessia Omenetti: alessia.omenetti@duke.edu; Wing-Kin Syn: wing-kin.syn@duke.edu; Youngmi Jung: youngmi.jung@duke.edu; Heather Francis: hfrancis@tamu.edu; Alessandro Porrello: porre001@duke.edu; Rafal P. Witek: rafalp.witek@duke.edu; Steve S. Choi: steve.choi@duke.edu; Liu Yang: yang.liu@mayo.edu; Marlyn Mayo: Marlyn.Mayo@UTSouthwestern.edu; M. Eric Gershwin: megershwin@ucdavis.edu; Gianfranco Alpini: galpini@tamu.edu; Anna Mae Diehl: annamae.diehl@duke.edu

¹ Department of Medicine, Division of Gastroenterology, Duke University Medical Center, Durham, NC, USA

² Department of Medicine, Texas A&M Health Science Center, College of Medicine, Scott & White Hospital

³ Systems Biology and Translational Medicine, Texas A&M University System Health Science Center, College of Medicine

⁴ Institute for Genome Sciences and Policy, Duke University, Durham, NC, USA

⁵ Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA

⁶ Division of Rheumatology, Allergy, and Clinical Immunology, School of Medicine, University of California Davis, CA, USA

⁷ Division of Research, Central Texas Veterans Health Care System

Abstract

The mechanisms mediating hepatic accumulation of inflammatory cells in cholestatic liver disease remain enigmatic. Our thesis is that Hedgehog (Hh) pathway activation promotes hepatic accumulation of immune cells that interact with cholangiocytes. We believe that myofibroblastic hepatic stellate cells (MF-HSC) release soluble Hh ligands that stimulate cholangiocytes to express chemokines that recruit mononuclear cell types with cognate receptors for these chemokines, thereby orchestrating a repair-related mechanism for liver inflammation. To address this thesis, we used three experimental systems that allow definition of Hh-dependent mechanisms that induce phenotypic changes in cholangiocytes. First, cholangiocytes were cultured alone or in the presence of Hh-producing MF-HSC in a transwell co-culture system and/or treated with MF-HSC-conditioned medium with or without Hh-neutralizing antibodies. Changes in cholangiocyte phenotype were then evaluated by microarray analysis, QRT-PCR, and/or ELISA for Cxcl16. Bile duct ligation was chosen to model biliary fibrosis in mice with an overly-active Hh pathway, control littermates, and healthy rats, and the gene profile was evaluated by QRT-PCR in whole liver tissue. Second, a transwell chemotaxis assay was used to examine NKT cell migration in response to cholangiocytes, particularly cholangiocyte-derived Cxcl16. Finally, we studied liver samples from PBC patients and controls by QRT PCR to compare differences in the Hh pathway and Cxcl16. Co-immunostaining of CK-7 and Cxcl16 was then performed to localize the

phenotypic source of the Cxcl16. We found that MF-HSC release soluble Hh ligands that stimulate cholangiocytes to produce Cxcl16 and recruit NKT cells. Hh pathway activation during cholestatic liver injury also induces cholangiocyte expression of Cxcl16.

Conclusion—During biliary injury, Hh pathway activation induces cholangiocyte production of chemokines that recruit NKT cells to portal tracts.

Keywords

Cholangiocytes; Myofibroblasts; Natural Killer T cells; Bile duct ligation; Primary biliary cirrhosis

Hepatic accumulation of various types of inflammatory cells is a hallmark of many chronic cholestatic liver diseases.(1–3) Such cells often cluster in and around “neo-cholangioles” (ductular-like structures) and admix with fibroblastic cells in portal triads, but sometimes they extend deeper into hepatic lobules, particularly when liver injury is accompanied by fibrosis. Foci of dead and injured liver epithelial cells are typically associated with these inflammatory infiltrates, suggesting that the inflammatory cells themselves, and/or mediators that they release, are hepatotoxic.(2,4) The resultant elimination of chronically infected and/or irrevocably-damaged hepatocytes and cholangiocytes may also be necessary for eventual recovery. In any case, evidence that the numbers of hepatic inflammatory cells and myofibroblasts dwindle as liver injury and fibrosis resolve supports the concept that hepatic accumulation of immune cells and fibrosing biliary injury are linked.(2,4)

Biliary fibrosis is accompanied by activation of the Hedgehog (Hh) pathway in rodents and humans.(5–8) Hh ligands are pleiotropic morphogens that regulate tissue remodeling responses during embryogenesis and adult tissue repair.(6,7,9,10) Two of the major cell types that are involved in repairing chronic biliary injury, immature ductular cells (cholangiocytes) and myofibroblastic hepatic stellate cells (MF-HSC), produce and respond to Hh ligands.(5,7,8,11) Hh pathway activation promotes proliferation and enhances viability of both cell types, permitting accumulation of myofibroblastic cells and immature ductular cells in fibro-ductular bridges. When unrestrained, this causes progressive fibrosis and hepatic architectural distortion.(6,7)

