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Original Article

Chitinase 3-like 1 deficiency ameliorates liver fibrosis by promoting hepatic macrophage apoptosis

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Aim: Chitinase 3-like 1 (CHI3L1), an 18-glycosyl hydrolase-related molecule, is a member of the enzymatically inactive chitinase-like protein family. Serum levels of CHI3L1 are strongly correlated with hepatic fibrosis progression during many liver diseases. Therefore, this protein could be involved in the development of hepatic fibrosis pathology; however, its role has not been elucidated. We aimed to elucidate its role in the pathophysiology of liver fibrosis.

Methods: Chitinase 3-like 1-deficient (*Chi3l1*$^{-/-}$) mice were given carbon tetrachloride twice per week for 4 weeks or fed a methionine choline-deficient diet for 12 weeks to generate mouse liver fibrosis models. Human fibrotic liver tissues were also examined immunohistochemically.

Results: In human and mouse fibrotic livers, CHI3L1 expression was mainly localized to hepatic macrophages, and the intrahepatic accumulation of CHI3L1^{+} macrophages was significantly enhanced compared to that in control livers. In the two mouse models, hepatic fibrosis was significantly ameliorated in *Chi3l1*$^{-/-}$ mice compared to that in wild-type mice, which was dependent on hepatic macrophages. The accumulation and activation of hepatic macrophages was also significantly suppressed in *Chi3l1*$^{-/-}$ mice compared to that in wild-type mice. Furthermore, apoptotic hepatic macrophages were significantly increased in *Chi3l1*$^{-/-}$ mice. Chitinase 3-like 1 was found to inhibit hepatic macrophage apoptosis by suppressing Fas expression and activating Akt signaling in an autocrine manner, which resulted in hepatic macrophage accumulation and activation, exaggerating liver fibrosis.

Conclusions: Chitinase 3-like 1 exacerbates liver fibrosis progression by suppressing apoptosis in hepatic macrophages. Therefore, this might be a potential therapeutic target for the treatment of liver fibrosis.

Key words: Akt, chitinase 3-like 1, Fas, liver fibrosis, macrophage

INTRODUCTION

SUSTAINED HEPATIC NORMAL wound healing responses cause liver fibrosis and lead to the abnormal continuation of fibrogenesis.[1] Liver diseases causing hepatic fibrosis include chronic hepatitis B, chronic hepatitis C, alcoholic liver disease, non-alcoholic fatty liver disease, autoimmune hepatitis, primary biliary cholangitis, primary sclerosing cholangitis, drug-induced liver disease, and hereditary hemochromatosis.[2] Liver fibrosis eventually progresses to liver cirrhosis, although such rates of progression differ depending on the cause; accordingly, both hepatic fibrosis and cirrhosis have a significant effect on the mortality rate among the global population.[1,2] A recent study showed that approximately 0.1% of the European population suffers from cirrhosis, corresponding to 14–

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26 new cases per 100 000 inhabitants per year or an estimated 170 000 deaths annually.[3] It has also been shown that the prevalence of advanced fibrosis in the general population over 40 years of age has reached approximately 2.8%.[2,4] Furthermore, a recent European population-based, cross-sectional study clarified that the prevalence of silent liver disease with advanced fibrosis is high.[5] Nevertheless, effective treatment for liver fibrosis has not been established, and therefore, elucidating the pathophysiology of liver fibrosis and establishing associated therapies are urgent tasks.[3]

Chitinase 3-like 1 (CHI3L1), an 18-glycosyl hydrolase-related molecule, is a member of the enzymatically inactive chitinase-like-protein family, and also called YKL-40 in humans and BRP-39 in mice.[6] Serum CHI3l1 levels are closely related to tissue injuries, such as various types of inflammation and tissue remodeling,[6] and also have a strong correlation with the degree of liver fibrosis and extracellular matrix synthesis in many chronic liver diseases, including chronic hepatitis B, chronic hepatitis C, alcoholic hepatitis, and non-alcoholic fatty liver disease.[7–10] Moreover, serum CHI3L1 has been assumed to be a useful biomarker for liver fibrosis and prognosis.[8–11] However, the detailed role of this marker in the pathophysiology of this disease has not been elucidated.

The aim of the present study was to elucidate the precise role of CHI3L1 in the pathophysiology of liver fibrosis using CHI3L1-deficient (*Chi3l1*$^{-/-}$) mice and human liver tissues.

METHODS

Human subjects

HUMAN FIBROTIC LIVER sections were obtained from three patients with liver fibrosis at hepatocellular carcinoma excision surgery. Control liver sections without liver fibrosis were obtained from three patients with metastatic liver cancer (two with colorectal cancer and one with gastric cancer) during hepatectomy.[12]

The human study was approved by the institutional review boards of the National Defense Medical College (Tokorozawa, Japan). All patients participating in this study provided written informed consent prior to the study.

Reagents

Carbon tetrachloride (CCl_4) and corn oil were from FUJIFILM Wako Pure Chemicals (Osaka, Japan). LY294002 was from Sigma-Aldrich (St. Louis, MO, USA). Recombinant mouse CHI3L1 (rCHI3L1) and recombinant mouse Fas ligand (rFasL) were from R&D Systems (Minneapolis, MN, USA). Anti-Mouse F4/80 antigen was from Tonbo Bioscience (San Diego, CA, USA). Anti-AKT1 (phosphorylated Akt [pAkt]), anti-Fas, anti-CHI3L1, and goat anti-rat IgG and L (Alexa Fluor 594) antibodies were from Abcam (Cambridge, UK). Goat anti-rabbit IgG and L (Alexa Fluor 488) and anti-mouse IgG and L (Alexa Fluor 594) antibodies were from Thermo Fisher Scientific (Waltham, MA, USA). Anti-F4/80 antibody was from Hycult Biotech (Uden, The Netherlands). Anti-CD68 monoclonal antibody (KP-1) was from Agilent Technologies (Santa Clara, CA, USA). Jo2 (purified NA/LE hamster anti-mouse CD95 antibody) was from BD Biosciences (San Jose, CA, USA).

Generation of *Chi3l1*$^{-/-}$ mice

The targeting vector was designed as previously described[13] and transfected into C57BL/6 J-derived embryonic stem cells (C57BL/6 ES cells). C57BL/6 ES cells with homologous recombination were selected as previously described[13] and injected into BALB/c-derived blastocysts to obtain chimeric mice. Crosses between these chimeric mice and C57BL/6 J mice were carried out to obtain the F1 generation with germline transmission as previously described[14] and further bred to generate C57BL/6 J-background *Chi3l1*$^{-/-}$ mice as noted in a previous study.[13]

Animal studies

Eight-week-old male C57BL/6 J were purchased from CLEA Japan (Tokyo, Japan). Eight-week-old male wild-type and *Chi3l1*$^{-/-}$ mice were given CCl_4 ($n = 4$/group) or corn oil (n = 4/group) twice per week for 4 weeks to induce liver fibrosis, as previously described.[15] A second group of 8-week-old male wild-type and *Chi3l1*$^{-/-}$ mice were fed a methionine–choline-deficient (MCD; cat. no. A02082002B; Research Diets, New Brunswick, NJ, USA) diet or a CE-2 (CLEA Japan) diet for 12 weeks to induce an alternate model of liver fibrosis, as previously described.[15] Animals were maintained under specific pathogen-free conditions at the Center for Laboratory Animal Science, National Defense Medical College. All animals received humane care in compliance with the National Research Council 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, USA). All experiments were carried out under the approval of the National Defense Medical College Animal Use and Care Committee, and all methods implemented were in accordance with relevant guidelines and regulations.

