Research Article

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CXCL10 plays a key role as an inflammatory mediator and a non-invasive biomarker of non-alcoholic steatohepatitis

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Background & Aims: Perpetuate liver inflammation is crucial in the pathogenesis of non-alcoholic steatohepatitis (NASH). Expression of CXCL10, a pro-inflammatory cytokine, correlates positively with obesity and type 2 diabetes. Whether CXCL10 plays a role in NASH was unknown. We aimed to investigate the functional and clinical impact of CXCL10 in NASH.

Methods: *Cxcl10* gene-deleted ($Cxcl10^{-/-}$) and C57BL/6 wild type (WT) mice were fed a methionine- and choline-deficient (MCD) diet for 4 or 8 weeks. In other experiments, we injected neutralizing anti-CXCL10 mAb into MCD-fed WT mice. Human serum was obtained from 147 patients with biopsy-proven non-alcoholic fatty liver disease and 73 control subjects.

Results: WT mice, fed the MCD diet, developed steatohepatitis with higher hepatic CXCL10 expression. $Cxcl10^{-/-}$ mice were refractory to MCD-induced steatohepatitis. We further revealed that CXCL10 was associated with the induction of important pro-inflammatory cytokines (TNF-α, IL-1β, and MCP-1) and activation of the NF-κB pathway. CXCL10 was linked to steatosis through upregulation of the lipogenic factors SREBP-1c and LXR, and also to oxidative stress (upregulation of CYP2E1 and C/EBPβ). Blockade of CXCL10 protected against hepatocyte injury *in vitro* and against steatohepatitis development in mice. We further investigated the clinical impact of CXCL10 and found circulating and hepatic CXCL10 levels were significantly higher in human NASH. Importantly, the circulating CXCL10 level was correlated with the degree of lobular inflammation and was an independent risk factor for NASH patients.

Conclusions: We demonstrate for the first time that CXCL10 plays a pivotal role in the pathogenesis of experimental steatohepatitis. CXCL10 maybe a potential non-invasive biomarker for NASH patients.

Keywords: Non-alcoholic fatty liver disease; Inflammation; Chemokine; Animal model; Biomarker.

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; CXCL, CXC chemokine ligand; TLR, toll-like receptor; NF-κB, nuclear factor-κB; WT, wild type; MCD, methionine- and choline-deficient; ALT, alanine aminotransferase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; FACS, fluorescence activated cell sorting; ROC, receiver operating characteristic; IQR, interquartile range; TBARS, thiobarbituric acid reactive substances; TNF-α, tumor necrosis factor-α; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; C/EBPβ, CCAAT/enhancer binding protein beta; COX-2, cyclooxygenase-2; ICAM-1, intercellular adhesion molecule-1; LXR, liver X receptors; SREBP-1c, sterol regulatory element binding protein isoform 1c; ChREBP, carbohydrate response element binding protein; SCD-1, stearoyl-CoA desaturase isoform-1; Cyp, cytochrome P450; mAb, monoclonal antibodies; BMI, body mass index; AUROC, area under the receiver operating characteristic curve; LDL-c, low density lipoprotein-cholesterol; HbA1c, glycated haemoglobin; ACK, ammonium chloride potassium.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become increasingly important worldwide due to changes in lifestyle and resultant over-nutrition [1]. Non-alcoholic steatohepatitis (NASH) is a severe form of NAFLD, characterized by necroinflammation and lipid accumulation [2,3]. Little is known about the factors responsible for the transition from benign steatosis to steatohepatitis in NAFLD/NASH. As a consequence, apart from addressing lifestyle issues, there are few effective interventions to treat patients with NASH. The present concept about NASH pathogenesis is that increased levels of toxic lipids, such as free fatty acids or free cholesterol provide initiating and propagating mechanism for hepatocellular injury and resultant inflammation. Inflammation may result from oxidative stress and pro-inflammatory chemokines and cytokines, which perpetuate liver injury and lead to fibrosis [4]. Identification of the pro-inflammatory cytokines, which are associated with lipotoxicity, may improve our understanding of

the pathogenesis of NASH, enabling the development of novel pharmacological treatments.

One particularly important pro-inflammatory cytokine associated with lipotoxicity is the CXC motif chemokine ligand 10 (CXCL10), which recruits inflammatory cells to the site of tissue damage [5,6]. CXCL10 has been implicated in the pathogenesis of hepatitis C virus infection through interactions with the toll-like receptor (TLR) 2 [7], and in hepatitis B virus-infection through the nuclear factor-κB (NF-κB) pathway [8]. In various types of liver injury, CXCL10 is secreted by hepatocytes in areas of lobular inflammation [9,10] and neutralization of CXCL10 accelerates liver regeneration [11]. These data indicate a potential role for CXCL10 in the development of intrahepatic inflammation. Moreover, CXCL10 is upregulated in NASH patients [12] and correlates positively with the incidence of obesity and type 2 diabetes [13,14]. These findings suggest that CXCL10 could be a pivotal molecule that facilitates transition from benign steatosis to progressively hepatocellular damage and inflammation in steatohepatitis.

We have recently reported that the anti-oxidant enzyme heme oxygenase-1 protects against development of experimental steatohepatitis in association with reduced production of CXCL10 [15]. In the present study, we first investigated the functional role of CXCL10 in the development of steatohepatitis using *Cxcl10* gene-deleted mice, and further explored the molecular mechanisms by which CXCL10 exerts its effects on inflammation, steatosis, oxidative stress and apoptosis. We demonstrated by *in vitro* and *in vivo* approaches that blockade of CXCL10 (neutralizing anti-CXCL10 mAb) protected against steatohepatitis. In particular, we tested the clinical impact of CXCL10 in 147 patients with biopsy-proven NAFLD and 73 control subjects and demonstrated that circulating CXCL10 is an independent risk factor for patients with NASH.

Materials and methods

Animals and treatments

Age-matched male *Cxcl10* knock out (*Cxcl10*$^{-/-}$) and C57BL/6 wild type (WT) mice (from Dr. Andrew D. Luster, Harvard Medical School) were fed either a methionine- and choline-deficient (MCD) diet or a control diet for 4 weeks to establish steatohepatitis, or for 8 weeks to establish fibrosing steatohepatitis [15,16].

For CXCL10 neutralization experiments, male C57BL/6 WT mice were given CXCL10-specific anti-CXCL10 mAb (R&D System, Minneapolis, MN) by intraperitoneal injection (50 μg in 200 μL PBS per mouse) at 12 h before MCD diet, and then the injection was repeated every 2 days for 5 cycles [10,14]. Mice were also given an isotype-matched rat IgG2A mAb (R&D System) at the same time as the controls. In a separate experiment, anti-CXCL10 mAb or control mAb were supplemented for 10 days under MCD diet after induction of steatohepatitis in mice fed the MCD diet for 3 weeks. All animals received humane care and all animal studies were performed in accordance with guidelines approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

Mice were sacrificed as previously described [17]. Biochemical determination of serum alanine aminotransferase (ALT) levels, triglycerides and lipid peroxidation rates were performed. Liver histology, liver collagen content analysis, cytokine profiling assay, cDNA expression array, nuclear DNA binding activity assay, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, fluorescence activated cell sorting (FACS) analysis, qPCR and western blot were performed.

Subjects and human sample collection

Serum samples were collected from 147 patients with biopsy-proven NAFLD and 73 healthy subjects as previously described [18,19]. Percutaneous liver biopsy specimens were collected from 11 patients with NASH, 11 patients with simple steatosis and 15 healthy controls in the Prince of Wales Hospital and the Queen Mary Hospital, Hong Kong. All subjects had given written informed consent and the study protocol was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong and the University of Hong Kong.

Statistical analysis

Differences between two groups were compared by the Mann-Whitney *U* test or Student's *t* test. Multiple group comparisons were made by the Kruskal-Wallis test or one-way ANOVA. Spearman's correlation coefficient was used to estimate the association of serum CXCL10 levels and several factors of interest, while multiple linear regression was used to determine the independent factors associated with levels of CXCL10. Multiple logistic regression was performed to identify the independent risk factors of NASH. A receiver operating characteristic (ROC) curve analysis was conducted to assess the performance of CXCL10 in the prediction of NAFLD/NASH. All statistical tests were performed using SPSS or GraphPad Software. Data were expressed as mean ± standard deviation or median (interquartile range [IQR]) and considered significant at p <0.05.

