Research Article

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Nod2 deficiency protects mice from cholestatic liver disease by increasing renal excretion of bile acids

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Background & Aims: Chronic liver disease is characterized by fibrosis that may progress to cirrhosis. Nucleotide oligomerization domain 2 (Nod2), a member of the Nod-like receptor (NLR) family of intracellular immune receptors, plays an important role in the defense against bacterial infection through binding to the ligand muramyl dipeptide (MDP). Here, we investigated the role of Nod2 in the development of liver fibrosis.

Methods: We studied experimental cholestatic liver disease induced by bile duct ligation or toxic liver disease induced by carbon tetrachloride in wild type and *Nod2*$^{-/-}$ mice.

Results: Nod2 deficiency protected mice from cholestatic but not toxin-induced liver injury and fibrosis. Most notably, the hepatic bile acid concentration was lower in *Nod2*$^{-/-}$ mice than wild type mice following bile duct ligation for 3 weeks. In contrast to wild type mice, *Nod2*$^{-/-}$ mice had increased urinary excretion of bile acids, including sulfated bile acids, and an upregulation of the bile acid efflux transporters MRP2 and MRP4 in tubular epithelial cells of the kidney. MRP2 and MRP4 were downregulated by IL-1β in a Nod2 dependent fashion.

Conclusions: Our findings indicate that Nod2 deficiency protects mice from cholestatic liver injury and fibrosis through enhancing renal excretion of bile acids that in turn contributes to decreased concentration of bile acids in the hepatocyte.

Keywords: Chronic liver disease; Bile acids transporter; IL-1β; Renal tubular epithelial cells; Bacterial translocation; Microbiome.

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Abbreviations: NASH, non-alcoholic steatohepatitis; Nod, nucleotide oligomerization domain; MDP, muramyl dipeptide; IL, interleukin; BDL, bile duct ligation; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TNF, tumor necrosis factor; LPS, lipopolysaccharide; CCl_4, carbon tetrachloride; GDCA, glycodeoxycholic acid; PI, propidium iodide; LDH, lactate dehydrogenase; MCA, muricholic acid; NTCP, Na-taurocholate cotransporting polypeptide; Slc, solute carrier family; OATP, organic anion transporting polypeptide; Slco, solute carrier organic anion transporter family; MRP, multidrug resistance-associated protein; Abc, ATP-binding cassette; OST, organic solute transporter; BSEP, bile salt export pump; MDR, multidrug resistance protein; CYP, Cytochrome P450; ASBT, sodium-dependent bile salt transporter.

Introduction

Liver fibrosis results from chronic liver injury commonly caused by cholestasis, toxins, alcohol abuse, viral infections or non-alcoholic steatohepatitis (NASH). Hepatic fibrosis can progress to cirrhosis, which is associated with a significant morbidity and mortality. Effective anti-fibrotic therapies are still lacking. Therefore, elucidating the molecular mechanisms initiating and driving liver fibrosis is crucial for the development of anti-fibrotic strategies that should prevent this fatal disease [1–3].

Nucleotide oligomerization domain 2 (Nod2) belongs to the Nod-like receptor (NLR) family which consists of intracellular innate immune receptors for bacterial peptidoglycans. These immune receptors play a crucial role in the host response to bacterial infection [4,5]. Muramyl dipeptide (MDP), the minimal motif of peptidoglycan from both Gram-positive and Gram-negative bacteria, was identified as the ligand of Nod2 [6]. MDP recognition by Nod2 activates transcription factor NF-κB and induces pro-inflammatory cytokine production by interacting with the RIP-like interacting CLARP kinase (RICK/RIP2) [7]. MDP stimulation also activates Nod2 to process and release mature interleukin (IL)-1β in a caspase-1-dependent fashion [8,9]. Genetic studies in humans have linked mutations in the Nod2 gene to higher susceptibility to Crohn's disease in a subset of patients [10]. Additionally, Nod2 plays an important role in intestinal microbial homeostasis [11], intestinal immunity [12] and gut barrier function [13,14].

