

## A High-Cholesterol Diet Exacerbates Liver Fibrosis in Mice via Accumulation of Free Cholesterol in Hepatic Stellate Cells

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**BACKGROUND & AIMS:** Some studies have indicated that dietary cholesterol has a role in the progression of liver fibrosis. We investigated the mechanisms by which dietary cholesterol might contribute to hepatic fibrogenesis. **METHODS:** C57BL/6 mice were fed a high-cholesterol diet or a control diet for 4 weeks; liver fibrosis then was induced by bile-duct ligation or carbon tetrachloride administration. Hepatic stellate cells (HSCs) were isolated from mice fed high-cholesterol diets or from Niemann-Pick type C1-deficient mice, which accumulate intracellular free cholesterol. **RESULTS:** After bile-duct ligation or carbon tetrachloride administration, mice fed high-cholesterol diets had significant increases in liver fibrosis and activation of HSCs compared with mice fed control diets. There were no significant differences in the degree of hepatocellular injury or liver inflammation, including hepatocyte apoptosis or Kupffer cell activation, between mice fed high-cholesterol or control diets. Levels of free cholesterol were much higher in HSCs from mice fed high-cholesterol diets than those fed control diets. In cultured HSCs, accumulation of free cholesterol in HSCs increased levels of Toll-like receptor 4 (TLR4), leading to down-regulation of bone morphogenetic protein and activin membrane-bound inhibitor (a pseudoreceptor for transforming growth factor [TGF] $\beta$ ); the HSCs became sensitized to TGF $\beta$ -induced activation. Liver fibrosis was not aggravated by the high-cholesterol diet in C3H/HeJ mice, which express a mutant form of TLR4; HSCs that express mutant TLR4 were not activated by accumulation of free cholesterol. **CONCLUSIONS:** Dietary cholesterol aggravates liver fibrosis because free cholesterol accumulates in HSCs, leading to increased TLR4 signaling, down-regulation of bone morphogenetic protein and activin membrane-bound inhibitor, and sensitization of HSC to TGF $\beta$ . This pathway might be targeted by antifibrotic therapies.

**Keywords:** Liver Disease; Mouse Model; Dyslipidemia; Lipopolysaccharide.

Liver fibrosis, a condition that indicates the progression of liver diseases, may progress to cirrhosis or hepatocellular carcinoma.<sup>1</sup> For this reason, it is important to thoroughly determine the pathologic mechanisms associated with this disorder.

Dietary factors are likely to be important determinants of liver fibrosis development. Data derived from 9221 participants in the first National Health and Nutrition Examination Survey in the United States showed that higher dietary consumption of cholesterol was associated with a higher risk of cirrhosis or liver cancer in both unadjusted and adjusted analyses.<sup>2</sup>

Several studies also have reported that statins and ezetimibe (cholesterol-lowering agents) improve liver fibrosis in patients with hypercholesterolemia.<sup>3,4</sup> In recent laboratory studies, rodents or rabbits developed liver fibrosis after long-term consumption of a high-cholesterol (HC) diet containing cholic acid (atherogenic diet) or a high-fat HC diet.<sup>5,6</sup> However, experiments using such diets are not suitable for explaining the exact role of cholesterol in the development of liver fibrosis because cholic acid and free fatty acids induce hepatic fibrosis genes,<sup>7</sup> hepatocyte apoptosis, and liver inflammation.<sup>8</sup> Although these studies are part of a growing accumulation of evidence showing the key role of cholesterol in the development and progression of liver fibrosis, the exact role of cholesterol in the mechanisms underlying liver fibrosis remains to be explored.

To clarify the precise impact of cholesterol in the pathophysiology of liver fibrosis, we therefore used experimental models involving administration of HC diets not containing cholic acid or an excessive amount of fatty

**Abbreviations used in this paper:** AcLDL, acetyl low-density lipoprotein; ALT, alanine aminotransferase; Bambi, BMP and activin membrane-bound inhibitor; BDL, bile duct ligation; CCl<sub>4</sub>, carbon tetrachloride; CE, cholesterol ester; compound 58035, acyl-CoA:cholesterol acyltransferase inhibitor 58035; FC, free cholesterol; HC, high cholesterol; HSCs, hepatic stellate cells; KO, knock-out; LPS, lipopolysaccharide; mRNA, messenger RNA; NPC1, Niemann-Pick type C1;  $\alpha$ SMA,  $\alpha$ -smooth muscle actin; TC, total cholesterol; TG, triglyceride; TGF $\beta$ , transforming growth factor  $\beta$ ; TLR4, Toll-like receptor 4; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; WT, wild-type.

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acids to mice in which liver fibrosis was induced by bile duct ligation (BDL) or carbon tetrachloride (CCl<sub>4</sub>) intoxication. Hepatic stellate cells (HSCs), the main producers of extracellular matrix in the fibrotic liver, play a key role in liver fibrosis, although liver fibrosis is strongly associated with some elements of liver injury, including hepatocyte death and Kupffer cell activation.<sup>1</sup> Our results show that consumption of an HC diet caused accumulation of free cholesterol in HSCs, which in turn significantly suppressed the expression of the transforming growth factor- $\beta$  (TGF $\beta$ ) pseudoreceptor bone morphogenetic protein and activin membrane-bound inhibitor (Bambi) through enhancement of Toll-like receptor 4 (TLR4) signaling, leading to aggravation of liver fibrosis.

## Materials and Methods

Please refer to the Supplementary Materials and Methods section for more detailed descriptions.

### Animal Model

Male 8-week-old wild-type (WT) C57BL/6, C3H/HeN, or C3H/HeJ mice were fed an HC (1% wt/wt) diet (TD 92181) or a corresponding control diet (Teklad no. 7001; Harlan Teklad, Madison, WI) for 4 weeks, and then either underwent BDL for 3 weeks, or were given CCl<sub>4</sub> at a dose of 5  $\mu$ L (10% CCl<sub>4</sub> in corn oil)/g body weight, by intraperitoneal injection twice a week for 4 weeks.

### Statistical Analysis

All data are expressed as the means  $\pm$  standard errors of the means. Statistical analyses were performed using the unpaired Student *t* test or 1-way analysis of variance (*P* < .05 was considered significant).

## Results

### HC Diet Significantly Accelerated BDL- and CCl<sub>4</sub>-Induced Liver Fibrosis

C57BL/6 mice were administered either an HC or control diet for 4 weeks and then divided into 2 groups: one group underwent BDL for 3 weeks and the other group received sham treatment. In a similar but separate experiment, mice were fed an HC or control diet for 4 weeks and then divided into 2 groups: one for 4-week treatment with CCl<sub>4</sub> and the other for treatment with corn oil.

The HC diet did not increase mean body or liver weight vs control (Supplementary Table 1). Although the HC diet significantly increased the serum concentration of total cholesterol (TC), no change was noted in serum triglyceride (TG) and glucose levels (Supplementary Table 1). In addition, the HC diet alone was not sufficient to cause hepatic steatosis or liver fibrosis (Figures 1 and 2). However, as shown by Masson trichrome staining and immunohistochemical staining of  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) in liver tissue, as well as by liver hydroxyproline quantitative measurement results, BDL significantly exacerbated liver fibrosis in the HC diet group as compared with the control (Figures 1A and B). The messenger RNA (mRNA) expres-

sions of collagen 1 $\alpha$ 1, collagen 1 $\alpha$ 2, and  $\alpha$ SMA were significantly enhanced with the development of BDL-induced liver fibrosis, which was more evident in the HC diet group than in the control group (Figure 1C). TGF $\beta$  mRNA levels showed no significant differences between the diet groups (Figure 1C). In a similar manner to the BDL model, the murine CCl<sub>4</sub> model of liver fibrosis showed a significant progression of liver fibrosis in the HC diet group vs control (Figures 1D and E). The mRNA expression of collagen 1 $\alpha$ 1, collagen 1 $\alpha$ 2, and  $\alpha$ SMA was significantly promoted as a result of the development of CCl<sub>4</sub>-induced liver fibrosis, and this was seen more clearly in the HC diet group than in the control group (Figure 1F). TGF $\beta$  mRNA levels showed no significant between-group differences (Figure 1F).

### HC Diet Did Not Accelerate BDL- or CCl<sub>4</sub>-Induced Hepatocellular Damage

Hepatic TC levels were increased significantly by HC diet consumption for both the BDL and sham groups (Figure 2A). However, liver TG levels showed no significant difference between the HC and control groups, and HC diet did not cause hepatic steatosis (Figures 1A and 2A).

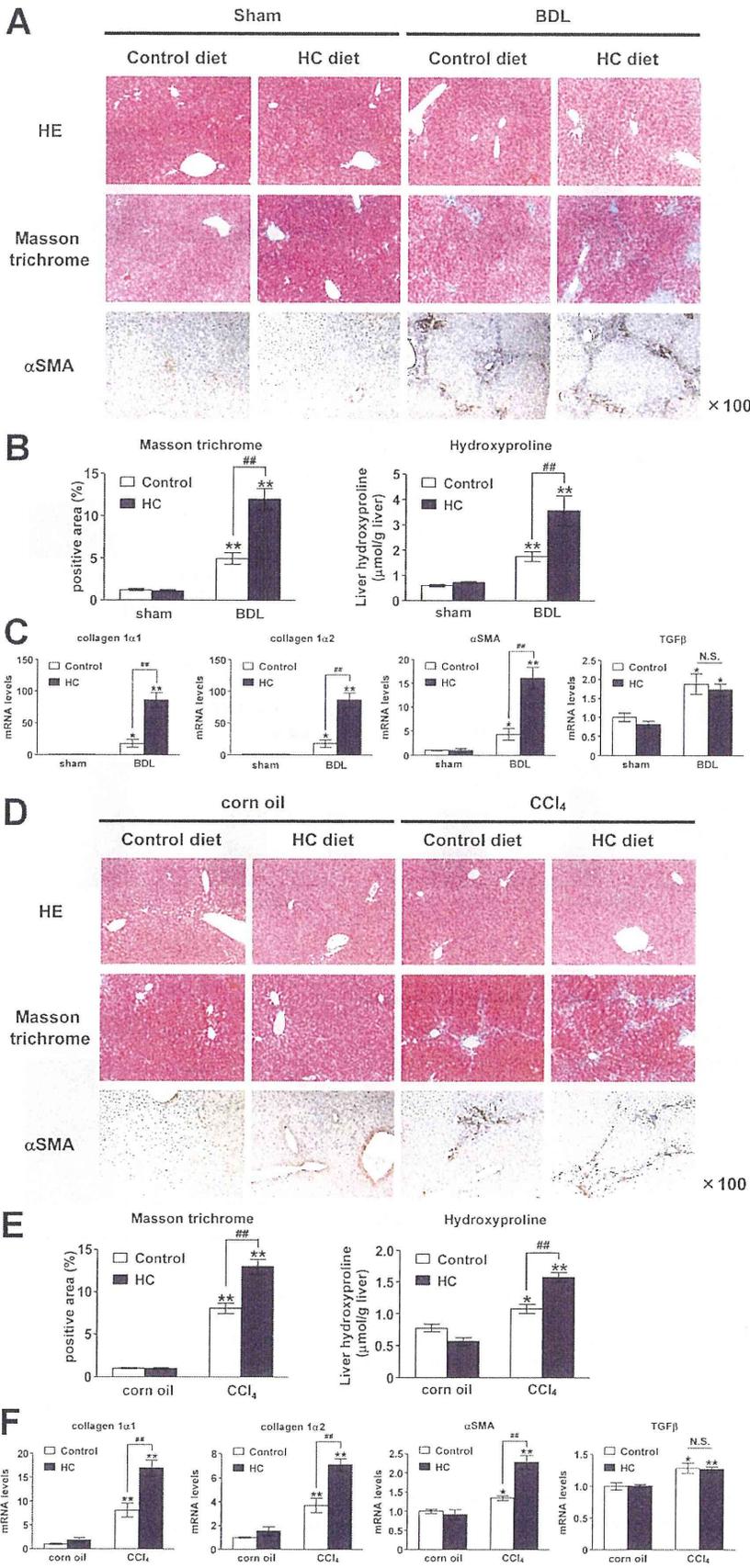
Serum alanine aminotransferase (ALT) levels, a biological marker of hepatocellular damage, were increased significantly in BDL-treated mice; however, this increase was not dependent on dietary cholesterol intake for either the sham or BDL group (Figure 2B). In addition, the HC diet did not significantly impact the mitochondrial inner membrane potentials or the numbers of terminal deoxynucleotidyl transferase-mediated deoxyuridine nick-end labeling-positive hepatocytes in the livers of BDL mice (Figure 2C).