Additional experimental procedures are provided in the Supplementary Materials and methods section.

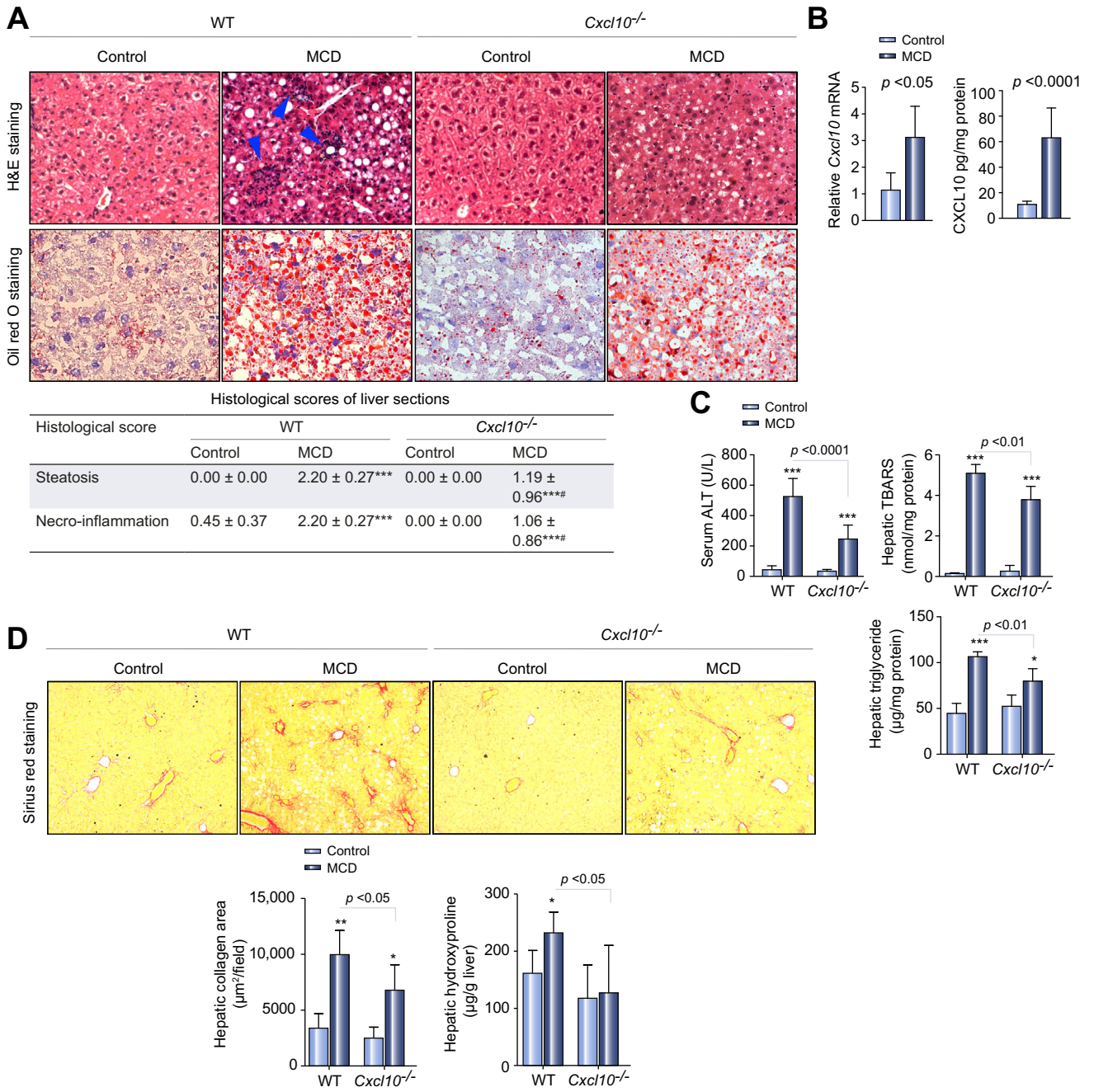
Results

Hepatic CXCL10 expression is upregulated in experimental steatohepatitis and is required for its development

To elucidate the role of CXCL10 in the development of steatohepatitis, *Cxcl10*$^{-/-}$ and WT mice were fed control or MCD diets for 4 weeks. MCD-fed WT mice developed steatosis, ballooning hepatocytes, scattered lobular inflammatory cell infiltration, and inflammatory foci (Fig. 1A), consistent with steatohepatitis. This was associated with increased hepatic *CXCL10* mRNA and protein levels compared with mice fed a control diet (Fig. 1B), which showed normal liver histology (Fig. 1A). Conversely, MCD-fed *Cxcl10*$^{-/-}$ mice showed significant less steatosis (p <0.01) and reduced inflammatory cell infiltration (p <0.01), as indicated by steatosis and necroinflammatory scores (Fig. 1A). Consistent with the histologic findings, measurement of serum ALT (p <0.0001), hepatic lipid peroxide by the thiobarbituric acid reactive substances (TBARS) assay (p <0.01) and hepatic triglyceride contents (p <0.01) revealed that loss of *CXCL10* protected mice from MCD diet-induced liver injury (Fig. 1C). The decreased lipid accumulation in MCD-fed *Cxcl10*$^{-/-}$ mice was confirmed by Oil red O staining (Fig. 1A). Taken together, these data suggest that CXCL10 contributes to the development of steatohepatitis.

CXCL10 is required for hepatic nutritional fibrosis

To examine whether CXCL10 plays a role in hepatic nutritional fibrosis, *Cxcl10*$^{-/-}$ mice and WT mice were fed with control or MCD diet for 8 weeks. Intraparenchymal pericellular fibrosis developed from steatohepatitis in WT mice fed with MCD for 8 weeks as shown by Sirius Red staining (Fig. 1D), whilst, MCD-fed *Cxcl10*$^{-/-}$ mice showed impressively reduced amounts of collagen fibres (Fig. 1D). Morphometric analysis yielded concordant results where the Sirius Red-stained collagen areas were significantly reduced in MCD-fed *Cxcl10*$^{-/-}$ mice compared to MCD-fed WT mice (p <0.05). Moreover, quantitation of collagen by measuring hepatic hydroxyproline content supported the improvement of liver fibrosis by *CXCL10* deficiency (Fig. 1D).



Histological scores of liver sections

Histological score	WT		*Cxcl10*$^{-/-}$	
	Control	MCD	Control	MCD
Steatosis	0.00 ± 0.00	2.20 ± 0.27***	0.00 ± 0.00	1.19 ± 0.96***#
Necro-inflammation	0.45 ± 0.37	2.20 ± 0.27***	0.00 ± 0.00	1.06 ± 0.86***#

Fig. 1. Deficiency of CXCL10 attenuates experimental steatohepatitis. (A) Representative H&E staining (arrows, inflammatory cells) and Oil red O staining from 4-week liver sections of *Cxcl10*$^{-/-}$ and WT mice fed a control or MCD diet. (B) Hepatic *CXCL10* mRNA and protein levels in liver tissues of WT mice. (C) Serum ALT, total hepatic lipid peroxide and liver triglyceride content in WT and *Cxcl10*$^{-/-}$ mice fed control or MCD diet for 4 weeks. (D) Collagen deposition by Sirius Red staining and hydroxyproline content of liver sections in mice fed a control or MCD diet for 8 weeks. Data are mean ± SD, n = 5–8/group. $^{*}p$ <0.05, $^{**}p$ <0.001, $^{***}p$ <0.0001 *vs.* same genotype mice fed control diet. $^{\#}p$ <0.01 *vs.* WT mice fed MCD diet.

