Biliary NIK promotes ductular reaction and liver injury and fibrosis in mice

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Excessive cholangiocyte expansion (ductular reaction) promotes liver disease progression, but the underlying mechanism is poorly understood. Here we identify biliary NF-κB-inducing kinase (NIK) as a pivotal regulator of ductular reaction. NIK is known to activate the noncanonical IKκα/NF-κB2 pathway and regulate lymphoid tissue development. We find that cholangiocyte NIK is upregulated in mice with cholestasis induced by bile duct ligation (BDL), 5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), or α-naphtyl-isothiocyanate (ANIT). DDC, ANIT, or BDL induces ductular reaction, liver injury, inflammation, and fibrosis in mice. Cholangiocyte-specific deletion of NIK, but not IKκα, blunts these pathological alterations. NIK inhibitor treatment similarly ameliorates DDC-induced ductular reaction, liver injury, and fibrosis. Biliary NIK directly increases cholangiocyte proliferation while suppressing cholangiocyte death, and it also promotes secretion of cholangiokines from cholangiocytes. Cholangiokines stimulate liver macrophages and hepatic stellate cells, augmenting liver inflammation and fibrosis. These results unveil a NIK/ductular reaction axis and a NIK/cholangiokine axis that promote liver disease progression.

Bile ducts provide conduits for bile flow from the liver to the gallbladder and along the journey, cholangiocytes—bile duct epithelial cells—modify bile compositions through absorption and/or secretion of various molecules. Biliary injury triggers cholangiocyte proliferation to compensate for bile duct loss and maintain biliary homeostasis. Bipotent cholangiocyte-like cells can differentiate into either cholangiocytes or hepatocytes, supporting liver regeneration1. However, excessive cholangiocyte expansion results in pathogenic ductular reaction, a hallmark of cholestasis, contributing to liver disease progression. Ductular reaction marks poor prognosis and increases the risk for cholangiocarcinoma3–5. Bile duct ligation (BDL) induces obstructive biliary injury to robustly stimulate ductular reaction and serves as a mouse ductular reaction model. Exposure to biliary toxicant 3, 5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or α-naphtyl-isothiocyanate (ANIT) also profoundly induces ductular reaction in rodents, leading to cholestasis6–8. However, cholangiocyte-intrinsic pathways mediating ductular reaction remain poorly understood.

NF-κB-inducing kinase (NIK), also known as MAP3K14, is activated by a subset of cytokines and required for the activation of the noncanonical NF-κB2 pathway9. TNF-like weak inducer of apoptosis (TWEAK) is a NIK-stimulating cytokine and interestingly, TWEAK stimulates cholangiocyte proliferation via its receptor Fn1410–13. NIK

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NIK inhibitors may have therapeutic potential for liver disease, previously unrecognized molecular driver for ductular reaction, shape liver microenvironments. Our results unveil biliary NIK as a stimulator of liver macrophages and hepatic stellate cells (HSCs) and langiocyte secretion of soluble factors, termed cholangikines, that mitigated DDC-induced ductular reaction, liver damage, and liver fibrosis (Fig. 1E, F). Additionally, mice treated with tamoxifen as well as olive oil vehicle control (hereafter referred to as vehicle). NIK-expressing (NIK+) cells were readily detected in NIK+/− mouse but not in NIK−/− mouse (Supplementary Fig. 1A). In human samples, we observed a few NIK+ cells and K19+ cholangiocytes in healthy livers (Control) (Fig. 1A). The numbers of K19+ cells, NIK+ cells, and K19+/NIK+ cells (NIK-expressing cholangiocytes) were considerably higher in primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), hepatitis B virus (HBV), hepatitis C virus (HCV), and alcoholic cirrhosis (Fig. 1A, B). NIK-expressing cholangiocytes are likely to be underestimated because anti-NIK antibody may not detect NIK at a relatively low level.

To determine whether biliary NIK is upregulated in mice with cholestasis, we treated C57BL/6j mice on DDC diet for 2 weeks to increase liver NIK levels and used global NIK knockout (NIK−/−) mice but not in NIK−/− mice (Supplementary Fig. 1A). In human samples, we observed a few NIK+ cells and K19+ cholangiocytes in healthy livers (Control) (Fig. 1A). The numbers of K19+ cells, NIK+ cells, and K19+/NIK+ cells (NIK-expressing cholangiocytes) were considerably higher in primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), hepatitis B virus (HBV), hepatitis C virus (HCV), and alcoholic cirrhosis (Fig. 1A, B). NIK-expressing cholangiocytes are likely to be underestimated because anti-NIK antibody may not detect NIK at a relatively low level.

To determine whether biliary NIK is upregulated in mice with cholestasis, we placed C57BL/6j mice on DDC diet for 4 weeks and stained liver sections with antibodies to NIK and cholangiocyte marker keratin-19 (K19). To validate anti-NIK antibody, we placed wild-type (NIK+/+) mice on DDC diet for 2 weeks to increase liver NIK levels and used global NIK knockout (NIK−/−) mice as negative control. Liver sections were stained with anti-NIK antibody. NIK-expressing (NIK+) cells were readily detected in NIK+/− mouse but not in NIK−/− mouse (Supplementary Fig. 1A). In human samples, we observed a few NIK+ cells and K19+ cholangiocytes in healthy livers (Control) (Fig. 1A). The numbers of K19+ cells, NIK+ cells, and K19+/NIK+ cells (NIK-expressing cholangiocytes) were considerably higher in primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), hepatitis B virus (HBV), hepatitis C virus (HCV), and alcoholic cirrhosis (Fig. 1A, B). NIK-expressing cholangiocytes are likely to be underestimated because anti-NIK antibody may not detect NIK at a relatively low level.