Certain types of T lymphocytes have been shown to be Hh-responsive in adults.(12,13) Whether or not liver cell-derived Hh ligands have any role in regulating immune responses to liver injury has not, to our knowledge, been examined. This merits investigation, however, because immune cells contribute to liver remodeling and typically localize near collections of immature ductular cells and myofibroblasts in injured livers.(2,14–16) Therefore, the current study focused on the thesis that the activation of the Hh pathway promotes the specific accumulation of immune cells that interact with cholangiocytes, and results in an orchestrated response that includes a repair-related mechanism for liver inflammation. Our results support the concept that paracrine Hh signaling between myofibroblasts and cholangiocytes promotes immune cell migration towards biliary epithelial cells. These findings identify a mechanism that mediates this process, namely Hh-dependent induction of chemokine production by ductular-type cells. Moreover, functional analysis of one of these Hh-regulated chemokines, Cxcl16, provides compelling evidence for the pathobiological significance of such repair-related inflammation in chronic biliary disease in rodents and humans.

METHODS

Human subjects

Anonymized liver samples were examined from 14 patients with PBC and 7 control healthy livers. Tissues were obtained from UC Davis (Davis, CA), UT Southwestern (Dallas, TX) and Duke University School of Medicine Tissue Bank Shared Resource (Durham, NC), and studied in accordance with NIH guidelines for human subjects research.

Animals

Ptc-deficient mice (Ptc^{+/-}) and their wild type (WT) littermates were obtained from P.A. Beachy (Johns Hopkins University, Baltimore, MD). (17) Fisher rats were obtained from Charles River (Wilmington, MA).

Cell lines

Murine cholangiocyte 603B line was provided by Yoshiyuki Ueno (Tohoku University, Sendai, Japan) and G. Gores (Mayo Clinic, Rochester, MN). (18,19) Clonally-derived rat Myofibroblastic Hepatic Stellate Cell (MF-HSC) line 8B was obtained from M. Rojkind (George Washington University, Washington D.C.) (20) Normal rat cholangiocyte line (NRC) was a gift of N. LaRusso (Mayo Clinic, Rochester, MN). (21) Murine invariant NKT hybridoma cell line (DN32) was provided by Dr. Albert Bendelac (University of Chicago, Chicago, IL). (22)

Animal experiments

Mice and rats were subjected to bile duct ligation (BDL) or sham surgery (n=6 per group) and sacrificed after 1 week to obtain liver tissues (from mice) (5,7) or cholangiocytes (from rats). Cholangiocytes were purified using a monoclonal antibody (from R. Faris, Brown University, Providence, RI) against a membrane antigen expressed by all intrahepatic cholangiocytes. (23) Purity of cholangiocytes was confirmed by cytochemistry for γ glutamyl transpeptidase (γ GT), a cholangiocyte-specific marker. (5,23) As assessed by trypan blue exclusion, cell viability was greater than 97% (5,23). All animal experiments were approved by the Institutional Animal Care and Use Committees of Duke University and Scott and White Hospital as set forth in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

Cell Culture Experiments

Assessment of MF-HSC-derived factors on cholangiocyte phenotype—

Immature (7) cholangiocytes (603B cells) were cultured in 6 well plates for 6 days alone (monoculture) or in a trans-well co-culture system with MF-HSC (MF-HSC-8B) as described. (5,7) In each experiment, mRNA and conditioned medium were pooled from all 6 wells, and used for subsequent microarray, QRT-PCR analysis, or ELISA. MF-HSC 8B cells were also mono-cultured for 6 days to obtain MF-HSC-conditioned medium for other studies (see below). All experiments were repeated three times.

Pharmacological inhibition of Hh signaling *in vitro*—Hh neutralizing antibody (SE1, Developmental Studies Hybridoma Bank, Iowa City, IA) or control IgG (R&D)(10 μ g/ml) was added to MF-HSC-conditioned medium, and used to treat cholangiocyte monocultures that had been serum-starved for 18h. (5) Cholangiocytes that were treated with unconditioned medium served as controls. 24h later, supernatants and cell pellets were harvested. Cxcl16 protein in supernatants was quantified by ELISA and mRNA extracted from cell pellets was analyzed by QRT-PCR for chemokine-related genes. (5,7) These studies were repeated using freshly isolated, primary rat cholangiocytes, (23) and

supernatants were harvested after 6 h for Cxcl16 quantification. All experiments were repeated three times.