CXCL10 induces hepatic chemokines, cytokines and other proinflammatory molecules

We next determined the mechanisms of CXCL10 in regulating hepatic inflammation by analysing chemokines and cytokines involved in inflammation and cell recruitment. In keeping with the improved liver histology and reduction of liver injury, loss of *CXCL10* significantly reduced the production of key pro-inflammatory chemokines and cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and monocyte chemoattractant protein-1 (MCP-1), as indicated initially by a cytokine profiling assay (Fig. 2A) and confirmed by qRT-PCR (Supplementary Fig. 1A–C). We then conducted a cDNA expression assay to identify molecules involved in CXCL10-mediated pathogenesis of

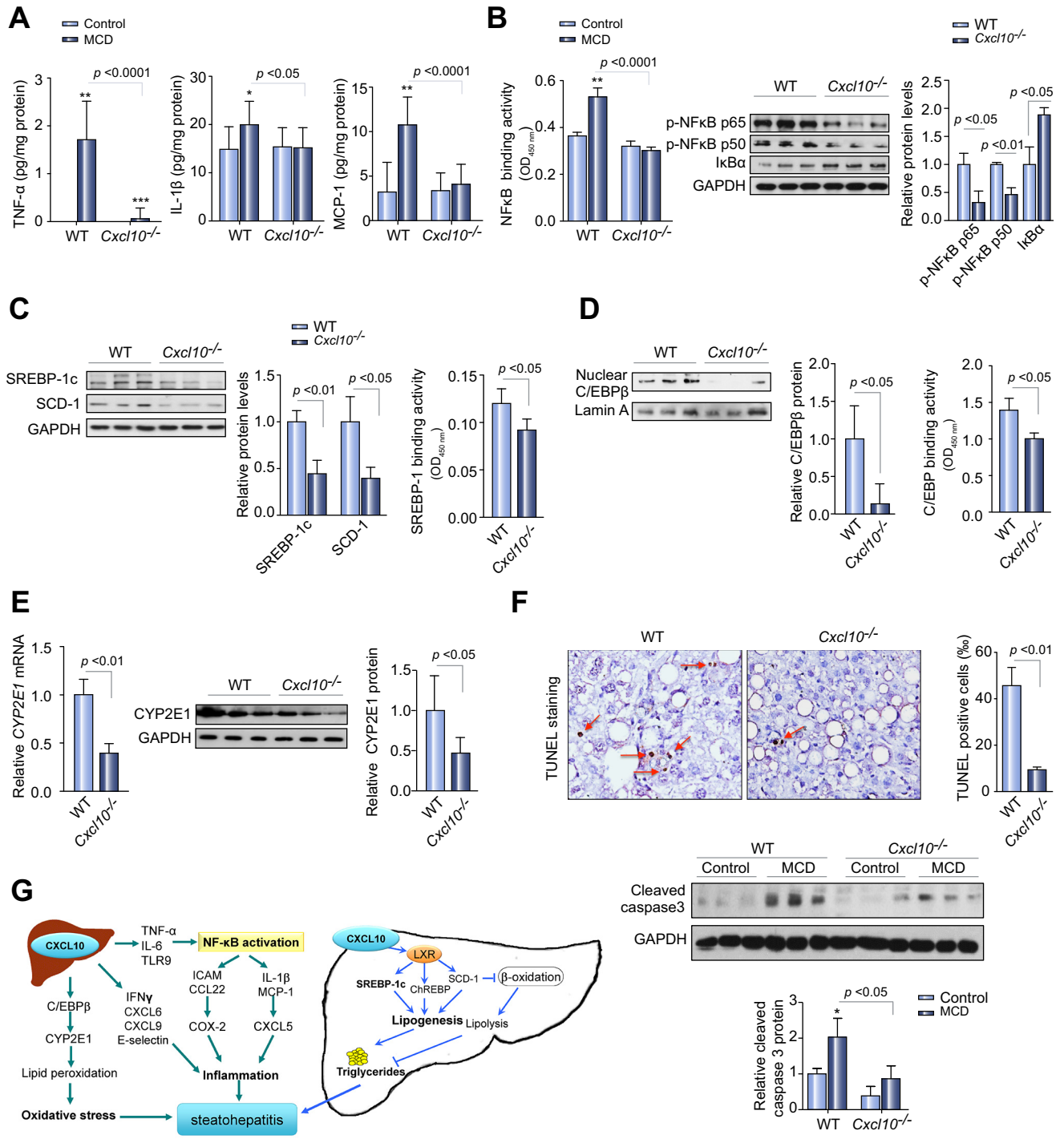


Fig. 2. CXCL10 induces steatohepatitis through hepatic inflammatory molecules, lipogenic factors and oxidative stress. (A) Hepatic TNF-α, IL-1β, and MCP-1 protein levels in mice fed control or MCD diet. (B) NF-κB nuclear binding activity and protein levels of phosphorylated NF-κB subunits p65, p50 and NF-κB suppressor IκBα, (C) protein levels of SREBP-1c, SCD1 and nuclear SREBP-1c DNA binding activity, (D) protein levels of C/EBPβ and nuclear C/EBP DNA binding activity, (E) hepatic *CYP2E1* mRNA and protein expression in MCD-fed mice. (F) TUNEL positive cells per 1000 cells and cleaved caspase 3 expression in liver tissues. (G) Schematic diagram for the mechanisms of CXCL10 in the promotion of steatohepatitis. *p <0.05, **p <0.0001 *vs.* same genotype mice fed control diet.

steatohepatitis using an additional panel of inflammatory response factors and comparing their expression in MCD-fed *Cxcl10*$^{-/-}$ and WT mouse livers. Loss of *CXCL10* was associated with substantially increased expression of *CXCL6* (63.2-fold), *CXCL9* (27.7-fold), E-selectin (*SELE*), *IFN-γ*, oxidative stress-associated transcription factor CCAAT/enhancer binding protein beta (*C/EBPβ*), and NF-κB signalling components including *IL-6* (24.1-fold), *CCL22* (13.2-fold), *TLR9*, *CXCL5* and cyclooxygenase-2

Table 1. The effect of CXCL10 on gene expression profiles of inflammatory response in mice liver tissues.

Gene name	Fold change†	Gene function
CXCL6	63.2	Pro-inflammatory cytokine
CXCL9	27.7	Inflammatory response
E-Selectin	7.4	Inflammatory response
IFN-γ	5.3	Pro-inflammatory cytokine
C/EBPβ	4.2	Oxidative stress
IL-6	24.1	Acute-phase response
CCL22	13.2	Inflammatory response
TLR9	4.2	Inflammatory response
CXCL5	2.8	Inflammatory response
COX-2	2.7	Acute-phase response

†MCD-fed WT mice *vs.* MCD-fed *Cxcl10*$^{-/-}$ mice.

(*COX-2*) (Table 1). These data support that *CXCL10* plays a critical role in liver inflammation.

CXCL10 activates NF-κB

Given the crucial role of NF-κB signalling in the pathogenesis of steatohepatitis [20,21], we examined whether CXCL10 played any role in modulation of this pathway in steatohepatitis. NF-κB nuclear binding activity was increased in MCD-fed WT mice compared with control diet (p <0.0001), but not in MCD-fed *Cxcl10*$^{-/-}$ mice (Fig. 2B). This was confirmed by enhanced levels of phosphorylated NF-κB subunits p65 and p50, decreased cytosolic NF-κB suppressor IκBα (Fig. 2B) and upregulation of NF-κB downstream factor intercellular adhesion molecule-1 (ICAM-1) in MCD-fed WT mice compared to corresponding *Cxcl10*$^{-/-}$ mice (Supplementary Fig. 1D). Our findings indicate that CXCL10 employs NF-κB signalling to mediate inflammation in steatohepatitis.

