Research Article
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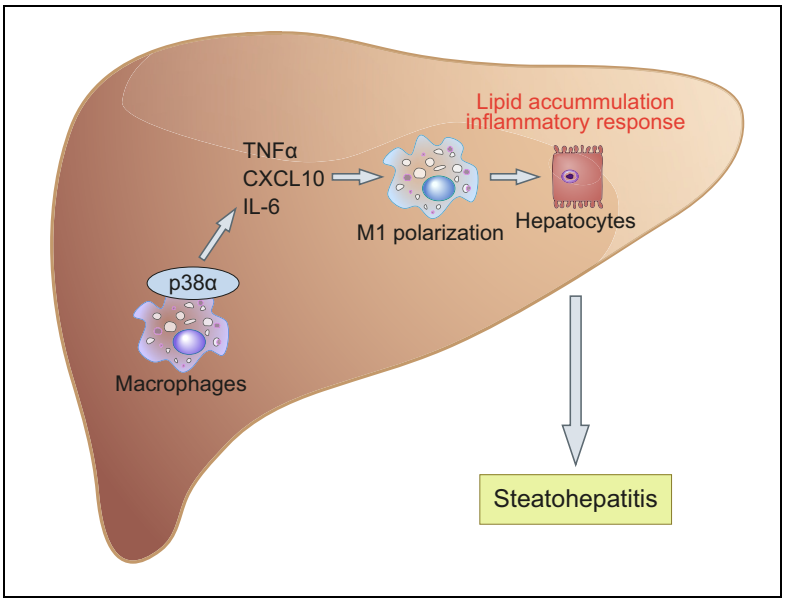
Graphical abstract



Highlights

- p38α expression is increased in livers of human patients with non-alcoholic fatty liver diseases.
- Macrophage p38α induces experimental steatohepatitis.
- Macrophage p38α causes M1 macrophage polarization.
- p38α deleted macrophages attenuate steatohepatitic changes in hepatocytes.
- Pharmacological p38 inhibitors prevent steatohepatitis in mice.

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Lay summary

p38 mitogen-activated protein kinases are important inflammatory factors. In the present study, we demonstrated that p38α is upregulated in liver tissues of patients with non-alcoholic fatty liver diseases. Genetic deletion of p38α in macrophages led to ameliorated nutritional steatohepatitis in mice through decreased pro-inflammatory cytokine secretion and increased M2 macrophage polarization.

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Macrophage p38α promotes nutritional steatohepatitis through M1 polarization

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Background & Aims: p38 mitogen-activated protein kinases are important inflammatory factors. p38α alteration has been implicated in both human and mouse inflammatory disease models. Therefore, we aimed to characterize the cell type-specific role of p38α in non-alcoholic steatohepatitis (NASH).

Methods: Human liver tissues were obtained from 27 patients with non-alcoholic fatty liver disease (NAFLD) and 20 control individuals. NASH was established and compared between hepatocyte-specific p38α knockout ($p38\alpha^{\Delta Hep}$), macrophage-specific p38α knockout ($p38\alpha^{\Delta M\Phi}$) and wild-type ($p38\alpha^{fl/fl}$) mice fed with high-fat diet (HFD), high-fat/high-cholesterol diet (HFHC), or methionine-and choline-deficient diet (MCD). p38 inhibitors were administered to HFHC-fed wild-type mice for disease treatment.

Results: p38α was significantly upregulated in the liver tissues of patients with NAFLD. Compared to $p38\alpha^{fl/fl}$ littermates, $p38\alpha^{\Delta Hep}$ mice developed significant nutritional steatohepatitis induced by HFD, HFHC or MCD. Meanwhile, $p38\alpha^{\Delta M\Phi}$ mice exhibited less severe steatohepatitis and insulin resistance than $p38\alpha^{fl/fl}$ mice in response to a HFHC or MCD. The effect of macrophage p38α in promoting steatohepatitis was mediated by the induction of pro-inflammatory factors (CXCL2, IL-1β, CXCL10 and IL-6) secreted by M1 macrophages and associated signaling pathways. $p38\alpha^{\Delta M\Phi}$ mice exhibited M2 anti-inflammatory polarization as demonstrated by increased $CD45^+F4/80^+CD11b^+CD206^+$ M2 macrophages and enhanced arginase activity in liver tissues. Primary hepatocytes from $p38\alpha^{\Delta M\Phi}$ mice showed decreased steatosis and inflammatory damage. In a co-culture system, p38α deleted macrophages attenuated steatohepatitic changes in hepatocytes through decreased secretion of pro-inflammatory cytokines (TNF-α, CXCL10 and IL-6), which mediate M1 macrophage polarization in $p38\alpha^{\Delta M\Phi}$ mice. Restoration of TNF-α, CXCL10 or IL-6 induced lipid accumulation and inflammatory responses in $p38\alpha^{fl/fl}$ hepatocytes co-cultured with $p38\alpha^{\Delta M\Phi}$ macrophages. Moreover, pharmacological p38 inhibitors suppressed HFHC-induced steatohepatitis.

Conclusions: Macrophage p38α promotes the progression of steatohepatitis by inducing pro-inflammatory cytokine secretion and M1 polarization. p38 inhibition protects against steatohepatitis.

Lay summary: p38 mitogen-activated protein kinases are important inflammatory factors. In the present study, we demonstrated that p38α is upregulated in liver tissues of patients with non-alcoholic fatty liver diseases. Genetic deletion of p38α in macrophages led to ameliorated nutritional steatohepatitis in mice through decreased pro-inflammatory cytokine secretion and increased M2 macrophage polarization.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome and a major healthcare burden worldwide.[1] The clinicopathological spectrum of NAFLD ranges from hepatic steatosis to non-alcoholic steatohepatitis (NASH), a more aggressive form which can progress to cirrhosis and/or hepatocellular carcinoma.[2] However, there is currently no effective pharmacological therapy approved for NASH, and efforts to control complications arising from the condition are far from satisfactory.[3] A better understanding of the molecular mechanisms of NASH is essential for developing promising treatment strategies for this highly prevalent disease.

Although the molecular pathogenesis of NASH remains elusive, compelling evidence shows that the initiation and perpetuation of liver inflammation is crucial to the pathogenesis of NASH.[4] Central to inflammatory signaling is the reversible phosphorylation of protein regulators and effectors, particularly mitogen-activated protein kinases (MAPK).[5] p38 MAPK (MAPK14) consists of 4 isoforms: p38α, p38β, p38γ and p38δ, with p38α being the most abundant family member. p38α protein kinase transduces a variety of extracellular signals that

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regulate cellular processes, such as inflammation, differentiation, proliferation and apoptosis.[6] The activation of p38 MAPK requires phosphorylation by MAPK kinases or autophosphorylation at pT180/pY182.[7] p38α has been implicated in many inflammatory diseases,[5,8] displaying cell type-specific functions in skin injury[5] and inflammatory bowel disease.[8] It appears to have dual functions in colorectal tumorigenesis: suppressing colitis-associated colon cancer on the one hand but contributing to the proliferation and survival of tumor cells on the other.[9] In the liver, p38α can act as a negative regulator of hepatocyte proliferation but also an oncogenic factor mediating inflammation associated with liver injury.[10,11] Cell type-specific functions of p38α are also evident in the liver as hepatic p38α is a pivotal regulator of hepatic gluconeogenesis,[12] while macrophage p38α induces acute liver injury.[13] Pharmacological inhibitors of p38 have been tested for the treatment of inflammatory diseases.[14] While there are some hints that p38α may be involved in various liver processes, their possible role in NASH progression is still unknown. Therefore, clarifying the functional significance of p38α in NASH is of great clinical importance for the identification of specific therapeutic targets.