The role of Nod2 in liver fibrosis is not known. Using wild type and Nod2 deficient mice, the function of Nod2 in experimental cholestasis- or toxin-induced liver fibrosis was investigated.

Materials and methods

Material and methods are described in the Supplementary data section.

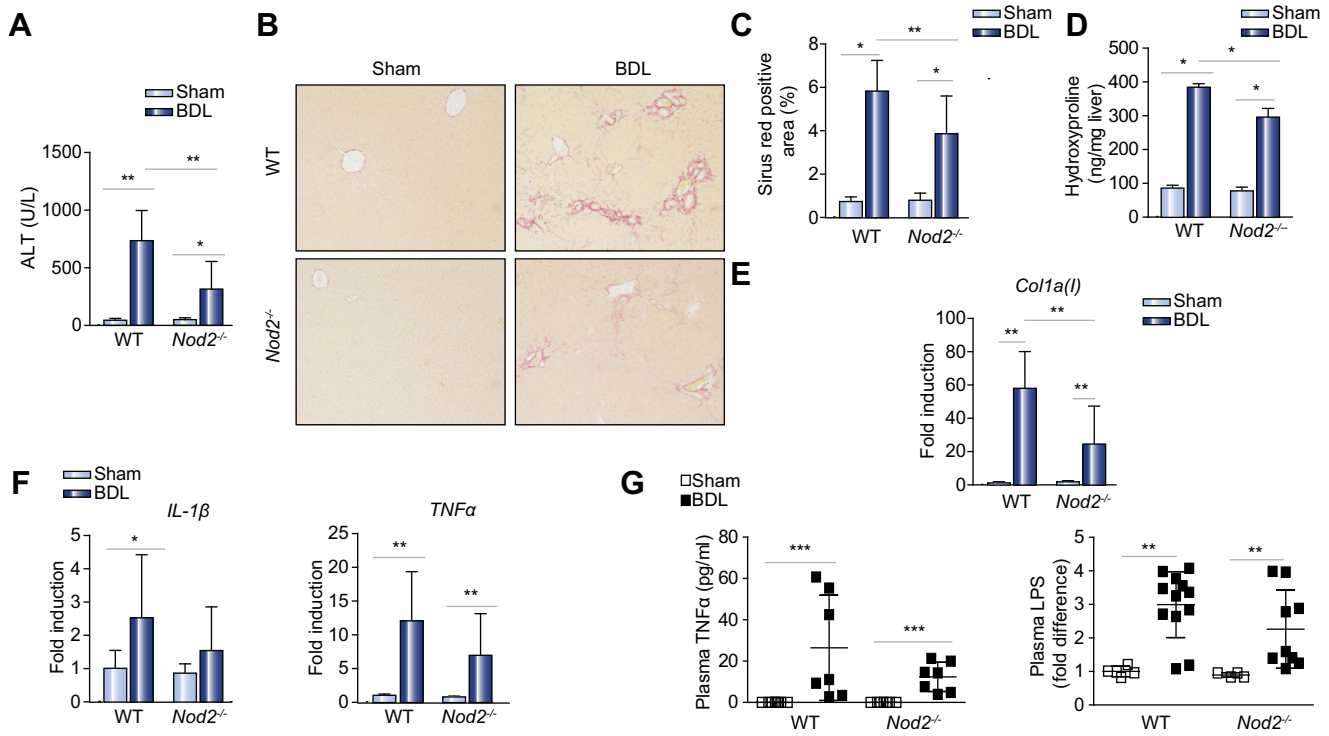

Fig. 1. Liver fibrosis is prevented in *Nod2*$^{-/-}$ mice following 3 weeks of bile duct ligation. Wild type and *Nod2*$^{-/-}$ mice underwent sham operation (n = 3–8 for wild type mice; n = 3–7 for *Nod2*$^{-/-}$ mice) or bile duct ligation (BDL; n = 7–13 for wild type mice; n = 7–9 *Nod2*$^{-/-}$ mice). (A) Plasma ALT levels. (B and C) Sirius red staining of liver sections are shown and quantitated by image analysis. (D) Hepatic hydroxyproline measurement. (E and F) Hepatic collagen α1(I), *IL1β* and *TNFα* gene expression. (G) Plasma TNFα and LPS levels $^{*}p$ <0.05;$^{**}p$ <0.01; $^{***}p$ <0.001. (This figure appears in colour on the web.)