Depletion of hepatic macrophages

Dichloromethylene diphosphonic acid (clodronate)-loaded liposomes (Encapsula NanoSciences, Brentwood, TN, USA) were injected i.v. into mice (200 μL/mouse), as previously described.[15]

Isolation and culture of hepatic macrophages

The isolation of hepatic macrophages from mice and subsequent cell cultures were carried out as previously described.[16] After incubation with rCHI3L1 for 24 or 48 h, the expression of annexin V, pAkt, and Fas on hepatic macrophages was evaluated by fluorescence microscopy, and the percentage of annexin-positive, Fas-positive, and pAkt-positive cells was calculated.

+B:Histology and immunohistochemistry

Hematoxylin–eosin (HE) and Masson-trichrome staining were undertaken using paraffin-embedded liver sections as previously described.[14] For immunohistochemistry, the specimens were deparaffinized, autoclaved in 10 mM citric acid solution (pH 6.0), incubated in a 3% hydrogen peroxide solution, and blocked using Blocking I (Nacalai Tesque, Kyoto, Japan), as previously described.[14] The sections were incubated with primary antibody at 4°C for 16 h, washed with phosphate-buffered saline, and then incubated with peroxidase-labeled secondary antibody at room temperature for 30 min. After color development using 3,3′-diaminobenzidine-4HCl, counterstaining was undertaken with Mayer's hematoxylin solution (FUJIFILM Wako Chemicals). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was carried out using *in situ* Apoptosis Detection Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. Tissue samples were observed using a BZ-X700 microscope (Keyence, Osaka, Japan). For immunofluorescence double staining, the sections were reacted with fluorescently labeled secondary antibodies at room temperature for 2 h. Fluorescently stained specimens were observed using a confocal laser scanning microscope (A1R+; Nikon, Tokyo, Japan). Positively immunostained cells in more than 10 high-power fields (magnification, ×200) were counted and analyzed using ImageJ (NIH) as previously described.[14]

Biochemical analysis

Mouse serum CHI3L1 levels were measured with the Mouse Chitinase 3-like 1 Quantikine ELISA kit (R&D Systems) in accordance with the manufacturer's instructions.

Quantification of mRNA

The mRNA was isolated and purified from liver tissues using an RNeasy Mini Kit (Qiagen, Dusseldorf, Germany). Reverse transcription was carried out with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), and real-time polymerase chain reaction amplification was undertaken using a Thermal Cycler Dice Real Time System (Takara Bio), as previously described.[14]

Flow cytometry

Liver non-parenchymal mononuclear cells were isolated from the liver and subjected to flow cytometry, as previously described.[17] The involvement of CHI3L1 in hepatic F4/80$^+$ macrophage apoptotic functions was investigated by examining the expression of annexin V and propidium iodide (PI) by flow cytometry (BD FACSCanto II; Becton Dickinson, Franklin Lakes, NJ, USA) with an ApoAlert Annexin V-FITC Apoptosis Kit (Clontech Laboratories, Mountain View, CA, USA), following the manufacturer's protocol.

Statistical analysis

All values are expressed as the mean and standard error of the mean. Statistical analysis was undertaken with an unpaired Student's *t*-test or one-way ANOVA with Tukey's post hoc test. $P < 0.05$ was considered significant.

RESULTS

Hepatic CHI3L1, mostly derived from hepatic macrophages, is significantly increased in patients with liver fibrosis

PATIENTS WITH LIVER fibrosis showed enhanced Masson-trichrome staining in the livers (Fig. 1a). Immunofluorescence double staining for CHI3L1 and CD68 showed that CHI3L1 expression was significantly enhanced in patients with liver fibrosis compared to that in normal subjects, and that much of this expression was localized to hepatic macrophages (Fig. 1b). Furthermore, the number of CHI3L1$^+$ hepatic macrophages was significantly higher in patients with liver fibrosis than in normal subjects (Fig. 1c).

Hepatic CHI3L1, mostly derived from hepatic macrophages, is significantly increased in two mouse models of liver fibrosis

Hepatic and serum levels of CHI3L1 were significantly increased in a mouse CCl_4 model of liver fibrosis, compared to that in control mice (Fig. 2a). Moreover, CCl_4-treated mice showed enhanced Masson-trichrome staining in the