Hepatic TC levels were increased significantly by consumption of the HC diet vs the control diet for both the CCl<sub>4</sub>- and corn oil-treated groups (Figure 2D). However, liver TG levels showed no significant difference between the HC and control groups, and the HC diet did not cause hepatic steatosis (Figures 1D and 2D).

Treatment with CCl<sub>4</sub> significantly increased serum ALT levels relative to treatment with corn oil; however, consumption of the HC diet did not influence serum ALT levels in either the corn oil or CCl<sub>4</sub> group when compared with the control diet (Figure 2E). The HC diet did not significantly change the mitochondrial inner membrane potentials or the numbers of terminal deoxynucleotidyl transferase-mediated deoxyuridine nick-end labeling-positive hepatocytes in the livers of CCl<sub>4</sub>-treated mice (Figure 2F).

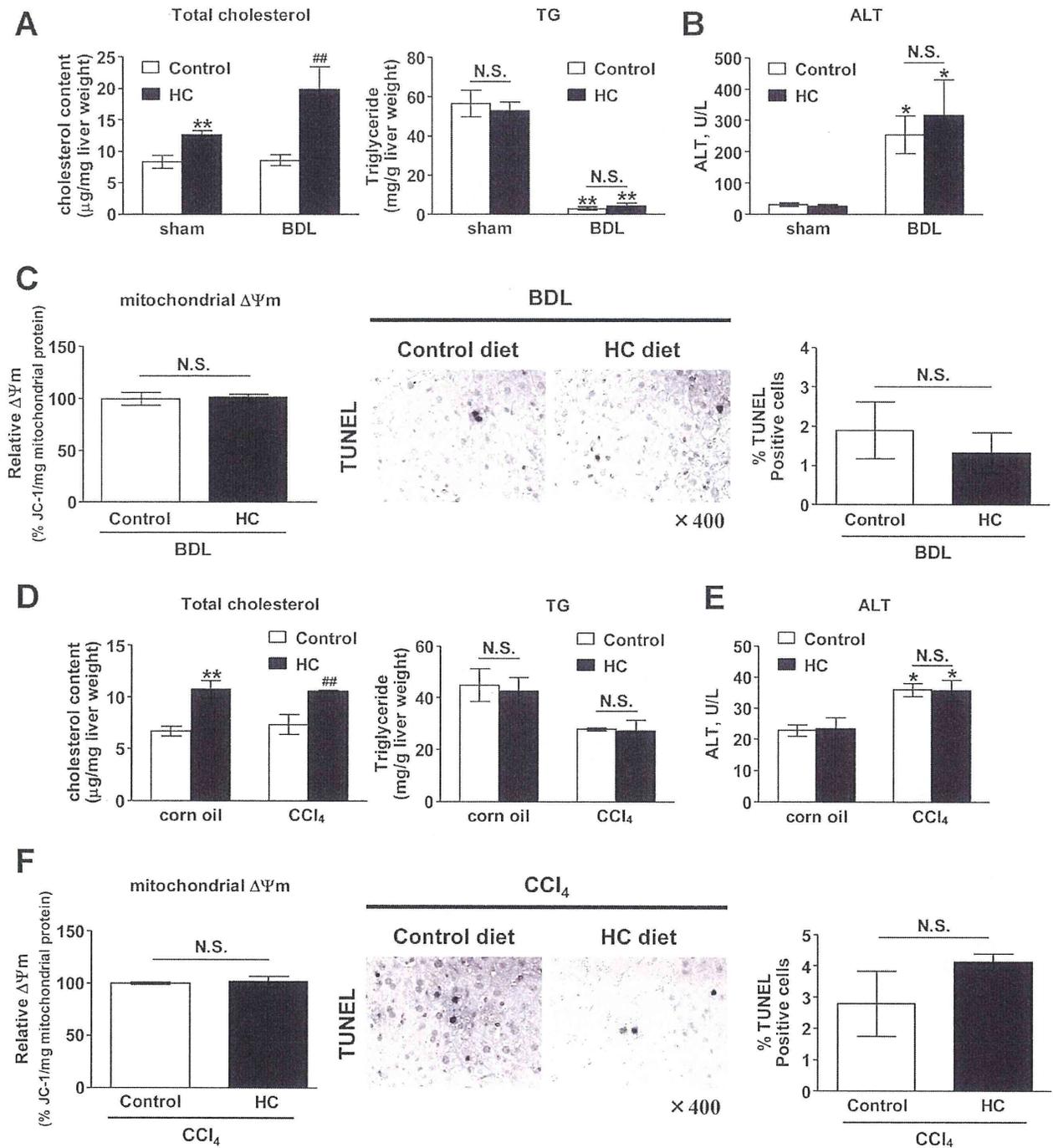
In addition, the HC diet did not aggravate acute BDL- or CCl<sub>4</sub>-induced hepatocellular damage, even at the time point when liver injury is known to peak (Supplementary Figures 1A and B and 2A and B).

These results show that the increase in hepatic cholesterol levels induced by intake of an HC diet did not aggravate BDL- or CCl<sub>4</sub>-induced hepatocellular damage.



**Figure 1.** Effects of the HC diet on liver fibrosis induced by BDL or CCl<sub>4</sub> treatment. After being fed a control or HC diet for 4 weeks, C57BL/6 mice were subjected to (A–C) 3-week BDL or (D–F) CCl<sub>4</sub> treatment twice a week for 4 weeks to induce liver fibrosis (N = 5–7/group). (A and D) H&E-stained sections, Masson trichrome-stained sections, and immunohistochemical detection of  $\alpha$ SMA in representative liver samples. (B and E) Quantification of Masson trichrome staining (left panel), and liver hydroxyproline concentrations (right panel). (C and F) Hepatic expression of collagen1 $\alpha$ 1, collagen1 $\alpha$ 2,  $\alpha$ SMA, and TGF $\beta$  mRNA (N = 5/group). \**P* < .05 and \*\**P* < .01 compared with the (B and C) control diet–sham-operated group or the (E and F) control diet–corn oil group. ##*P* < .01 compared with the (B and C) control diet–BDL group or the (E and F) control diet–CCl<sub>4</sub> group.

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**Figure 2.** Effects of HC diet on hepatocyte injury induced by BDL or CCl<sub>4</sub> treatment. (A and D) Hepatic TC and TG contents (N = 5–7/group). (B and E) Serum ALT activities (N = 5–7/group). (C and F, left panels) Electrochemical proton gradient ( $\Delta\Psi$ ) of the inner mitochondrial membrane (N = 5/group) (C, BDL-treated groups; F, CCl<sub>4</sub>-treated groups). The calculated relative  $\Delta\Psi$  was normalized to the values obtained in mice from the (C) control diet-BDL group or the (F) control diet-CCl<sub>4</sub> group. (C and F, right panels) The percentage of terminal deoxynucleotidyl transferase-mediated deoxyuridine nick-end labeling (TUNEL)-positive hepatocytes and the representative sections (N = 5–7/group). \*P < .05 and \*\*P < .01 compared with the (A and B) control diet–sham-operated group or the (D and E) control diet–corn oil group. ##P < .01 compared with the (A) control diet-BDL group or the (D) control diet-CCl<sub>4</sub> group.

**HC Diet Did Not Impact BDL- or CCl<sub>4</sub>-Induced Kupffer Cell Activation or Liver Inflammation**

Hepatic macrophage infiltration was evaluated by immunohistochemical staining using the Kupffer cell/

macrophage marker F4/80 antibody. The results show BDL-enhanced infiltration of macrophages into the liver in both control- and HC-diet-fed mice. However, consumption of the HC diet did not influence this infiltration (Figure 3A and B). Kupffer cells are the major source of



tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) production in the liver. Although BDL significantly promoted TNF $\alpha$  mRNA expression in the liver, the HC diet did not accelerate TNF $\alpha$  genetic expression. In addition, although BDL significantly increased mRNA expression of vascular cell adhesion molecule-1, intercellular adhesion molecule-1, CD68, and CD11b, this increase was not impacted by consumption of the HC diet (Figure 3C).

Mice treated with CCl<sub>4</sub> showed enhanced hepatic macrophage infiltration, similar to those treated with BDL. However, consumption of the HC diet did not impact the CCl<sub>4</sub>-mediated hepatic macrophage infiltration (Figure 3D and E). Treatment with CCl<sub>4</sub> significantly increased hepatic levels of TNF $\alpha$ , vascular cell adhesion molecule-1, intercellular adhesion molecule-1, CD68, and CD11b mRNA; however, these levels were not influenced by HC diet consumption (Figure 3F). In addition, the HC diet did not influence acute BDL- or CCl<sub>4</sub>-induced liver inflammation, even at the time point when liver inflammation peaks (Supplementary Figures 1C–E and 2C–E).

These results show that the HC diet used in this study did not influence the activation of Kupffer cells or the recruitment of macrophages to the liver. In addition, the HC diet did not impact the BDL- or CCl<sub>4</sub>-mediated infiltration of inflammatory cells such as T cells and neutrophils into the liver (Supplementary Figure 3). H&E staining and immunohistochemical staining for CD68 also showed that the HC diet did not induce the formation of hepatic macrophage foam cells or cause liver inflammation (Figure 1A and D and Supplementary Figure 4).