Biliary NIK is upregulated in mice and humans with chronic liver disease

To examine biliary NIK in humans, we immunostained liver sections with antibodies to NIK and cholangiocyte marker keratin-19 (K19). To validate anti-NIK antibody, we placed wild-type (NIK+/+) mice on DDC diet for 2 weeks to increase liver NIK levels and used global NIK knockout (NIK−/−) mice as negative control. Liver sections were stained with anti-NIK antibody. NIK-expressing (NIK+) cells were readily detected in NIK+/− mouse but not in NIK−/− mouse (Supplementary Fig. 1A). In human samples, we observed a few NIK+ cells and K19+ cholangiocytes in healthy livers (Control) (Fig. 1A). The numbers of K19+ cells, NIK+ cells, and K19+/NIK+ cells (NIK-expressing cholangiocytes) were considerably higher in primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), hepatitis B virus (HBV), hepatitis C virus (HCV), and alcoholic cirrhosis (Fig. 1A, B). NIK-expressing cholangiocytes are likely to be underestimated because anti-NIK antibody may not detect NIK at a relatively low level.

To determine whether biliary NIK is upregulated in mice with cholestasis, we treated C57BL/6j mice on DDC diet for 4 weeks and stained liver sections with antibodies to NIK and K19. We observed a few NIK+ cells and K19+ cholangiocytes in chow-fed mice (Fig. 1C). DDC feeding dramatically increased the numbers of NIK+ cells and K19+ cholangiocytes; importantly, the majority of cholangiocytes expressed NIK (NIK K19+) (Fig. 1C). It is worth mentioning that NIK K19+ cholangiocytes are likely to be underestimated because anti-NIK antibody may not recognize NIK at relatively low levels. In line with these data, DDC feeding markedly increased liver NIK mRNA abundance (Fig. 1D). ANIT feeding for 3 weeks (Supplementary Fig. 1B, C) or BDL for 7 days (Fig. 1E, F) also markedly increased NIK K19+ cholangiocyte number, liver NIK mRNA levels, and ductular reaction. Thus, biliary NIK upregulation is a signature of biliary injury regardless of etiology.

To test if cholangiocyte toxicants directly stimulate biliary NIK, we isolated primary cholangiocytes from NIK−/− mice, immortalized them using E1A lentiviral vectors, and purified individual lines through a series of dilutions. These lines expressed cholangiocyte-lineage genes, including K19, Cfr, and Hnf4α (Supplementary Fig. 2A, B), and were considered as cholangiocyte lines. We treated cholangiocytes with DCC or ANIT. Both DCC and ANIT significantly increased NIK mRNA abundance (Supplementary Fig. 2C). Endogenous NIK was below detection by anti-NIK antibody, so we transduced cholangiocytes with NIK adenoviral vectors. DCC or ANIT considerably increased NIK protein levels (Supplementary Fig. 2D). To assess NIK protein degradation, we pretreated cholangiocytes with DCC or ANIT and then treat them with protein translation inhibitor cycloheximide. DCC or ANIT pretreatment substantially increased NIK stability (Supplementary Fig. 2E, F). We loaded less lysates from DCC- or ANIT-treated cells (1/2 amounts of vehicle-treated cells) to avoid NIK overloading. To extend these observations, we examined the ubiquitin E3 ligase complex for NIK. Traf2 and Traf3 bind to NIK and recruit it to cIAP1 and cIAP2, and cIAP1/2 in turn catalyze NIK ubiquitination19. DDC treatment markedly decreased both cIAP1 and cIAP2, but increased Traf2 and Traf3, levels in cholangiocyte cultures (Supplementary Fig. 2G). To assess cIAP1/2, in vivo, we measured Traf2, Traf3, cIAP1, and cIAP2 levels in liver extracts by immunoblotting. DDC feeding markedly decreased liver cIAP1 and cIAP2 levels while increasing Traf2 and Traf3 levels (Supplementary Fig. 2H). To further confirm downregulation of hepatic cIAP1/2, we immunoblotted liver extracts with antibodies to cIAP1 and cIAP2 from a different resource. Liver cIAP1 and cIAP2 were profoundly down-regulated in DCC-treated mice (Supplementary Fig. 2I). cIAP1/2 downregulation explains, at least in part, increased NIK stability and perhaps, upregulation of Traf2/3 is a secondary response to cIAP1/2 deficiency. Collectively, these results indicate that biliary injury increases both expression and stability of cholangiocyte NIK.

Cholangiocyte-specific deletion of NIK suppresses DCC- and ANIT-induced ductular reaction

We sought to explore biliary NIK in vivo. We generated tamoxifen-induced, cholangiocyte-specific NIK knockout (NIKΔK19) mice by crossing NIK−/− mice with K19-CreERT drivers. NIK−/− and K19-CreERT mice have been described previously20,21. Because NIK and K19-CreERT genes reside in the same chromosome 11, NIK−/−K19-CreERT recombination occurs at a low frequency in germ cell meiosis. Nonetheless, we obtained NIK−/−K19-CreERT+ mice. NIK−/−K19-CreERT+ mice (7–10 weeks old) were treated with tamoxifen to delete NIK specifically in cholangiocytes, generating NIKΔK19 mice. To verify cholangiocyte-specific deletion of NIK, we prepared primary cholangiocytes and hepatocytes from NIK−/−K19-CreERT mice, treated them with tamoxifen, and measured the disrupted NIK allele (NIK−) by PCR-based genotyping. The NIK− allele was detected in tamoxifen-treated cholangiocytes, but not in tamoxifen-treated hepatocytes or vehicle-treated cholangiocytes (Supplementary Fig. 3A). We placed NIK−/− and control mice (2 weeks post tamoxifen treatment) on DDC diet for additional 4 weeks. We used 2 control groups. NIK−/−K19-CreERT+ mice were treated with olive oil vehicles as control (hereafter referred to as NIK−/−). As an additional control, NIK−/− mice were treated with tamoxifen for 2 weeks followed by a DCC feeding for 4 weeks. DDC-induced liver injury to a comparable level between tamoxifen-treated and olive oil vehicle-treated NIK−/− mice (Supplementary Fig. 3B), indicating that tamoxifen treatment alone did not affect the response of the liver to DCC. Given that tamoxifen-treated NIK−/− mice were indistinguishable from vehicle-treated NIK−/−K19-CreERT mice, they were not included in the following experiments.