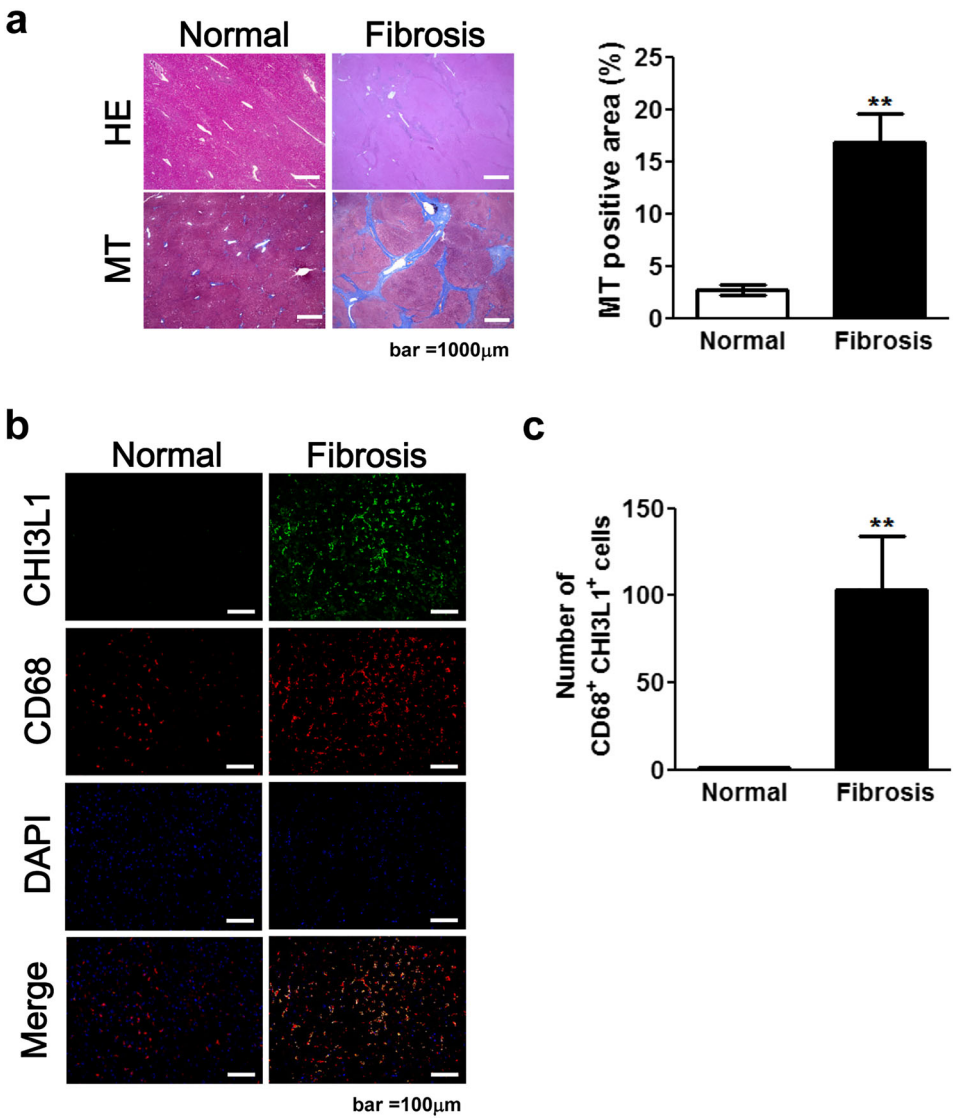

Figure 1 Hepatic chitinase 3-like 1 (CHI3L1), mostly derived from hepatic macrophages, is significantly increased in patients with liver fibrosis.(a) Representative hematoxylin–eosin (HE)- and Masson-trichrome (MT)-stained liver sections. (b) Representative immunofluorescence double-stained images for CHI3L1 (green) and CD68 (red) in liver tissue samples; yellow, co-staining. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). (c) Quantification of $CD68^+/CHI3L1^+$ cells. $^{**}P < 0.01$ vs. control liver samples. [Color figure can be viewed at wileyonlinelibrary.com]

livers (Fig. 2b). Immunofluorescence double staining for CHI3L1 and F4/80 showed that CHI3L1 expression was significantly enhanced in the liver of CCl_4-treated mice, and that much of this expression was localized to hepatic macrophages (Fig. 2c). Furthermore, the number of $CHI3L1^+$ hepatic macrophages was significantly higher in the livers of CCl_4-treated mice than in those of control mice (Fig. 2c).

Similarly, hepatic and serum levels of CHI3L1 were significantly increased in another mouse model of liver fibrosis, specifically, the mouse MCD diet model (Fig. 2d). Mice fed an MCD diet also showed enhanced Masson-trichrome staining in the liver (Fig. 2e). Immunofluorescence double staining for CHI3L1 and F4/80 showed that CHI3L1 expression was significantly enhanced in the livers of mice fed an MCD diet and that much of its expression was

