

Tumor Necrosis Factor- α -Mediated Hepatocyte Apoptosis Stimulates Fibrosis in the Steatotic Liver in Mice

Yosuke Osawa,^{1,2} Ekumi Kojika,¹ Yukiko Hayashi,¹ Masamichi Kimura,¹ Koji Nishikawa,¹ Sachiyo Yoshio,² Hiroyoshi Doi,² Tatsuya Kanto,² and Kiminori Kimura¹

Hepatocyte apoptosis has been implicated in the progression of nonalcoholic steatohepatitis. However, it is unclear whether the induction of tumor necrosis factor (TNF)- α -mediated hepatocyte apoptosis in the simple fatty liver triggers liver fibrosis. To address this question, high-fat diet-fed mice were repeatedly administered D-galactosamine, which increases the sensitivity of hepatocytes to TNF- α -mediated apoptosis. In mice treated with a high-fat diet plus D-galactosamine, hepatocyte apoptosis and liver fibrosis were induced, whereas both apoptosis and fibrosis were inhibited in these mice following gut sterilization with antimicrobials or knockout of TNF- α . Furthermore, liver fibrosis was diminished when hepatocyte apoptosis was inhibited by expressing a constitutively active inhibitor of nuclear factor κ B kinase subunit β . Thus, hepatocyte apoptosis induced by intestinal dysbiosis or TNF- α up-regulation in the steatotic liver caused fibrosis. Organ fibrosis, including liver fibrosis, involves the interaction of cyclic adenosine monophosphate-response element-binding protein-binding protein (CBP) and β -catenin. Here, hepatocyte-specific CBP-knockout mice showed reduced liver fibrosis accompanied by hepatocyte apoptosis diminution; notably, liver fibrosis was also decreased in mice in which CBP was specifically knocked out in collagen-producing cells because the activation of these cells was now suppressed. **Conclusion:** TNF- α -mediated hepatocyte apoptosis induced fibrosis in the steatotic liver, and inhibition of CBP/ β -catenin signaling attenuated the liver fibrosis due to the reduction of hepatocyte apoptosis and suppression of the activation of collagen-producing cells. Thus, targeting CBP/ β -catenin may represent a new therapeutic strategy for treating fibrosis in nonalcoholic steatohepatitis. (*Hepatology Communications* 2018;2:407-420)

Introduction

Nonalcoholic fatty liver disease (NAFLD)⁽¹⁾ is a component of metabolic syndrome and a spectrum of liver disorders ranging from simple steatosis to marked inflammation and nonalcoholic steatohepatitis (NASH), which might cause liver fibrosis. Although simple steatosis is benign, survival time is shorter in the case of patients with liver fibrosis than in those without fibrosis.⁽²⁾ The fibrogenic action of liver myofibroblasts is stimulated by hepatocyte apoptosis,^(3,4)

and patients with NASH present an increase in hepatocyte apoptosis in liver biopsy specimens⁽⁵⁾ or apoptosis biomarkers in plasma.⁽⁶⁾ Thus, hepatocyte apoptosis, induced by both death receptor-mediated and organelle-initiated mechanisms,⁽⁷⁾ is considered to be involved in disease progression in NAFLD.⁽⁸⁾

A multiple parallel hits model has been suggested for disease progression. Several factors, including lipopolysaccharide (LPS) and tumor necrosis factor- α (TNF- α), play critical roles in the progression from steatosis to NASH,⁽⁹⁾ and activation of the LPS/toll-like receptor

Abbreviations: α -SMA, α -smooth muscle actin; ALT, alanine aminotransferase; BDL, bile-duct ligation; CBP, cyclic adenosine monophosphate-response element-binding protein-binding protein; CDD, choline-deficient diet; fibro-KO, fibroblast specific knockout; GalN, D-galactosamine; hep-CA, hepatocyte specific expression of constitutively active; hep-KO, hepatocyte specific knockout; HFD, high-fat diet; HSC, hepatic stellate cells; IKK2, inhibitor of nuclear factor κ B kinase subunit β ; LPS, lipopolysaccharide; mac-KO, macrophage specific knockout; MCDD, methionine-choline-deficient diet; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NF, nuclear factor; TLR, toll-like receptor; TNF, tumor necrosis factor.

Received August 17, 2017; accepted January 22, 2018.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1158/full.

Supported by grants from the Takeda Science Foundation (YO), the Japan Agency for Medical Research and Development (AMED) Acceleration Transformative Research for Medical Innovation (JP17im0210105b) (KK), and AMED grant number JP17fk0210305b (TK).

(TLR)-4 pathway from bacterial overgrowth in the gut microbiota is involved in the progression to NASH.⁽¹⁰⁾ The roles of TNF- α in liver fibrosis have been reported. For example, the liver injury and fibrosis induced by bile duct ligation are reduced in TNF- $\alpha^{-/-}$ mice; however, exogenous administration of TNF- α decreases collagen $\alpha 1(I)$ messenger RNA (mRNA) expression in isolated rat hepatic stellate cells (HSCs),⁽¹¹⁾ which represent a major fibrogenic cell type in the liver. Moreover, TNF- α mediates hepatotoxic effects in mice harboring aberrant gut microbiota in the NASH model induced using the methionine-choline-deficient diet (MCDD).⁽¹²⁾ Liver macrophages respond to TLR ligands and produce TNF- α ,⁽¹³⁾ which is the predominant mediator of hepatocyte apoptosis in the acute liver injury model induced using D-galactosamine (GalN) plus LPS.⁽¹⁴⁾ TNF- α levels are increased in the serum of patients with NAFLD,⁽¹⁵⁾ and mice deficient in TNF receptors show diminished steatosis, inflammation, and fibrosis in the MCDD-induced NASH model.⁽¹⁶⁾ LPS or TNF- α stimulation alone does not trigger hepatocyte apoptosis because TNF- α induces anti-apoptotic signals,^(17,18) and TNF receptor-mediated hepatocyte apoptosis requires hepatocyte sensitization, such as through GalN treatment.⁽¹⁹⁾ In NAFLD, simple steatosis does not sensitize hepatocytes to LPS-induced liver injury and TNF- α -induced apoptosis.⁽²⁰⁾ Thus, although inhibition of TNF- α /TNF receptors has been reported to produce beneficial effects in animal models of

NASH,^(12,16,21,22) it remains unclear whether the induction of TNF- α -mediated hepatocyte apoptosis in the simple fatty liver triggers liver fibrosis.

Almost all aspects of embryonic development and the pathogenesis of numerous human diseases involve the molecule β -catenin.⁽²³⁾ Notably, knockout of β -catenin, specifically in hepatocytes, attenuates liver injury and hepatocyte apoptosis induced by GalN plus LPS or GalN plus TNF- α .^(24,25) Following liver injury, HSCs undergo activation and change phenotypically from quiescent retinoid-storing HSCs into collagen-producing and contractile myofibroblast-like cells. Notably in mice, an inhibitor of the interaction between β -catenin and cyclic adenosine monophosphate-response element-binding protein-binding protein (CBP) reduces liver fibrosis mediated by bile-duct ligation (BDL), CCl₄,⁽²⁶⁾ or hepatitis C virus⁽²⁷⁾ and prevents HSC activation, which results in a reduction of collagen expression *in vitro*.⁽²⁶⁾ Liver macrophages contribute to liver fibrosis; depletion of liver macrophages suppresses liver fibrosis following BDL⁽²⁸⁾ and decreases myofibroblasts in a liver tumor.⁽²⁹⁾ Moreover, inhibition of CBP/ β -catenin promotes liver fibrosis resolution by macrophages.⁽²⁶⁾ Although macrophages contribute to NASH development,⁽³⁰⁾ the role of CBP/ β -catenin in liver macrophages during liver fibrosis in NASH is unclear.

In this study, we sought to clarify the effects of hepatocyte apoptosis in fatty liver on liver fibrosis and the roles of CBP/ β -catenin in hepatocyte apoptosis

Copyright © 2018 The Authors. *Hepatology Communications* published by Wiley Periodicals, Inc., on behalf of the American Association for the Study of Liver Diseases. This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs License](#), which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep4.1158

Potential conflict of interest: Nothing to report.

ARTICLE INFORMATION:

From the ¹Department of Hepatology, Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Tokyo, Japan;

²The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Chiba, Japan.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Yosuke Osawa, M.D., Ph.D.
The Research Center for Hepatitis and Immunology
National Center for Global Health and Medicine
1-7-1 Kohnodai, Ichikawa
Chiba 272-8516, Japan
E-mail: osawa-gif@umin.ac.jp
Tel: +81-47-372-3501
Or

Kiminori Kimura, M.D., Ph.D.
Department of Hepatology
Tokyo Metropolitan Cancer and Infectious Diseases Center
Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku
Tokyo 113-8677, Japan
E-mail: kkimura@cick.jp
Tel: +81-3-3823-2101

and liver fibrosis. Thus, we developed a new animal model of liver fibrosis with steatosis by using a high-fat diet (HFD) plus GalN treatment and investigated how knocking out CBP in hepatocytes, fibroblasts, or macrophages affects hepatocyte apoptosis and liver fibrosis in the fibrosis model.