#### ***HC Diet-Induced Aggravation of Liver Fibrosis Was Kupffer Cell-Independent***

To determine whether the aggravation of liver fibrosis resulting from HC diet consumption required the presence of Kupffer cells, mice depleted of Kupffer cells with liposomal clodronate were treated with BDL or CCl<sub>4</sub> intoxication. In the BDL model, liposomal clodronate achieved almost complete depletion of Kupffer cells (Figure 4A), along with suppression of proinflammatory cytokines such as TNF $\alpha$  and interleukin-1 $\beta$  (Supplementary Figure 5A). Treatment with liposomal clodronate did not impact the BDL-induced hepatocellular injury (Figure 4A, bottom row). In mice treated with liposomal clodronate, intake of the HC diet significantly promoted the BDL-induced aggravation of liver fibrosis (Figure 4B). In agreement with these results, mice treated with liposomal clodronate showed a significant increase in BDL-induced expression of collagen 1 $\alpha$ 1, collagen 1 $\alpha$ 2, and  $\alpha$ SMA in the liver when fed an HC diet (Figure 4C).

Similarly, treatment with liposomal clodronate almost completely depleted Kupffer cells in the CCl<sub>4</sub> model (Figure 4D), and also suppressed proinflammatory cytokines such as TNF $\alpha$  and interleukin-1 $\beta$  (Supplementary Figure 5B). Administration of liposomal clodronate did not impact the CCl<sub>4</sub>-induced hepatocellular injury (Figure 4D, bottom row). In mice infused with liposomal clodronate, the HC diet significantly boosted the CCl<sub>4</sub>-induced pro-

gression of liver fibrosis (Figure 4E). In accord with these findings, mice dosed with liposomal clodronate showed a significant increase in CCl<sub>4</sub>-induced mRNA expression of collagen 1 $\alpha$ 1, collagen 1 $\alpha$ 2, and  $\alpha$ SMA in the liver when administered the HC diet (Figure 4F).

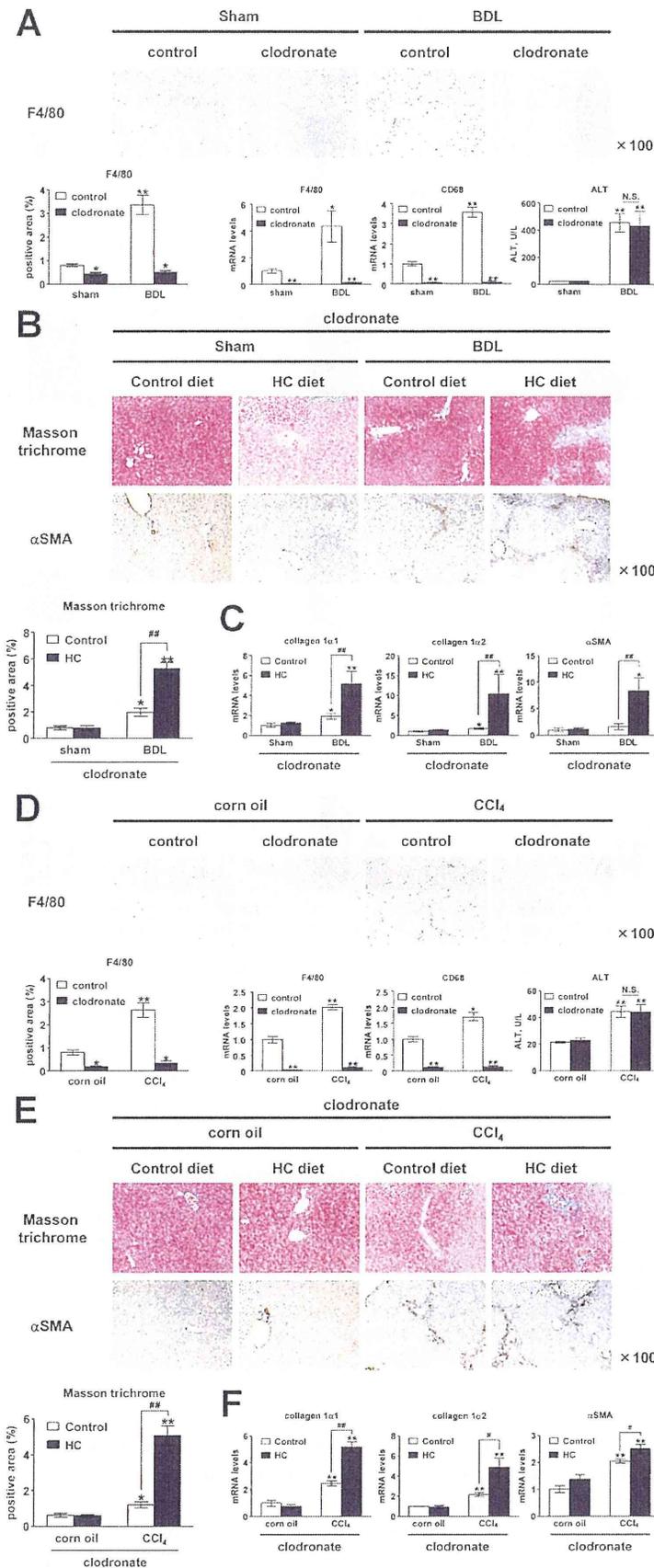
These results suggested that the HC diet promoted BDL- and CCl<sub>4</sub>-induced liver fibrosis in a Kupffer cell-independent manner.

#### ***Accumulation of Free Cholesterol Sensitized HSCs to TGF $\beta$ -Induced Activation***

To examine the effects of the HC diet on HSCs, these cells were isolated from mice given the control or HC diets. With HSCs from the control diet group, the mean ( $\pm$ SD) TC content was  $28.94 \pm 11.55 \mu\text{g}/\text{mg}$  cell protein. In HSCs from the HC diet group, the mean ( $\pm$ SD) TC content was increased significantly to  $59.90 \pm 22.93 \mu\text{g}/\text{mg}$  cell protein. In addition, free cholesterol (FC) and cholesterol ester (CE) levels in HSCs were determined. Consequently, FC levels were significantly higher in the HC diet group HSCs than in those from the control diet group; however, no significant difference was noted in the CE level between groups (Figure 5A).

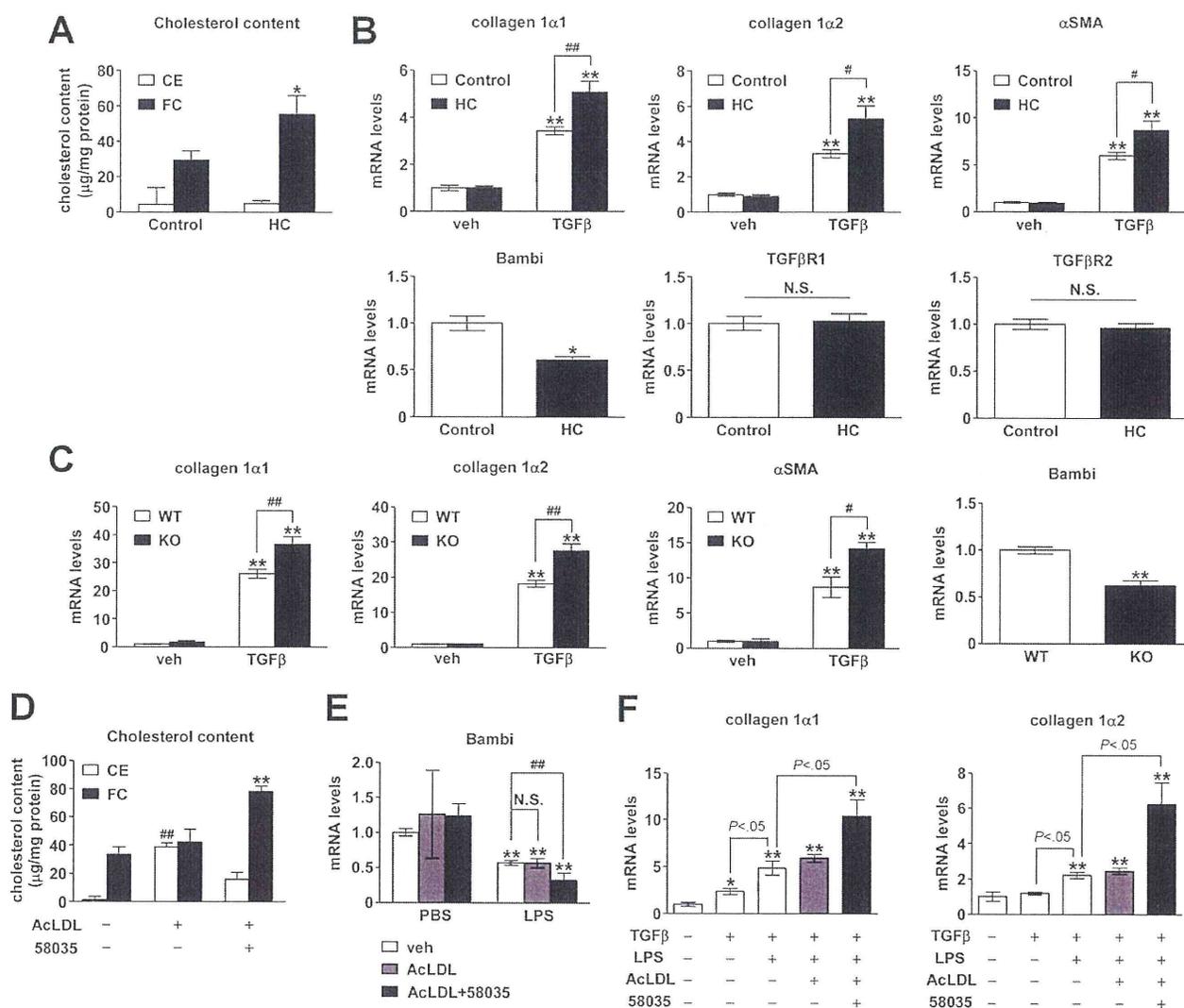
Second, to investigate the effects of HC diet on HSC activation, HSCs isolated from mice from both groups were stimulated with profibrogenic cytokine TGF $\beta$ . Samples of HSCs before treatment with TGF $\beta$ , collected from mice, showed that the HC diet did not affect mRNA levels of collagen 1 $\alpha$ 1, collagen 1 $\alpha$ 2, or  $\alpha$ SMA. Treatment with TGF $\beta$  significantly enhanced the levels of collagen 1 $\alpha$ 1, collagen 1 $\alpha$ 2, and  $\alpha$ SMA mRNA transcripts in HSCs. The enhancing effect was noted more prominently in the HC diet group than in controls (Figure 5B, top row). The HSC expression levels of TGF $\beta$  receptor-1 and TGF $\beta$  receptor-2 (regulating sensitivity to TGF $\beta$ ) and the TGF $\beta$  pseudoreceptor Bambi were compared quantitatively between the 2 diet groups. Expression of Bambi was significantly lower in the HC diet group than the control group (Figure 5B, bottom row), however, no significant difference was observed in the expression levels of the TGF $\beta$  receptors between groups. In accord with these findings, hepatic expression of Bambi mRNA also was significantly lower in the HC diet group than in the control group (Supplementary Figure 6).