Natural Killer T (NKT) cell migration assay—Mono-/co-cultures were repeated using 24 well plates.(5,7) After 6 days, inserts containing MF-HSC were removed, conditioned medium was collected, and kept at -80°C until ELISA for Cxcl16 was performed. Mono-/co-cultured cholangiocytes were then treated with NKT cell culture medium + anti-Cxcl16 antibody (R&D Systems) or irrelevant IgG ($5\mu\text{g/mL}$). After 1h, a modified chemotaxis assay(24,25) was performed. Briefly, new inserts ($5\mu\text{m}$ pore size) were placed in the wells, and NKT cells ($1.5\times 10^5/0.2\text{ ml}$) were added to the upper chambers. Cultures were incubated at 37°C in 5% CO_2 for 2 h, supernatants were collected from the bottom chamber, and NKT cells that had migrated through filters were quantified using a hemocytometer. Experiments were repeated three times.

Microarray analysis

Total RNA from mono- or co-cultured cholangiocytes (603B cells) was evaluated by microarray ($N=3$ samples per group).(5) After RNA quality was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), RNA was hybridized to Mouse 430-2 Affymetrix GeneChips® (Duke University Microarray Facility, Durham, NC).(5) The probe expression values of the GeneChips® were subsequently calculated applying the Robust Multichip Average (RMA) algorithm by means of RMAExpress (5,26–28) based on the Affymetrix CEL and CDF files as standard inputs. The RMA-based gene expression in the co-culture group was expressed as a ratio of the expression in the mono-culture control group. A 1.5-fold increase or decrease in relative gene expression (i.e., probes having an expression ratio in co-cultures/mono-cultures above 1.500 and below 0.666) was considered to be significant.(29,30) These were further evaluated by gene ontology (GO) analysis.(31) Each gene probe was assigned to its GO families, and EASE scores were calculated.(32) Chemokine genes that were differentially-expressed in the micro-array analysis were then validated by QRT-PCR.

Two-step Real-Time PCR

Total RNA from cholangiocyte cell lines (603B and NRC), primary cholangiocytes or liver tissues was reverse transcribed to cDNA templates after RNase-free DNase I treatment (Qiagen, Valencia, CA). Semi-quantitative QRT-PCR was then performed.(5) Species-specific primers are listed in Table 1. Target gene expression was normalized to housekeeping gene expression according to the $\Delta\Delta\text{C}_t$ method. In each experiment, data are expressed relative to the corresponding control group.(5) Amplicon products were also separated by electrophoresis on a 2.0% agarose gel buffered with $0.5\times$ Tris-borate-EDTA (TBE) and representative products were then visualized using an AlphaImager 3400 Gel Analysis System.(7)

Cxcl16 ELISA

Cxcl16 protein was detected by DuoSet ELISA assay (R&D Systems) as per the manufacturer. Results were expressed as absolute concentration of Cxcl16 (pg/ml).

Immunohistochemistry

Frozen human liver sections were fixed, permeabilized, saturated, and processed for double immunofluorescent staining with primary antibody against Cxcl16 (R&D Systems) and CK-7 (Dako, Carpinteria, CA). Alexa Fluor 568 and Alexa Fluor 488 (Molecular Probes, Carlsbad, CA) were used as secondary antibodies. Counterstaining with 4', 6-diamidino-2-phenylindole (DAPI) was employed to demonstrate nuclei.

Statistical Analysis

Results are expressed as mean $\pm$ Standard Error Mean (SEM). Comparisons between groups were performed using the Student's t-test and significance was accepted at the 5% level, unless stated otherwise.

RESULTS

Paracrine Hedgehog signaling between ductular and myofibroblastic cells induced chemokine gene expression in ductular cells

Ductular-type cells and myofibroblastic cells typically localize together and accumulate in concert during chronic liver injury.(2) Our previous studies of a transwell co-culture system of cholangiocytes and MF-HSC demonstrated that each of these cell types released soluble Hh ligands that promoted the proliferation and viability of the other type of cell.(7,11) To determine if MF-HSC generated soluble factors that also influenced the immunomodulatory properties of cholangiocytes, microarray analysis was done to compare genes expressed by co-cultured versus mono-cultured cholangiocytes. Interestingly, some of the most highly-induced genes in cholangiocytes regulated cytokine activity, inflammatory responses, chemotaxis, chemokines and antigen presentation/processing (Fig. 1A and Table 2). Since the major objective of this study was to determine if (and how) Hh pathway activation in liver cells might regulate hepatic accumulation of inflammatory cells, subsequent attention focused on differentially-expressed chemokines.