In this study, we used conditional hepatocyte-specific p38α knockout ($p38\alpha^{\Delta Hep}$) mice and macrophage-specific p38α knockout ($p38\alpha^{\Delta M\Phi}$) mice to investigate the mechanistic role of p38α during the evolution of NASH, including its impact on hepatic inflammation, lipid accumulation and insulin resistance.

Materials and methods

Human liver samples

Human liver tissue samples were collected by percutaneous liver biopsy. Liver samples were obtained from 27 patients with NAFLD (18 with frozen tissue for mRNA detection and 25 with paraffin-embedded slides for protein detection) from Prince of Wales Hospital, the Chinese University of Hong Kong and 20 control individuals who were liver transplant donors (17 with frozen tissue and 6 with paraffin-embedded slides) from Queen Mary Hospital, the University of Hong Kong (Table S1). Control individuals had no history of diabetes, alcohol use, or viral hepatitis. Histological slides were assessed and scored by 2 pathologists. Liver histology was reported by semiquantitative scoring of 4 histological features including steatosis (0–3), lobular inflammation (0–3), hepatocellular ballooning (0–2), and fibrosis (0–4). NAFLD score was the sum of steatosis, lobular inflammation and hepatocellular ballooning scores (Table S1). Patients with NAFLD were diagnosed by the presence of hepatic steatosis with or without lobular inflammation or ballooning while all controls had normal liver histology. All participants provided 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.

Animals and treatments

The p38α floxed allele was generated as previously described.[15] The floxed allele was bred into homozygosity to generate $p38\alpha^{fl/fl}$ mice. $p38\alpha^{\Delta hep}$ mice were generated by crossing $p38\alpha^{fl/fl}$ mice with Alb-Cre mice. $p38\alpha^{\Delta M\Phi}$ mice were generated by crossing $p38\alpha^{fl/fl}$ mice with LtrLys-Cre mice (all on C57BL/6 background).[8,15] Littermates with floxed alleles but without Cre were used as respective wild-type controls ($p38\alpha^{fl/fl}$).

Male $p38\alpha^{\Delta Hep}$ (Alb^{Cre}-$p38\alpha^{\Delta/\Delta}$) mice and $p38\alpha^{fl/fl}$ littermates (7–8 weeks of age) were randomly assigned to feeding with normal chow (NC), high-fat diet (HFD) (Specialty feeds, Glen Forrest, WA, Australia), high-fat/high-cholesterol diet (HFHC) (Specialty feeds) for 12 weeks, or methionine- and choline-deficient diet (MCD) (ICN Biomedical, Costa Mesa, CA) for 4 weeks (n = 4–7 per group).[16,17] Age-matched male $p38\alpha^{\Delta M\Phi}$ ($LtrLys^{Cre}$-$p38\alpha^{\Delta/\Delta}$) mice and $p38\alpha^{fl/fl}$ mice (7–8 weeks of age) were randomly assigned to feeding with HFHC with fructose-supplemented drinking water for 16 weeks or MCD for 4 weeks (n = 5–8 per group).[17,18] In a separate experiment, male C57BL/6 wild-type mice (7–8 weeks of age) were given intraperitoneal injections of p38 MAPK inhibitors SB203580 (50 mg/kg/day), BIRB796 (15 mg/kg/day)[12,14] (Selleckchem, Houston, TX) or vehicle (DMSO), for 10 consecutive days after 12 weeks of HFHC (n = 6–8 per group). Mice were fasted at the end of the experiments. Both serum and tissues were then harvested as previously described.[16] Visceral fat weights were determined by the summed weight of intra-abdominal fat deposits, including gonadal and retroperitoneal fat.[19] Livers were rapidly excised and weighed. All animal studies were performed in accordance with the guidelines of the Institutional Animal Use and the Animal Experimentation Ethics Committee at the Chinese University of Hong Kong and Xiamen University. All mice were maintained in ventilated cages under 12 h light/dark cycles with access to an enriched environment, water and *ad libitum* feeding.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Results

p38α expression is increased in livers of humans with NAFLD

We examined p38α expression in the liver tissues of 27 patients with NAFLD and 20 normal controls (Table S1). As determined by real-time PCR, p38α mRNA expression was significantly higher in patients with NAFLD than in normal controls (3.30 ± 3.52 *vs.* 1.18 ± 0.70, $p < 0.05$, Fig. 1A). The upregulated phosphorylated p38α (p-p38α) protein level in human NAFLD tissues was confirmed by immunohistochemistry (Fig. 1B). The activated form of p38α was widely expressed in hepatocytes and liver non-parenchymal cells but mainly concentrated around sinusoids and portal areas of livers from patients with NAFLD (Fig. 1B1). Further analysis showed that p-p38α positive hepatic non-parenchymal cells were mainly macrophages/or Kupffer cells by immunohistochemistry staining for human macrophage marker CD68 on serial sections (Fig. 1B2).

Hepatocyte p38α protects mice from steatohepatitis

We investigated the role of hepatocyte p38α in the development of steatohepatitis by feeding $p38\alpha^{\Delta hep}$ mice and $p38\alpha^{fl/fl}$ wild-type mice with HFD for 12 weeks. Hepatic p38α mRNA and p-p38α protein were readily expressed in $p38\alpha^{fl/fl}$ mice, but not in $p38\alpha^{\Delta Hep}$ mice (Fig. 1C). $p38\alpha^{\Delta Hep}$ mice fed with HFD showed significantly more body weight gain, higher visceral fat weight (Fig. 1D1) and higher liver weight (Fig. 1D1) than $p38\alpha^{fl/fl}$ mice fed with the same diet (Fig. 1D1). Moreover, $p38\alpha^{\Delta Hep}$ mice were more glucose intolerant than $p38\alpha^{fl/fl}$ mice, as determined by intraperitoneal glucose tolerance test (IPGTT) assay (Fig. 1D2). In keeping with this, both fasting and non-fasting insulin levels induced by glucose infusion were significantly higher in $p38\alpha^{\Delta Hep}$ mice than $p38\alpha^{fl/fl}$ mice ($p < 0.05$, Fig. 1D2). Histological examination of liver sections showed sig-