DDC feeding decreased body weight in both NIKΔK19 and NIK−/− littermates, but to a higher level in NIK−/− mice (Supplementary Fig. 3C). To grossly estimate biliary growth, we dissected intrahepatic bile ducts using collagenase. DDC feeding increased total bile duct weight in both NIKΔK19 and NIK−/− littermates, but to a markedly higher level in NIK−/−.
mice (Supplementary Fig. 3D). To directly examine ductular reaction, we co-stained liver sections with antibodies to K19 and NIK. As expected, DDC feeding induced dramatic ductular reaction and expansion of NIK-K19+ cholangiocytes in wild-type NIKf/f mice (Fig. 2A, B). K19+ cholangiocyte number was drastically reduced in NIKΔK19 mice (Fig. 2A). Liver NIK-K19+ cells were undetectable in NIKΔK19 mice (Fig. 2A, B), confirming that NIK was deleted specifically in cholangiocytes. To further verify NIK deletion in NIKΔK19 mice, we immunoblotted liver extracts with anti-NF-κB2 antibody. DDC feeding markedly increased NF-κB2 p100 levels and p100 conversion into p52 (NIK activation index) in NIKf/f mice, and p52 levels were dramatically lower in NIKΔK19 mice (Fig. 2C). To confirm that deficiency of biliary NIK blocks DDC-induced ductular reaction, we immunostained liver sections with anti-K19 antibody. We observed a few K19+ cholangiocytes in chow-fed
NIK and NIKΔK19 mice; importantly, DDC feeding increased K19 cholangiocyte number to a dramatically higher level in NIKΔK19 mice relative to NIK mice (Fig. 2D,E). Liver K19 mRNA levels were also significantly higher in NIKΔK19 than in NIKΔK19 mice (Fig. 2F). NIKΔK19 hepatocytes were barely detected in chow-fed mice and modestly increased after DDC feeding (Supplementary Fig. 3E, F). Unlike NIKΔK19 cholangiocytes, NIKΔK19 hepatocytes were comparable between NIKΔK19 and NIKΔK19 mice (Supplementary Fig. 3F). To extend these findings, we placed NIKΔK19 and NIKΔK19 littermates on an ANIT diet for 3 weeks. ANIT-induced ductular reaction was also dramatically attenuated in NIKΔK19 mice compared to NIKΔK19 mice (Fig. 2G and Supplementary Fig. 3G). These results unveil NIK as an indispensable cholangiocyte-intrinsic inducer of ductular reaction.

In vivo, NIKΔK19 mice are resistant to BDL-induced ductular reaction and liver injury, inflammation, and fibrosis

We next sought to examine the pathological consequence of ductular reaction. Body weight, plasma alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bilirubin were normal in NIKΔK19 mice on chow diet (Supplementary Fig. 6A, B). We placed NIKΔK19 and NIKΔK19 littermates on an ANIT diet for 4 weeks. Plasma ALT increased within a week post DDC diet and declined afterwards, and plasma ALP and bilirubin levels (biliary injury markers) increased progressively on DDC diet (Fig. 4A). Importantly, plasma ALT, ALP, and bilirubin levels were significantly lower in NIKΔK19 than in NIKΔK19 mice post DDC diet (Fig. 4A). DDC feeding induced severe liver damage and inflammation as detected by histological examinations; strikingly, liver damage and immune cell infiltration were substantially lower in NIKΔK19 than in NIKΔK19 mice (Fig. 4B). Liver CD11b+ macrophages and Gr-1+ myeloid cells were significantly less in NIKΔK19 than in NIKΔK19 mice (Fig. 4B, C). NIKΔK19 cells were comparable between NIKΔK19 and NIKΔK19 mice on DDC diet (Supplementary Fig. 3F). Liver CD4 and CD8 T cells were slightly lower in NIKΔK19 mice (Supplementary Fig. 4B). To corroborate these results, we placed NIKΔK19 and NIKΔK19 littermates on an ANIT diet for 3 weeks. ANIT feeding increased plasma ALT, ALP, and bilirubin to a significantly higher level in NIKΔK19 mice than in NIKΔK19 mice (Supplementary Fig. 7A). NIKΔK19 mice were also markedly resistant to ANIT-induced liver damage and inflammation (Fig. 4D, E and Supplementary Fig. 7B).

We examined liver fibrosis by Sirius red staining of liver sections and measuring liver hydroxyproline content, and we also examined smooth muscle actin (αSMA)-expressing HSCs that are responsible for liver fibrosis. Sirius red-positive fibers and αSMA+ HSCs were barely detectable in chow-fed mice and were robustly increased after DDC feeding (Fig. 5A). Sirius red areas, hydroxyproline content, and αSMA+ HSC number were substantially lower in NIKΔK19 than in NIKΔK19 mice on DDC diet (Fig. 5A, B). Liver extract αSMA levels were markedly lower in NIKΔK19 than in NIKΔK19 mice post DDC feeding (Fig. 5C). To determine whether sex influences the phenotypes, we placed NIKΔK19 and NIKΔK19 female mice on a DDC diet for 4 weeks. NIKΔK19 females, like males, were resistant to DDC-induced ductular reaction, liver injury, inflammation, and fibrosis (Supplementary Fig. 8). To further validate these findings, we placed NIKΔK19 and NIKΔK19 mice on an ANIT diet for 3 weeks. ANIT robustly stimulated αSMA+ HSC activation and liver fibrosis in NIKΔK19 mice; HSC activation and liver fibrosis were largely blocked in NIKΔK19 mice (Fig. 5D). These results demonstrate, for the first time, that cholangiocyte-intrinsic NIK promotes liver damage, inflammation, and fibrosis, perhaps through inducing ductular reaction.