Both microarray (Fig. 1A) and QRT-PCR (Fig. 2, black bars) analysis demonstrated that compared to mono-cultured cholangiocytes, co-cultured cholangiocytes expressed 2-fold to almost 40-fold higher levels of mRNAs encoding chemokines for neutrophils (Ccl20/MIP3 α , Cxcl1, Cxcl2, Cxcl5)(33), monocytes/macrophages (Ccl2/MCP-1, Ccl20/MIP3 α) (33,34), and lymphocytes (Ccl20/MIP3 α , (35–38) Cxcl11 (33,39)), including natural killer (NK)T cells (Cxcl16 (40–42)). To assure that chemokine production was not a unique characteristic of the 603B cholangiocyte line, basal expression of chemokine mRNAs was also assessed in a second cholangiocyte line (NRC) (Fig 1B) and in primary rat cholangiocytes (Fig. 1B). Effects of biliary injury on cholangiocyte chemokine production was then examined by comparing levels of chemokine mRNAs in freshly isolated, primary cholangiocytes from rats that had undergone either sham surgery or BDL one week earlier. BDL significantly influenced cholangiocyte expression of all of the chemokine mRNAs that had been induced in the co-culture experiments (Fig 2, white bars).

To determine if Hh ligands released from MF-HSC mediated induction of chemokine genes in neighboring cholangiocytes, the studies in cultured 603B cells were repeated with MF-HSC-conditioned medium containing either control IgG or Hh-neutralizing antibody. Chemokine gene expression was consistently inhibited by adding Hh neutralizing antibody to MF-HSC-conditioned medium (Fig. 3A), proving that soluble Hh ligands mediated much of the stimulatory effects that MF-HSC exerted on cholangiocyte chemokine expression.

Enhanced induction of chemokine mRNAs after bile duct ligation in mice with excessive Hh pathway activity

To determine the role of Hh signaling on chemokine production during liver injury, we next compared expression of the same chemokine genes in Ptc^{+/-} mice and their WT littermates one week after BDL. Mice with haplo-insufficiency of Ptc have an impaired ability to turn off Hh pathway signaling because Ptc normally restrains the activity of Smoothened, the signaling competent Hh co-receptor.(43) Hence, Ptc^{+/-} mice are prone to develop diseases that result from excessive Hh pathway activity,(17,44,45) and we reported previously that Ptc^{+/-} mice developed worse liver fibrosis than WT mice after BDL,(5,7) a potent stimulus

for activation of Hh signaling in the liver.(6,7) In the current study, *Ptc*^{+/-} mice exhibited generally greater expression of the chemokines that were Hh-responsive in the cholangiocyte cell line, demonstrating significantly greater expression of 6 of the 7 putative Hh-responsive chemokines than WT-BDL mice (Fig. 3B).

Hh-dependent paracrine mechanism stimulated ductular cells to synthesize and secrete Cxcl16 that chemoattracts NKT cells

Next we performed a more in depth functional analysis of Cxcl16, one of the chemokines that had proven to be Hh-inducible in both cultured cholangiocytes and BDL liver (Fig. 3). Cxcl16 was selected for scrutiny because it is a chemokine for NKT cells, and a series of elegant studies had already demonstrated that NKT cells accumulate in primary biliary cirrhosis (PBC)(46) and proven that such NKT cell accumulation drives local production of interferon (IFN)- γ , resulting in PBC-related bile duct injury.(47) The amount of Cxcl16 soluble protein was examined by ELISA in supernatants of freshly isolated primary cholangiocytes (Fig 4A–B), and in 603B cholangiocytes that were cultured alone or with MF-HSC in the transwell co-culture system (Fig 4C–D). Primary cholangiocytes from BDL rats released almost 16 times more Cxcl16 than cholangiocytes from sham controls (Fig. 4A), and co-cultured 603B cholangiocytes released about twice as much Cxcl16 protein as 603B mono-cultures (Fig. 4C). No Cxcl16 was detected in conditioned medium from mono-cultured MF-HSC (data not shown). Hence, this Cxcl16 was produced by the cholangiocytes in the co-culture. In both freshly isolated cholangiocytes and 603B cells, MF-HSC-derived Hh ligands were largely responsible for stimulating cholangiocyte production of Cxcl16 because Cxcl16 secretion was significantly inhibited by pre-treating MF-HSC conditioned medium with Hh-neutralizing antibody (but not control IgG) (Fig. 4B, D). Hh-neutralizing antibodies also blocked cholangiocyte mRNA expression of Adam10,(48,49) an enzyme that is required to release membrane-associated Cxcl16 from cells that synthesize this chemokine (Fig. 4E).