Materials and Methods

STUDY APPROVAL

All experiments were conducted in accordance with institutional guidelines (Guide for the Care and Use of Laboratory Animals, prepared by the National Academy of Sciences), and the protocol was approved by the Research Committee of Komagome Hospital.

ANIMALS AND TREATMENTS

Five-week-old male wild-type (C57BL/6J) mice were obtained from Japan SLC (Shizuoka, Japan). Detailed information regarding the genetically modified mice is provided in the [Supporting Experimental Procedures](#). Hepatic steatosis was induced by feeding the animals an HFD (62.2% calories from fat, HFD-60; Oriental Yeast, Tokyo, Japan) for 8 weeks. Control mice were fed a normal diet (12.6% calories from fat, CE-2; CLEA Japan, Tokyo, Japan). The animals were intraperitoneally injected with or without GalN (20 mg/mouse; Nacalai Tesque, Kyoto, Japan) twice a week for 8 weeks. For Cre recombinase expression in fibroblast-knockout CBP (CBP^{fibro-KO}) mice, tamoxifen (4 mg/mouse, T5648; Sigma-Aldrich, St. Louis, MO) was intraperitoneally administered twice a week for 8 weeks. In these mice, CBP expression is known to diminish in collagen-producing cells, such as activated HSCs. For gut sterilization, mice were orally administered an antimicrobial mixture containing ampicillin (1 g/L), neomycin (1 g/L), metronidazole (1 g/L), and vancomycin (0.5 g/L), according to a published protocol.⁽³¹⁾ The antimicrobial treatment was started concurrently with HFD feeding and was continued for 8 weeks; the treatment induced swelling of the cecum (data not shown), which indicated effective gut sterilization. After recording body weight, the mice were anesthetized and humanely killed through blood withdrawal from the inferior vena cava or the portal vein. The liver was immediately removed, and part of the dissected liver tissue was frozen in liquid nitrogen and part was fixed with 10% formalin for histologic analysis. For recording hepatocellular damage, serum

alanine aminotransaminase (ALT) levels were measured (Oriental Yeast).

OTHER EXPERIMENTAL PROCEDURES

Other experimental procedures are described in the [Supporting Experimental Procedures](#). These include histologic analysis, hydroxyproline measurement, measurement of triglycerides, western blotting, LPS measurement, quantitative real-time reverse-transcription polymerase chain reaction, and statistical analysis.

Results

INDUCTION OF HEPATOCYTE APOPTOSIS IN STEATOSIS STIMULATES LIVER FIBROSIS IN MICE

Hepatocytes were sensitized by repetitive administration of GalN to HFD-fed mice in order to investigate whether liver fibrosis is triggered by the induction of TNF- α -mediated hepatocyte apoptosis in the simple fatty liver. Liver fibrosis and HSC activation were induced in mice treated with an HFD plus GalN, as demonstrated by sirius red staining and hydroxyproline content (Fig. 1A, upper panel, 1B; [Supporting Fig. S1A, upper panel](#)), mRNA and protein expression of collagen (Fig. 1C, left panel, 1D), mRNA expression of the fibrosis-related marker secreted protein acidic and rich in cysteine (SPARC) (Fig. 1C, right panel), and expression of the HSC-activation marker α -smooth muscle actin (α -SMA) (Fig. 1A, lower panel, 1E; [Supporting Fig. S1A, lower panel](#)). In contrast to the combination treatment, the HFD or GalN treatment alone did not induce HSC activation or liver fibrosis. Liver steatosis, as examined by Sudan IV staining and triglyceride measurement in the liver, was induced by both an HFD and an HFD plus GalN in mice, and this was accompanied by an increase in body weight (Fig. 2A-C). However, infiltration of inflammatory cells and inflammatory foci were only observed in the liver of mice treated with an HFD plus GalN (Fig. 2A; [Supporting Fig. S1B](#)), suggesting that the steatosis induced by an HFD is not sufficient for induction of inflammation and fibrosis. Moreover, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling-positive (apoptotic) cells were increased only in the mice

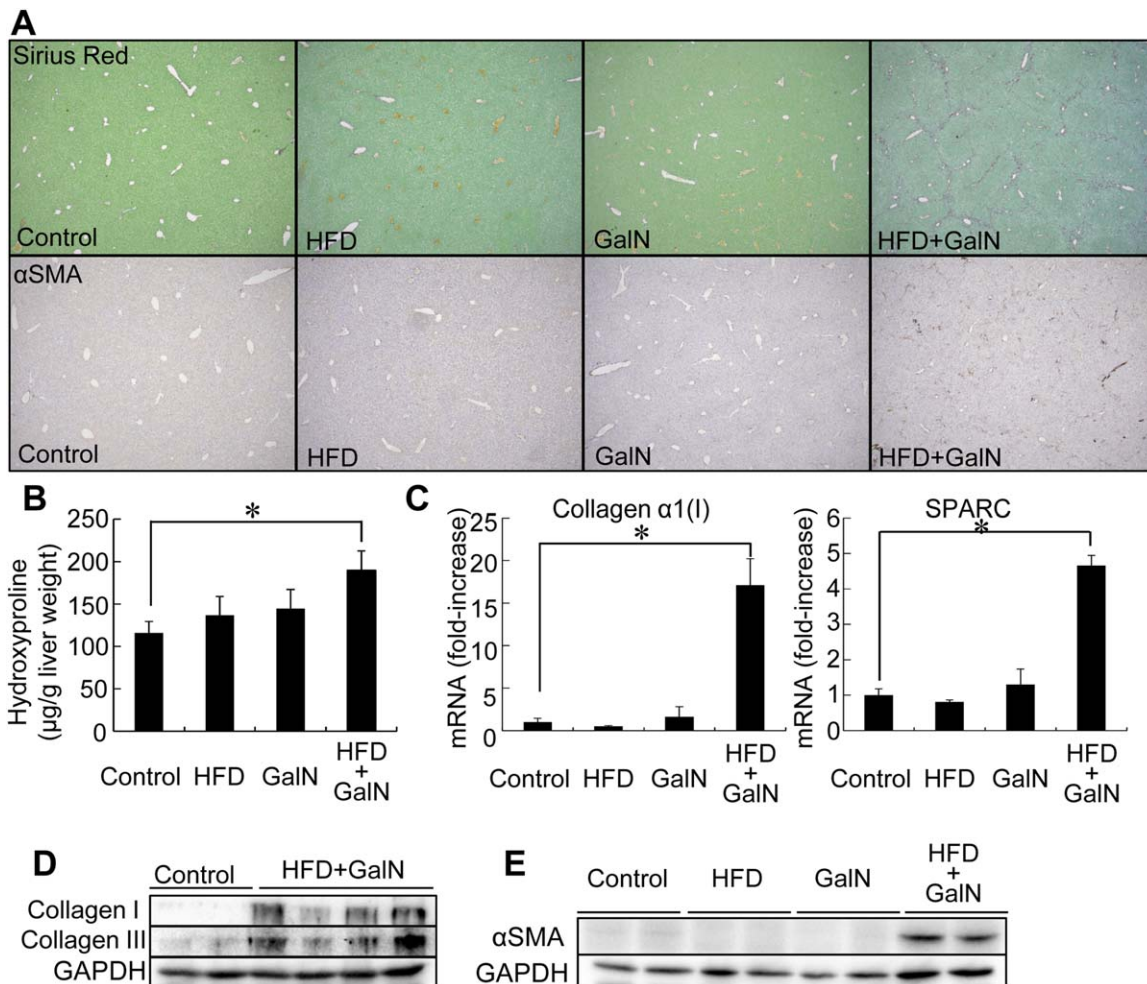


FIG. 1. Liver fibrosis is induced by GalN administration in HFD-fed mice. Mice were fed a normal diet or an HFD and were or were not intraperitoneally injected with GalN. (A,B) Collagen deposition was assessed by staining with sirius red (A, upper panels; original magnification $\times 40$) and by measuring the hydroxyproline content (B). α -SMA expression was examined through immunohistochemical staining with an anti- α -SMA antibody (A, lower panels; $\times 40$). (C) Expression of the indicated mRNA variants in the liver was determined using quantitative real-time reverse-transcription polymerase chain reaction. (D,E) Liver protein extracts were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and then immunoblotting was performed with antibodies against collagen 1, collagen 3, α -SMA, and GAPDH. Results are presented as the means \pm SD of data collected from at least four independent experiments. * $P < 0.05$, one-way analysis of variance (B) and Kruskal–Wallis test (C). Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

treated with an HFD plus GalN, and examination under high magnification revealed that the apoptotic cells were hepatocytes (Fig. 2A,D). In accordance with apoptosis induction, mRNA expression of TNF- α was increased only in mice treated with an HFD plus GalN (Fig. 2E). These results suggest that GalN treatment triggers TNF- α -mediated hepatocyte apoptosis in the steatotic liver induced by an HFD and that liver fibrosis develops in the fatty liver in which hepatocyte apoptosis has been induced. In the well-

established NASH model developed using the choline-deficient diet (CDD), we observed hepatocyte ballooning and severe inflammatory cell infiltration together with liver fibrosis (Supporting Fig. S2). However, in the HFD plus GalN model, these effects were milder than those in the CDD model and we did not detect hepatocyte ballooning (Fig. 2A) or ALT elevation (Fig. 2F), suggesting that severe hepatocellular injury is not necessary for the induction of liver fibrosis.