Third, the effect of FC on the HSC sensitivity to TGF $\beta$  was evaluated. Niemann-Pick C1 (NPC1) is a late endosomal protein that regulates intracellular cholesterol transport. Homozygous NPC1-deficient cells have been shown to accumulate intracellular FC.<sup>9,10</sup> Therefore, HSCs isolated from NPC1 knock-out (KO) mice were used for analysis. Before treatment with TGF $\beta$ , no significant differences were found between WT and NPC1 KO HSCs in the expression levels of collagen 1 $\alpha$ 1, collagen 1 $\alpha$ 2, or  $\alpha$ SMA mRNA transcripts. Treatment with TGF $\beta$  significantly increased the levels of collagen 1 $\alpha$ 1, collagen 1 $\alpha$ 2, and  $\alpha$ SMA mRNA transcripts. The positive effect was seen more markedly in NPC1 KO HSCs than in WT HSCs (Figure 5C, left three panels). Bambi mRNA levels were



**Figure 4.** Depletion of Kupffer cells does not abrogate the effects of the HC diet on hepatic fibrosis induced by BDL or CCl<sub>4</sub> treatment. WT C57BL/6 mice were injected with liposomal clodronate or vehicle. Thereafter, animals were subjected to (A–C) BDL or (D–F) CCl<sub>4</sub> intoxication to induce liver fibrosis. (A and D, upper panels) Immunohistochemical staining for F4/80. (A and D, lower panels) Quantification of immunohistochemical staining for F4/80 (left). Hepatic expression of F4/80 and CD68 mRNA (N = 4–7/group) (middle), and serum ALT levels (right). \*P < .05 and \*\*P < .01 compared with the (A) vehicle-treated–sham-operated group or the (D) vehicle-treated–corn oil group. (B and E, upper panels) Masson trichrome staining. (B and E, lower panels) Immunohistochemical detection of αSMA. (B and E, middle panels) Quantification of Masson trichrome staining. (C and F) Hepatic expression of collagen 1α1, collagen 1α2, and αSMA mRNA (N = 4–7/group). \*P < .05 and \*\*P < .01 compared with the (B and C) control diet–sham-operated group or the (E and F) control diet–corn oil group. #P < .05 and ##P < .01 compared with the (B and C) control diet–BDL group or the (E and F) control diet–CCl<sub>4</sub> group.

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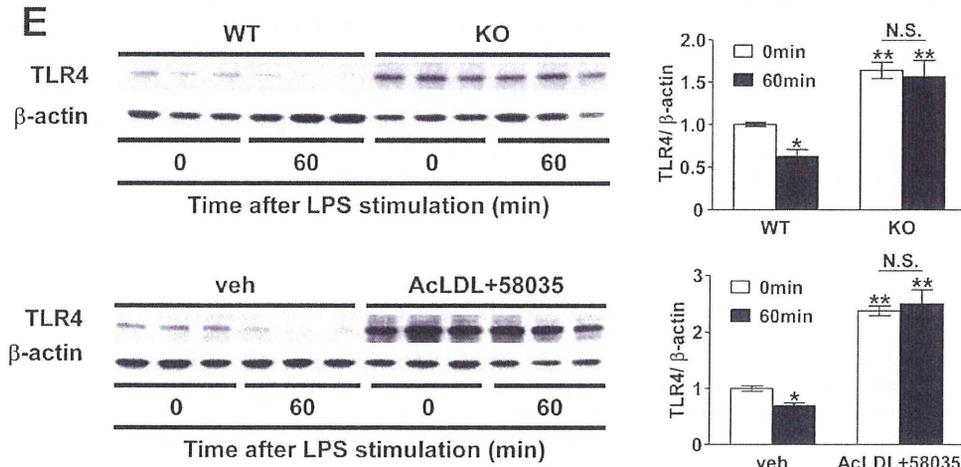
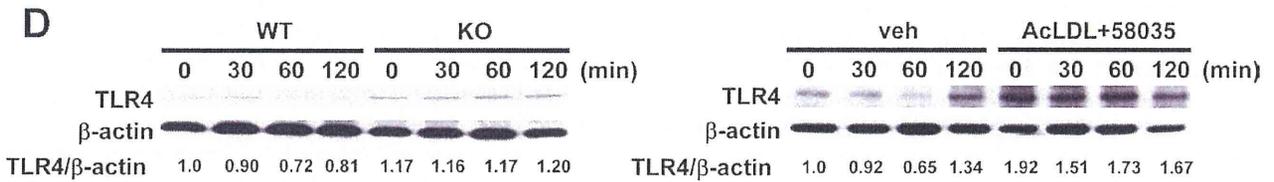
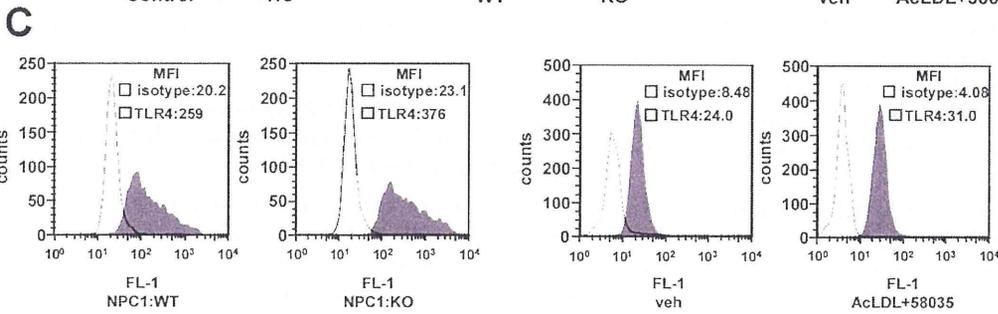
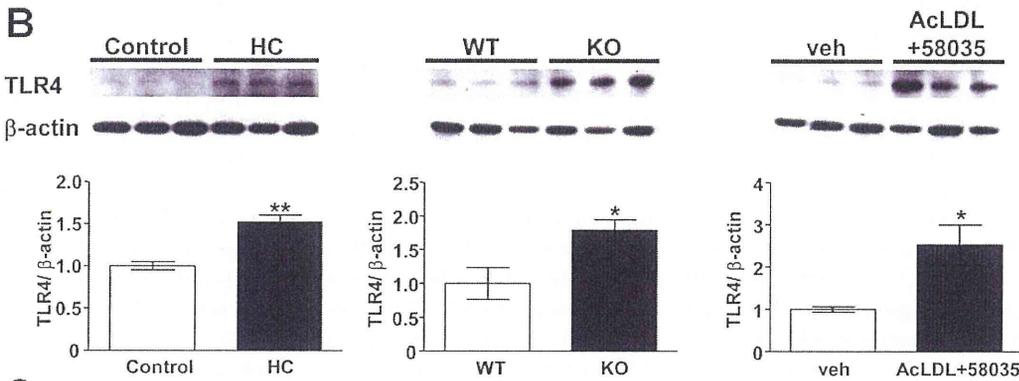
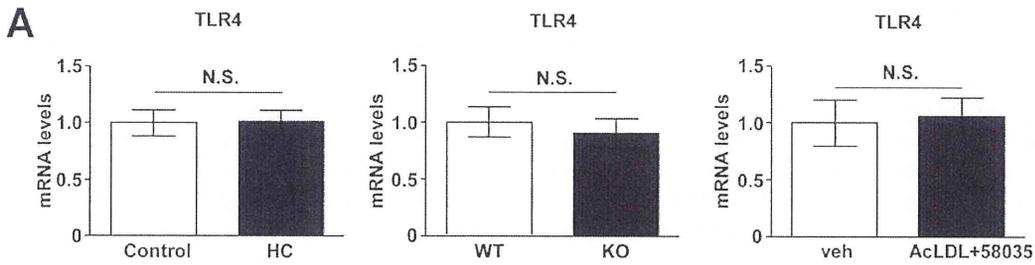


**Figure 5.** FC, but not CE, promotes TGFβ-induced HSC activation. (A) Quantification of cellular FC and CE in HSCs immediately after isolation from control diet-fed or HC diet-fed mice (N = 5–7/group). \*P < .05 vs control-diet group. (B) Expression of (upper panels) collagen 1α1, collagen 1α2, αSMA, and (lower panels) Bambi, TGFβR1, and TGFβR2 mRNA in primary HSC cultures. (Upper panels) Primary HSCs isolated from control diet-fed or HC diet-fed mice were analyzed with real-time polymerase chain reaction (N = 5–7/group). \*P < .05 and \*\*P < .01 vs the control diet–control culture. #P < 0.05 and ##P < 0.01 vs the control diet–TGFβ-treated culture. (C) Expression of (left three panels) collagen 1α1, collagen 1α2, αSMA, and (far right panel) Bambi mRNA in primary HSC cultures. (Left three panels) Primary HSCs isolated from WT mice or NPC1-deficient mice were treated with TGFβ (1 ng/mL) or not for 6 hours. (Far right panel) HSCs separated from WT mice or NPC1-deficient mice were analyzed by real-time polymerase chain reaction (N = 5–7/group). \*\*P < 0.01 vs the WT mice–control culture. #P < .05 and ##P < .01 vs the WT mice–TGFβ-treated culture. (D) Quantification of cellular FC and CE in primary cultured HSCs. HSCs were incubated with vehicle, AcLDL (50 μg/mL), or AcLDL plus compound 58035 (10 μg/mL) for 16 hours (N = 5–7/group). \*\*P < .01 vs cellular FC content in the vehicle-treated culture. ##P < .01 vs cellular CE content in the vehicle-treated culture. (E) Expression of Bambi mRNA in primary HSC cultures. HSCs were incubated with vehicle, AcLDL, or AcLDL plus compound 58035 for 16 hours, and then treated with LPS (100 ng/mL) or not for 6 hours (N = 5–7/group). \*\*P < .01 vs the corresponding culture without LPS treatment in each group. ##P < .01 vs the LPS-treated control culture. (F) Expression of collagen 1α1 and collagen 1α2 mRNA in primary HSC cultures. HSCs were incubated with vehicle, AcLDL, or AcLDL plus compound 58035 for 16 hours, and then treated with LPS (100 ng/mL) or not for 6 hours, before the addition of TGFβ for an additional 6 hours (N = 5–7/group). \*P < .05 and \*\*P < .01 vs the vehicle-treated control culture.

significantly lower in NPC1 KO HSCs relative to WT HSCs (Figure 5C, far right panel). It has been reported that cholesterol accumulates predominantly in late endosomes/lysosomes of cells in NPC1 KO mice.<sup>9</sup> Our study also found that FC levels in late endosomes/lysosomes were significantly higher in NPC1 KO HSCs than in WT HSCs (Supplementary Figure 7A). Similarly, FC levels in

late endosomes/lysosomes were significantly higher in the HSCs from the HC diet group than in those from the control diet group (Supplementary Figure 7B).