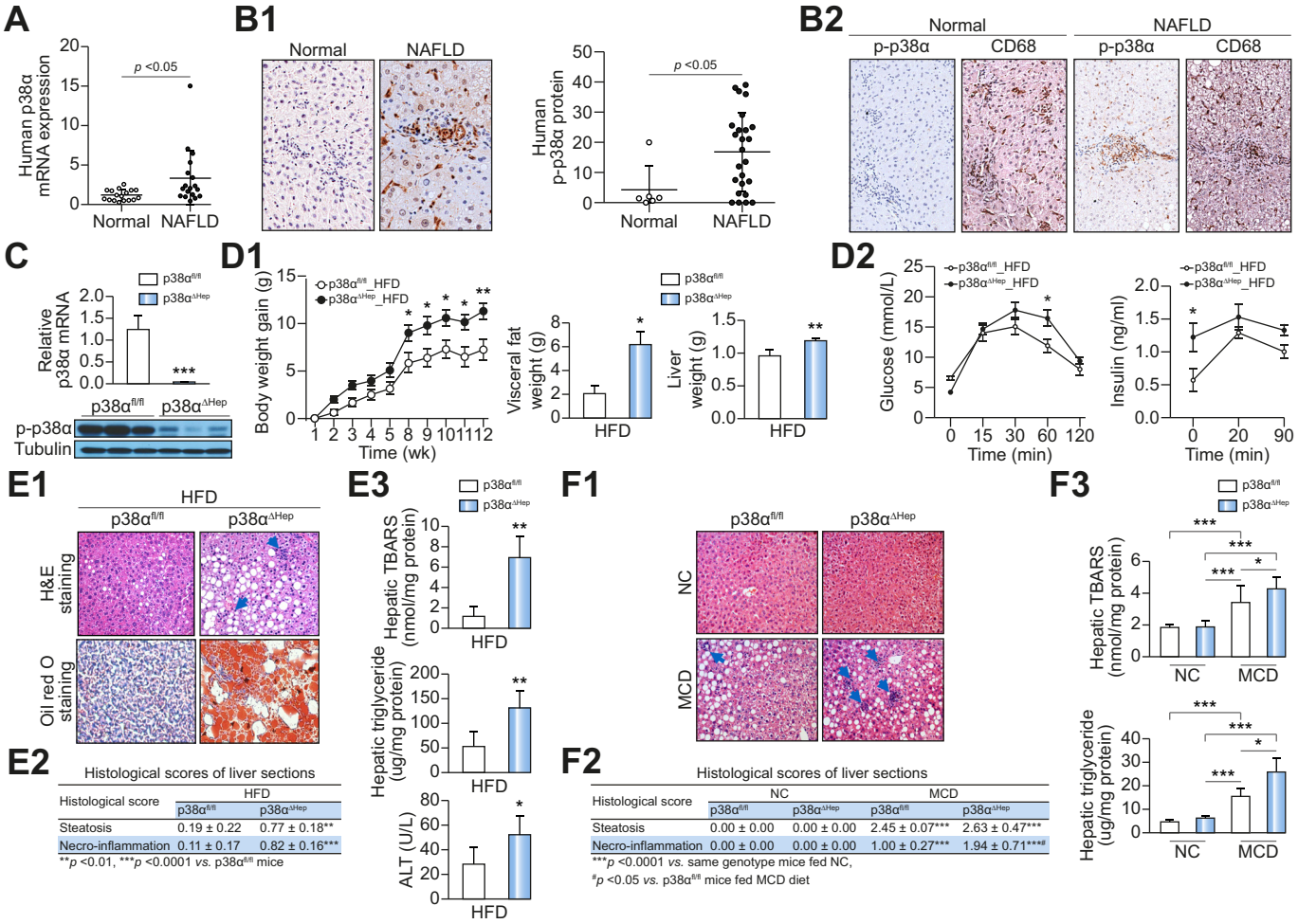


Fig. 1. p38α is upregulated in human NAFLD liver tissues and hepatocyte p38α deletion aggravates steatohepatitis in mice. (A) p38α mRNA expression in NAFLD (n = 18) and normal human liver tissues (n = 17); (B1) Representative immunohistochemistry images of p-p38α (200x) and (B2) CD68 and p-p38α (100x) in NAFLD (n = 25) and normal human liver tissues (n = 6); (C) p38α mRNA and protein expression in the liver tissues of p38α$^{fl/fl}$ and p38α$^{\Delta hep}$ mice; (D1) Body weight gain, visceral fat weight, liver weight, (D2) glucose tolerance test and insulin release test in p38α$^{fl/fl}$ and p38α$^{\Delta hep}$ mice fed with HFD; (E1) Representative pictures in H&E-stained (arrows, inflammatory cells), Oil Red-stained liver sections, (E2) histological scores, (E3) liver lipid peroxidation, hepatic triglyceride content and serum ALT levels in HFD-fed p38α$^{\Delta hep}$ mice and p38α$^{fl/fl}$ mice; (F1) Representative H&E staining (arrows, inflammatory cells), (F2) histological scores, (F3) hepatic lipoperoxide and triglyceride content in p38α$^{fl/fl}$ and p38α$^{\Delta hep}$ mice fed with NC or MCD. Data are expressed as mean ± SD, n = 4–7 per group. *p <0.05, **p <0.01, ***p <0.0001 (unpaired t test or Mann-Whitney U test or ANOVA). ALT, alanine aminotransferase; H&E, hematoxylin & eosin; HFD, high-fat diet; MCD, methionine-and choline-deficient diet; NAFLD, non-alcoholic fatty liver disease; NC, normal chow.

nificant steatohepatitis characterized by steatosis and lobular inflammation in p38α$^{\Delta Hep}$ mice fed with HFD for 3 months (Fig. 1E), while only minor steatosis was found in p38α$^{fl/fl}$ mice fed with the same diet. The increased hepatic steatosis in p38α$^{\Delta Hep}$ mice compared to p38α$^{fl/fl}$ mice was confirmed by Oil Red O staining (Fig. 1E). Consistent with the pronounced steatohepatitis seen on histology, hepatic lipid peroxide (p <0.01), hepatic triglyceride content (p <0.01) and serum alanine aminotransferase (ALT) levels (p <0.05) were all significantly higher in p38α$^{\Delta Hep}$ mice compared to p38α$^{fl/fl}$ mice (Fig. 1E), indicating that p38α deletion in hepatocytes caused liver injury in mice. The effect of hepatocyte p38α in steatohepatitis was further confirmed in 2 additional NAFLD mouse models: HFHC and MCD induced. p38α$^{\Delta Hep}$ mice fed with HFHC for 12 weeks showed more severe steatohepatitis, increased body weight, and greater impaired glucose intolerance compared to p38α$^{fl/fl}$ mice fed with the same diet (Fig. S1). Similarly, p38α$^{\Delta Hep}$ mice fed with MCD for 4 weeks displayed severe steatohepatitis with increased inflammatory cell infiltration (p <0.05), hepatic lipid peroxide (p <0.05) and hepatic triglyceride content (p <0.05) compared to p38α$^{fl/fl}$ mice fed with the same diet (Fig. 1F). Taken together, these results suggest that hepatocyte p38α protects mice from the development of steatohepatitis.