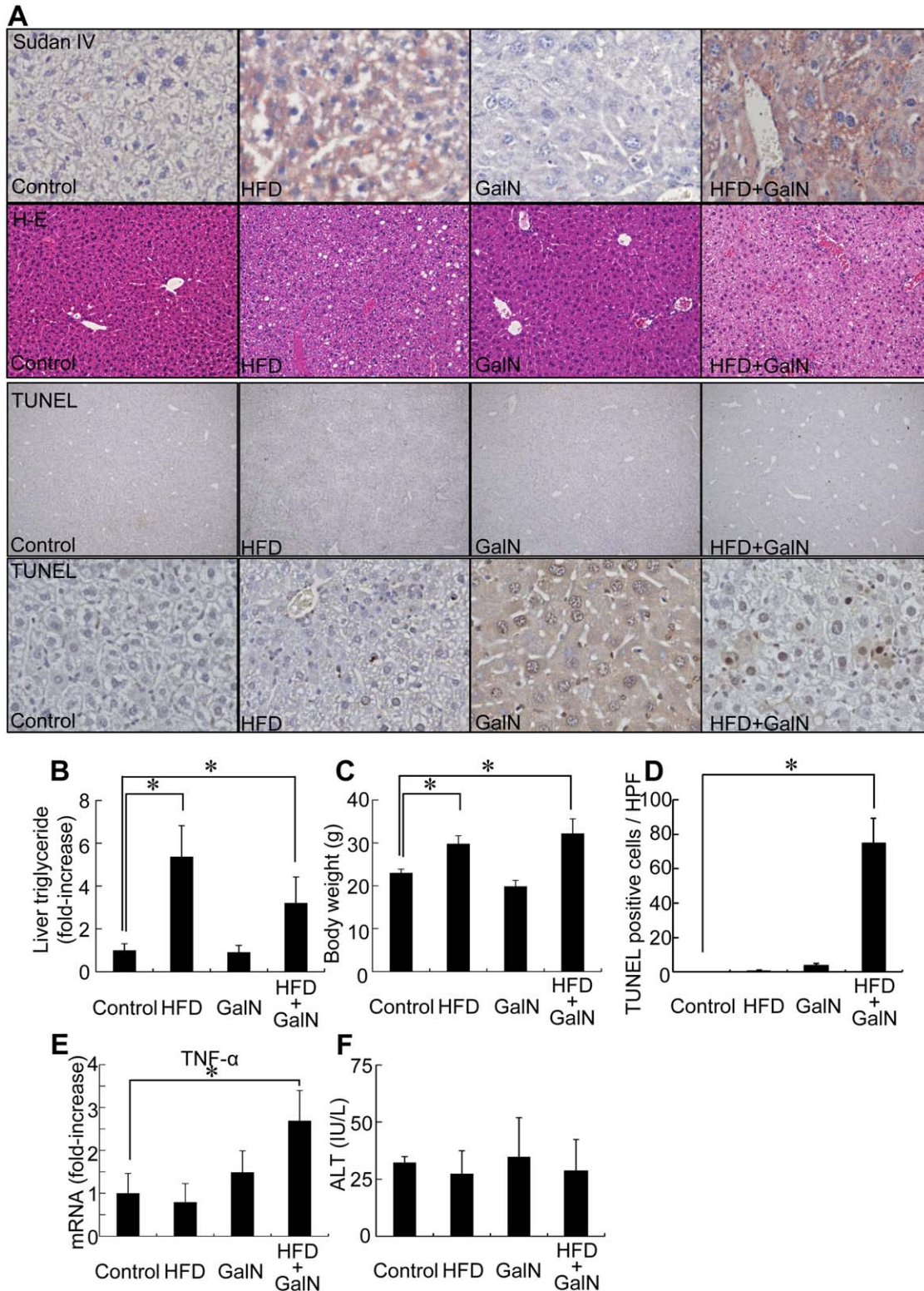


FIG. 2. Hepatocyte apoptosis is induced by GalN administration in HFD-fed mice. Mice were fed a normal diet or an HFD and were or were not intraperitoneally injected with GalN. (A) Hepatic lipid deposition was assessed by staining with Sudan IV (original magnification $\times 600$), and inflammatory cell infiltration and inflammatory foci were assessed using hematoxylin and eosin staining (magnification $\times 200$). Apoptotic nuclei were identified using TUNEL staining (magnification $\times 40$ and $\times 600$). (B) Hepatic lipid content was assessed by measuring triglycerides. (C) Body weight was measured. (D) TUNEL-positive cells identified in high-power fields were counted. (E) mRNA expression of TNF- α in the liver was measured using quantitative real-time reverse-transcription polymerase chain reaction. (F) Serum ALT levels were determined. Results are shown as the means \pm SD of data collected from at least four independent experiments. $*P < 0.05$, Kruskal–Wallis test (B,C,D) and one-way analysis of variance (E,F). Abbreviations: H-E, hematoxylin and eosin; HPF, high-power field; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

LPS AND TNF- α ARE INVOLVED IN HEPATOCYTE APOPTOSIS AND LIVER FIBROSIS IN MICE TREATED WITH AN HFD PLUS GalN

Bacterial overgrowth in the gut microbiota plays a role in the progression to NASH.⁽¹⁰⁾ To examine the involvement of the microbiota in liver fibrosis, we analyzed the LPS concentration in portal blood and the composition of the gut microbiota. In mice treated with an HFD or an HFD plus GalN, the LPS concentration in portal blood was elevated (Supporting Fig. S3A). The composition of the gut microbiota in the HFD and HFD plus GalN groups varied markedly from that in the control or GalN groups (Supporting Fig. S3B), and *Proteobacteria*, which produce LPS, were increased in these groups (Supporting Fig. S3C). Gut sterilization by antimicrobial treatment reduced liver fibrosis and α -SMA expression but did not affect liver steatosis (Fig. 3A-D; Supporting Fig. S1C). Gut sterilization prevented LPS elevation in mice treated with an HFD plus GalN (Fig. 3E). Similarly, TNF- α induction (Fig. 3C) and hepatocyte apoptosis (Fig. 3F) were reduced by the antimicrobial treatment. Moreover, liver fibrosis and hepatocyte apoptosis induced by the HFD plus GalN were inhibited in TNF- α ^{-/-} mice (Fig. 4; Supporting Fig. S1D) and expression of TNF- α was eliminated (Supporting Fig. S4A), whereas steatosis was induced in the TNF- α ^{-/-} mice. These results suggest that TNF- α is a key mediator of hepatocyte apoptosis and liver fibrosis. Thus, we propose that in mice treated with an HFD or HFD plus GalN, dysbiosis in the gut microbiota likely causes an elevation in the amount of LPS in the portal blood, which triggers TNF- α production from liver macrophages by acting through TLR4; the produced TNF- α then induces apoptosis in the GalN-sensitized hepatocytes. This initial hepatocyte apoptosis might result in further induction of TNF- α through macrophage activation, which in turn might induce further apoptosis in GalN-sensitized hepatocytes, mediating liver fibrosis.

HEPATOCYTE APOPTOSIS STIMULATES LIVER FIBROSIS IN MICE TREATED WITH AN HFD PLUS GalN

As noted above, liver fibrosis develops in the fatty liver in which hepatocyte apoptosis has been induced

in mice treated with an HFD plus GalN. Because nuclear factor (NF)- κ B activation prevents TNF- α -mediated hepatocyte apoptosis,⁽³²⁾ we used inhibitor of NF- κ B kinase subunit β (IKK2)^{hep-CA} mice in which NF- κ B was activated in the liver,⁽³³⁾ as determined by an increase in phosphorylated I κ B and the NF- κ B target molecule A1 and a decrease of nonphosphorylated I κ B (Supporting Fig. S4B) as previously reported, to determine whether hepatocyte apoptosis is causally related to liver fibrosis. Untreated IKK2^{hep-CA} mice showed no liver injury or inflammation⁽³⁴⁾ and resisted hepatocyte apoptosis and HSC activation induced by hepatocyte-specific knockout of NF- κ B regulatory subunit IKK-gamma (also known as NEMO).⁽³³⁾ In accordance with these previous reports, untreated IKK2^{hep-CA} mice exhibited no liver fibrosis (data not shown). Notably in these mice, liver fibrosis and hepatocyte apoptosis triggered by treatment with an HFD plus GalN were inhibited despite steatosis induction (Fig. 5; Supporting Fig. S1E). These results suggest that inhibition of hepatocyte apoptosis by hepatocyte-specific expression of constitutively active IKK2 reduced liver fibrosis in mice treated with an HFD plus GalN. Thus, hepatocyte apoptosis stimulates liver fibrosis in this disease model.