NIKΔK19 mice are resistant to BDL-induced ductular reaction and liver injury

To test if biliary NIK provides a common route to ductular reaction and liver disease, we performed BDL for 7 days on NIKΔK19 and NIKΔK19 mice. Plasma ALT, ALP, and bilirubin levels were comparable between sham-treated NIKΔK19 and NIKΔK19 mice (Supplementary Fig. 9A). BDL increased...
plasma ALT, ALP, and bilirubin levels to a significantly higher extent in NIK\textsuperscript{f/f} than in NIK\textsuperscript{ΔK19} littermates (Fig. 6A). BDL-induced liver injury, ductular reaction (K19 staining), liver inflammation (H&E, CD11b, and Gr-1 staining), and fibrosis (Sirius red and αSMA staining) in NIK\textsuperscript{f/f} mice (Fig. 6B), and NIK\textsuperscript{ΔK19} mice were resistant to BDL-induced liver damage, ductular reaction, inflammation, and fibrosis (Fig. 6B, C).

Cholangiocyte proliferation (K19\textsuperscript{Ki67\textsuperscript{+}}) was lower while cholangiocyte apoptosis (K19\textsuperscript{caspase-3\textsuperscript{+}}) was higher in NIK\textsuperscript{ΔK19} mice relative to NIK\textsuperscript{f/f} mice post BDL (Fig. 6B and Supplementary Fig. 9B). Collectively, these findings suggest that biliary NIK integrates, as a converging point, information from diverse biliary insults to induces ductular reaction, liver injury, inflammation, and fibrosis.
Biliary NIK promotes secretion of pro-inflammatory and pro-fibrosis cholangiokines

We next set out to investigate the mechanism by which biliary NIK-elicited ductular reaction promotes liver inflammation and fibrosis. We postulated that NIK might stimulate secretion of cholangiokines (cholangiocyte-derived mediators) that activate liver immune cells and HSCs, augmenting liver inflammation and fibrosis. We stimulated NIK+/− and NIK−/− cholangiocyte cultures with TWEAK to activate NIK and measured expression of various cytokines known to be involved in liver inflammation and fibrosis. Ablation of NIK significantly decreased expression of II-β, II-4, II-6, iNos, Tnfα (also called Cc12), and Tgflβ1 in NIK−/− cholangiocytes relative to NIK+/− cholangiocytes (Fig. 7A). To test if NIK-upregulated cholangiokines promote liver inflammation, we cultured mice cholangiocytes cocultured with macrophages (BMDMs) and cocultured them with either NIK+/− or NIK−/− cholangiocytes in transwells as we described previously24. Cholangiocytes were pretreated with TWEAK to activate NIK. BMDM activity was assessed by measuring expression of inflammatory mediators. Expression of II-β, II-4, II-6, iNos, Tnfα, Mcp1, and Cc15 was significantly lower in NIK-cholangiocyte-cocultured BMDMs relative to NIK+/− cholangiocyte-cocultured BMDMs (Fig. 7B). To further verify that biliary NIK stimulates secretion of cholangiokines that activate BMDMs, we stimulated NIK+/− and NIK−/− cholangiocyte-conditioned medium and their expression of inflammatory mediators was measured. Expression of II-β, II-4, II-6, iNos, Tnfα, Mcp1, and Cc15 was significantly lower in NIK−/− conditioned medium-treated BMDMs than in NIK+/− conditioned medium-treated BMDMs (Fig. 7C). To determine whether NIK-upregulated cholangiokines stimulate HSCs and liver fibrosis, we treated HSCs with NIK+/− or NIK−/− cholangiocyte-conditioned medium and measured their expression of fibrosis-related genes. Expression of αSMA, Collα1, Timp1, Mmp9, Cgfl, and Tgflβ1 was significantly lower in NIK−/− than in NIK+/− conditioned medium-stimulated HSCs (Fig. 7D). These results provide proof of concept evidence that biliary NIK potently stimulates secretion of cholangiokines that activate liver immune cells and HSCs, thereby promoting liver inflammation and fibrosis.

Ablation of biliary IKKα does not affect DDC-induced ductular reaction and cholestatic liver injury

To test if IKKα mediates NIK action in cholangiocytes, we generated tamoxifen-induced, cholangiocyte-specific IKKα knockout (IKKα−/−) mice by crossing IKKαfl/fl with K19-CreERT drivers. IKKαfl/fl/K19-CreERT mice were treated with tamoxifen (IKKα−/−/fl) or a vehicle (referred to as IKKαfl/fl). Two weeks later, mice were fed a DDC diet for 4 weeks. IKKα was detected in K19 cholangiocytes in IKKαfl/fl but not IKKα−/− mice by immunostaining (Supplementary Fig. 10A), confirming cholangiocyte-specific IKKα knockout. IKKα−/− mice did not phenocopy NIK−/− mice. Plasma ALT, ALP, and bilirubin levels were indistinguishable between IKKα−/− and IKKαfl/fl mice (Supplementary Fig. 10B). Cholangiocyte expansion, αSMA+ HSC number, and liver fibrosis were also comparable between IKKα−/− and IKKαfl/fl mice (Supplementary Fig. 10C). Unlike biliary NIK, biliary IKKα is not required for DDC-induced ductular reaction and cholestasis. Because IKKα−/− mice harbor the K19-CreERT transgene, these data indicate that tamoxifen activation of CreERT does not affect DDC-induced ductular reaction. Therefore, IKKα−/− mice also served as an additional control for NIK−/− mice.