It has been reported that FC accumulates in cells treated with the combination of acetyl low-density lipoprotein (AcLDL) and acyl-CoA:cholesterol acyltransferase inhibitor 58035 (compound 58035), whereas CE accumulates in cells



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treated with AcLDL alone.<sup>11</sup> In our study, treatment of HSCs with AcLDL plus compound 58035 significantly increased FC accumulation, and treatment with AcLDL alone significantly promoted CE accumulation (Figure 5D). However, the Bambi expression levels were not decreased when HSCs were treated with either AcLDL alone or the combination of AcLDL and compound 58035 (Figure 5E). The expression of the Bambi gene in HSCs has been reported to depend on TLR4 signaling and decreases with the addition of lipopolysaccharide (LPS).<sup>12</sup> In our study as well, Bambi mRNA expression levels were decreased significantly when HSCs were treated with LPS (Figure 5E). The decrease in Bambi mRNA levels was enhanced significantly when cells were treated with AcLDL plus compound 58035, whereas cells treated with AcLDL alone did not show a significant reduction compared with controls (Figure 5E). The accumulation of FC in HSCs also intensified LPS-mediated TLR4 signal transduction to induce proinflammatory cytokines such as monocyte chemoattractant protein-1 and macrophage inflammatory protein-2 (Supplementary Figures 8A and B), which are known to be up-regulated by the LPS-mediated TLR4 signal pathway in HSCs.<sup>12,13</sup> HSCs pretreated with LPS showed a significant enhancement of collagen 1 $\alpha$ 1 and 1 $\alpha$ 2 mRNA expression when stimulated with TGF $\beta$  (Figure 5F). These cells showed a further increase in the mRNA expression of these genes when loaded with AcLDL plus compound 58035, whereas no significant increase was observed when cells were incubated with AcLDL alone (Figure 5F). These results clearly indicate that FC accumulation in HSCs sensitized them to TGF $\beta$ -induced signals by down-regulating Bambi gene expression.

Unlike HSCs, there were no significant differences in Kupffer cell FC levels between the HC diet group and the control diet group; however, CE levels were significantly higher in Kupffer cells from the HC diet group than in those from the control diet group (Supplementary Figure 9A). An accumulation of CE did not accelerate TNF $\alpha$  mRNA expression in Kupffer cells (Supplementary Figure 9B), nor did it enhance LPS-induced TNF $\alpha$  mRNA expression in these cells (Supplementary Figure 9B). These results show that the HC diet did not cause Kupffer cells to trigger hepatic fibrosis, although it did increase Kupffer cell CE levels.

#### **Accumulation of FC in HSCs Up-Regulated TLR4 Expression**

Consumption of the HC diet did not affect TLR4 mRNA expression levels in HSCs. However, the HC diet

increased the amount of TLR4 protein expressed in HSCs (Figure 6A and B). In addition, increased levels of TLR4 gene expression in terms of the amount of protein (but not mRNA) also were observed in NPC1 KO HSCs and HSCs stimulated with AcLDL plus compound 58035 (Figure 6A and B). Moreover, NPC1 KO HSCs showed higher TLR4 protein membrane expression relative to WT HSCs (Figure 6C). Similar results were obtained for HSCs treated with AcLDL plus compound 58035 (Figure 6C).

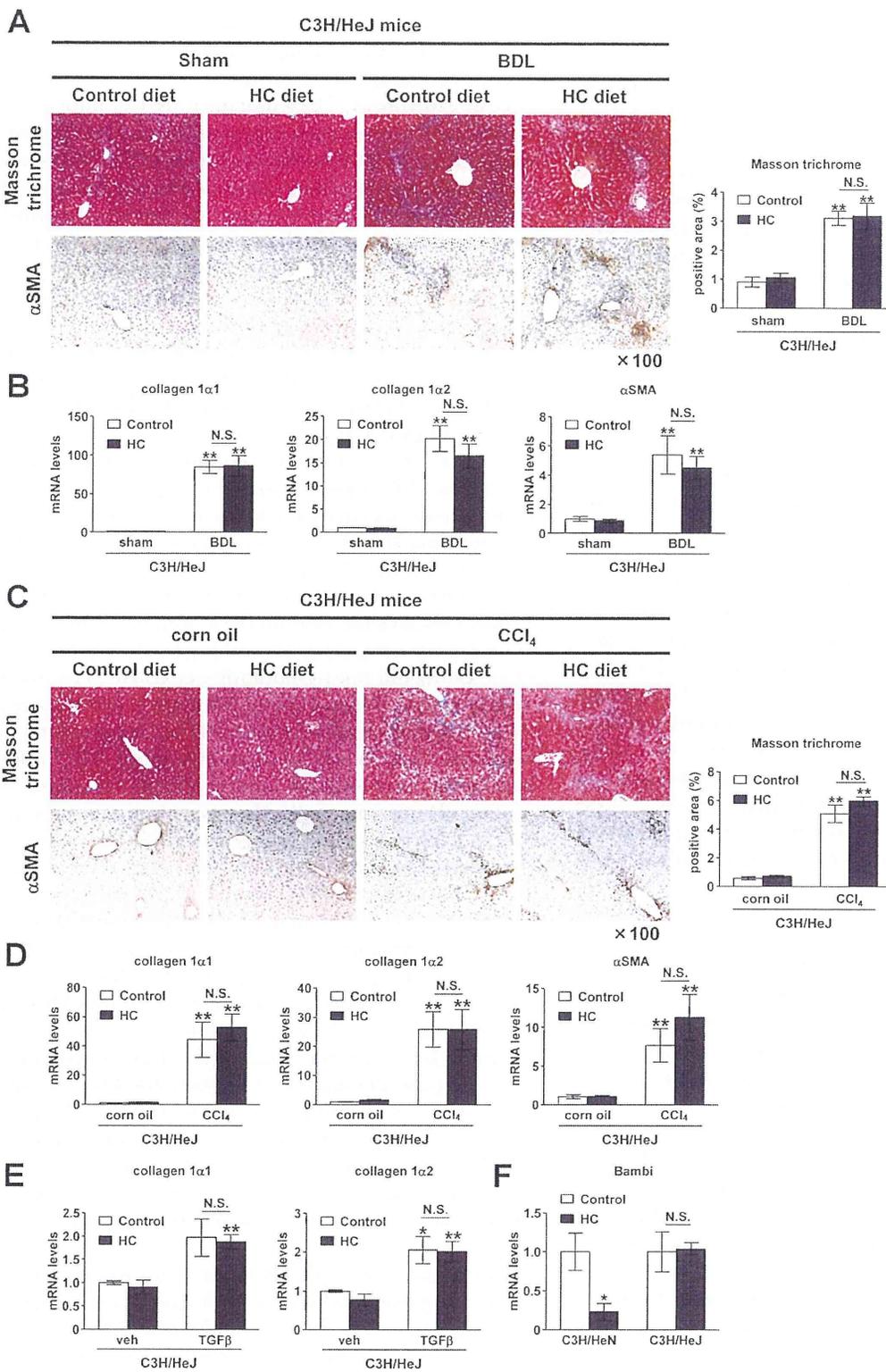
Under normal conditions, membrane proteins are internalized into the cytoplasm by endocytosis, where they are degraded by endosomal-lysosomal or proteasomal pathways. Ligand formation enhances the endocytotic activity, and, consequently, degradation of membrane proteins is accelerated.<sup>14</sup> To investigate the role of FC in TLR4 expression, we examined the dynamic change in the quantity of TLR4 protein in cells treated with LPS. We found that TLR4 protein expression was decreased 60 minutes after LPS treatments in WT HSCs, whereas that in NPC1 KO HSCs remained at a high level after LPS treatments (Figure 6D, left row, and E, top row). Similar results were obtained for HSCs treated with AcLDL plus compound 58035 (Figure 6D, right row, and E, bottom row). These results clearly show that HSC accumulation of FC significantly increased TLR4 protein content. We conjectured that intercellular FC accumulation probably suppressed the ligand-mediated enhanced degradation of TLR4.

#### **HC Diet-Induced Aggravation of Liver Fibrosis Was Dependent on TLR4 Signal Transduction in HSCs**

In the last part of the experiment, we used LPS-unresponsive C3H/HeJ mice (TLR4 mutant) to assess whether HC diet-induced aggravation of liver fibrosis was dependent on TLR4 signal transduction. Unlike the results obtained with WT mice, HC diet consumption did not enhance the progression of BDL-induced liver fibrosis in C3H/HeJ mice (Figure 7A and B). Similarly, the HC diet did not hasten the progression of CCL<sub>4</sub>-induced liver fibrosis (Figure 7C and D).

Next, we examined whether HSC activation by accumulated FC required TLR4 signaling in HSCs. Samples of HSCs were collected from C3H/HeJ mice given the control or HC diet for 4 weeks, and used for study. Treatment with TGF $\beta$  significantly enhanced the levels of collagen 1 $\alpha$ 1, collagen 1 $\alpha$ 2, and  $\alpha$ SMA mRNA transcripts in HSCs. However, unlike the results obtained with WT mouse HSCs, no significant difference was found between the HC and control diet

**Figure 6.** FC enhances protein expression of TLR4 in HSCs. TLR4 (A) mRNA and (B) protein expression (HSCs isolated from control diet-fed or HC-fed mice [left], WT or NPC1-deficient HSCs [middle], vehicle-treated or FC-loaded HSCs [right]); (B, lower panels) Quantification of TLR4 protein expression. \*\**P* < .01 vs control-diet group (left); \**P* < .05 vs WT HSCs (middle); and \**P* < .05 vs vehicle-treated HSCs (right). (C) Fluorescence-activated cell sorter assay of TLR4 expression on plasma membranes of WT or NPC1-deficient HSCs (left) and vehicle-treated or FC-loaded HSCs (right). The mean fluorescence intensity (MFI) is also shown at the upper right corner of each panel. (D) Dynamic changes and (E) quantification of TLR4 protein expression in WT or NPC1-deficient HSCs (D, left panels; E, upper panels), and vehicle-treated or FC-loaded HSCs (D, right panels; E, lower panels) shown at the time after LPS (100 ng/mL) treatment. The relative levels of TLR4 to  $\beta$ -actin are indicated below the corresponding bands. \**P* < .05 and \*\**P* < .01 vs the WT HSCs or vehicle-treated HSCs before LPS treatment. HSCs cultured 6 days after isolation from mice or rats were used (C–E).