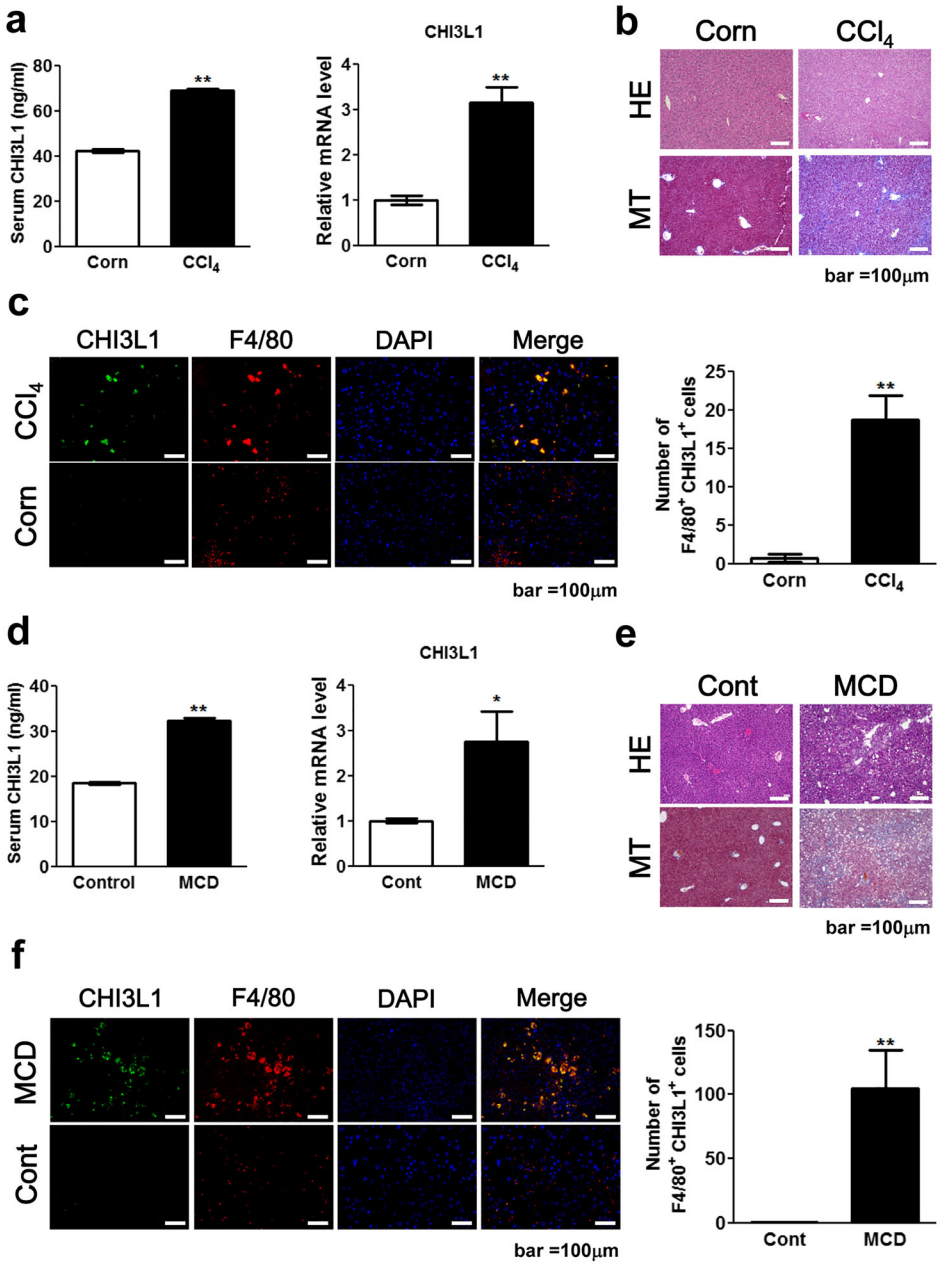

Figure 2 Hepatic chitinase 3-like 1 (CHI3L1), mostly derived from hepatic macrophages, is significantly increased in two mouse models of liver fibrosis.(a–c) Eight-week-old male C57BL/6 mice were given CCl_4 ($n = 5$/group) or corn oil (n = 5/group) twice per week for 4 weeks to induce liver fibrosis. (a) Left panel, serum levels of CHI3L1. Right panel, hepatic levels of *CHI3L1* mRNA. $^{**}P < 0.01$ vs. corn oil-treated group. (b) Representative hematoxylin–eosin (HE)- and Masson-trichrome (MT)-stained liver sections. (c) Left panel, representative immunofluorescence double-stained images of CHI3L1 (green) and F4/80 (red) in liver tissue samples; yellow, co-staining. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Right panel, quantification of $F4/80^+/CHI3L1^+$ cells. $^{**}P < 0.01$ vs. corn oil-treated group. (d–f) Eight-week-old male C57BL/6 mice were fed a methionine-choline-deficient (MCD; $n = 10$/group) or control (Cont; $n = 4$/group) diet for 12 weeks to induce liver fibrosis. (d) Left panel, serum levels of CHI3L1. Right panel, hepatic levels of *CHI3L1* mRNA. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control diet-fed group. (e) Representative HE- and MT-stained liver sections. (f) Left panel, representative immunofluorescence double-stained images of CHI3L1 (green) and F4/80 (red) in liver tissue samples; yellow, co-staining. Nuclei were stained with DAPI (blue). Right panel, quantification of $F4/80^+/CHI3L1^+$ cells. $^{**}P < 0.01$ vs. control diet-fed group. [Color figure can be viewed at wileyonlinelibrary.com]

localized to hepatic macrophages (Fig. 2f). Finally, the number of CHI3L1$^+$ hepatic macrophages was significantly higher in the livers of mice fed an MCD diet than in those of control mice (Fig. 2f).

Chitinase 3-like 1 deficiency ameliorates liver fibrosis in a mouse CCl_4 model of liver fibrosis

Using the mouse CCl_4 model of liver fibrosis, hepatic fibrosis was significantly ameliorated in *Chi3l1*$^{-/-}$ mice compared to that in wild-type mice, as shown by Masson-trichrome and α-smooth muscle actin (αSMA) staining (Fig. 3a,b). The hepatic expression of collagen1α1 (*Col1a1*), collagen1α2 (*Col1a2*), and αSMA (*Acta2*) mRNA was also significantly increased in the mouse CCl_4 model of liver fibrosis; hepatic mRNA levels of these molecules were significantly lower in *Chi3l1*$^{-/-}$ mice than in wild-type mice (Fig. 3c). Hepatic levels of tumor necrosis factor-α (*Tnfa*) mRNA were also significantly increased in a mouse CCl_4 model of liver fibrosis; however, in the model, hepatic *Tnfa* mRNA was significantly lower in *Chi3l1*$^{-/-}$ mice than in wild-type mice (Fig. 3d). There was no difference in hepatic levels of transforming growth factor-β (*Tgfb*) mRNA between genotypes (Fig. S1). Immunohistochemical staining for F4/80 showed that the number of hepatic macrophages was significantly diminished in CCl_4-treated *Chi3l1*$^{-/-}$ mice compared to that in CCl_4-treated wild-type mice (Fig. 3e).

Chitinase 3-like 1 deficiency ameliorates liver fibrosis in a mouse MCD model of liver fibrosis

Similarly, in a mouse MCD model of liver fibrosis, hepatic fibrosis was significantly ameliorated in *Chi3l1*$^{-/-}$mice compared to that in wild-type mice, as shown by Masson-trichrome and αSMA staining (Fig. 4a,b). Furthermore, the hepatic expression of *Col1a1*, *Col1a2*, and *Acta2* mRNA was significantly increased in the MCD model of liver fibrosis, whereas hepatic mRNA levels of these molecules were significantly lower in *Chi3l1*$^{-/-}$ mice than in wild-type mice (Fig. 4c). Hepatic *Tnfa* mRNA expression was also significantly increased in MCD-treated animals and similar to that observed in the previous model, levels were significantly decreased in *Chi3l1*$^{-/-}$ mice compared to those in wild-type mice (Fig. 4d). There was no difference in hepatic levels of *Tgfb* mRNA between genotypes (Fig. S1). Immunohistochemical staining for F4/80 showed that the number of hepatic macrophages was significantly increased in MCD mice with liver fibrosis, but that this was significantly diminished in *Chi3l1*$^{-/-}$ mice compared to that in wild-type mice (Fig. 4e).

Effect of CHI3L1 deficiency on ameliorating hepatic fibrosis is hepatic macrophage-dependent

Next, to clarify whether the effect of CHI3L1 on hepatic fibrosis is mediated by hepatic macrophages, mice that were depleted of hepatic macrophages by liposomal clodronate treatment were subjected to CCl_4-induced (Fig. S2) or MCD-induced liver fibrosis (Fig. S3). Immunohistochemical staining for F4/80 showed that, with clodronate treatment, most hepatic macrophages disappeared to the same extent in both *Chi3l1*$^{-/-}$ and wild-type mice and in both mouse models of liver fibrosis (Figs. S2,S3). In the two models of liver fibrosis depleted of hepatic macrophages, we found no differences in the progression of liver fibrosis between *Chi3l1*$^{-/-}$ and wild-type mice (Figs. S2, S3). We also did not detect any differences in the hepatic expression of *Col1a1*, *Col1a2*, and *Acta2* mRNA between *Chi3l1*$^{-/-}$ and wild-type mice (Figs. S2,S3). There were also no differences in hepatic mRNA levels of *Tnfa* in either mouse model after the depletion of hepatic macrophages (Figs. S2,S3).

Chitinase 3-like 1 deficiency enhances hepatic macrophage apoptosis in two mouse models of liver fibrosis

Flow cytometry analysis using hepatic non-parenchymal mononuclear cells from *Chi3l1*$^{-/-}$ or wild-type mice showed that the number of F4/80$^+$ PI$^-$/annexin V$^+$ hepatic macrophages was significantly increased in the liver of CCl_4-treated *Chi3l1*$^{-/-}$ mice, compared to that in CCl_4-treated wild-type mice (Fig. 5a). Moreover, the median fluorescence intensity (MFI) of annexin V in PI$^-$ hepatic macrophages was significantly higher in CCl_4-treated *Chi3l1*$^{-/-}$ mice than in CCl_4-treated wild-type mice (Fig. 5b). Immunohistochemical staining for F4/80 and TUNEL using serial sections also clarified that *Chi3l1*$^{-/-}$ mice had a higher proportion of apoptotic hepatic macrophages than wild-type mice in the mouse CCl_4 model of liver fibrosis (Fig. S4).