Akt has been known to promote cholangiocyte expansion3, prompting us to test if it is a downstream mediator of the NIK pathway. We stimulated NIK+/− and NIK−/− cholangiocyte cultures with TWEAK and measured Akt phosphorylation by immunoblotting. TWEAK-stimulated Akt phosphorylation/activation to a higher level in NIK+/− than in NIK−/− cells (Supplementary Fig. 5E). Next, we tested if blocking Akt activation attenuates NIK-stimulated cholangiocyte proliferation, using BrdU assay. Cholangiocyte cultures were pretreated with PI-3 kinase inhibitor wortmannin or LY294002 and then stimulated with TWEAK. TWEAK increased proliferation rates to a higher level in NIK+/− cholangiocytes relative to NIK−/− cholangiocytes; importantly, inhibition of Akt by either wortmannin or LY294002 abrogated TWEAK-stimulated proliferation of NIK+/− cholangiocytes (Fig. 3F). In contrast, wortmannin and LY294002 did not affect NIK−/− cholangiocyte proliferation. Thus, NIK promotes cholangiocyte expansion at least in part by enhancing Akt activation.

NIK inhibitor treatment ameliorates DDC-induced ductular reaction, liver injury, and fibrosis

NIK-selective inhibitor Compound 33 (C33) has been developed to treat cancer29. We evaluated C33’s therapeutic potential in liver disease treatment. To validate C33, we cotransfected HEK293 cells with NF-κB/p100 and NIK plasmids and then treated them with C33. C33 inhibited cleavage of p100 into p52 (stimulated by NIK) in a dose-dependent manner (Fig. 8A). C33 also inhibited Akt phosphorylation/activation to a higher level in NIK−/− cells (Supplementary Fig. 5E). Next, we tested if blocking Akt activation attenuates NIK-stimulated cholangiocyte proliferation, using BrdU assay. Cholangiocyte cultures were pretreated with PI-3 kinase inhibitor wortmannin or LY294002 and then stimulated with TWEAK. TWEAK increased proliferation rates to a higher level in NIK+/− cholangiocytes relative to NIK−/− cholangiocytes; importantly, inhibition of Akt by either wortmannin or LY294002 abrogated TWEAK-stimulated proliferation of NIK+/− cholangiocytes (Fig. 3F). In contrast, wortmannin and LY294002 did not affect NIK−/− cholangiocyte proliferation. Thus, NIK promotes cholangiocyte expansion at least in part by enhancing Akt activation.

Discussion

Ductular reaction commonly occurs in chronic liver diseases, including cholestasis, alcoholic liver disease, and nonalcoholic steatohepatitis (NASH). Ductular reaction associates with poor prognosis and is an important risk factor for intrahepatic cholangiocarcinoma30,31. Bilirubin insults, in concert with aberrant liver microenvironments, drive cholangiocyte expansion and ductular reaction. In this study, we have identified cholangiocyte-intrinsic NIK as a molecular bridge connecting bilirubin insults to ductular reaction, liver injury, inflammation, and fibrosis in mice.
We demonstrated that DDC, ANIT, or BDL treatment markedly upregulated biliary NIK in mice through increasing both its expression and stability. Consistently, DDC and ANIT directly increased the expression and stability of NIK in cholangiocyte cultures.

In wild-type mice, DDC, ANIT, or BDL induced ductular reaction, liver injury, inflammation, and fibrosis as expected. Strikingly, cholangiocyte-specific ablation of NIK blocked DDC-, ANIT-, and BDL-induced ductular reaction, liver injury, and fibrosis in NIKΔK19 mice. Likewise, pharmacological inhibition of NIK by C33 also mitigated DDC-induced ductular reaction, liver injury, inflammation, and fibrosis in mice.

Cholangiocyte proliferation was lower while its death was higher in NIKΔK19 mice relative to NIKf/f mice post DDC, ANIT, or BDL treatment, and this may explain the resistance of NIKΔK19 mice to cholangiocyte expansion and ductular reaction. NIK can be activated by cytokines,
intracellular stress, and cell-damaging factors\cite{4, 2}, raising the possibility that biliary NIK may integrate a wide spectrum of biliary damaging-related signals to promote ductular reaction, liver injury, inflammation, and fibrosis (Fig. 7E). Of note, biliary NIK was profoundly upregulated in humans with PBC, PSC, HCV, or alcoholic liver disease, raising the intriguing possibility that a similar biliary NIK/ductular reaction/liver inflammation/liver fibrosis cascade may also operate in human liver disease progression.

Given that IKKα acts downstream of NIK to activate the non-canonical NF-κB pathway, it is unexpected that IKKα−/− mice did not phenocopy NIK−/− mice. In line with these findings, ablation of cholangiocyte reLB, a NF-κB partner, also does not affect DDC-induced ductular reaction\cite{3}. Hence, biliary NIK appears to promote ductular reaction by an IKKα/NF-κB-independent mechanism. We observed that TWEAK stimulated both NF-κB and Akt pathways in NIK−/− cholangiocytes. As expected, NIK deficiency completely blocked TWEAK-stimulated activation of the noncanonical NF-κB pathway in NIK−/− cholangiocytes. NIK deficiency also substantially attenuated Akt phosphorylation in TWEAK-stimulated NIK−/− cholangiocytes, indicating that TWEAK, or related cytokines, stimulate a NIK/Akt pathway in cholangiocytes. TWEAK has been known to stimulate biliary growth and expansion, and ablation of TWEAK receptor Fn14 suppresses ductular reaction\cite{1}. Akt is also believed to mediate biliary proliferation\cite{17}. Pharmacological inhibition of the PI 3-kinase/Akt pathway blocked TWEAK-stimulated proliferation of NIK−/− cholangiocytes, but not NIK+/− cholangiocytes, suggesting that the biliary NIK/Akt pathway mediates, at least in part, ductular reaction (Fig. 7E).