CBP DEFICIENCY REDUCES LIVER FIBROSIS INDUCED BY AN HFD PLUS GalN IN MICE

We had previously reported that PRI-724, a selective inhibitor of the CBP/ β -catenin interaction, attenuates CCl₄ or BDL-induced liver fibrosis.⁽²⁶⁾ Similarly, PRI-724 treatment reduced the liver fibrosis induced by an HFD plus GalN despite steatosis induction (Supporting Fig. S5). As β -catenin associates with TNF- α -mediated hepatocyte apoptosis,⁽²⁵⁾ we next examined the contribution of CBP/ β -catenin signaling in our model. In the liver of mice treated with an HFD plus GalN, expression levels of Wnt/ β -catenin target genes were increased compared to levels in control diet-fed mice (Fig. 6A). mRNA expression of glutamine synthetase and S100A4, which are regulated by β -catenin signaling, was increased in the liver of mice treated with an HFD plus GalN; however, an HFD or GalN alone did not affect the expression (Fig. 6B), suggesting that β -catenin signaling is activated in livers in which hepatocyte apoptosis and liver fibrosis are induced.

Glutamine synthetase and S100A4 protein expression were increased and were observed in parenchymal

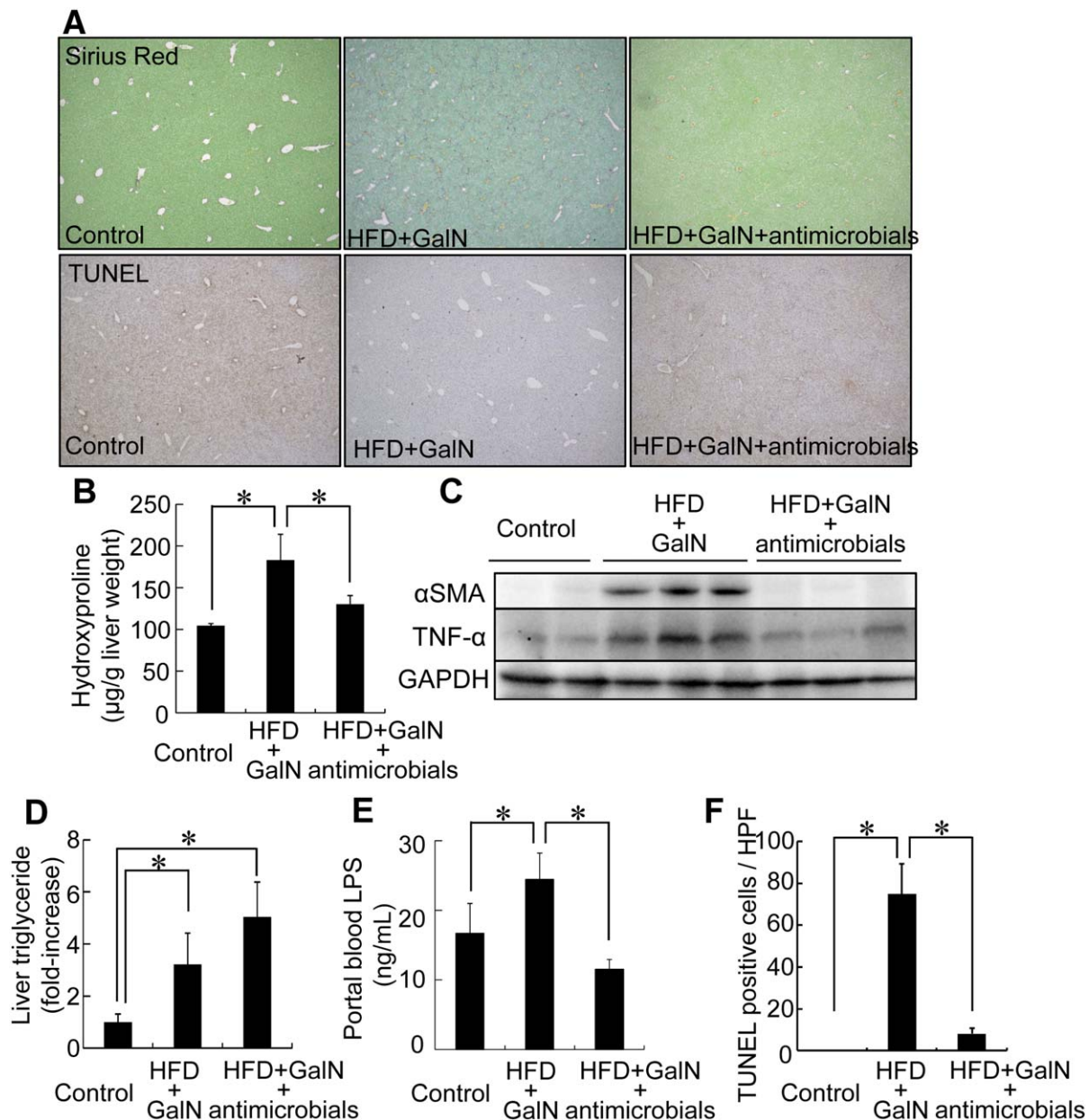


FIG. 3. Gut sterilization attenuates liver fibrosis induced by an HFD plus GalN. Mice were treated with an HFD plus GalN supplemented with or without antimicrobials for 8 weeks. (A,B) Collagen deposition was assessed by staining with sirius red (A, upper panels; original magnification $\times 40$) and by measuring the hydroxyproline content (B). (C) Liver protein extracts were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with antibodies against α -SMA, TNF- α , and GAPDH. (D) Hepatic lipid content was assessed through triglyceride measurement. (E) LPS levels in portal blood were measured. (A,F) Apoptotic nuclei were identified using TUNEL staining (A, lower panels; magnification $\times 40$). TUNEL-positive cells identified in high-power fields were counted (F). Results are presented as the means \pm SD of data collected from at least four independent experiments. * $P < 0.05$, Kruskal–Wallis test (B,D,F) and one-way analysis of variance (E). Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPF, high-power field; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

and nonparenchymal cells, respectively (Fig. 6C,D). This suggests that β -catenin signaling is activated in both parenchymal and nonparenchymal cells. To directly investigate the involvement of β -catenin

signaling in liver fibrosis, hepatocyte specific β -catenin knockout (β -catenin^{hep-KO}) mice were treated with an HFD plus GalN. Here, liver fibrosis, infiltration of inflammatory cells, and hepatocyte apoptosis were