Similarly, flow cytometry of hepatic non-parenchymal mononuclear cells from *Chi3l1*$^{-/-}$ or wild-type mice based on the mouse MCD model of liver fibrosis showed that the number of PI$^-$/annexin V$^+$ hepatic macrophages was significantly increased in the liver of MCD-fed *Chi3l1*$^{-/-}$ mice, compared to that in MCD-fed wild-type mice (Fig. 5c). The MFI of annexin V in PI$^-$ hepatic macrophages was also significantly higher in MCD-fed *Chi3l1*$^{-/-}$ mice than in MCD-fed wild-type mice (Fig. 5d). Immunohistochemical staining for F4/80 and TUNEL using serial sections also clarified that *Chi3l1*$^{-/-}$

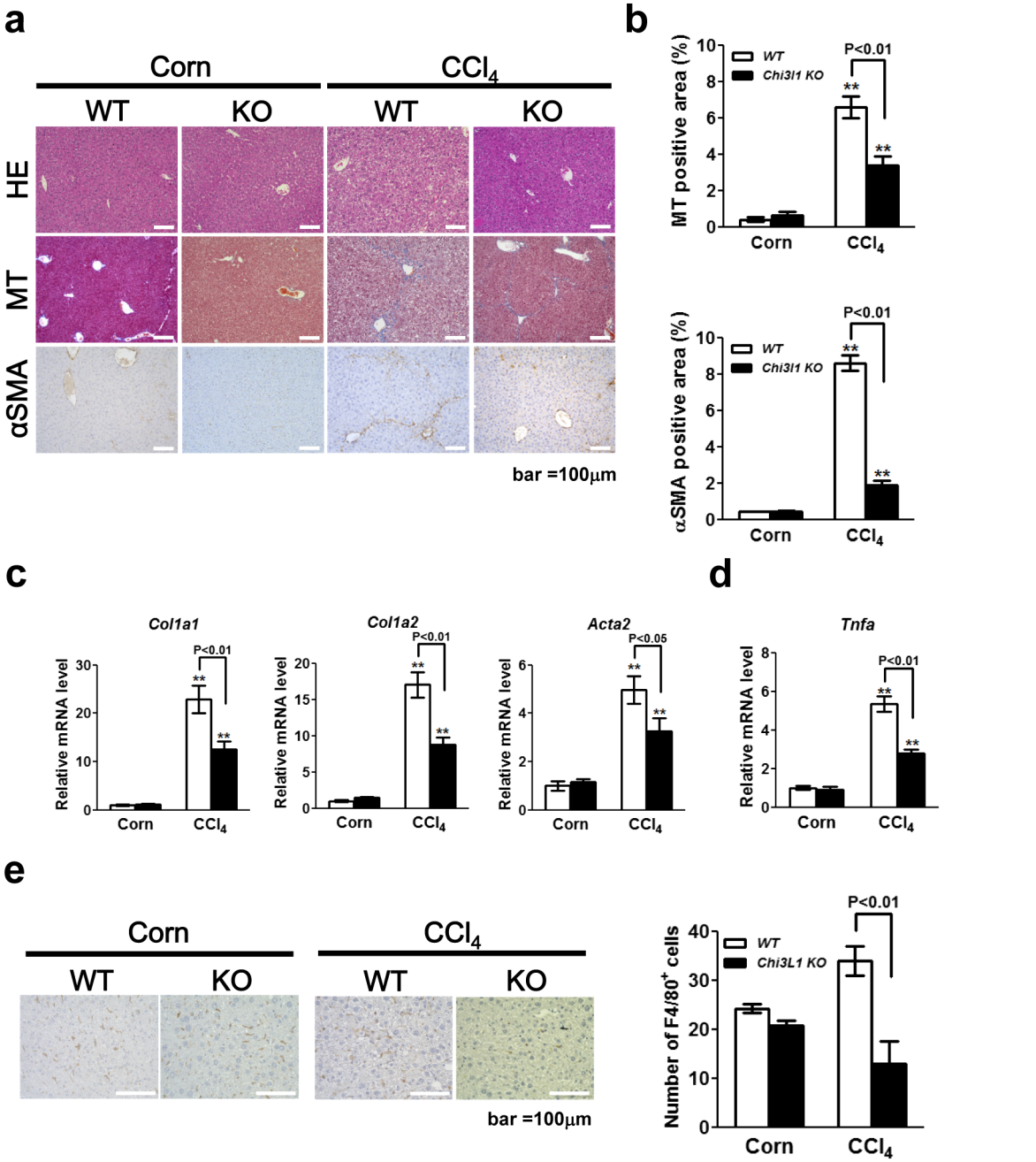

Figure 3 Chitinase 3-like 1 (CHI3L1) deficiency ameliorates liver fibrosis in a mouse CCl_4 model of liver fibrosis. Eight-week-old male wild-type (WT) and *Chi3l1*$^{-/-}$ (KO) mice were given CCl_4 ($n = 5$/group) or corn oil ($n = 5$/group) twice per week for 4 weeks to induce liver fibrosis. (a) Representative hematoxylin–eosin (HE)-stained, Masson-trichrome (MT)-stained, and α-smooth muscle actin (αSMA)-immunostained liver sections. (b) Quantification of MT staining and αSMA immunostaining in liver sections. (c) Hepatic levels of *Col1a1*, *Col1a2*, and *Acta2* mRNA. (d) Hepatic levels of *Tnfa* mRNA. (e) Left panel, representative immunohistochemical images of F4/80 in liver tissue samples. Right panel, quantification of F4/80$^+$ cells. $^{**}P < 0.01$ vs. corn oil-treated WT mice. [Color figure can be viewed at wileyonlinelibrary.com]