Hepatocyte p38α deletion inhibits lipolysis, and induces hepatic ER stress signaling and pro-inflammatory cytokine production in p38α$^{\Delta hep}$ mice

To understand the reason behind hepatocyte p38α deletion causing severe steatosis, we assessed the hepatic expression of fatty acid oxidation and lipogenic genes. Compared to p38α$^{fl/fl}$ mice fed with MCD, MCD-fed p38α$^{\Delta Hep}$ mice showed decreased mRNA levels of *Ppara*, *Cyp4a14*, *Aco* [or *Acox*] and *Lcad* (or *Acadl*), but not genes regulating fatty acid synthesis and metabolism (*Srebp-1c*, *ChREBP* [or *Mlxipl*] and *Fasn*) (Fig. 2A). These results suggest that hepatocyte p38α deletion may inhibit hepatic β- and ω-oxidation, thereby suppressing hepatic lipolysis, leading to hepatic lipid accumulation (Fig. 2B).

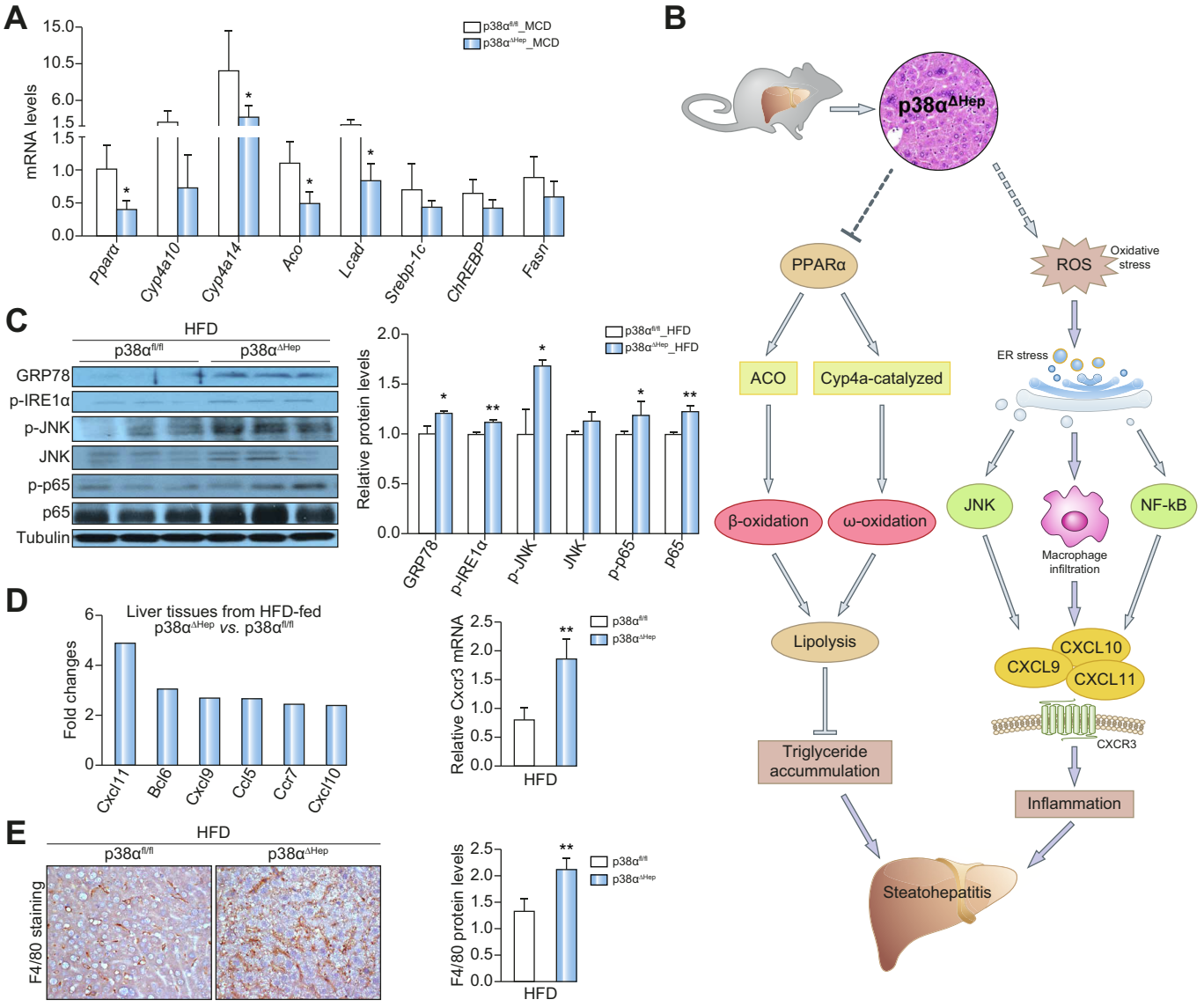


Fig. 2. Hepatocyte p38α deletion activates ER stress and its associated JNK and NF-κB signaling cascades in mice liver tissues. (A) Fatty acid regulation genes in p38α$^{fl/fl}$ and p38α$^{\Delta hep}$ mice fed with MCD; (B) Schematic diagram for the mechanisms of steatohepatitis induction in p38α$^{\Delta hep}$ mice; (C) Protein levels of GRP78, p-IRE1α, p-JNK, JNK, phosphorylated NF-κB subunit p65 and total p65 in p38α$^{fl/fl}$ and p38α$^{\Delta hep}$ mice fed with HFD; (D) cDNA expression array, *Cxcr3* mRNA expression, and (E) F4/80 protein (Immunohistochemistry) expression in p38α$^{fl/fl}$ and p38α$^{\Delta hep}$ mice fed with HFD. Data are expressed as mean ± SD, n = 4–7 per group. *p <0.05, **p <0.01 (unpaired t test or Mann-Whitney U test). ER, endoplasmic reticulum; HFD, high-fat diet; MCD, methionine-and choline-deficient diet.

To clarify the mechanism(s) underlying the anti-inflammatory effect of hepatocyte p38α, we investigated the involvement of endoplasmic reticulum (ER) stress and its related pro-inflammatory factors JNK and NF-κB in steatohepatitis mediated by hepatocyte p38α deletion. We found that protein expressions of ER stress markers GRP78 (HSPA5), phosphorylated IRE1α (ERN1), phosphorylated-JNK, NF-κB subunits p-p65 and p65 were significantly induced in p38α$^{\Delta Hep}$ mice compared to p38α$^{fl/fl}$ mice (Fig. 2C). As the synthesis of cytokines is mediated by NF-κB[16] and JNK pathways,[20] we further analyzed the cytokines involved in steatohepatitis resulting from hepatocyte p38α deletion using the Inflammatory Response & Autoimmunity PCR Array. As shown in Fig. 2D, pro-inflammatory cytokines such as *Cxcl11*, *Cxcl9* and *Cxcl10*, and their receptor *Cxcr3* were significantly induced in HFD-fed p38α$^{\Delta Hep}$ mice compared to corresponding p38α$^{fl/fl}$ mice. It is known that CXCL10 is secreted by macrophages and CXCR3 is mainly expressed on macrophages in steatohepatitis.[16,18] We evaluated hepatic macrophage infiltration by F4/80 immunostaining and revealed that macrophage infiltration was significantly increased in HFD-fed p38α$^{\Delta Hep}$ mice compared to p38α$^{fl/fl}$ mice (p <0.01, Fig. 2E). These results suggest that enhanced macrophage infiltration and related cytokine production contribute to the development of steatohepatitis in p38α$^{\Delta Hep}$ mice (Fig. 2B).