To examine the biological activity of cholangiocyte-derived Cxcl16, murine iNKT cells were then added to the upper well of transwell systems that contained cholangiocytes (that had either been grown for 6 days in mono-culture or co-culture with HSC-MF) in the bottom chamber. NKT cell migration was monitored for 2 hours in the absence or presence of anti-Cxcl16 antibody. Mono-cultured cholangiocytes evoked some NKT cell migration, but significantly more NKT cells migrated towards the co-cultured cholangiocytes. In both cases, anti-Cxcl16 blocked migration, proving that the NKT cells were chemoattracted by cholangiocyte-derived Cxcl16 (Fig. 4F). Together with the data displayed in Fig. 3, these results identify a Hh-dependent, paracrine mechanism by which MF-HSC stimulate cholangiocytes to synthesize and secrete Cxcl16, which then chemoattracts NKT cells to the Cxcl16-producing ductular cells.

Hh-associated induction of Cxcl16 production by ductular cells in PBC patients with NKT cell-mediated bile duct injury

PBC is characterized by chronic non-suppurative cholangitis that eventually destroys intralobular bile ducts, causing ductopenia and biliary-type fibrosis. It is not entirely clear why portal tracts accumulate iNKT cells in PBC patients and murine models of PBC, but it is known that the iNKT cells produce IFN- γ and contribute to bile duct injury because both IFN- γ and bile duct injury are prevented in PBC-prone mice that are genetically deficient in iNKT cells.(46,47) Previously, we reported that Hh pathway activity is increased in the livers of PBC patients, resulting in induction of Hh-target genes in many of the ductular-type cells.(5,8) Here we examined liver samples from 13 patients with PBC and 7 control healthy livers. QRT-PCR analysis showed that *Gli2* mRNA level was virtually undetectable in control livers, while it was significantly induced in samples from PBC patients ($P < 0.05$ PBC

vs controls); Gli2 induction was also accompanied by a 70% down-regulation of the Hh pathway inhibitor Hhip (Hh interacting protein) in PBC samples ($P < 0.05$, data not shown), confirming our previous report that the Hh pathway is activated in patients with PBC.(5,8)

Cxcl16 is a Hh-regulated gene in cholangiocytes (Fig. 3). To clarify whether or not the reactive Cxcl16-producing cholangiocytes reside in the same CK-7(+) compartment that has been shown to be Hh-responsive,(5,50) we performed hepatic double immunofluorescent staining to localize Cxcl16 expression in adults with PBC. Cxcl16 expression co-localized with that of CK-7, a marker of immature ductular cells (Fig. 4A–D). Hepatic expression of Cxcl16 mRNA and IFN- γ were also significantly greater in PBC patients than in controls without chronic liver disease (Fig. 4E–F). These findings complement and extend results obtained by studying $Ptc^{+/-}$ (Fig. 3B), strengthening support for the concept that activation of the Hh pathway during biliary injury induces immune responses, including recruitment of NKT cells, that amplify cholestatic liver damage.

DISCUSSION

The healthy liver is an immune organ, harboring large numbers of resident macrophages, dendritic cells, and various types of lymphocytes. During many types of chronic liver injury, populations of immune cells expand and some of these cells clearly contribute to liver damage by exerting direct cytotoxicity, generating reactive oxygen and/or reactive nitrogen species, and/or releasing hepatotoxic cytokines, such as IFN- γ . Several processes appear to be involved in expanding and activating immune cells in the liver, including presentation of foreign antigens (or altered self epitopes) by various types of resident liver cells, release of factors from dying liver cells, and hepatic induction of various chemokines and other factors that promote adhesion and viability of different types of immune cells. The present study identifies a unifying mechanism that may help to initiate and coordinate these diverse responses, namely injury-related activation of the Hedgehog pathway.

Two of the major cell types that are involved in adult liver repair, myofibroblastic stellate cell and ductular cells, produce and respond to Hh ligands.(5–8,11,50) Herein we demonstrated that Hh ligands dramatically up-regulate ductular cell expression of genes that promote antigen presentation and processing, inflammatory responses, and chemotaxis of neutrophils, monocytes/macrophages, B cells, T cells, NKT cells, and dendritic cells. A more in-depth evaluation of one of these Hh-regulated gene products, Cxcl16, provides evidence that these gene expression changes are likely to have functional relevance. Hh-dependent induction of Cxcl16 resulted in NKT cell chemotaxis towards cholangiocytes *in vitro*. Moreover, Hh-related induction of Cxcl16 was demonstrated in both bile duct ligated rats and humans with PBC, types of biliary injury in which NKT cells play a pivotal pathogenic role.

In both BDL mice(51) and humans with PBC,(46) bile duct destruction involves NKT cell-mediated increases in IFN- γ because inhibiting IFN- γ production is protective. Both BDL and PBC promote biliary fibrosis and may ultimately result in biliary-type cirrhosis.(2) The mechanisms that couple chronic biliary injury to fibrosis are not fully understood, however. Indeed, IFN- γ (the putative mediator of cholangiocyte death) has significant anti-fibrotic actions. Hence, other factors must be involved.