CXCL10 contributes to hepatic steatosis by inducing lipogenic genes

To seek an explanation for the reason why deletion of *CXCL10* caused less steatosis, we assessed hepatic expression of lipogenic regulators and genes, including liver X receptor (LXR)α, LXRβ, sterol regulatory element binding protein isoform 1c (SREBP-1c), carbohydrate response element binding protein (ChREBP) and stearoyl-CoA desaturase isoform-1 (SCD-1) as well as genes and regulators of hepatic fatty acid oxidation, such as adiponectin, peroxisome proliferator-activated receptor-alpha and its downstream target molecules, acyl-CoA oxidase, long-chain acyl-CoA dehydrogenase, cytochrome P450 (CYP) 4a10 and 4a14. Compared to MCD-fed WT mice, MCD-fed *Cxcl10*$^{-/-}$ mice showed significantly lower mRNA expression of *LXRα*, *LXRβ*, *SREBP-1c*, *ChREBP*, and *SCD-1* (Table 2). Western blot confirmed the downregulation of SREBP-1c and SCD-1 protein expression in liver lysates (Fig. 2C). Concomitantly, the nuclear DNA-binding activity of SREBP-1c was decreased in *Cxcl10*$^{-/-}$ mice (Fig. 2C). Expression of lipolytic genes, regulating fatty acid oxidation, was similar between *Cxcl10*$^{-/-}$ and WT mice fed MCD (Table 2). These findings indicate that CXCL10 either directly or indirectly (such as via MCP-1) can influence hepatic lipogenesis, thereby contributing to steatosis as well as its inflammatory consequences.

CXCL10 contributes to oxidative stress through CYP2E1 and C/EBPβ

In addition to the accumulation of hepatic triglycerides in response to choline deficiency and lipogenesis [22,23], and impaired antioxidant defences in response to methionine deficiency, induction of CYP2E1 (or CYP4A) [24] and C/EBPβ [17] may induce oxidative stress in the MCD model of steatohepatitis. To establish whether the latter factors contributed to the protection against steatohepatitis afforded by *CXCL10* deletion, we evaluated the levels of C/EBPβ and CYP2E1. Both C/EBPβ and CYP2E1 mRNA and protein expression were significantly less in MCD-fed *Cxcl10*$^{-/-}$ compared to corresponding WT mice (Fig. 2D and E, Table 1). Nuclear C/EBP DNA-binding activity was also decreased in *Cxcl10*$^{-/-}$ mice (Fig. 2D). Thus, CXCL10 could contribute to hepatic oxidative stress in steatohepatitis by regulating C/EBP and its downstream target CYP2E1.

CXCL10 contributes to hepatic apoptosis

As the elevation of apoptotic cell death is closely associated with the severity of NASH [25], we assessed the role of CXCL10 in regulating hepatic apoptosis in steatohepatitis by TUNEL assay. We found that TUNEL-positive cells were significantly less in *Cxcl10*$^{-/-}$ mice compared to WT mice fed MCD (0.94% *vs.* 4.56%, p <0.01) (Fig. 2F). Consistent with the impaired apoptosis, the protein expression of the active form of the apoptosis regulator caspase-3 was significantly downregulated in *Cxcl10*$^{-/-}$ mice compared with WT mice (Fig. 2F).

Phenotypic analysis of immune cells in the spleen and peripheral blood of Cxcl10$^{-/-}$ *and WT mice*

In order to investigate the major immune cell populations in CXCL10$^{-/-}$ and WT mice, we performed FACS analysis in the spleen and peripheral blood. Consistent with a previous report of *Cxcl10*$^{-/-}$ mice [26], the frequencies of B cells ($CD19^+CD3^-$), T cells ($CD3^+CD19^-$), NK cells ($NK1.1^+CD3^-$), NKT cells ($NK1.1^+CD3^+$), macrophages ($CD11b^+F4/80^+$) and neutrophils ($CD11b^+Ly6G^+$) in the spleen and peripheral blood of *Cxcl10*$^{-/-}$ mice were not changed compared to WT mice under MCD or control diet (Supplementary Figs. 2 and 3). Only a slight reduction of $CD8^+$ T lymphocytes ($CD8^+CD4^-$) was observed in the spleen of MCD-fed *Cxcl10*$^{-/-}$ mice compared to WT mice fed with the same diet (34.3 ± 2.26 *vs.* 38.5 ± 1.34) (Supplementary Fig. 2).

Inactivation of CXCL10 by anti-CXCL10 mAb antagonizes MCD-induced steatohepatitis

The above results indicate essential and multiple roles of CXCL10 in steatohepatitis pathogenesis. If this is the case, specific CXCL10 inhibition should dampen or abrogate the development of this type of liver pathology. To test this, we first examined the functional effect of an anti-CXCL10 monoclonal antibody (mAb) on steatosis and injury to hepatocyte-derived cells *in vitro*. As reported [15,17], incubation of the immortalized murine hepatocyte cell line AML-12 with MCD medium for 24 h increased medium ALT, cellular triglyceride and oxidative stress, detected by TBARS and lipid hydroperoxide assays (Supplementary Fig. 4). Conversely, anti-CXCL10 mAb added to AML-12 cells incubated in MCD medium significantly reduced medium ALT, cellular triglycerides, cellular TBARS and lipid hydroperoxide levels

Table 2. Hepatic mRNA expression of genes involved in fatty acid regulation in *Cxcl10*$^{-/-}$ mice.

Gene	WT mice		*Cxcl10*$^{-/-}$ mice	
	Control	MCD	Control	MCD
Lipogenic genes				
LXRα	1.05 ± 0.33	1.52 ± 0.31**	0.99 ± 0.12	0.97 ± 0.23##
LXRβ	1.05 ± 0.30	1.91 ± 0.68**	1.28 ± 0.31	1.30 ± 0.34#
SREBP-1c	1.01 ± 0.16	0.63 ± 0.30*	0.53 ± 0.23	0.30 ± 0.11#
ChREBP	1.01 ± 0.18	0.66 ± 0.26*	1.25 ± 0.31	0.32 ± 0.08***#
SCD-1	1.035 ± 0.33	0.012 ± 0.006***	0.570 ± 0.213	0.006 ± 0.004***#
Lipolytic genes				
Adiponectin	1.44 ± 1.09	0.30 ± 0.29	2.15 ± 0.97	1.08 ± 0.91
PPARα	1.01 ± 0.14	0.69 ± 0.23*	1.17 ± 0.14	0.59 ± 0.09***
ACO	1.04 ± 0.35	0.28 ± 0.07**	1.27 ± 0.49	0.29 ± 0.06
LCAD	1.04 ± 0.31	1.19 ± 0.53	0.74 ± 0.29	0.71 ± 0.22
CYP4A10	0.91 ± 0.55	2.79 ± 1.20*	1.30 ± 0.68	1.46 ± 0.61
CYP4A14	2.03 ± 2.45	79.5 ± 34.2***	1.69 ± 1.16	50.2 ± 25.4**

Specific mRNA expression values were normalized to the expression of GAPDH. Data are mean ± SD, n = 5–8/group. *p <0.05, **p <0.01, ***p <0.0001 compared with corresponding mice fed control diet. #p <0.05, ##p <0.0001 compared with WT mice fed the MCD diet.

compared to AML-12 cells in MCD medium exposed to control IgG2A mAb (Supplementary Fig. 4).

We next examined whether administration of the anti-CXCL10 mAb by intraperitoneal injection could prevent MCD-induced steatohepatitis *in vivo*. Administration of the anti-CXCL10 mAb to MCD-fed WT mice reduced steatosis and inflammatory cell infiltration (Fig. 3A), with concordant reduction of serum ALT, hepatic triglyceride and lipid hydroperoxide levels (Fig. 3B) compared to MCD-fed mice administered control mAb. Likewise, CXCL10 neutralization suppressed NF-κB binding activity (p <0.01), and reduced the expression of phosphorylated NF-κB subunits p65 and p50 (p <0.05) and *ICAM-1* mRNA (p <0.05) (Fig. 3C). Moreover, blocking CXCL10 significantly decreased the levels of CYP2E1 (p <0.01) and SREBP-1c (p <0.05) (Fig. 3D).

After confirming a preventive effect on steatohepatitis, we further examined whether CXCL10 neutralization could treat steatohepatitis after it has been established. After induction of steatohepatitis in mice fed the MCD diet for 3 weeks, anti-CXCL10 mAb or control mAb was supplemented for 10 days under MCD diet. Histological analysis of livers by H&E and Oil red O staining showed significantly reduced lipid accumulation and inflammatory cell infiltration in MCD-fed mice treated with anti-CXCL10 mAb (Fig. 3E). Anti-CXCL10 mAb treatment in MCD-fed mice also significantly decreased hepatic triglyceride and lipid peroxide levels compared to MCD-fed mice administrated with control mAb (Fig. 3F). Moreover, CXCL10 neutralization suppressed hepatic *TNF-α* (p <0.05) and *ICAM-1* (p <0.05) mRNA expression (Fig. 3G). These data added further weight to the effects of CXCL10 in mediating inflammation, oxidative stress and steatosis in the evolution of steatohepatitis.