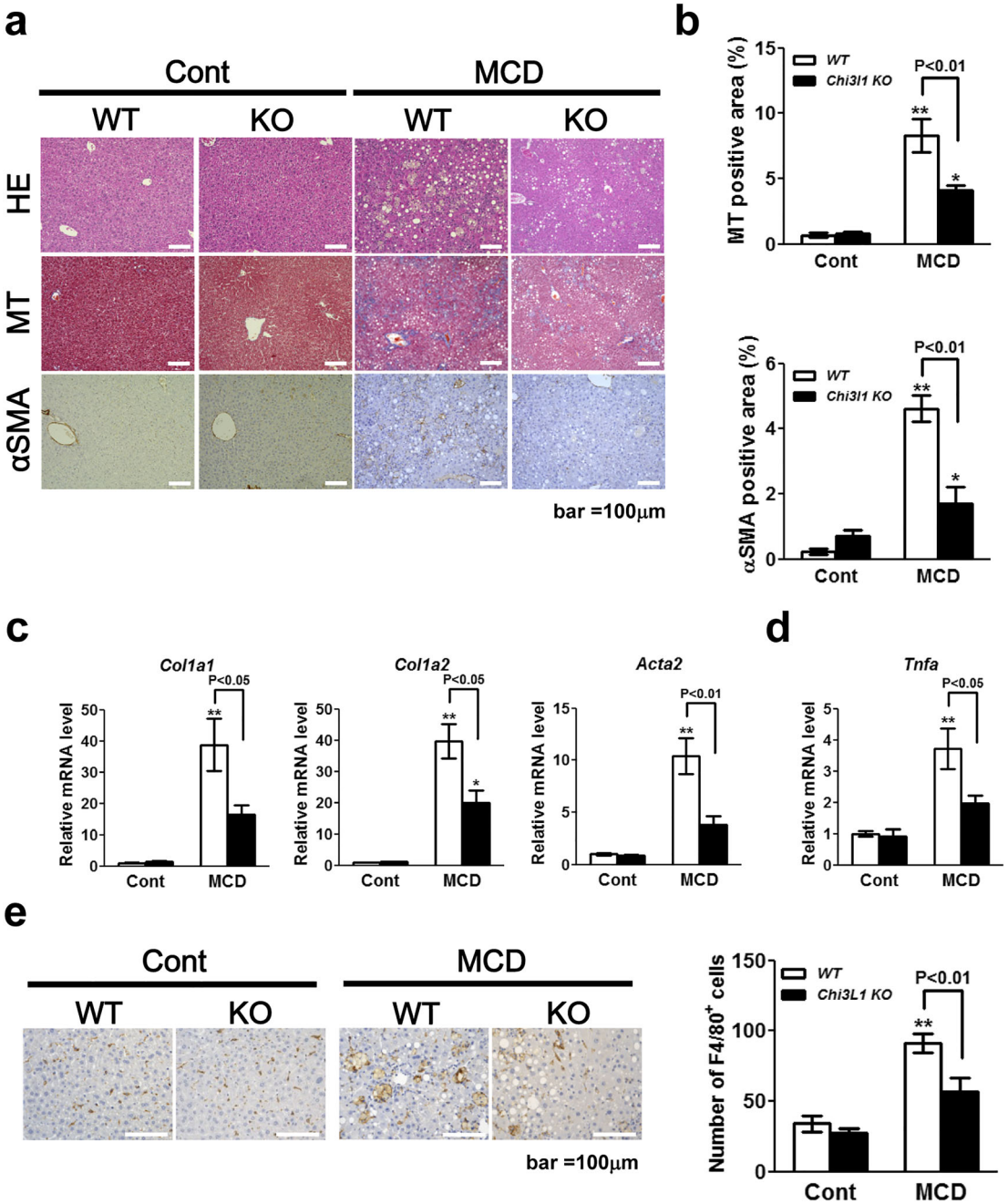

Figure 4 Chitinase 3-like 1 (CHI3L1) deficiency ameliorates liver fibrosis in a mouse methionine–choline-deficient (MCD) model of liver fibrosis. Eight-week-old male wild-type (WT) and *Chi3l1*$^{-/-}$ (KO) mice were fed an MCD ($n = 5$/group) or control ($n = 3$/group) diet for 12 weeks to induce liver fibrosis. (a) Representative hematoxylin–eosin (HE)-stained, Masson-trichrome (MT)-stained, and α-smooth muscle actin (αSMA)-immunostained liver sections. (b) Quantification of MT staining and αSMA immunostaining in liver sections. (c) Hepatic levels of *Col1a1*, *Col1a2*, and *Acta2* mRNA. (d) Hepatic levels of *Tnfa* mRNA. (e) Left panel, representative immunohistochemical images of F4/80 in liver tissue samples. Right panel, Quantification of F4/80^{+} cells. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control diet-fed WT mice. [Color figure can be viewed at wileyonlinelibrary.com]