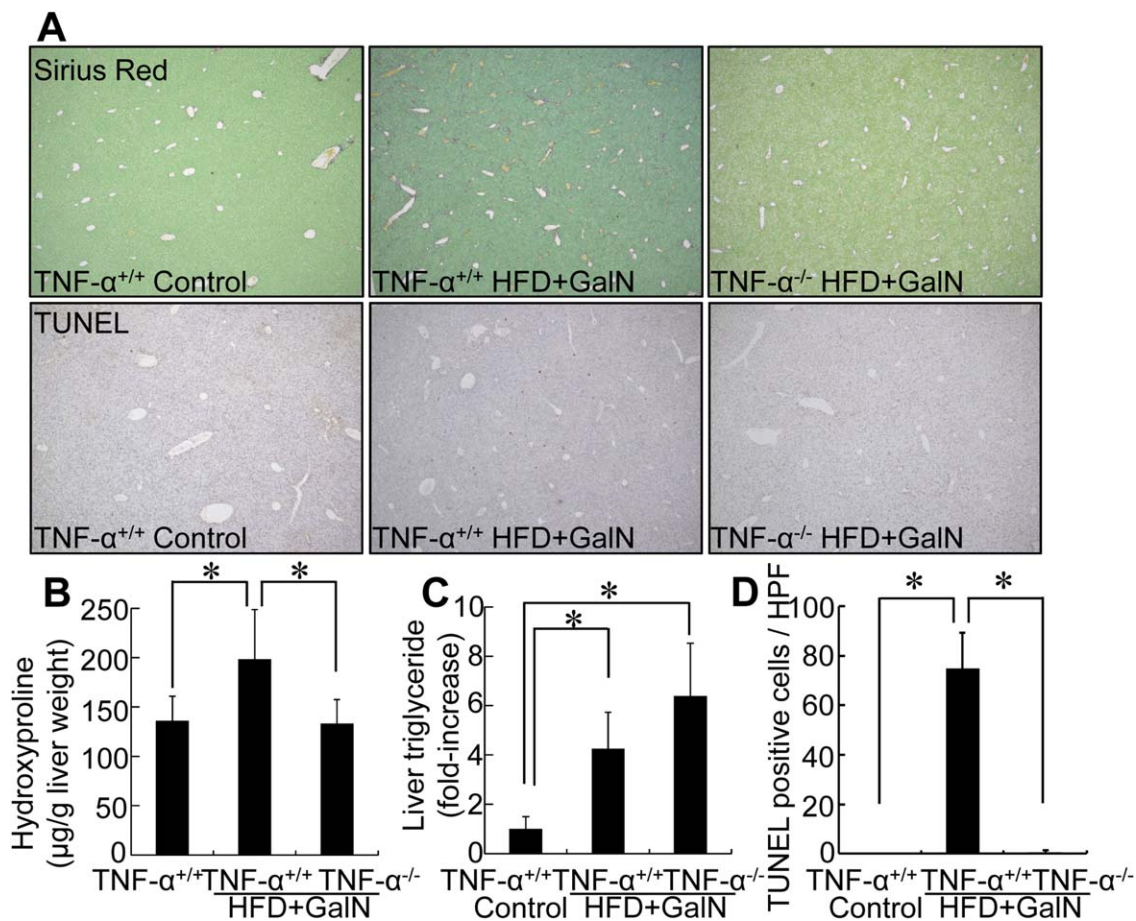


FIG. 4. TNF- α deficiency reduces liver fibrosis and hepatocyte apoptosis induced by an HFD plus GalN. TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice were treated with an HFD plus GalN. (A,B) Collagen deposition was assessed using sirius red staining (A, upper panels; original magnification $\times 40$) and by measuring the hydroxyproline content (B). (C) Hepatic lipid content was assessed by measuring triglycerides. (A,D) Apoptotic nuclei were identified using TUNEL staining (A, lower panels; magnification $\times 40$). TUNEL-positive cells identified in high-power fields were counted (D). Results are shown as the means \pm SD of data collected from at least four independent experiments. * $P < 0.05$, one-way analysis of variance (B) and Kruskal–Wallis test (C,D). Abbreviations: HPF, high-power field; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

attenuated (Supporting Fig. S6A), although fibrosis in the steatotic liver could not be evaluated using these mice because liver triglyceride concentration and body weight did not increase relative to the control (Supporting Fig. S6B,C).

Metabolic abnormality might exist in β -catenin^{hep-KO} mice, and so we developed conditional CBP-knockout mice. CBP^{hep-KO} mice, in which expression of CBP was eliminated in hepatocytes (Supporting Fig. S4C), showed reduced liver fibrosis (Fig. 7A,B; Supporting Fig. S1F) accompanied by a lack of α -SMA expression (Fig. 7C) compared to control CBP-flox mice after treatment with an HFD plus GalN. In addition, induction of hepatocyte apoptosis was also attenuated

in the CBP^{hep-KO} mice (Fig. 7A,D). Moreover, the increase in glutamine synthetase and S100A4 expression in the liver induced by an HFD plus GalN was inhibited in these mice. These results suggest that CBP knockout in hepatocytes results in the suppression of hepatocyte apoptosis and of subsequent liver fibrosis. Furthermore, CBP^{fibro-KO} mice, in which expression of CBP was eliminated in activated HSCs (Supporting Fig. S4D), also showed reduced liver fibrosis (Fig. 7A,B) accompanied by a lack of increase in α -SMA and S100A4 expression (Fig. 7C). In contrast to CBP^{hep-KO} mice, the CBP^{fibro-KO} mice exhibited hepatocyte apoptosis and glutamine synthetase expression (Fig. 7A,C,D). Thus, the fibrotic pathway

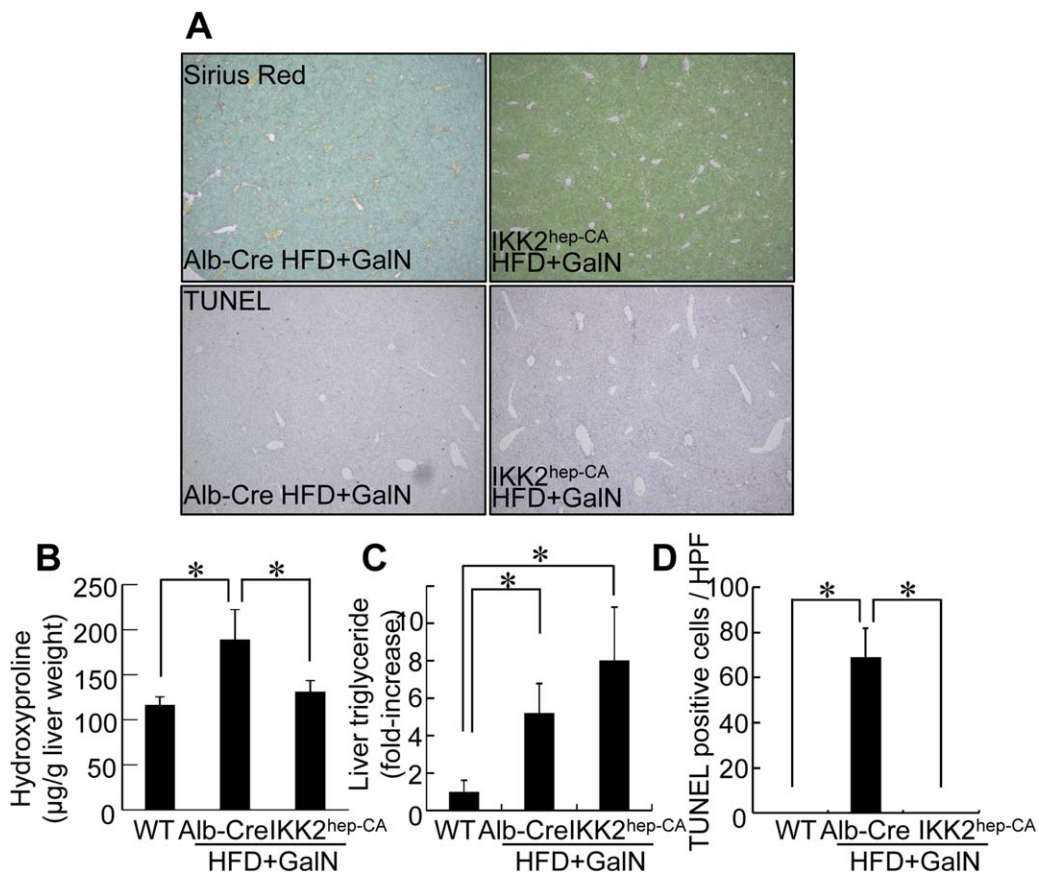


FIG. 5. IKK2^{hep-CA} mice show reduced liver fibrosis and hepatocyte apoptosis induced by an HFD plus GalN. Alb-Cre and IKK2^{hep-CA} mice were treated with an HFD plus GalN. (A,B) Collagen deposition was assessed by staining with sirius red (A, upper panels; original magnification $\times 40$) and by measuring the hydroxyproline content (B). (C) Hepatic lipid content was assessed by measuring triglycerides. (A,D) Apoptotic nuclei were identified using TUNEL staining (A, lower panels; magnification $\times 40$). TUNEL-positive cells identified in high-power fields were counted (D). Results are presented as the means \pm SD of data collected from at least four independent experiments. * $P < 0.05$, one-way analysis of variance (B) and Kruskal-Wallis test (C,D). Abbreviations: Alb-Cre, albumin promoter-driven Cre recombinase; HPF, high-power field; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; WT, wild type.

from hepatocyte apoptosis induced by an HFD plus GalN was blocked at collagen-producing cells, such as activated HSCs in CBP^{fibro-KO} mice. These results suggest that the S100A4-expressing cells were collagen-producing cells.

The activated CBP/ β -catenin complex functions in liver fibrosis both by promoting hepatocyte apoptosis and by activating HSCs. Unlike the aforementioned two conditional knockout mice, macrophage specific CBP-knockout (CBP^{mac-KO}) mice, in which expression of CBP was eliminated in macrophages (Supporting Fig. S4E), showed liver fibrosis and hepatocyte apoptosis at levels comparable to those in CBP-flox mice after treatment with an HFD plus GalN (Fig. 7A,B,D). Increased expression of α -SMA, glutamine

synthetase, and S100A4 in the liver was also observed in these mice. These results suggest a minor role for macrophage CBP/ β -catenin in hepatocyte apoptosis and liver fibrosis.

Furthermore, in contrast to β -catenin^{hep-KO} mice, the three types of conditional CBP-knockout mice showed liver steatosis (Fig. 7E). Elevation of mRNA expression of TNF- α by an HFD plus GalN was observed in the liver of CBP^{fibro-KO} and CBP^{mac-KO} mice. Conversely, there was no significant difference in TNF- α between control and CBP^{hep-KO} mice (Supporting Fig. S7). Because apoptosis was reduced in CBP^{hep-KO} mice, any subsequent additional response, including TNF- α production mediated by initial hepatocyte apoptosis, might be attenuated in these animals.