Several lines of evidence suggest that the Hh pathway modulates the sequelae of cholangiocyte death. Hh ligands promote the expansion of both liver myofibroblastic cells and progenitor cell populations.(6,7,10,11,52) The former occurs, at least in part, via Hh-mediated induction of epithelial-to-mesenchymal transition (EMT) in ductular-type progenitor cells.(5) Hence, sustained Hh pathway activation, as occurs during chronic biliary

injury, diverts progenitors away from bile duct cell replacement and promotes their differentiation into myofibroblastic cells. Myofibroblastic liver cells produce Hh ligands (7,11) and the latter help to maintain their myofibroblastic phenotype by up-regulating Snail and inducing mesenchymal gene expression, while repressing expression of bone morphogenic protein (BMP-7), E-cadherin, and other factors that support epithelial differentiation (Choi SS, unpublished data). Therefore, eventual ductopenia and biliary-type fibrosis are the predicted outcomes of persistent Hh pathway activation during conditions that cause repeated death of mature biliary epithelial cells. Other Hh-regulated factors may fuel this process. For example, Hh-mediated induction of β 2-microglobulin might amplify cholangiocyte presentation of altered self-antigens, while increased expression of Hh-regulated T and B cell chemokines may recruit cytotoxic lymphocytes into the liver and/or stimulate local production of antibodies that trigger further bile duct injury. Similarly, Hh-related increases in Ccl2/MCP1 might enhance hepatic recruitment of bone marrow-derived fibrocytes, and myofibroblast trans-differentiation of portal fibroblasts,(53) worsening liver fibrosis.

Although the current study focused on Hh signaling during chronic cholestatic liver damage, Hh pathway activation has been demonstrated in other types of adult liver disease, including nonalcoholic and alcoholic fatty liver diseases,(52) cholangiocarcinoma,(54) and hepatocellular carcinoma.(10,55) Hepatic accumulation of inflammatory cells, myofibroblasts, liver progenitors, and fibrosis may also occur in each of these conditions. The results of the present study provide a compelling rationale for investigating the causes and consequences of Hh pathway activation in these other disorders. Such work may identify novel biomarkers and therapeutic targets that could improve the outcomes of an array of chronic diseases for which, at present, there are few effective treatments.

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List of Abbreviations

Hh	Hedgehog
MF-HSC	Myofibroblastic Hepatic Stellate Cell
Cxcl16	chemokine (C-X-C) motif ligand 16
NKT	Natural Killer T
IFNγ	Interferon γ
PBC	Primary Biliary Cirrhosis
CK-7	Cytokeratin-7
Ptc	Patched

WT	Wild type
BDL	Bile Duct Ligation
γ GT	γ glutamyl transpeptidase, DAPI, 4',6-diamidino-2-phenylindole
Ccl2/MCP-1	chemokine (C-C) motif ligand 2/monocyte chemotactic protein-1
Ccl5	chemokine (C-C) motif ligand 5
Ccl20/MIP3α chemokine (C-C) motif ligand 20/macrophage inflammatory protein-3α	Cxcl1, chemokine (C-X-C) motif ligand 1
Cxcl2	chemokine (C-X-C) motif ligand 2
Cxcl5	chemokine (C-X-C) motif ligand 5
Cxcl10	chemokine (C-X-C) motif ligand 10
Cxcl11	chemokine (C-X-C) motif ligand 11
BMP-7	bone morphogenic protein-7

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Fig. 1. Paracrine signaling between myofibroblastic and ductular cells induced chemokine gene expression in ductular cells

(A) Chemokine related probe analysis Microarray analysis was performed in mono-/co-culture samples derived from 3 independent experiments. Each probe having an expression ratio (average expression in co-cultures/average expression in monocultures) above 1.500 and below 0.666, was considered for the gene ontology (GO) analysis and assigned to its GO families. Probes belonging to Chemokine Activity, Chemokine receptor binding and Chemotaxis GeneOntology families are displayed as ratio of the expression in co-cultured vs mono-cultured cholangiocytes. **(B) 2% Agarose gel of representative amplicon products** QRT-PCR was performed in the murine cholangiocyte 603B line, rat NRC line and primary rat cholangiocytes using species-specific primers. Amplicon products were separated by electrophoresis on a 2% agarose gel buffered with 0.5 $\times$ Tris-borate-EDTA (TBE) and then visualized using an AlphaImager 3400 Gel Analysis System. Ccl2/MCP-1 (chemokine C-C motif ligand 2/monocyte chemotactic protein-1); Ccl5 (chemokine C-C motif ligand 5); Ccl20/MIP3 α (chemokine C-C motif ligand 20/macrophage inflammatory protein-3 α), Cxcl1 (chemokine C-X-C motif ligand 1); Cxcl2 (chemokine C-X-C motif ligand 2); Cxcl5 (chemokine C-X-C motif ligand 5); Cxcl10 (chemokine C-X-C motif ligand 10); Cxcl11 (chemokine C-X-C motif ligand 11); Cxcl16 (chemokine C-X-C motif ligand 16).