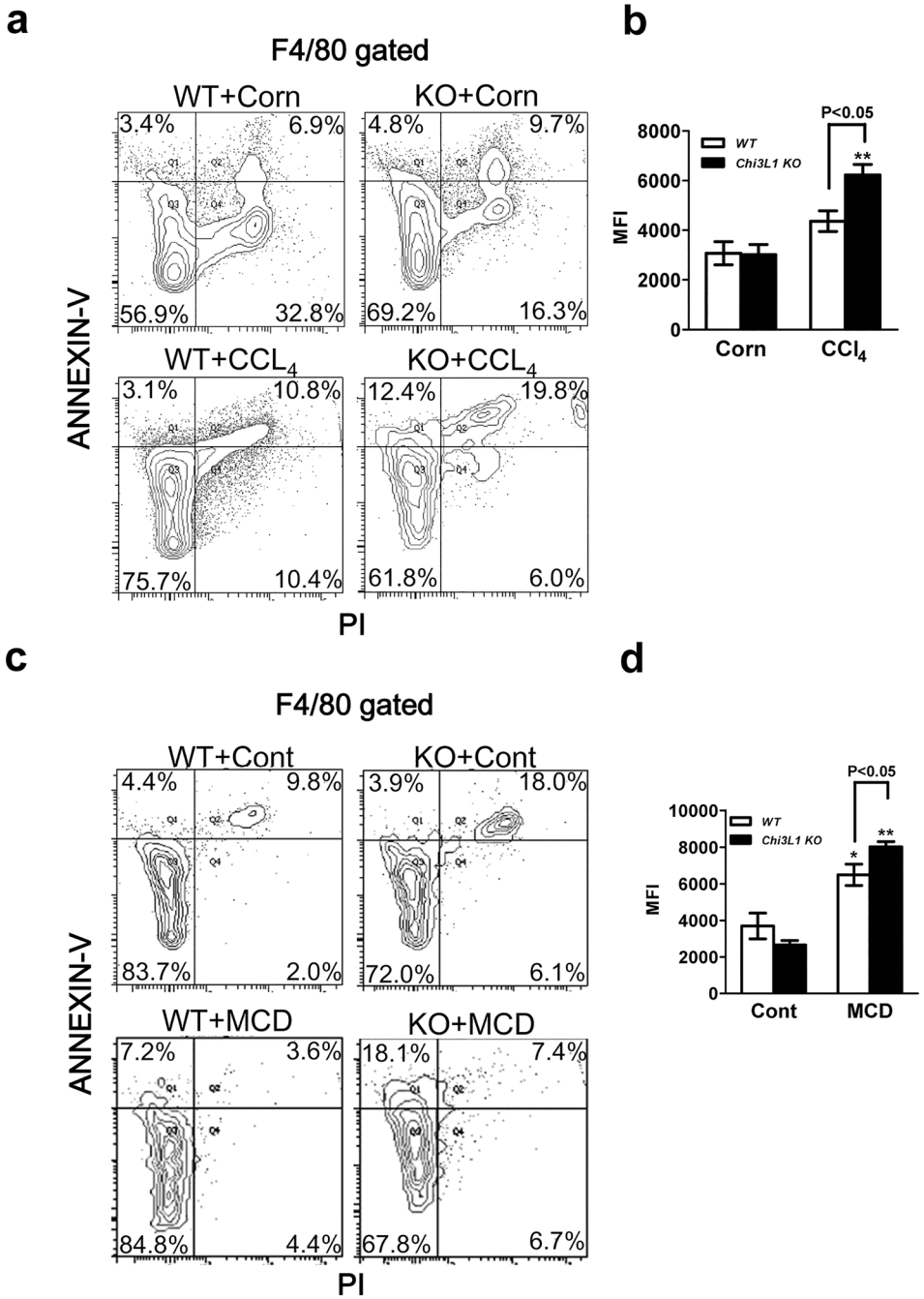

Figure 5 Chitinase 3-like 1 (CHI3L1) deficiency enhances hepatic macrophage apoptosis in two mouse models of liver fibrosis. (a,b) Eight-week-old male wild-type (WT) and *Chi3l1*$^{-/-}$ (KO) mice were given CCl_4 ($n = 4$/group) or corn oil (n = 4/group) twice per week for 4 weeks to induce liver fibrosis. (a) Representative annexin V and propidium iodide (PI) staining of F4/80$^+$ hepatic macrophages based on flow cytometry. (b) Quantification of mean fluorescence intensity (MFI) of annexin V in PI$^-$F4/80$^+$ hepatic macrophages. $^{**}P < 0.01$ vs. PI$^-$F4/80$^+$ hepatic macrophages from corn oil-treated WT mice. (c,d) Eight-week-old male wild-type and *Chi3l1*$^{-/-}$ mice were fed a methionine–choline-deficient (MCD; n = 4/group) or control (n = 4/group) diet for 12 weeks to induce liver fibrosis. (c) Representative annexin V and PI staining of F4/80$^+$ hepatic macrophages based on flow cytometry. (d) Quantification of MFI of annexin V in PI$^-$F4/80$^+$ hepatic macrophages. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. PI$^-$F4/80$^+$ hepatic macrophages from control diet-fed (Cont) wild-type mice.

mice had a higher proportion of apoptotic hepatic macrophages than wild-type mice in this model of liver fibrosis (Fig. S4).

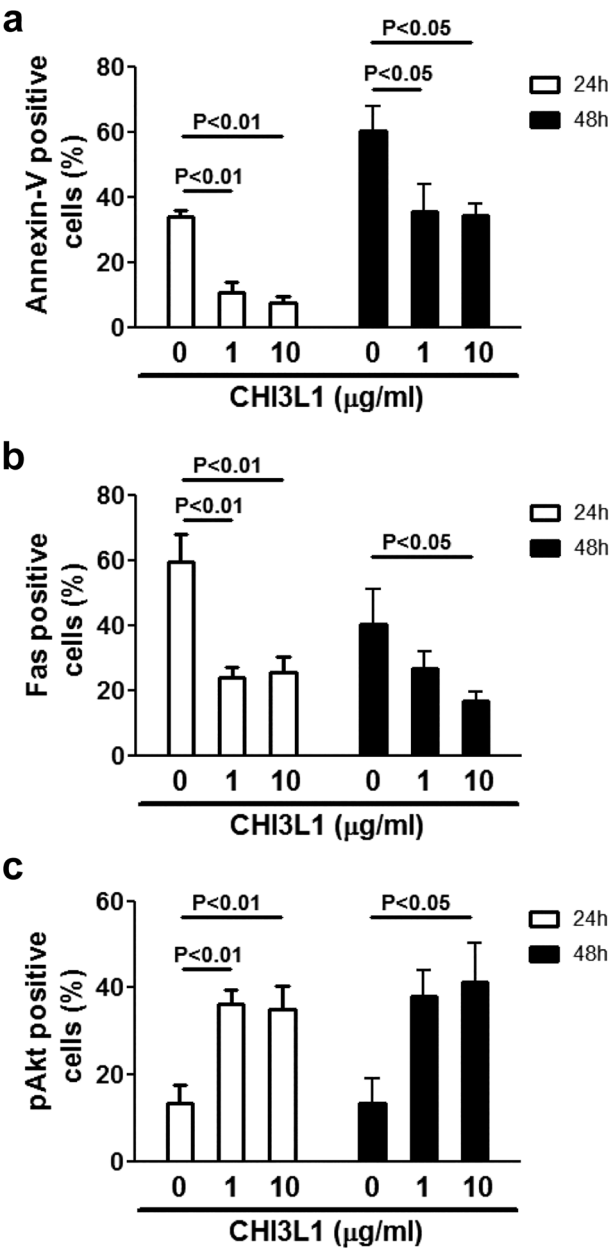

Figure 6 Chitinase 3-like 1 (CHI3L1) prevents hepatic macrophage apoptosis by decreasing Fas expression and enhancing phosphorylated Akt (pAkt) expression. Hepatic macrophages, freshly isolated from *Chi3l1*$^{-/-}$ mice, were incubated with recombinant CHI3L1 at the indicated concentration for 24 or 48 h (n = 3/group). Mean percentages of annexin V^{+} (a), Fas^{+} (b), and pAkt^{+} (c) hepatic macrophages.

Chitinase 3-like 1 significantly prevents the apoptosis of hepatic macrophages by decreasing Fas expression and enhancing pAkt expression

We detected significantly more CHI3L1 secreted into the media from activated primary cultured hepatic macrophages (Fig. S5) than from control cells. Treatment with rCHI3L1 for 24 and 48 h significantly decreased the number of apoptotic primary cultured hepatic macrophages from *Chi3l1*$^{-/-}$ mice (Fig. 6a). Treatment with the rFasL or Jo2 Fas-activating antibody significantly increased the number of apoptotic *Chi3l1*$^{-/-}$ hepatic macrophages (Fig. S5), which indicated that the Fas-mediated pathway is involved in the apoptosis of hepatic macrophages. Treatment with rCHI3L1 significantly decreased the ratio of Fas-positive *Chi3l1*$^{-/-}$ hepatic macrophages (Fig. 6b).

Treatment with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 significantly increased the number of apoptotic *Chi3l1*$^{-/-}$ hepatic macrophages (Fig. S5), which suggested that the PI3K/Akt pathway plays a role in the apoptosis of hepatic macrophages. Incubation with rCHI3L1 for 24 and 48 h significantly increased the ratio of pAkt^{+} *Chi3l1*$^{-/-}$ hepatic macrophages (Fig. 6c).

DISCUSSION

THE PRESENT STUDY showed that CHI3L1 is localized predominantly to hepatic macrophages in the liver, and that the accumulation of CHI3L1-positive hepatic macrophages is significantly enhanced during liver fibrosis in humans and mice, which could be the cause of elevated serum CHI3L1 levels. We also clarified that, with respect to the pathogenesis of liver fibrosis, CHI3L1 inhibits hepatic macrophage apoptosis by suppressing Fas expression and activating Akt signaling in an autocrine manner. Accordingly, CHI3L1 deficiency ameliorated the progression of liver fibrosis by inhibiting the accumulation of hepatic macrophages by promoting apoptosis.

Many studies have reported that the accumulation of hepatic macrophages plays a role in the pathogenesis of liver fibrosis by secreting pro-inflammatory cytokines such as TNFα.[18,19] Recent reports have also shown that dysregulated macrophage function contributes to the development of fibrosis and cell death and that apoptosis is a common response of hepatic macrophages to toxic insults.[20,21] It was also shown that hepatic macrophages undergo apoptosis in fibrotic livers, possibly through the Fas-mediated pathway.[22] Another study[23] reported that

the apoptosis of M1 hepatic macrophages, which secrete pro-inflammatory mediators such as TNFα, plays a role in the pathogenesis of liver injury. Apoptosis in hepatic macrophages was found to be preponderant in individuals with mild liver injury, compared to that in individuals with severe liver injury.[23] Furthermore, hepatic macrophage apoptosis was reported to be protective against the progression of steatohepatitis.[24] The depletion of hepatic macrophages was also found to ameliorate liver fibrosis, specifically in the progressive inflammatory injury phase,[25] similar to our results. These studies and our results suggest that hepatic macrophage apoptosis could play an important role in regulating the accumulation and activation of these cells during the pathogenesis of liver fibrosis, leading to the amelioration of this disease.