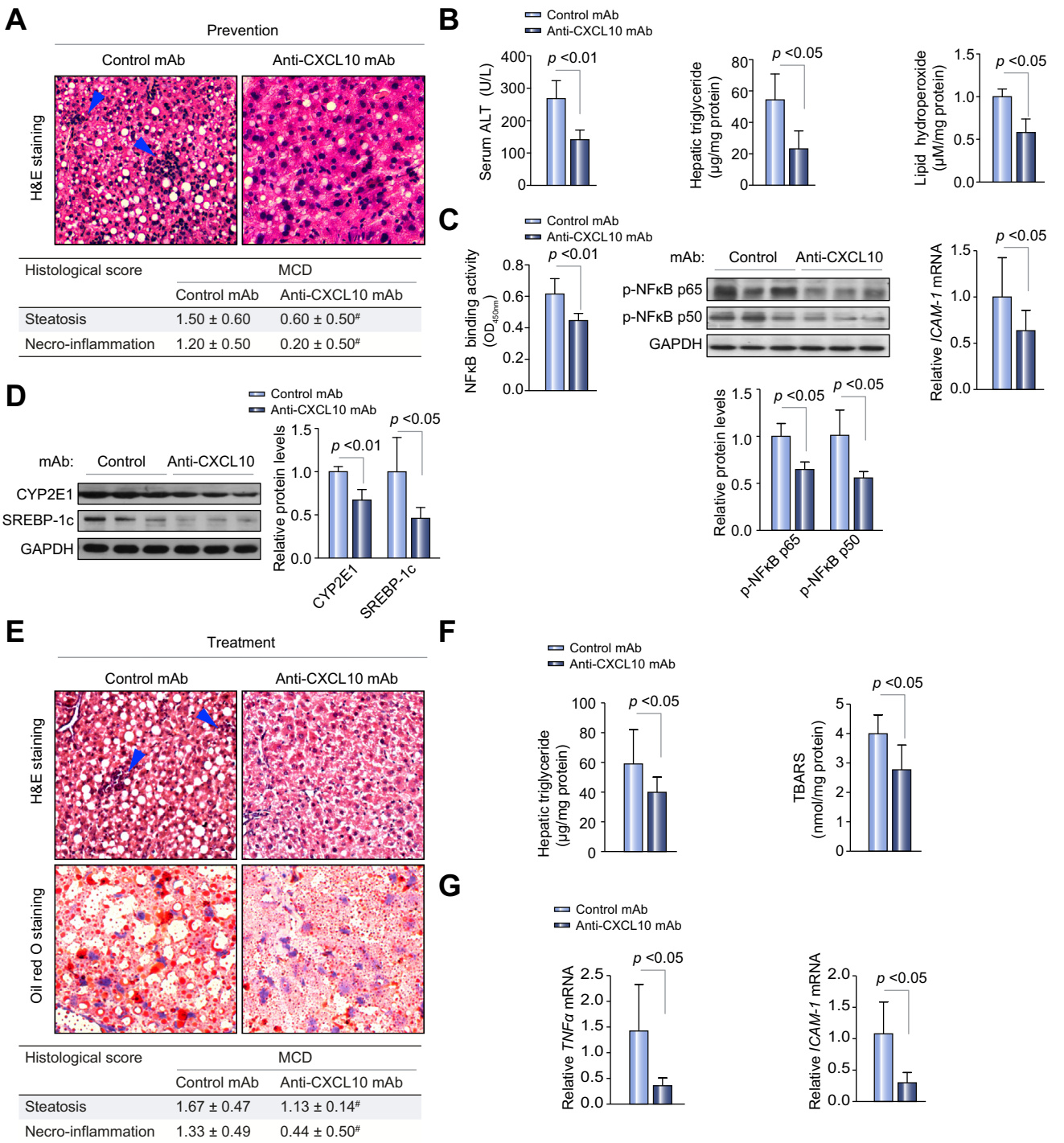
CXCL10 is associated with lobular inflammation and acts as an independent risk factor of human NASH

Since the MCD model reflects pathologically severe steatohepatitis with choline and amino acid nutritional deficiency and a context of "lipid trapping" in the liver with severe oxidative stress, it remains important to establish whether human NASH related to over-nutrition is also associated with increased liver expression and circulating levels of CXCL10. To this end, we assayed *CXCL10* mRNA in liver biopsy from 15 control subjects and 22 NAFLD patients (11 simple steatosis patients and 11 human NASH patients). The results showed that hepatic *CXCL10* mRNA levels were significantly higher in primary NASH tissue compared to simple steatosis (p <0.05) and normal controls (p <0.001) (Fig. 4A), inferring that hepatic CXCL10 production is prominent in patients with NASH.

We next ascertained the clinical impact of CXCL10 in NASH patients. We enrolled a well-established prospective cohort of 73 control subjects without fatty liver measured by proton-magnetic resonance spectroscopy and 147 age and gender matched biopsy-proven NAFLD patients, 69 of whom were diagnosed as NASH [18,19]. We found that serum CXCL10 was significantly increased in a stepwise fashion from control subjects (111 [IQR: 98–146] pg/ml), patients with simple steatosis (170 [133–225] pg/ml) to patients with NASH (248 [154–310] pg/ml) (Fig. 4B, all p <0.0001). In NAFLD patients (simple steatosis and NASH), CXCL10 was significantly and positively correlated with lobular inflammation (rho: 0.26, p = 0.002) and hepatocyte ballooning degeneration (rho: 0.24, p = 0.004), which are two major histological features of NASH (Table 3). Multivariable linear regression analysis also demonstrated that the serum CXCL10 level was positively associated with lobular inflammation (Beta: 47.9; 95% CI: 15.0–80.8; p = 0.005) and ballooning (Beta: 51.1; 95% CI: 20.0–82.1; p = 0.001) independent of metabolic syndrome, body mass index (BMI), ALT, triglyceride, fasting glucose and cholesterol. Moreover, we performed a multivariate logistic regression analysis on these subjects and identified that CXCL10 was an independent risk factor for NASH in NAFLD patients (OR: 1.008, 95% CI: 1.004–1.013, p <0.001) (Table 4, with factors included in the regression model listed).

CXCL10 is a potential biomarker for the clinical diagnosis of NASH

To evaluate the utility of CXCL10 as a biomarker in the diagnosis of NAFLD and NASH, a ROC curve was constructed. CXCL10



Histological score	MCD	
	Control mAb	Anti-CXCL10 mAb
Steatosis	1.50 ± 0.60	0.60 ± 0.50#
Necro-inflammation	1.20 ± 0.50	0.20 ± 0.50#

Histological score	MCD	
	Control mAb	Anti-CXCL10 mAb
Steatosis	1.67 ± 0.47	1.13 ± 0.14#
Necro-inflammation	1.33 ± 0.49	0.44 ± 0.50#

Fig. 3. CXCL10 neutralization protects against steatohepatitis *in vivo*. (A) Representative H&E staining, (B) serum ALT, hepatic triglyceride, lipid hydroperoxide, (C) NF-κB binding activity, phospho NF-κB p65, p50, ICAM-1 levels, (D) CYP2E1 and SREBP-1c expression in mice administrated with anti-CXCL10 or control mAb at 12 h before feeding MCD. (E) Liver sections with H&E staining and Oil red O staining, respectively, (F) hepatic triglyceride and lipid peroxidation products (TBARS), (G) *TNF-α* and *ICAM-1* mRNA expression from mice injected with anti-CXCL10 or control mAb at 3 weeks after MCD feeding. #p <0.05 *vs.* mice treated with control mAb. Data are mean ± SD, n = 5/group.