Fig. 2. Microarray data validation and comparison with freshly isolated cholangiocytes by QRT-PCR analysis

Chemokine gene mRNA levels were normalized to housekeeping gene expression in 603B mouse cholangiocytes or primary rat cholangiocytes. Data are displayed as fold-change in co-cultured 603B cholangiocytes relative to that in 603B mono-cultures (black bars), or in primary cholangiocytes isolated from 1 week BDL rat relative to sham controls (white bars). Data are representative of 3 experiments and shown as mean±SEM (*P<0.05, ** P<0.005).

Fig. 3. Cholangiocyte chemokine gene induction is Hedgehog-dependent (A) QRT-PCR analysis of cholangiocytes cultured in myofibroblast-conditioned medium with or without Hh-neutralizing antibody

MF-HSC 8B were cultured for 6 days and conditioned medium was collected. This MF-HSC-conditioned medium was then added to monocultures of cholangiocyte cell line 603B that had been cultured without serum for 18 hours. Cholangiocytes were incubated with MF-HSC conditioned medium in presence of Hh-neutralizing antibody (5E1) (grey bars) or control IgG (black bars) (10 µg/mL) for an additional 24 hours. 603B cells cultured in unconditioned medium (white bars) provided an additional control group. QRT-PCR was then performed to analyze the expression of chemokine-related genes that had been altered when the cholangiocytes were co-cultured with MF-HSC line 8B. Data are representative of 3 independent experiments and expressed as mean±SEM. (* P <0.05, ** P <0.005) **(B) QRT-PCR analysis of whole liver tissue from bile duct ligated (BDL) mice.** Ptc-deficient

(Ptc^{+/-}) mice (black bars) (n=6) and their WT littermates (white bars) (n=6) underwent BDL and were analyzed for the same chemokines that were Hh-dependent *in vitro*. Data are shown as mean±SEM (**P*<0.05, ** *P*<0.005).

Fig. 4. Hh-dependent paracrine signaling caused ductular cells to secrete the NKT chemokine Cxcl16 and chemoattracted NKT cells *in vitro*

(A) Cxcl16 soluble protein analysis in primary cholangiocytes. Primary cholangiocytes were freshly isolated from sham or BDL rats. Supernatants were collected after 6 hours of incubation and analyzed for Cxcl16 by ELISA assay. **(B) Effect of Hh ligand neutralization on Cxcl16 protein release by primary cholangiocytes.** Freshly isolated cholangiocytes from normal rats were treated for 6 hours with either unconditioned medium (white bars) or 6 day MF-conditioned medium in presence of Hh ligand neutralizing antibody (5E1) (grey bars) or control IgG (black bars) (10µg/ml). Cxcl16 protein released into the supernatants was assessed by ELISA assay. **(C) Cxcl16 soluble protein analysis in**

603B cholangiocyte mono-/co-cultures. Conditioned medium was collected from 6 day mono- or co-cultures and Cxcl16 protein production was quantified by ELISA assay **(D)** **Effect of Hh ligand neutralization on Cxcl16 protein release by 603B cholangiocytes.** 6 day MF-conditioned medium was collected and added to mono-cultures of cholangiocytes in presence of Hh-neutralizing antibody (5E1) (grey bars) or control IgG (black bars) (10 µg/mL). 603B cells cultured with unconditioned medium (white bars) were used as controls. Supernatants were assessed by ELISA for Cxcl16 soluble protein levels, and cell pellets were analyzed by QRT-PCR for Adam10 gene expression. **(E) (F) NKT cell migration assay.** Cholangiocytes were cultured in absence/presence of MF-HSC. After 6 days, transwell filter inserts containing MF-HSC were removed. Cholangiocyte mono-/co-cultures were pre-treated for 1 hour with antibody neutralizing Cxcl16 or irrelevant IgG (5µg/mL). NKT cells were then added to the system using 5µm pore inserts, and their migration was monitored for 2 hours. Data are representative of 3 independent experiments and displayed as mean±SEM. (* $P<0.05$, ** $P<0.005$)

Fig. 5. Cxcl16 localizes in Hh-responsive immature epithelial cells in PBC
(A–D) Double immunofluorescent staining. Cxcl16 (A) and CK-7 (B) co-localized (C–D) in representative sections from PBC liver. Normal liver co-staining failed to demonstrate CK-7/Cxcl16 double positive cells (C, NL insert). In all merged images, blue DAPI-staining demonstrates cell nuclei. (A–C) Original magnification $\times 40$. (D) Magnified section of the indicated area in panel C. **(E–F) QRT-PCR Analysis** of whole liver tissue from 13 patients with PBC and 7 control healthy livers (NL). (E) Cxcl16; (F) IFN γ . Data are expressed as mean $\pm$ SEM (* $P < 0.05$ vs NL).