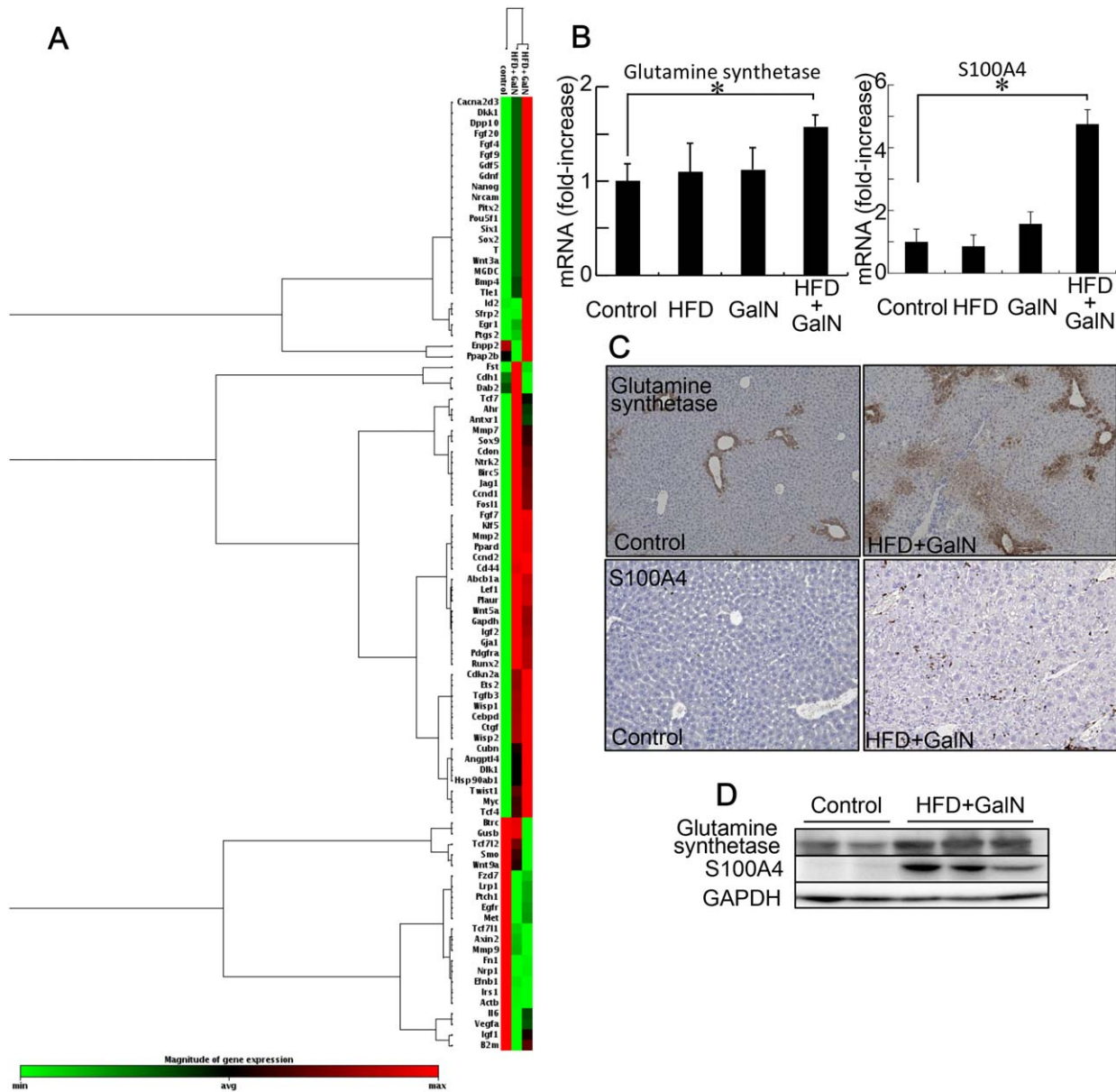


FIG. 6. β -catenin signaling in the liver is activated by an HFD plus GalN. Mice were fed a normal diet or an HFD and were or were not intraperitoneally injected with GalN. (A) mRNA expression levels of the indicated β -catenin target genes in the liver were determined using the RT² Profiler PCR Array. (B) mRNA expression of glutamine synthetase and S100A4 in the liver was determined using quantitative real-time RT-PCR. (C,D) Expression of glutamine synthetase and S100A4 was examined by performing immunohistochemical staining (C, upper panels; original magnification $\times 100$, lower panels; $\times 200$) and western blotting (D) with specific antibodies. Liver protein extracts were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then immunoblotted with antibodies against glutamine synthetase, S100A4, and GAPDH. Results are shown as the means \pm SD of data collected from at least four independent experiments. * $P < 0.05$, one-way analysis of variance. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse-transcription polymerase chain reaction.

Discussion

In this study, we investigated the contribution of hepatocyte apoptosis to the progression of liver fibrosis

in steatotic liver in mice. Our results suggest that hepatocyte apoptosis stimulates liver fibrosis and that the CBP/ β -catenin complex functions in liver fibrosis by promoting hepatocyte apoptosis and activating HSCs.

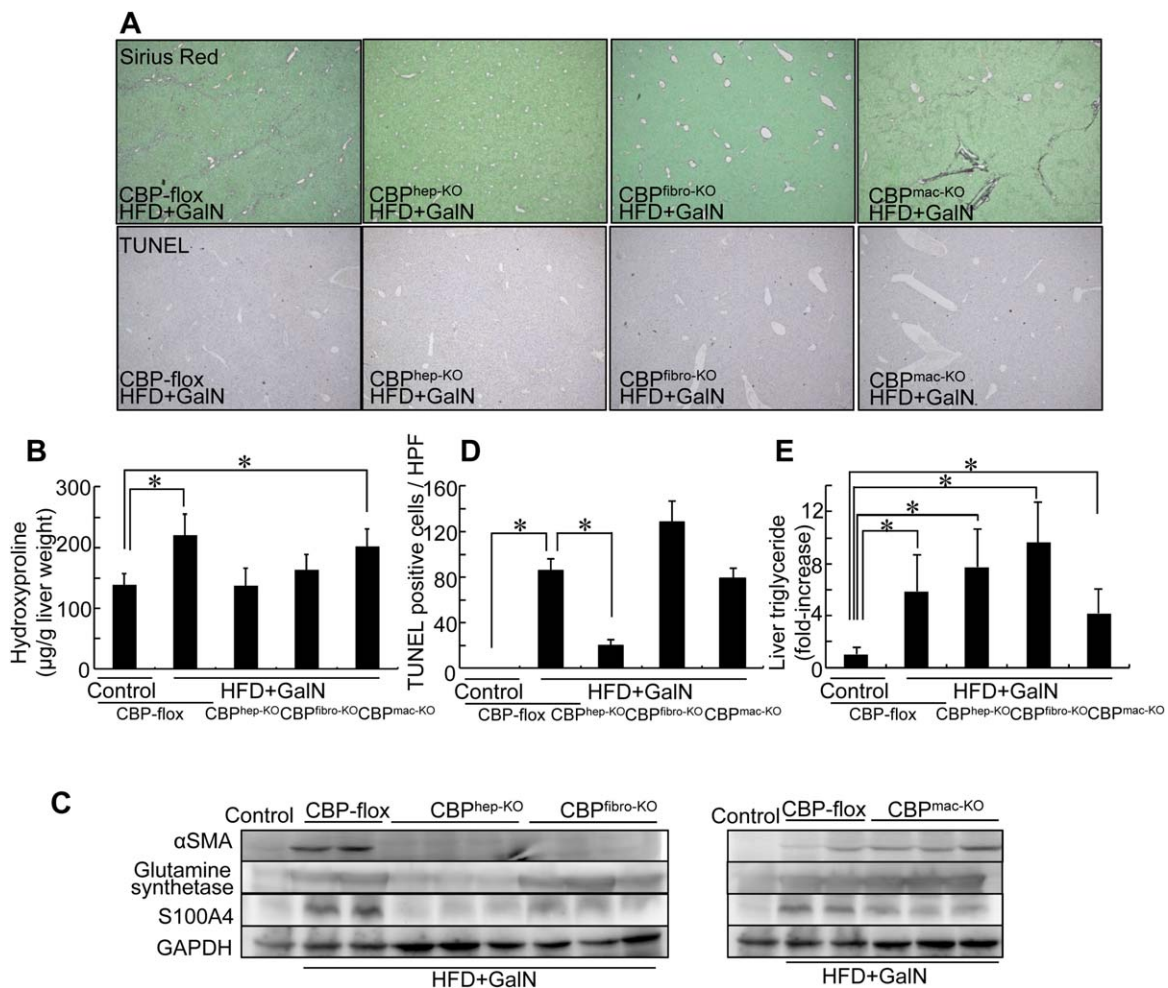


FIG. 7. CBP^{hep-KO} and CBP^{fibro-KO} mice show reduced liver fibrosis induced by an HFD plus GalN. CBP-flox, CBP^{hep-KO}, CBP^{fibro-KO}, and CBP^{mac-KO} mice were treated with an HFD plus GalN. (A,B) Collagen deposition was assessed through sirius red staining (A, upper panels; original magnification $\times 40$) and by measuring the hydroxyproline content (B). (C) Liver protein extracts were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then immunoblotted with antibodies against α -SMA, glutamine synthetase, S100A4, and GAPDH. (A,D) Apoptotic nuclei were identified using TUNEL staining (A, lower panels; magnification $\times 40$). TUNEL-positive cells identified in high-power fields were counted (D). (E) Hepatic lipid content was assessed by measuring triglycerides. Results are presented as the means \pm SD of data collected from at least four independent experiments. * $P < 0.05$, one-way analysis of variance (B) and Kruskal–Wallis test (D,E). Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPF, high-power field; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

These findings offer novel therapeutic possibilities for the treatment of liver fibrosis progression in NASH.