exhibited a high overall accuracy in discriminating NAFLD from control subjects with the area under the receiver operating characteristic curve (AUROC) of 0.81 (95% CI: 0.75–0.87) (Fig. 4C). In NAFLD patients, CXCL10 had a moderate accuracy with the AUROC of 0.68 (95% CI: 0.59–0.77) in discriminating NASH from simple steatosis (Fig. 4C). If control subjects were also added to

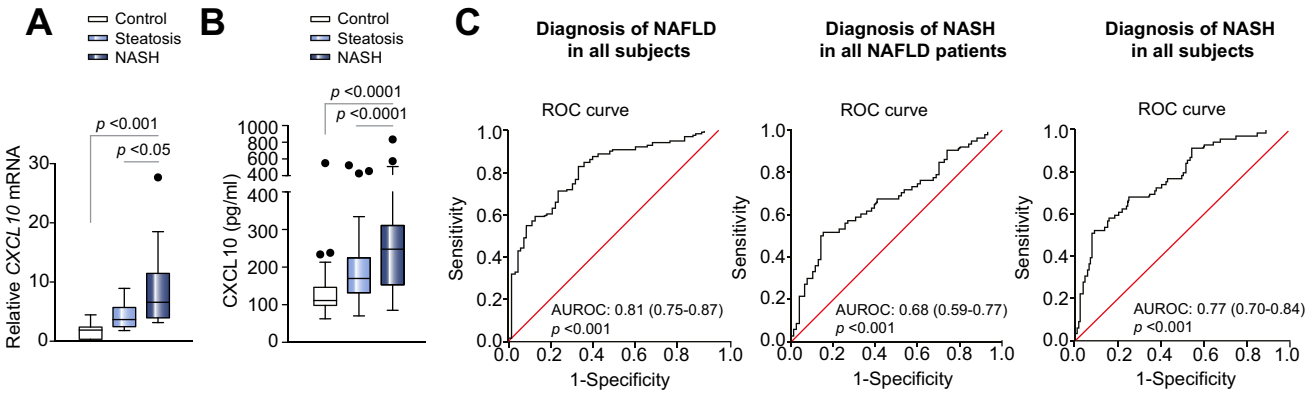


Fig. 4. CXCL10 in control and NAFLD patients. (A) Hepatic human *CXCL10* mRNA levels; (B) Serum CXCL10 protein levels; (C) Receiver-operating characteristics curves of CXCL10 in diagnosing NAFLD in all subjects, NASH in NAFLD patients and NASH in all subjects.

Table 3. Correlations with CXCL10 in NAFLD patients.

	CXCL10	
	rho	*p* value‡
Age	0.16	0.062
BMI	0.07	0.408
Total cholesterol	0.09	0.304
Triglyceride	0.09	0.286
Steatosis	0.15	0.070
Lobular inflammation	0.26	0.002
Ballooning	0.24	0.004
Fibrosis	0.25	0.002

‡*p* value corresponds to Ho: rho = 0.

Table 4. Multivariable analysis for independent risk factors for NASH in NAFLD patients.

	OR	95% CI	*p* value
CXCL10	1.008§	1.004-1.013	<0.001
Metabolic syndrome	3.083	1.203-7.903	0.019

Variables entered in the regression model: CXCL10, gender, age, body mass index (BMI), metabolic syndrome, alanine aminotransferase (ALT), fasting glucose, triglyceride, low density lipoprotein-cholesterol (LDL-c), glycated haemoglobin (HbA1c).
§For every 1 unit increase of CXCL10 level.

the analysis, the AUROC of diagnosing NASH increased to 0.77 (95% CI: 0.70–0.84) (Fig. 4C). Thus, CXCL10 can be a novel biomarker for the clinical diagnosis of NAFLD and NASH.

Discussion

The first novel finding in these studies is that *Cxcl10*$^{-/-}$ mice administrated with MCD diet showed significantly attenuated steatohepatitis compared with WT mice fed the same diet; these findings were corroborated by improved liver histology, lowered serum ALT, and hepatic triglyceride content. Moreover, *CXCL10* deletion was associated with a significant reduction of intrahepatic oxidative stress, as indicated by decreased lipid peroxide levels. This change was clearly associated with the attenuation of hepatic inflammation. In addition, *CXCL10* deficiency confers protection from hepatic nutritional fibrosis. Our data provide the first evidence that CXCL10 may contribute to lipogenesis, thereby influencing steatosis and possibly lipotoxicity, as well as hepatocellular injury and perpetuation of liver inflammation in steatohepatitis, at least in the MCD model.

The molecular mechanisms by which CXCL10 exerts its broad range of functions in steatohepatitis were subsequently studied. As a key pro-inflammatory cytokine, CXCL10 often amplifies the effects of other cytokines [5]. We therefore evaluated the effect of CXCL10 on other potential cytokines in steatohepatitis and showed that CXCL10 was associated with induction of TNF-α, IL-1β, and MCP-1. TNF-α is a key inflammatory factor involved in the development of human NASH [27] and experimental steatohepatitis [28]. TNF-α can activate neutrophils, cause insulin resistance and promote NASH development. TNF-α and IL-1β are able to induce MCP-1 *in vitro*, suggesting that these cytokines are functionally related [29]. MCP-1 is also an important molecule in NASH as it may bridge inflammatory responses with the induction of insulin resistance [30]. Moreover, MCP-1 can stimulate lipogenesis to promote steatosis in the liver, allowing inflammation to exacerbate steatosis [4]. This suggests that CXCL10 induces cytokine expression, leading to the development of steatohepatitis.

We further characterized the inflammatory factors, regulated by CXCL10 in steatohepatitis, by a cDNA array covering 84 well-known inflammatory genes. Our results show that pro-inflammatory factors, including *IFN-γ*, *TLR9*, *CXCL9*, *IL-6*, *SELE*, *CXCL6*, *CCL22*, *CXCL5*, and *COX-2*, were significantly higher in WT mice than in *Cxcl10*$^{-/-}$ mice fed with MCD. Each of these molecules could amplify the inflammatory recruitment in steatohepatitis. To be specific, IFN-γ is a major inducer of CXCL10 related to NASH pathogenesis [31]; TLR9 activates IFN regulatory factors that induce production of IL-1β, leading to NASH development in mouse model [32]; CXCL9, induced by IFN-γ, is increased in the livers of patients with NASH [33], while IL-6 is a key inflammatory factor involved in NASH development [34]. Serum levels of E-selectin (*SELE*) are also higher in patients with NASH similar to those of IL-6 [35]. CXCL6 is associated with the severity of hepatic inflammation in NAFLD patients and it can be used for predicting NASH progression [36]. Similarly, CCL22 and CXCL5, two small chemokines, are serum markers for NASH and the related obesity and metabolic syndrome [37]. Finally, COX-2,

another pro-inflammatory mediator, plays an important role in metabolic forms of steatohepatitis as reported earlier by us [28]. In addition to IFN-γ and TLR9, a close correlation between CXCL10 and TNF-α, MCP-1, IL-6 has been well documented [38,39]. Collectively, these data suggested that induction of pro-inflammatory cytokines and key inflammatory factors by CXCL10 is part of a mechanism for the inflammatory recruitment in MCD-induced steatohepatitis.

An important observation in the present study is that the majority of the above cytokines and inflammatory factors are regulated by NF-κB signalling (Fig. 2G). TNF-α is a potent activator of NF-κB, and in turn activated NF-κB induces TNF-α expression [40]. TLR9 and IL-6 can also activate NF-κB [32], while ICAM-1, CCL22, COX-2, IL-1β, MCP-1, and CXCL5 are downstream effectors of NF-κB activation (Fig. 2G) [20]. These data, when combined with the previous finding that NF-κB is a key regulator of early hepatic inflammatory recruitment and liver injury in NASH [21], implicate a collaborative interaction of CXCL10 and NF-κB to promote steatohepatitis. Therefore, CXCL10 may act as a lipotoxic molecule that activates NF-κB and its downstream inflammatory effectors to induce hepatocyte apoptosis and liver injury, leading to the progression of steatohepatitis.