Macrophage p38α induces experimental steatohepatitis

The above results suggest that specific deletion of p38α in hepatocytes promotes NASH progression. As macrophage infiltration is implicated in this process, we evaluated the role of macrophage p38α in the development of steatohepatitis. p38α$^{\Delta M\Phi}$ mice and wild-type p38α$^{fl/fl}$ mice were fed with NC

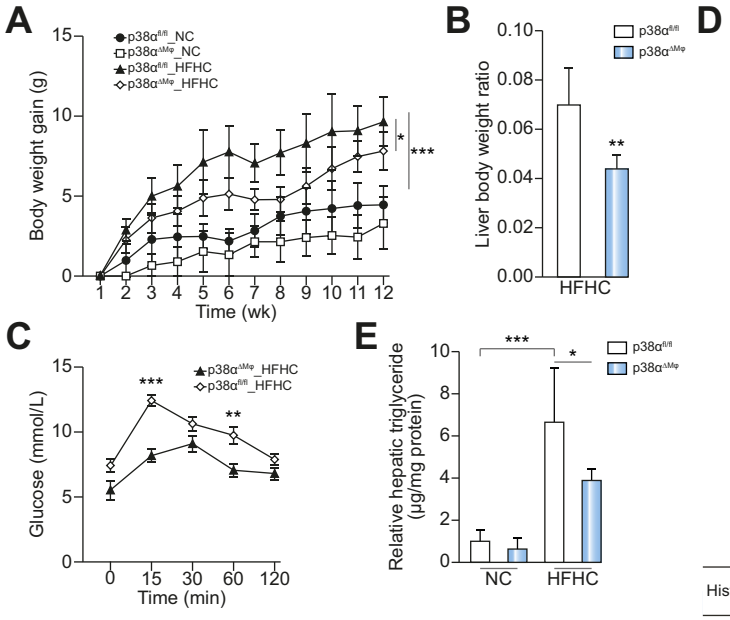
or HFHC. The body weight (Fig. 3A) and liver-to-body weight ratio (Fig. 3B) of p38α$^{\Delta M\Phi}$ mice fed with HFHC was lower compared to wild-type p38α$^{fl/fl}$ mice on the same diet. p38α$^{\Delta M\Phi}$ mice had improved glucose tolerance compared to wild-type p38α$^{fl/fl}$ mice, as determined by IPGTT assay (Fig. 3C). Histological examination of liver sections showed that p38α$^{\Delta M\Phi}$ mice had much improved liver histology with significantly less steatosis and inflammatory cell infiltration compared to p38α$^{fl/fl}$ mice fed with HFHC (Fig. 3D). Accordingly, hepatic triglyceride content (p <0.05) was significantly decreased compared to p38α$^{fl/fl}$ mice (Fig. 3E). These results indicated that p38α deletion in macrophages ameliorates steatohepatitis in mice.

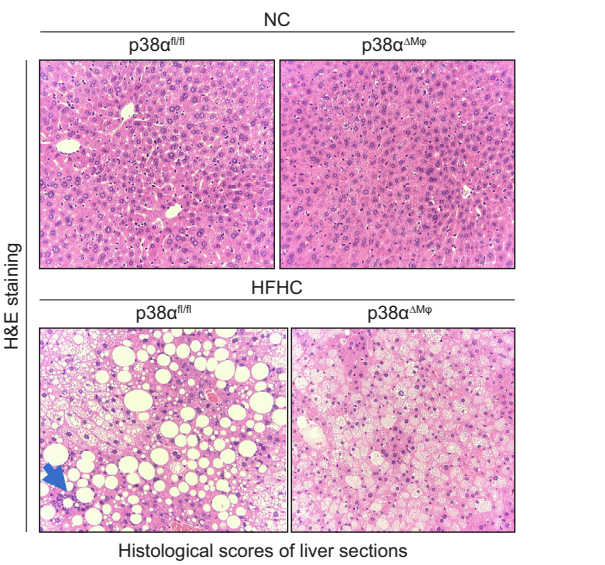
To confirm the effect of macrophage p38α deletion in steatohepatitis, the experiment was repeated using another mouse model of MCD-induced steatohepatitis. Consistent with the HFHC model, MCD-fed p38α$^{\Delta M\Phi}$ mice showed significantly less severe steatosis and inflammatory cell infiltration compared to p38α$^{fl/fl}$ mice (Fig. 4A). The decreased hepatic lipid accumulation in MCD-fed p38α$^{\Delta M\Phi}$ mice was confirmed by Oil Red O staining (Fig. 4B) and triglyceride quantification assay (Fig. 4C).

To characterize the mechanism involved in macrophage p38α-associated steatohepatitis, we screened the hepatic cytokine expression using the Inflammatory Response & Autoimmunity PCR array. p38α$^{\Delta M\Phi}$ mice fed with MCD showed significantly reduced levels of 44 pro-inflammatory cytokines in liver tissues compared to p38α$^{fl/fl}$ mice fed with the same diet (Fig. 4D). Further pathway analysis showed that toll-like receptor signaling, cytokine-cytokine interaction and immune response signaling pathways were enriched in macrophage p38α-associated steatohepatitis (Fig. 4E). Decreased mRNA expression of *Cxcl2*, *Il1b*, *Cxcl10* and *Il6* in liver tissues of MCD-fed p38α$^{\Delta M\Phi}$ mice were confirmed by quantitative reverse transcription PCR (qRT-PCR) (Fig. 4F). These results indicate that macrophage p38α promotes the secretion of pro-inflammatory cytokines, thereby inducing steatohepatitis development.