Ductular reaction has long been recognized to associate with chronic liver disease, but its contribution to liver disease progression remains obscure. Resistance of NIK−/− mice to DDC, ANIT, and BDL-induced liver inflammation and fibrosis strongly argues for the notion that biliary NIK-elicited ductular reaction is a causal factor for liver inflammation and fibrosis. In ductular reaction, reactive cholangiocytes gain proinflammatory and profibrosis properties\cite{18, 33, 35}. We observed that expression of inflammatory mediators (IL-1β, IL-4, IL-6, TNFα, MCP1) and profibrotic genes (Tgfb1) was markedly lower in NIK−/− cholangiocytes relative to NIK+/− cholangiocytes. In line with these results, macrophages were activated to a higher level by coculture with NIK−/− cholangiocytes relative to NIK+/− cholangiocytes, and to a higher level by stimulation with NIK−/− cholangiocyte-conditioned medium relative to NIK−/− cholangiocyte-conditioned medium. Likewise, NIK−/− cholangiocyte-conditioned medium, which harbored cholangiokines, was much more potent than NIK−/− cholangiocyte-conditioned medium to stimulate HSC activation and its expression of profibrotic genes (aSMA, ColIa1, Mmp1, Tgfβ, and Col1a1). Based on these findings, we are tempted to postulate that biliary NIK, which is upregulated in chronic liver disease, directly stimulates expression and secretion of cholangiokines that in turn activate liver immune cells and HSCs (Fig. 7E). The biliary NIK/cholangiokine/immune cell axis and the biliary NIK/cholangiokine/HSC axis drive the pathogenesis of liver inflammation and fibrosis. As discussed above, biliary NIK induces ductular reaction, and it is conceivable that expansion of NIK+ cholangiocytes further increases secretion of cholangiokines that shape pathogenic liver microenvironments and exacerbate liver disease progression.

C3 treatment substantially mitigated DDC-induced ductular reaction, liver inflammation, and fibrosis, raising the intriguing possibility that NIK inhibitors are anti-liver disease drug candidates. C33 was not as potent as genetic deletion of biliary NIK in protecting against liver disease, because it partially inhibited liver NIK under the experimental conditions. Of note, this study has limitations. We cannot exclude the possibility that C33 may target additional cell types in addition to cholangiocytes in vivo. Global or thymic epithelial cell-specific deletion of NIK impairs thymus development and central T cell tolerance in mice\cite{20, 21}, raising a concern about the safety of NIK inhibitors. However, the human thymus is degenerated in adults, arguing against the possibility that NIK inhibitors may impair T cell-based adaptive immunity in adults. The identities of individual cholangiokines, which mediate crosstalk between cholangiocytes and macrophages and between cholangiocytes and HSCs, remain to be defined. The contribution of the biliary NIK/Akt pathway to ductular reaction needs to be validated in vivo. Human liver biopsies need to be further increased to consolidate the conclusion about biliary NIK in human liver disease. These questions point to future research directions.

**Methods**

**Animals**

NIK−/−, IKKα−/− and K19-CreERT mice (C57BL/6J background) were described previously\cite{28, 33, 34}. NIK and K19 reside in the same Chromosome II. The NIK−/−;K19-CreERT++ genotypes were generated through homologous recombination in germ cell meiosis (low frequency). NIK−/−;K19-CreERT mice and IKKα−/−;K19-CreERT mice were treated with tamoxifen (5 mg/mouse, twice, 4 days apart, i.p) at 7–8 weeks of age to generate NIK−/− and IKKα−/− mice, respectively. Olive oil vehicle was used as control. NIK−/− mice were injected with tamoxifen as an additional control. Mice were housed on a 12:12 light-dark cycle and at 25°C of ambient temperature in the Unit for Laboratory Animal Medicine at the University of Michigan (ULAM) and fed ad libitum a chow diet (9% fat in calories; TestDiet, St. Louis, MO), DDC diet, or ANIT diet.

**Ethics statements**

Animal research was complied with all relevant ethic regulations. Animal experiments were conducted following the protocols approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC).

**Human liver biopsies**

De-identified human liver specimens were provided by Dr. Suthat Liangpunsakul under the IRB-approved protocol at the Indiana-Purdue University Indianapolis and by Dr. Anna S. Lok under the IRB-approved protocol at the University of Michigan. The study design and conduct were authorized by the Indiana-Purdue University Indianapolis and University of Michigan IRB committees and complied with all relevant regulations regarding the use of human study participants. Patients provided informed consent. The study was conducted in accordance with the criteria set by the Declaration of Helsinki.
Fig. 4 | Ablation of biliary NIK ameliorates DDC-induced liver injury and inflammation. A–C NIK<sup>−/−</sup> and NIK<sup>eff</sup> male mice were fed a DDC diet for 4 weeks. A Plasma ALT, ALP and total bilirubin levels. NIK<sup>−/−</sup>: n = 8 mice, NIK<sup>ΔK19</sup>: n = 9 mice. B, C Liver sections were stained with H&E or antibodies to CD11b, and Gr-1. B Representative images. Scale bar: 200 μm. C CD11b (n = 3 mice per group) and Gr-1 cells (n = 3 mice per group) were counted and normalized to total liver cell number (n = 3–4 mice per group). D, E NIK<sup>−/−</sup> and NIK<sup>eff</sup> male mice were fed an ANIT diet for 3 weeks. D Representative H&E staining of liver sections. Scale bar: 200 μm. E Liver sections were stained with anti-CD11b antibody. CD11b cells were counted and normalized to total liver cells. NIK<sup>−/−</sup>: n = 7 mice, NIK<sup>ΔK19</sup>: n = 6 mice. Data are presented as mean ± SEM. *p < 0.05, 2-way ANOVA (A) and 2-tailed student’s t-test (C, E). Source data are provided as a Source Data file.
Fig. 5 | Ablation of NIK in cholangiocytes protects against liver fibrosis.