The underlying causes of hepatic triglyceride accumulation in steatosis include enhanced uptake and synthesis of fatty acids, and inhibition of fatty acid oxidation. In our experimental steatohepatitis model, knockout of *CXCL10* significantly reduced hepatic triglyceride content and steatosis (Fig. 1A and C). This reduction was associated with reduced activity of SREBP-1c and downregulation of SREBP-1c, ChREBP, LXRs, and SCD-1 (Fig. 2 and Table 2), which are involved in *de novo* fatty acid synthesis. In addition to SREBP-1c and ChREBP, LXR is a major transcriptional activator for lipogenesis [41]; it modulates the expression of SREBP-1c through directly binding to the promoter of *SREBP-1c*. LXR also induces the transcription of the lipogenic genes *SCD-1* and *ChREBP* [41]. Thus, the likely pathways by which CXCL10 promotes hepatic steatosis include the upregulation of key fatty acid synthesis genes that promote fatty acid synthesis (Fig. 2G).

It is of general agreement that oxidative stress facilitates the advancement of steatosis to steatohepatitis. Among the common mediators of oxidative stress [42], CYP2E1 is an oxido-reductase that can promote NASH development by inducing oxidative/nitrosative stress, protein modifications, inflammation and insulin resistance [43]. Consistent with this, we confirmed earlier findings [17,24] that CYP2E1 expression is upregulated in MCD-induced steatohepatitis. Importantly, we showed that deletion of *CXCL10* completely abolished the MCD-dependent stimulation of CYP2E1, and significantly reduced the expression of its transcriptional activator C/EBPβ (Fig. 2D–E). These data were in accordance with the lower level of CYP2E1 in MCD-fed *Cebpb*$^{-/-}$ mice compared with MCD-fed WT mice [41], and demonstrate that CXCL10 can act upstream of C/EBPβ and CYP2E1 to modulate oxidative stress.

If CXCL10 plays a key part in the pathogenesis of steatohepatitis, it would be important to establish that its functional blockade ameliorates the severity of steatohepatitis. To test this, we used anti-CXCL10 mAb to neutralize CXCL10 *in vitro*. Such neutralization caused a dose-dependent decrease in triglyceride secretion and ALT release, together with a concomitant suppression of cellular oxidative stress in AML12 hepatocytes (Supplementary Fig. 4). Moreover, anti-CXCL10 mAb ameliorated the severity of fatty liver disease in MCD-fed mice. In the present work, CXCL10 neutralization using anti-CXCL10 mAb in mice showed significant improvements in the prevention and regression of steatohepatitis (Fig. 3). These effects were associated with reduced hepatic triglyceride and lipid peroxide levels (Fig. 3). Thus, CXCL10 is a potential target for the prevention and treatment of steatohepatitis.

These mechanistic findings of CXCL10 in the evolution of experimental steatohepatitis encouraged us to explore the clinical impact of CXCL10 in patients with NAFLD and NASH. We first demonstrated that CXCL10 was significantly upregulated both in liver and serum samples of NASH patients. Moreover, the circulating level of CXCL10 in NASH patients was associated with lobular inflammation, which is supported by a previous study that showed that increased CXCL10 levels were correlated with the degree of chronic liver inflammatory damage caused by hepatitis C virus infection [9]. Early identification of patients with NASH may allow intervention that may alter the course of the disease. Currently, liver biopsy remains the standard method for the diagnosis of NASH and differentiation from simple steatosis. However, biopsy is an invasive diagnostic procedure that has been associated with sampling error and observer variability. Thus, the development of a non-invasive test is paramount to the management of NASH. To date, there are no reliable serologic tests for the identification of NASH. Identification of such biomarker would aid clinicians in the identification of patients with NASH, and allow for non-invasive frequent monitoring of disease progression and response to therapy. Building on the significantly elevated CXCL10 level in NASH patients, we tested the clinical utility of CXCL10 as a serologic biomarker for the diagnosis of NASH. Base on a multivariate Cox regression analyses in a study cohort of 147 NAFLD patients and 73 control subjects, CXCL10 was revealed to be a novel risk factor of NASH independent of metabolic syndrome, ALT, diabetes and triglycerides (Table 4). Moreover, the AUROC indicated an overall accuracy of 81% to diagnose NAFLD and an accuracy of 77% to diagnose NASH, suggesting that circulating CXCL10 production could be regarded as a valuable new diagnostic factor for NAFLD and NASH. However, it should be noted that a few prediction models such as the NAFLD fibrosis score have also been developed to predict advanced fibrosis [44,45], These scores are comprised of predicting factors of fibrosis such as age, BMI and metabolic factors. While it is interesting that CXCL10 may serve as a marker of NASH, the finding warrants independent validation. Furthermore, it would also be important to explore its role in conjunction with other predicting factors to improve the diagnosis.

The MCD diet model is a classic and widely adopted dietary model for studying NASH. It can induce hepatic steatohepatitis with inflammation, oxidative stress, mitochondrial DNA damage, apoptosis and fibrosis [46]. Therefore, it is considered as one of the best-established models for studying NASH-associated inflammation, oxidative stress and fibrosis. However, it does not fully manifest all human NASH features. Mice fed with MCD diet lose weight instead of being obese and lack insulin resistance [47]. In the future, high-fat and high-fructose model (also termed as American Lifestyle-Induced Obesity Syndrome [ALIOS]), which may result in an obese animal with severe steatosis, inflammation, oxidative stress and insulin resistance at 16 weeks [46,48], could be used to support our findings.

In conclusion, these observations and interventions demonstrate for the first time that CXCL10 plays an essential role in

the development of steatohepatitis in the context of fatty liver disease. Further, the mechanism of this effect is through regulation of lipogenesis and oxidative stress either directly or indirectly via pathway modulation and pro-inflammatory signalling, altering the expression of other key chemokines, cytokines and pro-inflammatory molecules. Circulating CXCL10 may be a potential biomarker for patients with NAFLD and NASH.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

XZ was involved in study design, experiments conduct, and data analysis; JS, ESHC, TOY, JCYS, MYYG, and JD performed the research; KM, LL, and VWSW provided material support; JJYS and GF commented on the study and revised the paper. JY designed, supervised the study and wrote the paper.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.07.006.