Macrophage p38α causes M1 macrophage polarization

We subsequently attempted to identify the macrophage subtypes responsible for regulating NASH pathogenesis by macrophage p38α. We analyzed total macrophages (CD45$^+$F4/80$^+$CD11b$^+$), M1 (CD45$^+$F4/80$^+$CD11b$^+$MHCII$^+$CD206$^-$) and M2 (CD45$^+$F4/80$^+$CD11b$^+$ MHCII$^-$CD206$^+$) macrophages from liver tissues of p38α$^{fl/fl}$ and p38α$^{\Delta M\Phi}$ mice by flow cytometry. Compared to p38α$^{fl/fl}$ mice fed with the MCD diet, p38α$^{\Delta M\Phi}$ mice fed with the same diet showed decreased total macrophages (macrophages/CD45$^+$ lymphocytes: 16.3% *vs.* 12.9%, p <0.05), and M1 macrophages (M1 macrophages/total macrophages: 51.8% *vs.* 42.3%, p <0.01); but increased M2 macrophages (M2 macrophage/total macrophages: 2.6% *vs.* 7.7%, p <0.01) (Fig. 5A). Consistently, hepatic expression of M1 macrophage markers *Cd11c* and *Tlr4* was reduced in HFHC-fed p38α$^{\Delta M\Phi}$ mice and hepatic expression of *Cxcl2*, *Il1b*, *Cxcl10* and *Il6* was reduced in MCD-fed p38α$^{\Delta M\Phi}$ mice compared to p38α$^{fl/fl}$ mice fed with the same diet (Figs. 5B and 4F). Moreover, arginase activity was significantly increased in the liver tissues of HFHC-fed p38α$^{\Delta M\Phi}$ mice compared to p38α$^{fl/fl}$ mice (Fig. 5C). In addition, we isolated primary bone marrow-derived macrophages (BMDMs) and peritoneal macrophages from p38α$^{\Delta M\Phi}$ and p38α$^{\Delta M\Phi}$ mice with *in vitro* stimulation of IL-4 (Fig. 5D). p38α deletion increased the ability of IL-4-induced M2 macrophage polarization (F4/80$^+$CD11b$^+$CD206$^+$) in both BMDMs and





Histological scores of liver sections

Histological score	NC		HFHC	
	p38α$^{fl/fl}$	p38α$^{\Delta M\Phi}$	p38α$^{fl/fl}$	p38α$^{\Delta M\Phi}$
Steatosis	0.00 ± 0.00	0.00 ± 0.00	1.45 ± 0.15**	0.73 ± 0.30**##
Necro-inflammation	0.00 ± 0.00	0.00 ± 0.00	0.32 ± 0.15**	0.11 ± 0.01**#

**p <0.01 *vs.* same genotype mice fed NC, #p <0.05, ##p <0.01 *vs.* p38α$^{fl/fl}$ mice fed HFHC

Fig. 3. Deficiency of macrophage p38α attenuates experimental steatohepatitis in HFHC-fed mice. (A) Body weight gain in p38α$^{\Delta M\Phi}$ and p38α$^{fl/fl}$ mice fed with NC or HFHC; (B) Liver-to-body weight ratio, and (C) glucose tolerance test in p38α$^{fl/fl}$ and p38α$^{\Delta M\Phi}$ mice fed with HFHC; (D) Representative liver H&E staining (arrows, inflammatory cells) and histological scores of steatosis and inflammation of liver sections of p38α$^{\Delta M\Phi}$ and p38α$^{fl/fl}$ mice with fed NC or HFHC; (E) Hepatic triglyceride content in 38α$^{\Delta M\Phi}$ and p38α$^{fl/fl}$ mice fed with NC or HFHC. Data are expressed as mean ± SD, n = 5–8 per group. *p <0.05, **p <0.01, ***p <0.0001 (unpaired t test or ANOVA). H&E, hematoxylin & eosin; HFHC, high-fat/high-cholesterol diet; NC, normal chow.