Hepatic steatosis, hepatocyte ballooning, and lobular inflammation are features of NASH.⁽³⁵⁾ Patients with NAFLD and liver fibrosis, regardless of steatohepatitis or NAFLD activity score, present shorter survival times than do those without fibrosis,⁽²⁾ and hepatic fibrosis is the only histologic feature that predicts liver-related mortality in these patients.^(2,36) Thus, fibrosis is the most important therapeutic target in NASH. In the injured liver, initial hepatocyte death stimulates

subsequent inflammatory responses, which lead to further liver injury and fibrosis. HSC activation and fibrogenesis are mediated by a complex crosstalk between damaged parenchymal and nonparenchymal cells, such as macrophages, which activate HSCs.⁽³⁷⁾ Hepatocyte apoptosis has been implicated in disease progression in NAFLD,⁽⁸⁾ and in our model, the induction of TNF- α -mediated hepatocyte apoptosis by GalN in the steatotic liver stimulated liver fibrosis. In contrast to other well-established NASH models (such as those induced using MCDD, CDD, a high-fat high-cholesterol diet,

and a high-fat high-carbohydrate diet), treatment with an HFD plus GalN in our model induced liver fibrosis without an increase in ALT. Elevated ALT levels are generally considered to represent increased risk of NASH. However, the ALT level does not correlate with hepatocyte apoptosis,⁽³⁸⁾ and patients with NAFLD presenting normal levels of ALT might also exhibit severe fibrosis.⁽³⁹⁻⁴¹⁾ These reports and our results suggest that for the induction of liver fibrosis, hepatocellular injury accompanied by a deviation of liver enzyme levels is not necessary whereas hepatocyte apoptosis is sufficient. Intestinal dysbiosis is implicated in disease progression in NAFLD, and gut-derived LPS directly affects macrophages and HSCs,⁽⁴²⁾ which results in liver fibrosis. Accordingly, gut sterilization ameliorated liver fibrosis and concurrently reduced hepatocyte apoptosis in mice treated with an HFD plus GalN. Thus, the gut microbiota-derived LPS in portal blood increase TNF- α , which induces apoptosis in the hepatocytes sensitized by GalN. However, in mice treated with an HFD alone, TNF- α expression was not increased in the liver despite the occurrence of LPS elevation in the portal blood, which suggests that HFD-induced intestinal dysbiosis is not adequate for triggering TNF- α production and hepatocyte apoptosis. Damage-associated molecular-pattern molecules are released from immunogenic apoptotic cells and stimulate macrophages⁽⁴³⁾ that produce proinflammatory cytokines, such as TNF- α .⁽¹³⁾ Thus, initial hepatocyte apoptosis might result in further induction of TNF- α -mediated apoptosis through macrophage activation in addition to the response to LPS. Consistent with this concept, the elevation of TNF- α by an HFD plus GalN was not observed in the liver of CBP^{hep-KO} mice in which hepatocyte apoptosis was attenuated.

Because simple steatosis does not sensitize hepatocytes,⁽²⁰⁾ the process of sensitization to apoptosis in patients with NASH is unclear. GalN has been reported to block hepatic mRNA transcription by metabolic depletion of uridine nucleotides,^(44,45) leading to transcriptional inhibition of anti-apoptotic molecules. Based on RNA fingerprinting analysis using arbitrarily primed polymerase chain reaction, we previously reported that the majority of complementary DNA bands do not display significant changes in the liver after GalN treatment, indicating that expression of the majority of the mRNA population in the liver is not influenced by GalN treatment.⁽⁴⁶⁾ In addition, GalN treatment increases antioxidant selenoprotein P and glutathione peroxidase-1 expression in the liver, and H₂O₂ treatment sensitizes Huh-7 cells against

TNF- α -induced apoptosis, suggesting possible involvement of reactive oxygen species in the sensitization.⁽⁴⁶⁾ Thus, reactive oxygen species may contribute to the sensitization mechanisms for patients with NASH.

As indicated by the preceding discussion, control of hepatocyte apoptosis and liver fibrosis is crucial for the treatment of NASH. We previously reported that PRI-724, an inhibitor of CBP/ β -catenin interaction, reduces liver fibrosis mediated by BDL, CCl₄,⁽²⁶⁾ or hepatitis C virus⁽²⁷⁾ in mice and directly prevents HSC activation, which results in a reduction of collagen expression *in vitro*.⁽²⁶⁾ In accordance with our previous results, CBP^{fibro-KO} mice showed reduced liver fibrosis after treatment with an HFD plus GalN, which indicates that the activation of CBP/ β -catenin signaling stimulates collagen production by HSCs. Liver macrophages contribute to both liver fibrosis and fibrosis resolution,^(28,47,48) and PRI-724 likely enhances the production of matrix metalloproteinases from M1 macrophages, which accelerates fibrosis resolution.⁽²⁶⁾ However, in CBP^{mac-KO} mice, hepatocyte apoptosis and liver fibrosis were comparable to those in control CBP-flox mice, which suggests that CBP/ β -catenin signaling in macrophages plays a minor role in the model used here. In addition to the role of CBP in fibroblasts, our results revealed the involvement of CBP/ β -catenin signaling in hepatocyte apoptosis after treatment with an HFD plus GalN. β -catenin^{hep-KO} mice are resistant to TNF- α -mediated hepatocyte apoptosis because of increased NF- κ B activation.⁽²⁵⁾ However, an inhibitor of CBP/ β -catenin interaction, ICG-001, reduced NF- κ B activation in the hepatoma cell line Hep3B,⁽²⁵⁾ which suggests that the anti-apoptotic effect in CBP^{hep-KO} mice does not depend on NF- κ B activation. In addition to inactivation of NF- κ B, various other pro-apoptotic mechanisms of β -catenin have been reported. In melanoma cells, activation of β -catenin signaling enhances the apoptosis induced by TNF-receptor death-inducing ligand, and this is accompanied by increased levels of pro-apoptotic molecules BCL2L1 and BBC3 and a decreased level of the anti-apoptotic molecule MCL1.⁽⁴⁹⁾ Moreover, conditional expression of an active form of β -catenin increases apoptosis frequency in hematopoietic stem/progenitor cells.⁽⁵⁰⁾ Thus, anti-apoptotic mechanisms of some type potentially exist in CBP^{hep-KO} mice.

Our data do not reveal the source of TNF- α , and the roles of macrophages in liver fibrosis in the HFD plus GalN model have not been investigated. The mechanisms of β -catenin activation in the liver of mice

treated with an HFD plus GalN also remain unclear as does the mechanism by which CBP knockout prevents hepatocyte apoptosis and HSC activation. Thus, further investigation is required to resolve these uncertainties. In conclusion, induction of TNF- α -mediated hepatocyte apoptosis stimulates collagen-producing cells to trigger liver fibrosis in the steatotic liver. The inhibition of CBP/ β -catenin signaling attenuates the liver fibrosis due to the reduction of hepatocyte apoptosis and suppression of HSC activation. Thus, targeting CBP/ β -catenin represents a new therapeutic strategy for treating fibrosis in NASH.