In our study, CHI3L1 deficiency significantly decreased hepatic levels of TNFα but did not affect hepatic levels of TGFβ, which is known to be produced by M2-like hepatic macrophages.[26] This result suggested that CHI3L1 inhibits M1-like but not M2-like hepatic macrophage apoptosis, which warrants further investigation.

The death receptor Fas and downstream signaling are known as a representative extrinsic pathway of apoptosis in macrophages.[27] In contrast, Akt signaling, which is constitutively active in macrophages, is known to be a major anti-apoptotic pathway in macrophages.[28,29] The present study showed that Fas-mediated signaling and the PI3K/Akt pathway play a role in the apoptosis of hepatic macrophages, and that CHI3L1 promotes the apoptosis of hepatic macrophages by decreasing Fas expression and activating Akt signaling. Furthermore, CHI3L1, secreted mainly from hepatic macrophages in the liver, enhanced their resistance to apoptotic stimuli during the pathogenesis of liver fibrosis in an autocrine manner. This resulted in the accumulation and activation of hepatic macrophages, leading to the exaggeration of liver fibrosis. In this manner, CHI3L1 was found to be an exacerbating factor for liver fibrosis.

Although CHI3L1 is known as a useful fibrosis marker for various liver diseases,[8–11] its effect on the pathogenesis of liver fibrosis had previously not been clarified. In the present study, we found that CHI3L1 exaggerates liver fibrosis through the suppression of hepatic macrophage apoptosis and their subsequent accumulation and activation. Therefore, this pathway could be a promising therapeutic target for patients with liver fibrosis, and especially those with high serum levels of this marker, as serum levels could reflect hepatic levels of this protein. Large-scale clinical research is expected to be carried out in the future to confirm this.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found online in the Supporting Information section at the end of the article.

Figure S1 Hepatic levels of *Tgfb* mRNA do not differ between genotypes in mouse models of liver fibrosis. (a) Hepatic levels of *Tgfb* mRNA in 8-week-old male wild-type and *Chi3l1*$^{-/-}$mice given CCl_4 ($n = 5$/group) or corn oil ($n = 5$/group) twice per week for 4 weeks to induce liver fibrosis. $^{**}P < 0.01$ vs. corn oil-treated wild-type mice. (b) Hepatic levels of *Tgfb* mRNA in 8-week-old male wild-type and *Chi3l1*$^{-/-}$mice fed a methionine–choline-deficient (MCD; $n = 5$/group) or control ($n = 3$/group) diet for 12 weeks to induce liver fibrosis. $^{**}P < 0.01$ vs. control diet-fed wild-type mice.

Figure S2 Following hepatic macrophage depletion by clodronate treatment, Chitinase 3-like 1 deficiency does not affect liver fibrosis in a mouse CCl_4 model of liver fibrosis. Eight-week-old male wild-type and *Chi3l1*$^{-/-}$mice were injected with liposomal clodronate to deplete hepatic macrophages and then given CCl_4 ($n = 4$/group) or corn oil ($n = 4$/group) twice per week for 4 weeks to induce liver fibrosis. (a) Left panel, representative immunohistochemical images of F4/80 in liver tissue samples. Right panel, quantification of F4/80-positive cells. (b) Left panel, representative hematoxylin–eosin (HE)- and Masson-trichrome (MT)-stained liver sections. Right panel, quantification of MT-stained areas in liver sections. (c) Hepatic levels of *Col1a1*, *Col1a2*, and *Acta2* mRNA. (d) Hepatic levels of *Tnfa* mRNA. $^{**}P < 0.01$ vs. corn oil-treated wild-type mice.

Figure S3 Following hepatic macrophage depletion by clodronate treatment, chitinase 3-like 1 deficiency does not affect liver fibrosis in a mouse methionine–choline-deficient (MCD) model of liver fibrosis. Eight-week-old male wild-type and *Chi3l1*$^{-/-}$mice were injected with liposomal clodronate to deplete hepatic macrophages, and then fed an MCD ($n = 4$/group) or control ($n = 4$/group) diet for 8 weeks to induce liver fibrosis. At 2, 4, and 6 weeks after the initiation of feeding, they were injected with liposomal clodronate as previously described.[15] (a) Left panel, representative immunohistochemical images of F4/80 in liver tissue samples. Right panel, Quantification of F4/80^{+} cells. (b) Left panel, representative hematoxylin–eosin (HE)- and Masson-trichrome (MT)-stained liver sections. Right panel, quantification of MT-stained areas of liver sections. (c) Hepatic levels of *Col1a1*, *Col1a2*, and *Acta2* mRNA. (d) Hepatic

levels of *Tnfa* mRNA. $^{**}P < 0.01$ and $^{*}P < 0.05$ vs. control diet-fed wild-type mice.

Figure S4 Chitinase 3-like 1 deficiency increases apoptotic hepatic macrophages in two mouse models of liver fibrosis. (a,b) Eight-week-old male wild-type and *Chi3l1*$^{-/-}$mice were given CCl_4 ($n = 4$/group) twice per week for 4 weeks to induce liver fibrosis. (a) Representative immunohistochemical staining of F4/80 and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) using serial liver sections. Arrows indicate apoptotic hepatic macrophages. (b) Quantification of the proportion of apoptotic hepatic macrophages. $^{**}P < 0.01$ vs. CCl_4-treated wild-type mice. (c,d) Eight-week-old male wild-type and *Chi3l1*$^{-/-}$mice were fed a methionine–choline-deficient (MCD; $n = 4$/group) diet for 12 weeks to induce liver fibrosis. (c) Representative immunohistochemical images of F4/80 and TUNEL using serial liver sections. Arrows indicate apoptotic hepatic macrophages. (d) Quantification of the proportion of apoptotic hepatic macrophages. $^{**}P < 0.01$ vs. MCD-fed wild-type mice.

Figure S5 Enhanced Fas-mediated signaling and phosphatidylinositol 3-kinase (PI3K)/Akt pathway inhibition promote apoptosis of hepatic macrophages. (a) Chitinase 3-like 1 concentration in the culture media in which hepatic macrophages separated from wild-type mice 24 h after treatment with CCl_4 or vehicle were cultured for 48 h ($n = 6$/group). ND, not detected. (b) Mean percentage of annexin V^{+} *Chi3l1*$^{-/-}$ hepatic macrophages, incubated with 100 ng/mL recombinant Fas ligand (rFasL), 10 μg/mL Jo2 Fas-activating antibody, 100 μM LY294002 (PI3K inhibitor [PI3KI]), or vehicle for 24 h ($n = 3$/group). $^{**}P < 0.01$ vs. control group.