Table 1
Sequences of primers used for QRT-PCR

Ccl2/MCP-1 (chemokine C-C motif ligand 2/monocyte chemotactic protein-1); Ccl5 (chemokine C-C motif ligand 5); Ccl20/MIP3 α (chemokine C-C motif ligand 20/macrophage inflammatory protein-3 α), Cxcl1 (chemokine C-X-C motif ligand 1); Cxcl2 (chemokine C-X-C motif ligand 2); Cxcl5 (chemokine C-X-C motif ligand 5); Cxcl10 (chemokine C-X-C motif ligand 10); Cxcl11 (chemokine C-X-C motif ligand 11); Cxcl16 (chemokine C-X-C motif ligand 16).

Target Gene	Forward Sequence	Reverse Sequence
Ccl2/MCP1 *	TGATCCCAATGAGTAGGCT	TCAGATTACGGGTCAACTT
Ccl5 *	CCCTCACCATCATCCTCACT	CCCTCACCATCATCCTCACT
Ccl20/MIP3 α *	CCCAGCACTGAGTACATCAA	GTATGTACGAGAGGCAACAGTC
Cxcl1 *	CCCAAACCGAAGTCATAGCC	TCAGAAGCCAGCGTTCACC
Cxcl2 *	GCCGACAGAAAGTCATAGCC	TTCTCTTGGTTCTTCCGTTGA
Cxcl5 *	TCATGAGAAGGCAATGCT	ACATTATGCCATACTACGAAGA
Cxcl10 *	TGAATCCGGAATCTAAGACCAT	AAGACCAAGGGCAATTAGGACT
Cxcl11 *	CCTGGCTGCGATCATCT	GCATTATGAGGCGAGCTT
Cxcl16 *	TTCTTGTGGCGCTGCTGAC	TGCGTCAAAACAGTCCACTAG
S9 *	GACTCCGGAACAAAGGTGAGGT	CTTCATCTTGCCCTCGTCCA
Ccl2/MCP1 **	CAGTTAATGCCCCACTCACC	TTCTTATTGGGGTCAGCAC
Ccl20/MIP α **	GCGAGAATTTTTGTGGGTTT	CGGATCTTTTCGACTTCAGG
Cxcl1 **	AGACAGTGGCAGGGATTAC	TGGCTATGACTTCGGTTTGG
Cxcl2 **	GGCTTCAGGGTTGAGACAAA	AGGGTACAGGGGTGTTGTG
Cxcl5 **	AGTCGATTCCGCTTTGTTTT	AACCCCAAAATGATCGCTAA
Cxcl11 **	TCAAAATGGCAGCAATCAAG	CCAGGCACCTTTGTCCTTA
Cxcl16 **	CCAGCAGTCCACTTTTCCAT	GCTCCTGGTTCTTTTGCTG
S9 **	GACTCCGGAACAAAGGTGAGGT	CTTCATCTTGCCCTCGTCCA
IFN γ ***	TGACCAGAGCATCCAAAAGA	CTCTTCGACCTCGAAACAGC
Cxcl16 ***	ACTCGTCCCAATGAAACCAC	ATGAAGATGATGGCCAGGAG
β actin ***	TGGCATCCACGAAACTACCT	ACGGAGTACTTGCGCTCAG

* mouse,

** rat

*** human

Table 2
Significative change in chemokine related GeneOntology (GO) gene sets

Gene probes having an expression ratio (co-cultures/mono-cultures) above 1.500 (upper threshold) and below 0.666 (lower threshold) respectively, were considered for the gene ontology (GO) analysis. Each gene probe was then assigned to its multiple GO families and significance (EASE score) was evaluated.

GO Family ^A	Gene Count ^B	EASE Score ^C
Cytokine activity	13	0.033000
Inflammatory response	10	0.042000
Taxis	10	0.003000
Chemotaxis	10	0.003000
Chemokine receptor binding	9	0.000007
Chemokine activity	9	0.000007
Antigen processing, endogenous antigen via MHC class I	5	0.039000
Antigen presentation, endogenous antigen	5	0.042000

^A Chemokine related GO families.

^B Absolute number of altered genes.

^C EASE Score.