REFERENCES

- Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; 346:1221-1231.
- Angulo P, Kleiner DE, Dam-Larsen S, Adams LA, Bjornsson ES, Charatcharoenwittaya P, et al. Liver fibrosis, but no other histologic features, is associated with long-term outcomes of patients with nonalcoholic fatty liver disease. *Gastroenterology* 2015;149:389-397.e310.
- Canbay A, Friedman S, Gores GJ. Apoptosis: the nexus of liver injury and fibrosis. *Hepatology* 2004;39:273-278.
- Takehara T, Tatsumi T, Suzuki T, Rucker EB 3rd, Hennighausen L, Jinushi M, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. *Gastroenterology* 2004;127:1189-1197.
- Feldstein AE, Canbay A, Angulo P, Taniai M, Burgart LJ, Lindor KD, et al. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology* 2003;125:437-443.
- Wieckowska A, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *Hepatology* 2006;44:27-33.
- Alkhoufi N, Carter-Kent C, Feldstein AE. Apoptosis in nonalcoholic fatty liver disease: diagnostic and therapeutic implications. *Expert Rev Gastroenterol Hepatol* 2011;5:201-212.
- Feldstein A, Gores GJ. Steatohepatitis and apoptosis: therapeutic implications. *Am J Gastroenterol* 2004;99:1718-1719.
- Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* 2010;52:1836-1846.
- Miura K, Seki E, Ohnishi H, Brenner DA. Role of toll-like receptors and their downstream molecules in the development of nonalcoholic fatty liver disease. *Gastroenterol Res Pract* 2010; 2010:362847.
- Osawa Y, Hoshi M, Yasuda I, Saibara T, Moriwaki H, Kozawa O. Tumor necrosis factor- α promotes cholestasis-induced liver fibrosis in the mouse through tissue inhibitor of metalloproteinase-1 production in hepatic stellate cells. *PLoS One* 2013;8:e65251.
- Henaoui-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* 2012;482:179-185.
- Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. *Cell Mol Immunol* 2016;13:316-327.
- Luster MI, Germolec DR, Yoshida T, Kayama F, Thompson M. Endotoxin-induced cytokine gene expression and excretion in the liver. *Hepatology* 1994;19:480-488.
- Hui JM, Hodge A, Farrell GC, Kench JG, Kriketos A, George J. Beyond insulin resistance in NASH: TNF- α or adiponectin? *Hepatology* 2004;40:46-54.
- Tomita K, Tamiya G, Ando S, Ohsumi K, Chiyo T, Mizutani A, et al. Tumor necrosis factor alpha signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice. *Gut* 2006;55:415-424.
- Osawa Y, Banno Y, Nagaki M, Brenner DA, Naiki T, Nozawa Y, et al. TNF- α -induced sphingosine 1-phosphate inhibits apoptosis through a phosphatidylinositol 3-kinase/Akt pathway in human hepatocytes. *J Immunol* 2001;167:173-180.
- Kodama Y, Taura K, Miura K, Schnabl B, Osawa Y, Brenner DA. Antiapoptotic effect of c-Jun N-terminal kinase-1 through Mcl-1 stabilization in TNF-induced hepatocyte apoptosis. *Gastroenterology* 2009;136:1423-1434.
- Nagaki M, Sugiyama A, Osawa Y, Naiki T, Nakashima S, Nozawa Y, et al. Lethal hepatic apoptosis mediated by tumor necrosis factor receptor, unlike Fas-mediated apoptosis, requires hepatocyte sensitization in mice. *J Hepatol* 1999;31:997-1005.
- Mari M, Caballero F, Colell A, Morales A, Caballeria J, Fernandez A, et al. Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. *Cell Metab* 2006;4:185-198.
- Li Z, Yang S, Lin H, Huang J, Watkins PA, Moser AB, et al. Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. *Hepatology* 2003; 37:343-350.
- Feldstein AE, Werneburg NW, Canbay A, Guicciardi ME, Bronk SF, Rydzewski R, et al. Free fatty acids promote hepatic lipotoxicity by stimulating TNF- α expression via a lysosomal pathway. *Hepatology* 2004;40:185-194.
- Clevers H. Wnt/ β -catenin signaling in development and disease. *Cell* 2006;127:469-480.
- Zou SS, Yang W, Yan HX, Yu LX, Li YQ, Wu FQ, et al. Role of β -catenin in regulating the balance between TNF- α and Fas-induced acute liver injury. *Cancer Lett* 2013;335:160-167.
- Nejak-Bowen K, Kikuchi A, Monga SP. β -catenin-NF- κ B interactions in murine hepatocytes: a complex to die for. *Hepatology* 2013;57:763-774.
- Osawa Y, Oboki K, Imamura J, Kojika E, Hayashi Y, Hishima T, et al. Inhibition of cyclic adenosine monophosphate (cAMP)-response element-binding protein (CREB)-binding protein (CBP)/ β -catenin reduces liver fibrosis in mice. *EBioMedicine* 2015;2:1751-1758.
- Tokunaga Y, Osawa Y, Ohtsuki T, Hayashi Y, Yamaji K, Yamane D, et al. Selective inhibitor of Wnt/ β -catenin/CBP signaling ameliorates hepatitis C virus-induced liver fibrosis in mouse model. *Sci Rep* 2017;7:325.
- Osawa Y, Seki E, Adachi M, Suetsugu A, Ito H, Moriwaki H, et al. Role of acid sphingomyelinase of Kupffer cells in cholestatic liver injury in mice. *Hepatology* 2010;51:237-245.
- Osawa Y, Suetsugu A, Matsushima-Nishiwaki R, Yasuda I, Saibara T, Moriwaki H, et al. Liver acid sphingomyelinase inhibits growth of metastatic colon cancer. *J Clin Invest* 2013;123: 834-843.
- Tacke F. Targeting hepatic macrophages to treat liver diseases. *J Hepatol* 2017;66:1300-1312.

- 31) Ishioka M, Miura K, Minami S, Shimura Y, Ohnishi H. Altered gut microbiota composition and immune response in experimental steatohepatitis mouse models. *Dig Dis Sci* 2017;62:396-406.
- 32) Nagaki M, Naiki T, Brenner DA, Osawa Y, Imose M, Hayashi H, et al. Tumor necrosis factor alpha prevents tumor necrosis factor receptor-mediated mouse hepatocyte apoptosis, but not fas-mediated apoptosis: role of nuclear factor-kappaB. *Hepatology* 2000;32:1272-1279.
- 33) Kondylis V, Polykratis A, Ehlken H, Ochoa-Callejero L, Straub BK, Krishna-Subramanian S, et al. NEMO prevents steatohepatitis and hepatocellular carcinoma by inhibiting RIPK1 kinase activity-mediated hepatocyte apoptosis. *Cancer Cell* 2015;28:582-598.
- 34) Lu H, Lei X, Zhang Q. Moderate activation of IKK2-NF-kB in unstressed adult mouse liver induces cytoprotective genes and lipogenesis without apparent signs of inflammation or fibrosis. *BMC Gastroenterol* 2015;15:94.
- 35) Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* 2012;55:2005-2023.
- 36) Younossi ZM, Stepanova M, Rafiq N, Makhlof H, Younossi Z, Agrawal R, et al. Pathologic criteria for nonalcoholic steatohepatitis: interprotocol agreement and ability to predict liver-related mortality. *Hepatology* 2011;53:1874-1882.
- 37) Bataller R, Brenner DA. Hepatic stellate cells as a target for the treatment of liver fibrosis. *Semin Liver Dis* 2001;21:437-451.
- 38) Canbakan B, Senturk H, Canbakan M, Toptas T, Tabak O, Balci H, et al. Is alanine aminotransferase level a surrogate biomarker of hepatic apoptosis in nonalcoholic fatty liver disease? *Biomark Med* 2010;4:205-214.
- 39) Verma S, Jensen D, Hart J, Mohanty SR. Predictive value of ALT levels for non-alcoholic steatohepatitis (NASH) and advanced fibrosis in non-alcoholic fatty liver disease (NAFLD). *Liver Int* 2013;33:1398-1405.
- 40) Fracanzani AL, Valenti L, Bugianesi E, Andreoletti M, Colli A, Vanni E, et al. Risk of severe liver disease in nonalcoholic fatty liver disease with normal aminotransferase levels: a role for insulin resistance and diabetes. *Hepatology* 2008;48:792-798.
- 41) Mofrad P, Contos MJ, Haque M, Sargeant C, Fisher RA, Luketic VA, et al. Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. *Hepatology* 2003;37:1286-1292.
- 42) Leung C, Rivera L, Furness JB, Angus PW. The role of the gut microbiota in NAFLD. *Nat Rev Gastroenterol Hepatol* 2016;13:412-425.
- 43) Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. *Nat Rev Immunol* 2009;9:353-363.
- 44) Decker K, Keppler D. Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. *Rev Physiol Biochem Pharmacol* 1974;77-106.
- 45) Leist M, Gantner F, Bohlinger I, Tiegs G, Germann PG, Wendel A. Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. *Am J Pathol* 1995;146:1220-1234.
- 46) Osawa Y, Nagaki M, Banno Y, Yamada Y, Imose M, Nozawa Y, et al. Possible involvement of reactive oxygen species in D-galactosamine-induced sensitization against tumor necrosis factor-alpha-induced hepatocyte apoptosis. *J Cell Physiol* 2001;187:374-385.
- 47) Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest* 2005;115:56-65.
- 48) Friedman SL. Mac the knife? Macrophages- the double-edged sword of hepatic fibrosis. *J Clin Invest* 2005;115:29-32.
- 49) Zimmerman ZF, Kulikauskas RM, Bomsztyk K, Moon RT, Chien AJ. Activation of Wnt/beta-catenin signaling increases apoptosis in melanoma cells treated with trail. *PLoS One* 2013;8:e69593.
- 50) Ming M, Wang S, Wu W, Senyuk V, Le Beau MM, Nucifora G, et al. Activation of Wnt/beta-catenin protein signaling induces mitochondria-mediated apoptosis in hematopoietic progenitor cells. *J Biol Chem* 2012;287:22683-22690.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1158/full.