**Figure 7.** HC diet-induced aggravation of liver fibrosis was dependent on TLR4 signal transduction in HSCs. TLR4 mutant (C3H/HeJ) mice were fed a control or HC diet for 4 weeks. Then, while remaining on the same diet, animals were subjected to (A and B) BDL or (C and D) CCl<sub>4</sub> intoxication for induction of liver fibrosis (N = 4–7/group). (A and C) Masson trichrome staining (left upper panels) and immunohistochemical staining for α-SMA (left lower panels). Quantification of Masson trichrome staining (right panels). (B and D) Hepatic expression of collagen 1α1, collagen 1α2, and αSMA mRNA (N = 4–7/group). \*\*P < .01 compared with the (B) control diet–sham-operated group or the (D) control diet–corn oil group. (E) Expression of collagen 1α1 and collagen 1α2 mRNA in primary HSCs. HSCs isolated from control diet–fed or HC diet–fed C3H/HeJ mice were treated or not treated with TGFβ (1 ng/mL) for 6 hours (N = 5–7/group). \*P < .05 and \*\*P < .01 vs the control diet–control culture. (F) Expression of Bambi mRNA in primary HSCs isolated from C3H/HeN mice or C3H/HeJ mice, fed a control or an HC diet (N = 5–7/group). \*P < .05 vs the control diet–C3H/HeN mice group.

groups (Figure 7E). Moreover, although HC diet consumption significantly attenuated Bambi gene expression in HSCs isolated from C3H/HeN mice (WT control for C3H/HeJ mice), the HSC Bambi mRNA level was not affected in C3H/HeJ mice (Figure 7F).

These findings clearly show that HC diet-induced aggravation of liver fibrosis was dependent on TLR4 signal transduction. Our study suggested that HC diet consumption attenuated HSC Bambi expression via TLR4 signaling, which led to the aggravation of BDL- or CCl<sub>4</sub>-induced liver fibrosis.

## Discussion

Our present results clearly show that an HC diet aggravated BDL- and CCl<sub>4</sub>-induced liver fibrosis, although an HC diet alone was not sufficient for inducing liver fibrosis. TGF $\beta$ , the most potent factor predisposing to human fibrogenesis, has been shown to play a central role in the pathophysiology of liver fibrosis.<sup>1</sup> Moreover, these results showed that major causes for exacerbation of liver fibrosis involved HSC accumulation of cholesterol in the form of FC, which sensitized HSCs to TGF $\beta$ -induced activation.

Recent research has shown that intracellular FC accumulation increased TLR4 protein levels in the membrane fraction to facilitate TLR4 signaling activation.<sup>15</sup> Our results showed that HSC accumulation of FC increased cytomembrane-bound TLR4 protein levels; however, the amounts of TLR4 mRNA transcripts were similar. Under normal conditions, cytomembrane TLR4 protein molecules are transferred into the cytoplasm by endocytosis, and degraded by endosomal–lysosomal or proteasomal pathways. Degradation of cytomembrane TLR4 proteins is accelerated when internalization of these molecules is promoted by ligand formation. Inhibition of the degradation pathways intensifies ligand-mediated TLR4 signal transduction.<sup>14</sup> In our study, the level of the TLR4 protein in HSCs was lowered significantly in cells incubated with LPS, the major ligand for TLR4. Moreover, the LPS-induced decrease in the HSC level of the TLR4 protein was inhibited prominently by FC accumulated in HSCs. These results suggest that FC accumulated in HSCs inhibited the TLR4 degradation pathway, thereby increasing TLR4 protein levels.

In the present study, FC levels in late endosomes/lysosomes were significantly higher in HSCs from the HC diet group or NPC1 KO mice than in those from the control diet group or WT mice. Recent studies have reported that FC modulated the endosomal–lysosomal pathway of endocytosis through regulation of endosome motility.<sup>16</sup> NPC1 KO mice, which accumulate intracellular FC predominantly in late endosomes/lysosomes, were found to retain amyloid precursor proteins as a result of endosomal dysfunction.<sup>9</sup> NPC1 KO mice also were found to show autophagic–lysosomal dysfunction in the brain.<sup>10</sup> These findings suggest that FC accumulation in HSCs is involved in endosomal–lysosomal dysfunction, leading to TLR4 protein accumulation.

The expression of the TGF $\beta$  pseudoreceptor Bambi in HSCs was solely dependent on TLR4 signaling.<sup>12</sup> The activation of TLR4 signaling in HSCs, which down-regulates the expression of the downstream Bambi gene, was reported to sensitize HSCs to TGF $\beta$ -induced activation, contributing to advancement of liver fibrosis.<sup>12</sup> In our study, HSCs also showed a significantly decreased expression of the Bambi gene when incubated with the TLR4 ligand LPS. The HSC accumulation of FC (not CE) significantly promoted LPS-mediated Bambi down-regulation and markedly accelerated (LPS-mediated) enhance-

ment of HSC sensitivity against TGF $\beta$  signaling. We contend that these changes activated HSCs further, thereby promoting liver fibrosis.

In our experiment, TLR4-mutant C3H/HeJ mice given the HC diet did not show aggravation of liver fibrosis. Accumulation of FC in HSCs collected from TLR4-mutant mice did not give rise to Bambi down-regulation, and no change was observed in the HSC sensitivity against TGF $\beta$  signaling. Based on these results, we concluded that the activation of the TLR4 signal pathway mediated by FC accumulated in HSCs played a critical role in HC diet-induced exacerbation of liver fibrosis.

In the murine liver fibrosis models reported here, consumption of an HC diet neither affected hepatocyte injury nor influenced the pathophysiology of liver inflammation, including Kupffer cell activation.

Marí et al<sup>17</sup> found that FC accumulated in hepatocytes exacerbated LPS-mediated acute liver injury in a manner that induced susceptibility of hepatocytes to TNF $\alpha$ -mediated apoptosis. However, several other researchers claimed that TNF $\alpha$ -mediated hepatocyte apoptosis was not involved in the progression of liver fibrosis,<sup>18,19</sup> and their findings seem to shed light on the reason why cholesterol accumulation in hepatocytes resulting from consumption of an HC diet did not significantly exacerbate hepatocyte damage, as shown in our study. Wouters et al<sup>20</sup> reported that administration of a high-fat HC diet in low-density lipoprotein receptor KO and apolipoprotein E KO mice caused liver inflammation and the transformation of Kupffer cells into foam cells. However, HC diet consumption did not trigger macrophage foam cell formation in the models used in our study. We also clearly showed that an HC diet aggravated BDL- and CCl<sub>4</sub>-induced liver fibrosis in mice depleted of Kupffer cells by administration of clodronate. Altogether, these results suggest that HSCs, rather than hepatocytes or Kupffer cells, should be focused on as the primary site of alterations in liver fibrosis resulting from HC diet consumption.

In summary, our study has provided new insights into the mechanisms linking HC diet uptake and liver fibrosis. The HC diet-induced accumulation of FC in HSCs promoted TLR4 signal transduction by increasing membrane TLR4 levels, and thereby suppressed the HSC expression of the Bambi gene. Consequently, HSC TGF $\beta$  signaling was boosted, resulting in HSC activation and progression of liver fibrosis.

Our present work indicates that in the process of liver fibrosis progression, cholesterol functions as a signal-enhancing factor FC that accumulates in HSCs, rather than as an extracellular activation-inducible factor for HSCs. The findings of this study warrant further investigations that focus on FC in HSCs as the target of new therapeutic strategies for the treatment of liver fibrosis.

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of

*Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: 10.1053/j.gastro.

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T. Teratani and K. Tomita contributed equally to this work and share first authorship.

#### Conflicts of interest

The authors disclose no conflicts.

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## Supplementary Materials and Methods

### Reagents

Reagents were obtained as follows: AcLDL was from Biomedical Technologies (Stoughton, MA). Compound 58035 and LPS were from Sigma (St. Louis, MO). TGF $\beta$  was from R&D Systems (Minneapolis, MN). CCl $_4$  was from Wako Pure Chemical Industries (Osaka, Japan).

### Animal Model

Male WT C57BL/6, C3H/HeN, and C3H/HeJ mice and Sprague-Dawley rats were purchased from Sankyo Laboratories (Tokyo, Japan). NPC1 $^{-/-}$  mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were bred and housed in a temperature- and light-controlled facility with unlimited access to food and water. For BDL, we anesthetized mice and after midline laparotomy we ligated the common bile duct twice with silk sutures and closed the abdomen. We performed the sham surgery similarly, except that the bile duct was not ligated. Mice were killed 3 weeks after BDL. For acute liver injury, mice were fed a control or an HC diet for 4 weeks, and then were killed 5 days after BDL or 24 hours after a single injection of CCl $_4$ . All animals received humane care in compliance with the National Research Council's criteria outlined in the "Guide for the Care and Use of Laboratory Animals," prepared by the US National Academy of Sciences and published by the US National Institutes of Health (Bethesda, MD).

### Biochemical and Histologic Analysis

Serum concentrations of ALT, TGs, glucose, and cholesterol were determined as previously described. Hepatic TG content and liver hydroxyproline concentrations were measured as previously described.<sup>1</sup> Liver cholesterol levels or the cholesterol content of HSCs or Kupffer cells were measured using the Cholesterol/Cholesteryl Ester Quantitation Kit (BioVision, Mountain View, CA), following the manufacturer's instructions. We determined the cholesterol content of HSCs or Kupffer cells immediately after isolation. Liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and stained with H&E and a Masson trichrome solution. For protein or RNA analysis, tissues were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until needed.

### Kupffer Cell Depletion

We injected dichloromethylene diphosphonic acid (clodronate)-loaded or phosphate-buffered saline-loaded liposomes (Encapsula NanoSciences, Nashville, TN) intravenously into mice (200  $\mu\text{L}$  per mouse).

### HSC Isolation and Culture

HSCs were isolated from mice or rats as previously described.<sup>1</sup> We cultured HSCs on uncoated plastic tissue culture dishes in Dulbecco's modified Eagle me-

dium containing 1% or 10% fetal bovine serum, and used them as nonpassaged primary cultures only. For FC accumulation in HSCs, primary HSCs were incubated with AcLDL (50  $\mu\text{g}/\text{mL}$ ) plus compound 58035 (10  $\mu\text{g}/\text{mL}$ ) for 16 hours. We used enzyme-linked immunosorbent assay kits for mouse monocyte chemoattractant protein-1 (Thermo Scientific, Rodkford, IL) or macrophage inflammatory protein-2 (R&D Systems) for quantification of secreted monocyte chemoattractant protein-1 and macrophage inflammatory protein-2 in HSC cultures.

### Kupffer Cell Isolation and Culture

Kupffer cells were isolated from mice, and cultured as previously described.<sup>2</sup>

### Immunohistochemistry

Paraffinized sections were deparaffinized, rehydrated, blocked with normal horse serum, and incubated with anti- $\alpha\text{SMA}$  monoclonal antibody 1A4 (Dako Japan, Kyoto, Japan), anti-F4/80 monoclonal antibody (Serotec, Oxford, UK), or anti-CD3 monoclonal antibody (Abcam, Cambridge, UK) overnight at  $4^{\circ}\text{C}$ . The mouse F4/80 antigen is a 160-kilodalton glycoprotein expressed by mouse macrophages; antimouse F4/80 antibody binds mouse monocytes/macrophages and Kupffer cells. The antigen is not expressed by either lymphocytes or polymorphonuclear cells. Antibody binding was detected by incubation with biotinylated antimouse immunoglobulin G antibody and visualized with a Vectastain Elite ABC Kit (Vector Laboratories, Inc, Burlingame, CA) by reaction with Vectastatin DAB Substrate (Vector).