A. B NIKΔK19 and NIKf/f male mice were fed a DDC diet for 4 weeks. Liver sections were stained with Sirius red/fast green or antibody to αSMA. A Representative images. Scale bar: 200 μm. B Sirius red (NIKf/f: n = 6 mice, NIKΔK19: n = 7 mice) and αSMA HSC (n = 4 mice per group) areas were measured and normalized to total areas. Liver hydroxyproline levels were measured and normalized to liver weight (NIKf/f: n = 6 mice, NIKΔK19: n = 8 mice). C Mice were fed a chow or DDC diet for 4 weeks. Liver extracts were immunoblotted with anti-αSMA and p85 (loading control) antibodies. Each lane represents one individual animal (n = 3 mice per group). D NIKΔK19 (n = 6 mice) and NIKf/f (n = 7 mice) male mice were fed an ANIT diet for 3 weeks. Liver sections were stained with Sirius red/fast green or anti-αSMA. Scale bar: 200 μm. Sirius red and αSMA areas were normalized to total areas. Data are presented as mean ± SEM. * p < 0.05, 2-tailed student’s t-test. Source data are provided as a Source Data file.
Fig. 6 | Ablation of biliary NIK attenuates BDL-induced ductular reaction, liver injury, and inflammation. BDL was performed on NIKΔK19 and NIKf/f male mice, and livers were analyzed 7 days later. A Plasma ALT, ALP, and total bilirubin levels (n = 6 mice per group). B, C Liver sections were stained with the indicated antibodies. B Representative images. Scale bar: 200 μm. C Individual subpopulations were counted and normalized to total liver cells. Sirius red area was normalized to total view areas (n = 3 mice per group). Liver hydroxyproline content was normalized to liver weight (NIKf/f: n = 5 mice, NIKΔK19: n = 6 mice). K19: n = 3 mice per group; Casp3+K19+: n = 3 mice per group; Ki67+K19+: n = 3 mice per group; Gr-1: n = 4 mice per group; CD11b: n = 4 mice per group; αSMA: n = 4 mice per group. Data are presented as mean ± SEM. *p < 0.05, 2-tailed student’s t-test (C) and 2-way ANOVA (A). Source data are provided as a Source Data file.
BDL

NIK<sup>−/−</sup> and NIK<sup>fl/fl</sup> mice (9–10 weeks) were anesthetized with isoflurane and subjected to midline laparotomy (~1 cm) to expose the common bile duct. Two knots were made on the common bile duct using a 5–0 silk suture. The bile duct was cut between the two knots. The peritoneum was re-aligned. The skin and the underneath muscle layers were closed individually using a 5–0 silk suture.

Compound 33 treatment

C57BL/6j male mice (8 weeks) were fed a DDC diet and simultaneously treated with NIK inhibitor Compound 33 (Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai) at 10 mg/kg body weight (twice a week, i.p.) for 3 weeks. Compound 33 was prepared in a vehicle of 10% PEG400 (polyethylene glycol, molecular weight 400, Sigma #P3265) and 3% Cremophor EL (Sigma #C5135). Body weight, blood chemistry, and liver pathology were analyzed.

Blood chemistry

Blood samples were collected from tail veins. Plasma ALT, ALP, and bilirubin levels were measured using commercial kits (Pointe Scientific Inc., Canton, MI; ALT: A7526625; ALP: A7516150; bilirubin: B7576120).

Hydroxyproline assays

Liver samples were homogenized in 6 N HCl, hydrolyzed at 100 °C for 18 h, and centrifuged at 9391xg for 5 min. The supernatant was dried in a speed-vacuum, dissolved in H2O, and neutralized with 10 N NaOH. Samples were incubated in a chloramine-T solution [60 mM chloramines-T (Sigma, 857319), 20 mM citrate, 50 mM acetate, pH 6.5] for 25 min at room temperatures, and then in Ehrlich’s reagents using an In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN, 11684817910). Hydroxyproline content was measured using a Beckman Coulter AD 340 Plate Reader (570 nm) and normalized to liver weight.

Staining of liver sections

Liver paraffin sections were stained with 0.1% Sirius-red (Sigma, 365548) and 0.1% Fast-green (Sigma, F7252) (in saturated picric acid). Liver frozen sections were prepared using a Leica cryostat (Leica Biosystems Nussloch GmbH, Nussloch, Germany), fixed in 4% paraformaldehyde for 30 min, blocked for 3 h with 5% normal goat serum (Life Technologies) supplemented with 1% BSA, and incubated with the indicated antibodies overnight at 4 °C. Antibodies were listed in Supplementary Table 1. Liver frozen sections were stained with TUNEL reagents using an In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN, 11684817910).

Immunoblotting

Cells or tissues were homogenized in a L-RIPA lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 2 mM EGTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5% sodium deoxycholate, 1% Triton X-100, 10 mM β-glycerophosphate, 10 μg/ml aprotinin, 1 mM PMSF, and 1 mM sodium orthovanadate) and subjected to midline laparotomy (~1 cm) to expose the common bile duct. Two knots were made on the common bile duct using a 5–0 silk suture. The bile duct was cut between the two knots. The peritoneum was re-aligned. The skin and the underneath muscle layers were closed individually using a 5–0 silk suture.

DDC diet and ANIT diet

Two weeks after tamoxifen treatment (deleting biliary NIK or IKKa), NIK<sup>−/−</sup>, IKKa<sup>−/−</sup>, and control mice were fed a DDC diet for 4 weeks or with an ANIT diet for 3 weeks. DDC diet and ANIT diet were prepared by adding 0.1% DDC (Sigma-Aldrich, 137030) or 0.08% ANIT (Sigma-Aldrich, 18951) to chow diet powder (LabDiet 5001).
Fig. 8 | NIK inhibitor treatment ameliorates DDC-induced ductular reaction and liver injury. A HEK293 cells were cotransfected with NF-κB2 and NIK expression plasmids and treated with C33. Cell extracts were immunoblotted with antibodies to NF-κB2 and NIK. B HEK293 cells were cotransfected with NF-κB luciferase reporter and NIK expression plasmids and treated with C33. Luciferase activity was assessed 48 h after C33 treatment (n = 3 repeats per group). C–E C57BL/6J males were treated with DDC and C33 for 3 weeks. C Plasma ALT, ALP, and total bilirubin levels (n = 8 mice per group). D, E Liver sections were immunostained with the indicated antibodies. D Representative liver images. Scale bar: 200 μm. E K19+ and MPO+ cells were counted and normalized to total liver cells. Sirius red and αSMA HSC areas were normalized to total areas. K19, MPO, and αSMA: n = 8 mice per group. Sirius red: n = 4 mice per group. Data are presented as mean ± SEM. *p < 0.05, 2-tailed student’s t-test (E) and 1-way (B) or 2-way (C) ANOVA. Source data are provided as a Source Data file.
100 mM NaCl, 10 mM Na2HPO4, 1 mM benzamidine, 10 µg ml−1 aprotenin, 10 µg ml−1 leupeptin, 1 mM phenylmethylsulfonyl fluoride). Proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Antibodies were listed in Supplementary Table 1. HEK293 cell lines were from the ATCC (CRL-1573).