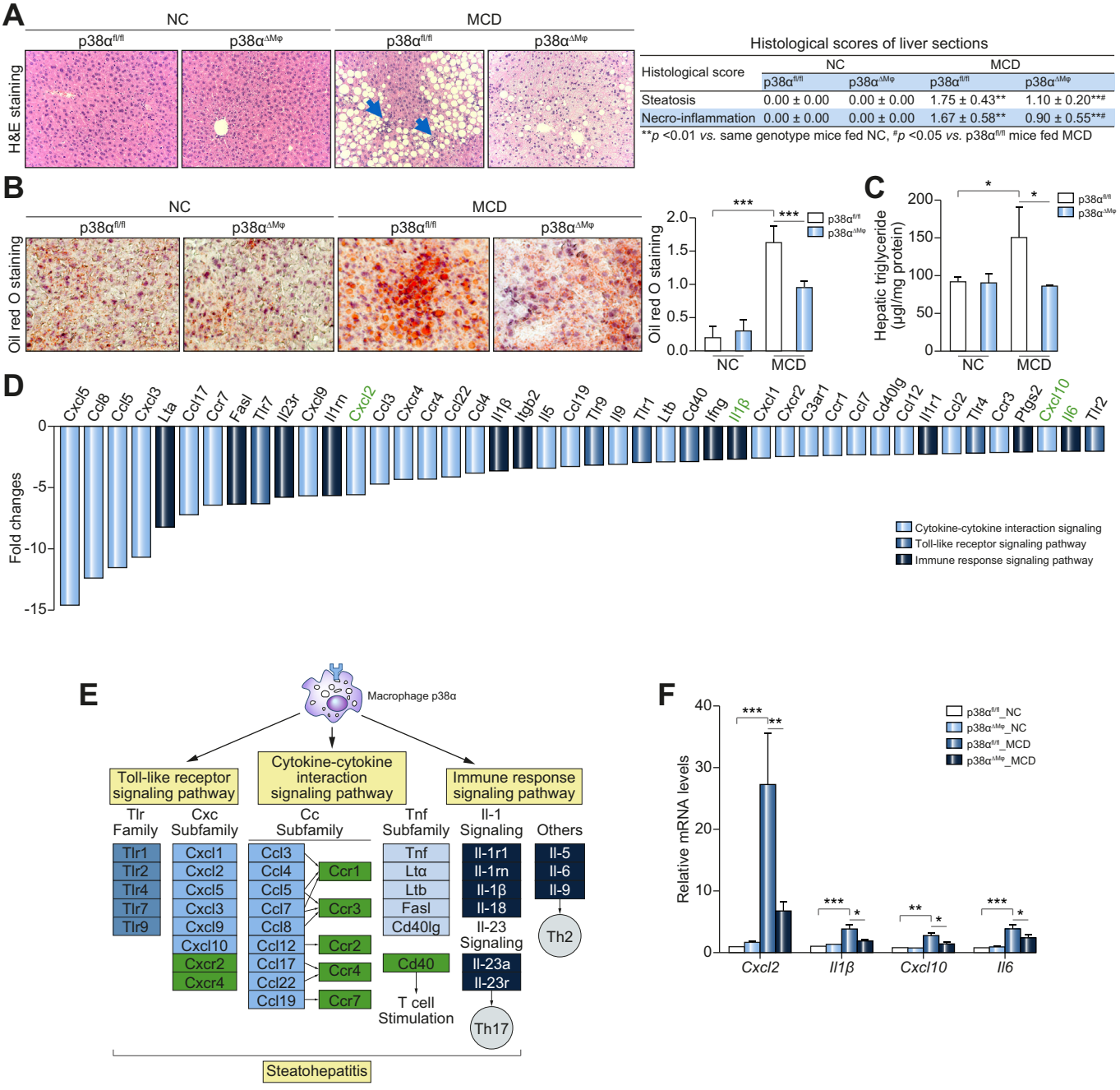


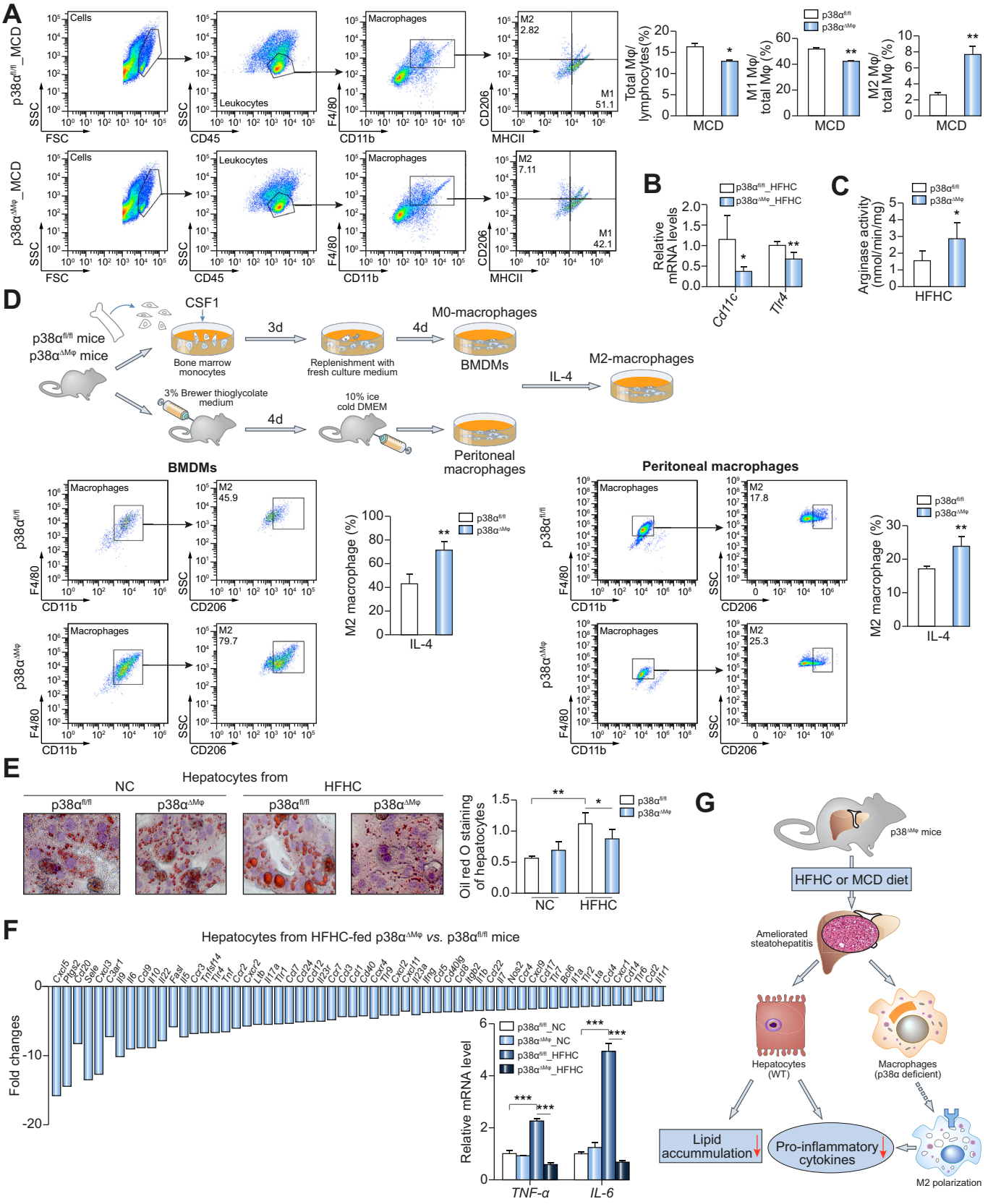
Fig. 4. Deficiency of macrophage p38α attenuates experimental steatohepatitis in MCD-fed mice. (A) Representative H&E staining (arrows, inflammatory cells), histological scores, (B) Oil red O staining from 4-week liver sections and (C) liver triglyceride content in p38α$^{\Delta M\Phi}$ and p38α$^{fl/fl}$ mice fed with NC or MCD; (D) cDNA expression array in the liver tissues of MCD-fed p38α$^{\Delta M\Phi}$ mice compared to p38α$^{fl/fl}$ mice; (E) Schematic diagram for the induction of pro-inflammatory pathways and cytokines by macrophage p38α; (F) Real-time PCR analysis of *Cxcl2, Il1b, Cxcl10* and *Il6* in p38α$^{\Delta M\Phi}$ and p38α$^{fl/fl}$ mice fed with NC or MCD. Data are expressed as mean ± SD, n = 5–8 per group. *p <0.05, **p <0.01, ***p <0.0001 (unpaired t test or ANOVA). H&E, hematoxylin & eosin; MCD, methionine-and choline-deficient diet; NC, normal chow.

peritoneal macrophages (Fig. 5D). These results collectively confirmed the M2 polarization in p38α$^{\Delta M\Phi}$ mice.

Moreover, loss of p38α in macrophages led to decreased lipid accumulation (Fig. 5E) and decreased cDNA expression of most pro-inflammatory cytokines (Fig. 5F) in the hepatocytes of HFHC-fed p38α$^{\Delta M\Phi}$ mice compared to p38α$^{fl/fl}$ mice. The decreased mRNA expression of *Tnfα* and *Il6* in hepatocytes of HFHC-fed p38α$^{\Delta M\Phi}$ mice was confirmed by qRT-PCR (Fig. 5F). On the other hand, macrophages isolated from p38α$^{\Delta hep}$ mice fed with HFD or MCD showed an increased proportion of the M1 pro-inflammatory phenotype compared to macrophages isolated from p38α$^{fl/fl}$ mice fed with the same diet (Fig. S2). These results suggest that macrophage p38α deletion protects against the evolution of steatohepatitis partly through the restoration of M2 macrophage polarization (Fig. 5G).

Macrophage p38α induces lipid accumulation and pro-inflammatory cytokine production in hepatocytes *in vitro*

To investigate whether p38α deleted macrophages with M2 polarization can ameliorate the steatohepatitic changes of


A
Cells
Leukocytes
Macrophages
M2
2.82
M1
51.1
M2
7.11
M1
42.1
FSC
SSC
CD45
CD11b
F4/80
CD206
MHCII
Total Mφ/ lymphocytes(%)
M1 Mφ/ total Mφ (%)
M2 Mφ/ total Mφ (%)
MCD
B
Relative mRNA levels
Cd11c
Tlr4
HFHC
C
Arginase activity (nmol/min/mg)
D
CSF1
Bone marrow monocytes
3d
Replenishment with fresh culture medium
4d
M0-macrophages
BMDMs
IL-4
M2-macrophages
3% Brewer thioglycolate medium
10% ice cold DMEM
Peritoneal macrophages
M2
45.9
M2
79.7
M2
17.8
M2
25.3
M2 macrophage (%)
E
NC
HFHC
Hepatocytes from
Oil red O staining of hepatocytes
F
Hepatocytes from HFHC-fed p38α^ΔMφ vs. p38α^fl/fl mice
Fold changes
Relative mRNA level
TNF-α
IL-6
G
p38^ΔMφ mice
HFHC or MCD diet
Ameliorated steatohepatitis
Hepatocytes (WT)
Macrophages (p38α deficient)
Lipid accummulation
Pro-inflammatory cytokines
M2 polarization