Fresh-frozen liver sections were cut 6-mm thick on a cryostat, collected on slides, and immediately dried. The sections were fixed with acetone. The slides were incubated overnight with anti-CD68 Ab (Serotec), followed by incubation with Histofine Simple Stain Mouse MAX-PO (Nichirei, Tokyo, Japan) for 1 hour.

### Neutrophil Infiltration

Neutrophils in the liver were stained using the naphthol AS-D chloroacetate esterase technique. Paraffin-embedded liver sections were stained using the naphthol AS-D chloroacetate kit (Sigma Chemical Co, St. Louis, MO) following the manufacturer's instructions.

### Detection of Apoptosis

Terminal deoxynucleotidyl transferase-mediated deoxyuridine nick-end labeling staining (Chemicon International, Temecula, CA) was performed on specimens to assess apoptosis. Apoptosis was quantified by counting positively stained cells in 10 random fields at 200 $\times$  magnification. Apoptosis was measured for each specimen as a percentage of total cells per field. Antibody binding was detected by incubation with biotinylated anti-mouse immunoglobulin G antibody and visualized with a Vectastain Elite ABC Kit (Vector Laboratories, Inc) by reaction with Vectastatin DAB Substrate (Vector).

### *Mitochondrial Isolation and Characterization*

A mitochondrial fraction was enriched from 100-mg liver specimens with the Mitochondria Isolation Kit (Sigma) by 2 consecutive centrifugation steps at 600 g and 11,000 g. The electrochemical proton gradient ( $\Delta\Psi$ ) of the inner mitochondrial membrane was tested by measuring the uptake of the fluorescent carbocyanine dye JC-1 (Sigma) into mitochondria, as specified by the manufacturer.<sup>1</sup> Relative  $\Delta\Psi$  was calculated in comparison with values obtained in control-diet-fed mice.

### *Real-Time Quantitative and Reverse-Transcription Polymerase Chain Reaction Analysis*

Total RNA was extracted from total liver homogenates or HSCs using TaKaRa RNAiso Reagent (TaKaRa Bio, Ohtsu, Japan), according to the manufacturer's instructions. Reverse transcription was performed as previously described.<sup>1</sup> For quantitation of mRNA expression, the following real-time polymerase chain reaction amplifications were performed in duplicate, using the SYBR Premix Ex Taq (Perfect Real Time) kit (TaKaRa Bio) in a Thermal Cycler Dice Real Time system (TaKaRa Bio).

### *Western Blot Analysis*

Preparation of whole-cell protein extracts from HSCs, electrophoresis of whole-cell protein extracts (5  $\mu$ g), and subsequent blotting were performed using antibodies against TLR4 and  $\beta$ -actin, as previously described.<sup>1</sup>

### *Flow Cytometry Analysis*

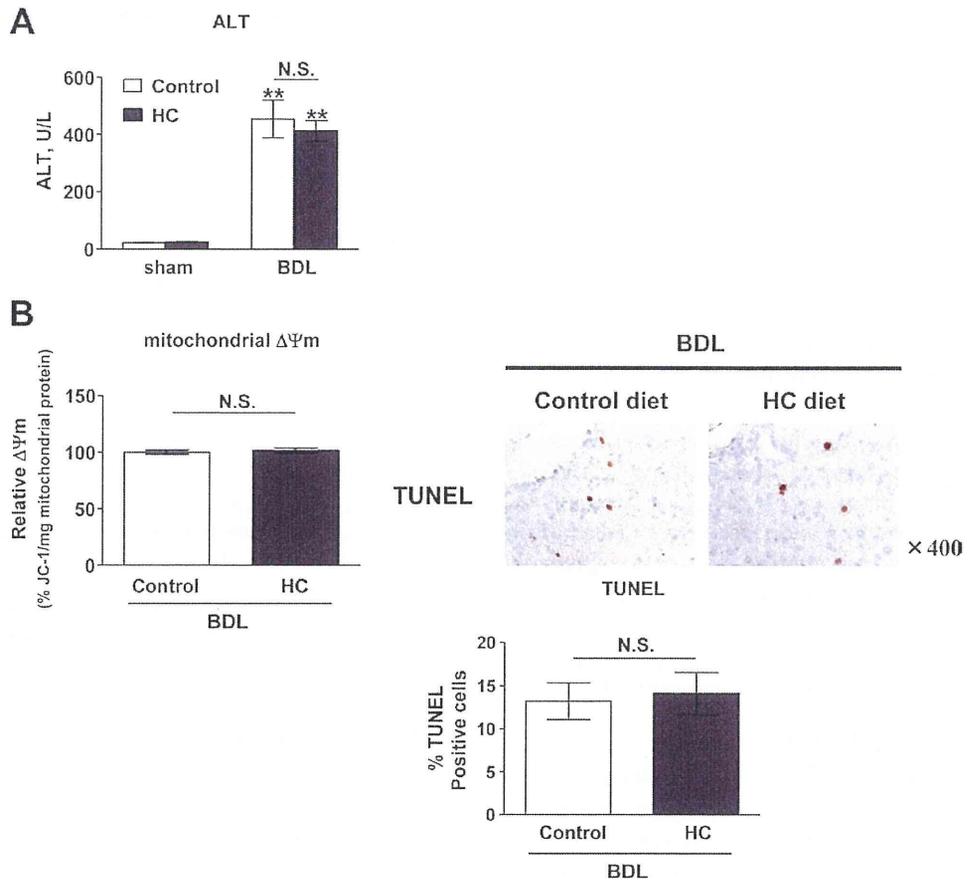
HSC surface expression of TLR4 was detected by flow cytometry of live cells stained with phycoerythrin-conjugated anti-TLR4 antibody (Abcam) or phycoerythrin-conjugated anti-immunoglobulin G isotype control. A total of 10,000 cells/condition were analyzed in a FACScan, using the FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

### *Isolation of Late Endosomes/Lysosomes From HSCs*

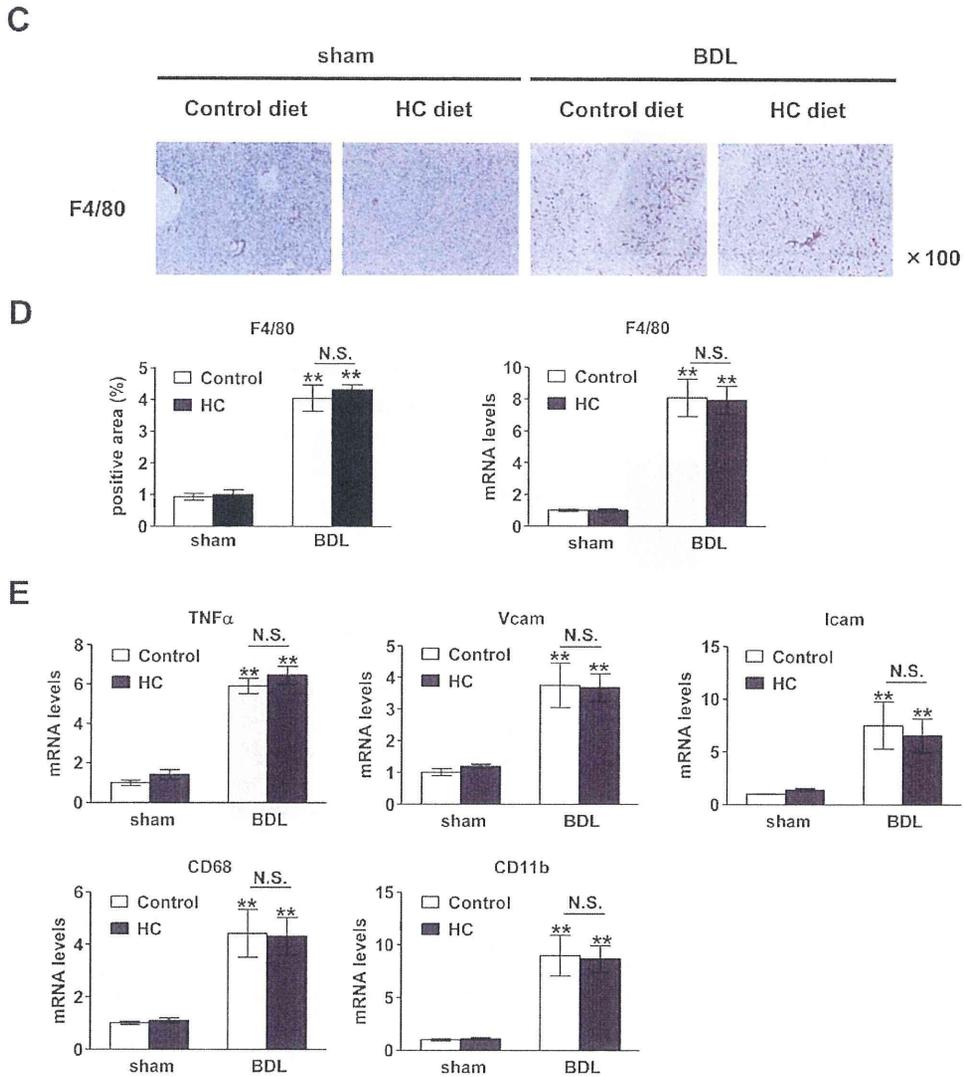
Late endosomal/lysosomal fractions were prepared from HSCs using the lysosome isolation kit (Sigma) following the manufacturer's instructions.