Results

Nod2 deficiency protects mice from cholestasis-induced liver fibrosis

Wild type and Nod2 deficient mice were subjected to cholestatic liver injury induced by bile duct ligation. Following 3 weeks of bile duct ligation, liver injury as determined by ALT levels was dramatically lower in Nod2 deficient mice as compared with wild type mice (Fig. 1A). Plasma alkaline phosphatase (ALP) was also lower in *Nod2*$^{-/-}$ mice than wild type mice following bile duct ligation (Supplementary Fig. 1A). Liver weight and plasma bilirubin levels were not significantly different between wild type and *Nod2*$^{-/-}$ mice following bile duct ligation (Supplementary Fig. 1B and C). As a measurement of liver fibrosis, fibrillar collagen deposition was determined by Sirius red staining. Hepatic fibrosis was lower in *Nod2*$^{-/-}$ mice as compared with wild type mice after bile duct ligation (Fig. 1B). The lower level of Sirius red staining was confirmed by morphometric analysis (Fig. 1C) and hydroxyproline measurement (Fig. 1D). A similar reduction of *collagen α1*(I) mRNA expression was found in *Nod2*$^{-/-}$ mice as compared with wild type mice (Fig. 1E).

As Nod2 induces pro-inflammatory cytokines [9], *IL1β* and tumor necrosis factor α (*TNFα*) gene expression were measured. *Nod2*$^{-/-}$ mice showed a trend towards lower hepatic *IL1β* and *TNFα* mRNA expression following bile duct ligation for 3 weeks compared with wild type mice (Fig. 1F). This trend, however, was not significant suggesting that attenuated fibrosis results from decreased liver damage rather than as a direct consequence of Nod2 deficiency on liver inflammation. Plasma IL-1β levels were below the detection limit of the ELISA in wild type or *Nod2*$^{-/-}$ mice after sham operation or bile duct ligation for 3 weeks (data not shown), while plasma TNFα was not significantly different between *Nod2*$^{-/-}$ and wild type mice (Fig. 1G). Since the amount of translocated bacterial products is dependent on the intestinal bacterial burden and since the progression of liver fibrosis is dependent on translocated bacterial products [15], bacterial overgrowth and translocation of microbial products from the gut lumen to the systemic circulation was investigated. There was no significant difference in bacterial overgrowth between wild type and *Nod2*$^{-/-}$ mice after bile duct ligation (data not shown). Plasma LPS levels were also not significantly different between wild type and *Nod2*$^{-/-}$ mice following bile duct ligation for 3 weeks (Fig. 1G). Taken together, Nod2 deficient mice are protected from bile duct ligation-induced liver injury and fibrosis which is not explained by a stabilized intestinal mucosal barrier.

Nod2 deficient mice are not protected from toxin-induced liver injury and fibrosis

To determine whether Nod2 deficiency suppresses hepatic fibrosis induced by a different etiology, toxic liver injury was induced by repeated intraperitoneal injections of carbon tetrachloride (CCl_4). In contrast to cholestatic liver injury, carbon tetrachloride-induced liver injury was not different in *Nod2*$^{-/-}$ mice compared with wild type mice as evidenced by plasma ALT levels (Supplementary Fig. 2A). Similarly, hepatic collagen deposition (Supplementary Fig. 2B and C) and *collagen α1* (I) mRNA expres-