References

[1] Farrell GC, Wong VW, Chitturi S. NAFLD in Asia – As common and important as in the West. Nat Rev Gastroenterol Hepatol 2013;10:307–318.
[2] Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. Science 2011;332:1519–1523.
[3] Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. Hepatology 2006;43:S99–S112.
[4] Farrell GC, van Rooyen D, Gan L, Chitturi S. NASH is an inflammatory disorder: pathogenic, prognostic, and therapeutic implications. Gut Liver 2012;6:149–171.
[5] Neville LF, Mathiak G, Bagasra O. The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily. Cytokine Growth Factor Rev 1997;8:207–219.
[6] Luster AD, Unkeless JC, Ravetch JV. Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. Nature 1985;315:672–676.
[7] Abe T, Fukuhara T, Wen X, Ninomiya A, Moriishi K, Maehara Y, et al. CD44 participates in IP-10 induction in cells in which hepatitis C virus RNA is replicating, through an interaction with Toll-like receptor 2 and hyaluronan. J Virol 2012;86:6159–6170.
[8] Zhou Y, Wang S, Ma JW, Lei Z, Zhu HF, Lei P, et al. Hepatitis B virus protein X-induced expression of the CXC chemokine IP-10 is mediated through activation of NF-kappaB and increases migration of leukocytes. J Biol Chem 2010;285:12159–12168.
[9] Harvey CE, Post JJ, Palladinetti P, Freeman AJ, Ffrench RA, Kumar RK, et al. Expression of the chemokine IP-10 (CXCL10) by hepatocytes in chronic hepatitis C virus infection correlates with histological severity and lobular inflammation. J Leukoc Biol 2003;74:360–369.
[10] Hintermann E, Bayer M, Pfeilschifter JM, Luster AD, Christen U. CXCL10 promotes liver fibrosis by prevention of NK cell mediated hepatic stellate cell inactivation. J Autoimmun 2010;35:424–435.
[11] Yoneyama H, Kai Y, Koyama J, Suzuki K, Kawachi H, Narumi S, et al. Neutralization of CXCL10 accelerates liver regeneration in carbon tetrachloride-induced acute liver injury. Med Mol Morphol 2007;40:191–197.
[12] Bertola A, Bonnafous S, Anty R, Patouraux S, Saint-Paul MC, Iannelli A, et al. Hepatic expression patterns of inflammatory and immune response genes associated with obesity and NASH in morbidly obese patients. PLoS One 2010;5:e13577.
[13] Schulthess FT, Paroni F, Sauter NS, Shu L, Ribaux P, Haataja L, et al. CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling. Cell Metab 2009;9:125–139.
[14] Morimoto J, Yoneyama H, Shimada A, Shigihara T, Yamada S, Oikawa Y, et al. CXC chemokine ligand 10 neutralization suppresses the occurrence of diabetes in nonobese diabetic mice through enhanced beta cell proliferation without affecting insulitis. J Immunol 2004;173:7017–7024.
[15] Yu J, Chu ES, Wang R, Wang S, Wu CW, Wong VW, et al. Heme oxygenase-1 protects against steatohepatitis in both cultured hepatocytes and mice. Gastroenterology 2010;138:694–704.
[16] Yu J, Zhang S, Chu ES, Go MY, Lau RH, Zhao J, et al. Peroxisome proliferator-activated receptors gamma reverses hepatic nutritional fibrosis in mice and suppresses activation of hepatic stellate cells in vitro. Int J Biochem Cell Biol 2010;42:948–957.
[17] Shen B, Yu J, Wang S, Chu ES, Wong VW, Zhou X, et al. *Phyllanthus urinaria* ameliorates the severity of nutritional steatohepatitis both in vitro and in vivo. Hepatology 2008;47:473–483.
[18] Shen J, Chan HL, Wong GL, Chan AW, Choi PC, Chan HY, et al. Assessment of non-alcoholic fatty liver disease using serum total cell death and apoptosis markers. Aliment Pharmacol Ther 2012;36:1057–1066.
[19] Shen J, Chan HL, Wong GL, Choi PC, Chan AW, Chan HY, et al. Non-invasive diagnosis of non-alcoholic steatohepatitis by combined serum biomarkers. J Hepatol 2012;56:1363–1370.
[20] Baker RG, Hayden MS, Ghosh S. NF-kappaB, inflammation, and metabolic disease. Cell Metab 2011;13:11–22.
[21] Dela Pena A, Leclercq I, Field J, George J, Jones B, Farrell G. NF-kappaB activation, rather than TNF, mediates hepatic inflammation in a murine dietary model of steatohepatitis. Gastroenterology 2005;129:1663–1674.
[22] Rizki G, Arnaboldi L, Gabrielli B, Yan J, Lee GS, Ng RK, et al. Mice fed a lipogenic methionine-choline-deficient diet develop hypermetabolism coincident with hepatic suppression of SCD-1. J Lipid Res 2006;47:2280–2290.
[23] Larter CZ, Yeh MM, Haigh WG, Williams J, Brown S, Bell-Anderson KS, et al. Hepatic free fatty acids accumulate in experimental steatohepatitis: role of adaptive pathways. J Hepatol 2008;48:638–647.
[24] Leclercq IA, Farrell GC, Field J, Bell DR, Gonzalez FJ, Robertson GR. CYP2E1 and CYP4A as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis. J Clin Invest 2000;105:1067–1075.
[25] 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.
[26] Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD. IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. J Immunol 2002;168:3195–3204.
[27] Kugelmas M, Hill DB, Vivian B, Marsano L, McClain CJ. Cytokines and NASH: a pilot study of the effects of lifestyle modification and vitamin E. Hepatology 2003;38:413–419.
[28] Yu J, Ip E, Dela Pena A, Hou JY, Sesha J, Pera N, et al. COX-2 induction in mice with experimental nutritional steatohepatitis: role as pro-inflammatory mediator. Hepatology 2006;43:826–836.
[29] Petrasek J, Bala S, Csak T, Lippai D, Kodys K, Menashy V, et al. IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice. J Clin Invest 2012;122:3476–3489.

[30] Maher JJ, Leon P, Ryan JC. Beyond insulin resistance: innate immunity in nonalcoholic steatohepatitis. Hepatology 2008;48:670–678.
[31] Baranova A, Schlauch K, Elariny H, Jarrar M, Bennett C, Nugent C, et al. Gene expression patterns in hepatic tissue and visceral adipose tissue of patients with non-alcoholic fatty liver disease. Obes Surg 2007;17:1111–1118.
[32] Miura K, Kodama Y, Inokuchi S, Schnabl B, Aoyama T, Ohnishi H, et al. Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice. Gastroenterology 2010;139:323–334.
[33] Wasmuth HE, Lammert F, Zaldivar MM, Weiskirchen R, Hellerbrand C, Scholten D, et al. Antifibrotic effects of CXCL9 and its receptor CXCR3 in livers of mice and humans. Gastroenterology 2009;137:309–319.
[34] Wieckowska A, Papouchado BG, Li Z, Lopez R, Zein NN, Feldstein AE. Increased hepatic and circulating interleukin-6 levels in human nonalcoholic steatohepatitis. Am J Gastroenterol 2008;103:1372–1379.
[35] Musso G, Gambino R, Bo S, Uberti B, Biroli G, Pagano G, et al. Should nonalcoholic fatty liver disease be included in the definition of metabolic syndrome? A cross-sectional comparison with Adult Treatment Panel III criteria in nonobese nondiabetic subjects. Diabetes Care 2008;31:562–568.
[36] Mehta R, Birerdinc A, Neupane A, Shamsaddini A, Afendy A, Elariny H, et al. Expression of inflammation-related genes is altered in gastric tissue of patients with advanced stages of NAFLD. Mediators Inflamm 2013;2013:684237.
[37] Hammerich L, Heymann F, Tacke F. Role of IL-17 and Th17 cells in liver diseases. Clin Dev Immunol 2011;2011:345803.
[38] Xu W, Joo H, Clayton S, Dullaers M, Herve MC, Blankenship D, et al. Macrophages induce differentiation of plasma cells through CXCL10/IP-10. J Exp Med 2012;209:S1811–S1812.
[39] Tosello-Trampont AC, Landes SG, Nguyen V, Novobrantseva TI, Hahn YS. Kupffer cells trigger nonalcoholic steatohepatitis development in diet-induced mouse model through tumor necrosis factor-alpha production. J Biol Chem 2012;287:40161–40172.
[40] Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. J Clin Invest 2001;107:7–11.
[41] Jump DB, Botolin D, Wang Y, Xu J, Christian B, Demeure O. Fatty acid regulation of hepatic gene transcription. J Nutr 2005;135:2503–2506.
[42] Abdelmegeed MA, Banerjee A, Yoo SH, Jang S, Gonzalez FJ, Song BJ. Critical role of cytochrome P450 2E1 (CYP2E1) in the development of high fat-induced non-alcoholic steatohepatitis. J Hepatol 2012;57:860–866.
[43] Rahman SM, Schroeder-Gloeckler JM, Janssen RC, Jiang H, Qadri I, Maclean KN, et al. CCAAT/enhancing binding protein beta deletion in mice attenuates inflammation, endoplasmic reticulum stress, and lipid accumulation in diet-induced nonalcoholic steatohepatitis. Hepatology 2007;45:1108–1117.
[44] Angulo P, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, et al. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology 2007;45:846–854.
[45] Wong VW, Wong GL, Chim AM, Tse AM, Tsang SW, Hui AY, et al. Validation of the NAFLD fibrosis score in a Chinese population with low prevalence of advanced fibrosis. Am J Gastroenterol 2008;103:1682–1688.
[46] Kohli R, Feldstein AE. NASH animal models: are we there yet? J Hepatol 2011;55:941–943.
[47] Rinella ME, Green RM. The methionine-choline deficient dietary model of steatohepatitis does not exhibit insulin resistance. J Hepatol 2004;40:47–51.
[48] Tetri LH, Basaranoglu M, Brunt EM, Yerian LM, Neuschwander-Tetri BA. Severe NAFLD with hepatic necroinflammatory changes in mice fed trans fats and a high-fructose corn syrup equivalent. Am J Physiol Gastrointest Liver Physiol 2008;295:G987–G995.