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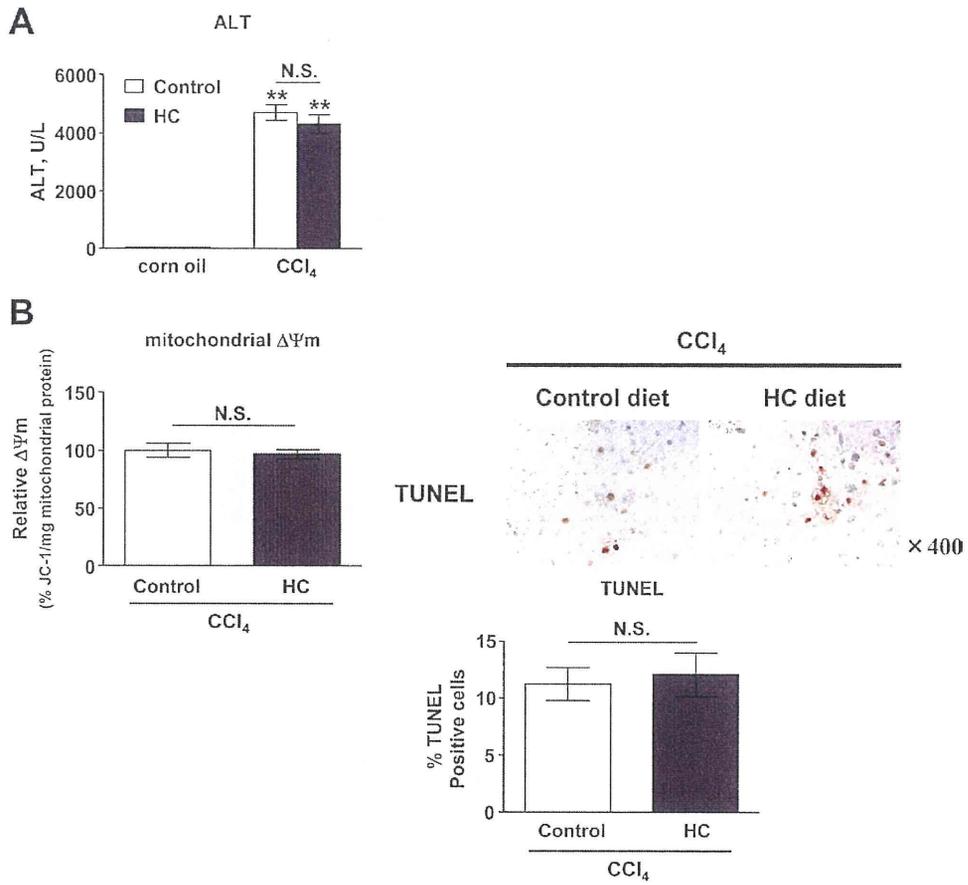
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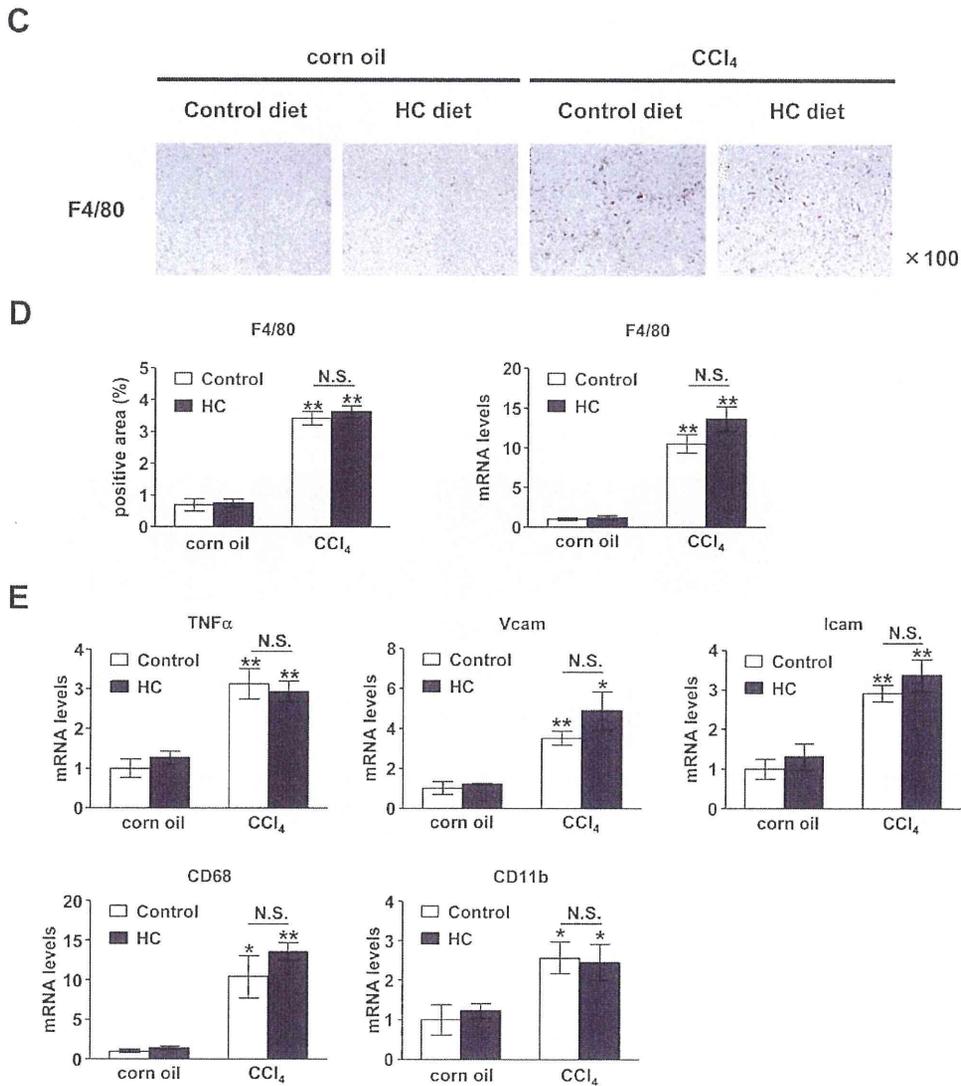
**Supplementary Figure 1.** Effects of the HC diet on acute liver injury induced by BDL treatment. After being fed a control or an HC diet for 4 weeks, C57BL/6 mice were subjected to acute liver injury, induced 5 days after BDL treatment (N = 5/group). (A) Serum ALT activities (N = 5/group). (B, left panels) Electrochemical proton gradient of the inner mitochondrial membrane (N = 5/group). The calculated relative  $\Delta\Psi$  was normalized to the values obtained in mice from the control diet–BDL group. (B, right panels) The percentage of terminal deoxynucleotidyl transferase–mediated deoxyuridine nick-end labeling (TUNEL)-positive hepatocytes (N = 5/group) and the representative sections.



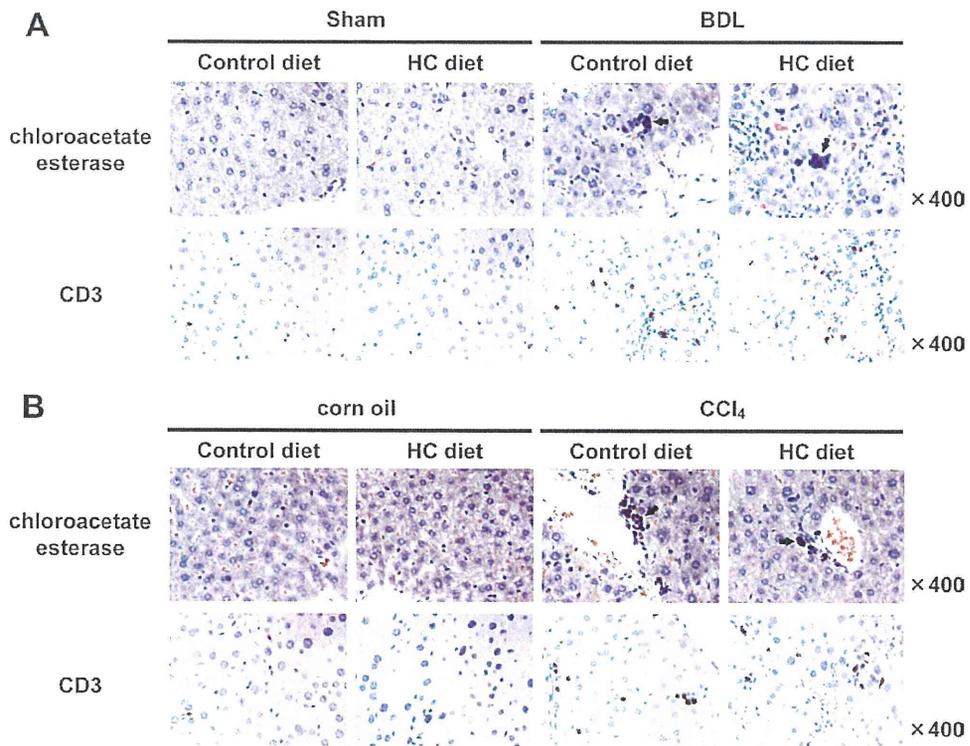
**Supplementary Figure 1 (Cont'd.).** (C) Immunohistochemical detection of F4/80-positive cells in livers. (D) Quantification of F4/80 by immunohistochemical staining and mRNA levels. (E) Hepatic expression of TNF $\alpha$ , vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), CD68, and CD11b mRNA (N = 5/group). \*\*P < .01 compared with control diet–sham-operated group.



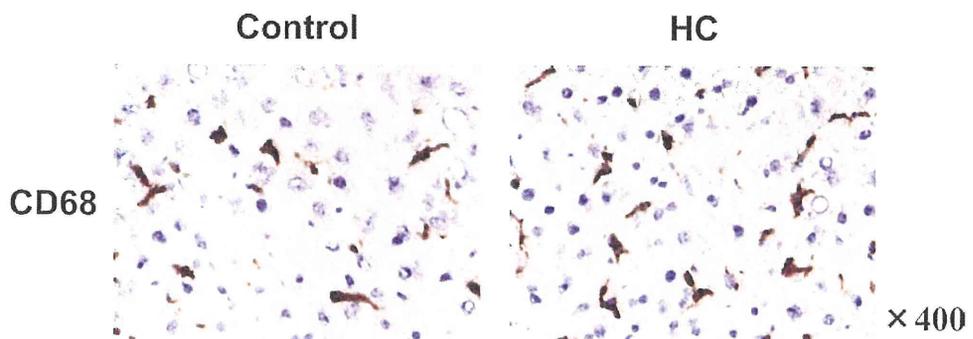
**Supplementary Figure 2.** Effects of the HC diet on acute liver injury induced by CCl<sub>4</sub> treatment. After being fed a control or an HC diet for 4 weeks, C57BL/6 mice were subjected to acute liver injury induced by a single injection of CCl<sub>4</sub> (N = 5/group). (A) Serum ALT activities (N = 5/group). (B, left panel/s) Electrochemical proton gradient of the inner mitochondrial membrane (N = 5/group). The calculated relative  $\Delta\Psi$  was normalized to the values obtained in mice from the control diet-CCl<sub>4</sub> group. (B, right panels) The percentage of terminal deoxynucleotidyl transferase-mediated deoxyuridine nick-end labeling (TUNEL)-positive hepatocytes (N = 5/group) and the representative sections.



**Supplementary Figure 2 (Cont'd.).** (C) Immunohistochemical detection of F4/80-positive cells in livers. (D) Quantification of F4/80 by immunohistochemical staining and mRNA levels. (E) Hepatic expression of TNF $\alpha$ , vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), CD68, and CD11b mRNA (N = 5/group). \**P* < .05 and \*\**P* < .01 compared with control diet-corn oil group.



**Supplementary Figure 3.** Effects of the HC diet on the BDL- or CCl<sub>4</sub>-mediated infiltration of T cells and neutrophils into the liver. After being fed a control or an HC diet for 4 weeks, C57BL/6 mice were subjected to (A) 3-week BDL or (B) CCl<sub>4</sub> treatment twice a week for 4 weeks to induce liver fibrosis (N = 5–7/group). (Upper panels) Staining by the naphtol AS-D chloroacetate esterase technique for detection of neutrophils and (lower panels) immunohistochemical staining for CD3 for detection of T cells.



**Supplementary Figure 4.** HC diet did not induce the formation of hepatic macrophage foam cells. Immunohistochemical detection of CD68-positive cells in livers: mice fed a control diet (left panel) or an HC diet (right panel) for 8 weeks.