Isolation of the biliary tree and generation of cholangiocyte lines
Nik−/− males (9 weeks) were anesthetized with isoflurane, and the liver was perfused with type II collagenase (Worthington Biochem, Lakewood, NJ). Bile duct trees were isolated, extensively washed with PBS, weighed, minced into 1-mm blocks, and cultured in DMEM (25 mM glucose) supplemented with 10% FBS and antibiotics. Proliferating cholangiocytes, portal fibroblasts, and HSCs emigrated from tissue blocks in 7–8 days. These cells were collected, transduced with E1A lentiviral vectors, and treated with puromycin for 1 week to select immortalized cells. Individual lines, derived from single cell lineage, were generated by a series of dilution. To characterize these lines, we measured expression of cholangiocyte markers (K7, K19, Cfr, Hnf4β) and HSC markers (colla1, αSMA) by qPCR. NIK−/− cholangiocytes (NIK−/−) were transduced with Cre adenoviral vectors to delete NIK (NIK−/−). Adenoviral vectors were subsequently removed by repeatedly passaging (>10 generations). Cholangiocytes were cultured in DMEM supplemented 10% FBS and antibiotics.

Cholangiocyte proliferation and death
Cholangiocyte cultures (-50% confluence) were deprived of serum overnight and stimulated with 1% FBS, 10% FBS, or TWEAK (20 ng/ml) for 24 h. Cells were counted. Cholangiocytes were stimulated for 24 h with FBS or TWEAK in the presence of BrdU and stained with anti-BrdU antibody. Cholangiocytes (-80% confluence) were cultured for 24 h in DMEM without FBS or in DMEM supplemented with 10% FBS and 200 µM palmitate. Cholangiocyte death and survival were assessed by TUNEL or MTT assays.

Cholangiocyte-conditioned medium and cholangiocyte/BMDM cocultures
Confluent NIK−/− and NIK−/− cholangiocytes were cultured for 8 h in DMEM supplemented with 1% FBS and 50 ng/ml TWEAK. Culture medium was replaced with fresh DMEM (1% FBS, no TWEAK) and collected 24 h later (cholangiocyte-conditioned medium). BMDMs were isolated from the femur and tibia of C57BL/6j male mice and cultured in DMEM supplemented with 20% L929 conditioned medium, 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. BMDMs were fully differentiated 6 days later. Differentiated BMDMs were grown in cholangiocyte-conditioned medium for 6 h, and their gene expression was assessed by qPCR. HSCs were cultured in DMEM supplemented with 5% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Confluent HSCs were grown for 24 h in cholangiocyte-conditioned medium supplemented with 50 ng/ml TGFβ1, and their gene expression was measured by qPCR. In transwells, cholangiocytes were seeded in the insert and pretreated with DMEM supplemented with 1% FBS and 50 ng/ml TWEAK for 8 h and then cocultured with BMDMs grown in DMEM supplemented 20% L929 conditioned medium, 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin in the bottom well. BMDMs were harvested 24 h after coculture with cholangiocytes, and their gene expressions were measured by qPCR.

NIK stability in cholangiocyte cultures
Cholangiocytes were transduced with β-galactosidase (β-gal) or NIK adenoviral vectors for 2 h and subsequently grown for 16 h in DMEM supplemented with 5% FBS. The medium was replaced with DMEM supplemented with DDC (100 µm), ANIT (50 µm) or DMSO, and 4 h later, cells were treated with cycloheximide (100 µg/ml) for 0.5, 1, 1.5, 2, 2.5, and 3 h. Cell extracts were prepared and immunoblotted with antibodies to NIK and β-actin. NIK levels were quantified, normalized to β-actin, and presented as a percentage of the initial values.

Real-time quantitative PCR (qPCR)
Total RNAs were extracted using TRIzol reagents (Life technologies). Relative mRNA abundance was measured using Radiant™ SYBR Green 2X Lo-ROX qPCR Kits (Alkali Scientific, Pompano Beach, FL) and StepOnePlus RT PCR Systems (Life Technologies Corporation, NY, USA). Primers were listed in Supplementary Table 2.

Statistics
Data were analyzed using Microsoft excel 2016 and presented as mean ± sem. The difference was analyzed between two groups by two-tailed Student’s t-test and between multiple groups by 1-way ANOVA/Tukey test (GraphPad Prism 8). Longitudinal data were analyzed by 2-way ANOVA/Sidak posttest (GraphPad Prism 8). P < 0.05 was considered statistically significant.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Source data are provided within this paper as a Source Data file. All data supporting the findings described in this paper are available in the article and in the Supplementary Information and from the corresponding author upon reasonable request. Source data are provided with this paper.

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**Author contributions**

Z.Z., X.Z., H.S., and L.S. conducted the experiments, X.Z., H.S., and L.R. designed the experiments and wrote the paper, X.Z., H.S., Z.Z., S.L., A.S.L., M.B.O., S.W., and L.R. edited the paper, and S.L., A.S.L., and M.B.O. provided human liver biopsies.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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