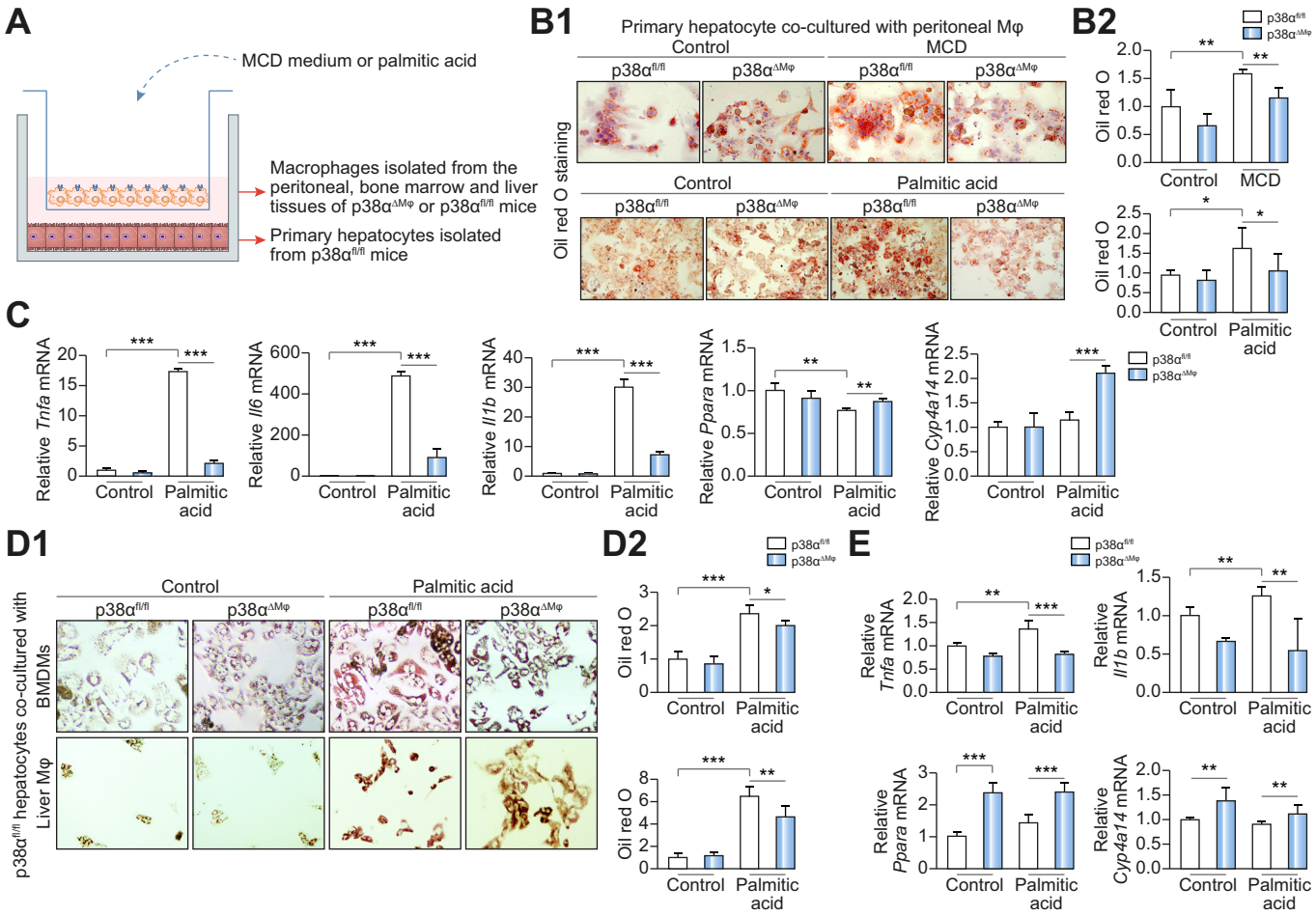


Fig. 6. Macrophage p38α induces lipid accumulation and pro-inflammatory cytokine production in hepatocytes *in vitro*. (A) Schematic showing that primary macrophages isolated from the peritoneal cavity, bone marrow or liver of p38α$^{\Delta M\Phi}$ or p38α$^{fl/fl}$ mice were co-cultured with primary p38α$^{fl/fl}$ hepatocytes with or without MCD medium or palmitic acid; (B1) Lipid accumulation and (B2) semiquantitative analysis of Oil Red O Staining in hepatocytes of the co-culture system; (C) mRNA levels of *Tnfa, Il6, Il1b, Ppara* and *Cyp4a14* in palmitic acid-treated hepatocytes co-cultured with peritoneal macrophages from p38α$^{\Delta M\Phi}$ or p38α$^{fl/fl}$ mice; (D1) Lipid accumulation and (D2) semi-quantitate analysis of Oil Red O Staining of palmitic acid-treated hepatocytes co-cultured with BMDMs or liver macrophages isolated from p38α$^{\Delta M\Phi}$ or p38α$^{fl/fl}$ mice; (E) *Tnfa, Il6, Il1b, Ppara* and *Cyp4a14* mRNA levels in hepatocytes co-cultured with BMDMs from p38α$^{\Delta M\Phi}$ or p38α$^{fl/fl}$ mice. Data are expressed as mean ± SD. **p* <0.05, ***p* <0.01, ****p* <0.0001 (unpaired *t* test or ANOVA). BMDMs, bone marrow-derived macrophages; HFHC, high-fat/high-cholesterol diet.

hepatocytes *in vitro*, we co-cultured primary hepatocytes isolated from p38α$^{fl/fl}$ mice with primary peritoneal macrophages isolated from p38α$^{\Delta M\Phi}$ and p38α$^{fl/fl}$ mice in MCD or palmitic acid medium (Fig. 6A). Lipid accumulation (Fig. 6B), and pro-inflammatory cytokines (*Tnfa, Il6* and *Il1b*) (Fig. 6C) were significantly increased while lipolytic genes (*Ppara*) (Fig. 6C) were reduced in hepatocytes isolated from p38α$^{fl/fl}$ mice cultured in MCD or palmitic acid-treated medium compared to control medium. However, the induction of lipid accumulation (Fig. 6B) and cytokines and reduction of lipolytic genes (*Ppara* and *Cyp4a14*) (Fig. 6C) in hepatocytes was blunted when they were co-cultured with p38α$^{\Delta M\Phi}$ macrophages as compared to p38α$^{fl/fl}$ macrophages. Similar results were observed in primary hepatocytes co-cultured with BMDMs and liver macrophages isolated from p38α$^{\Delta M\Phi}$ or p38α$^{fl/fl}$ mice (Fig. 6D-E). p38α$^{\Delta M\Phi}$ BMDMs and p38α$^{\Delta M\Phi}$ liver macrophages decreased lipid accumulation in hepatocytes isolated from p38α$^{fl/fl}$ mice (Fig. 6D). The pro-inflammatory cytokines (*Tnfa* and *Il1b*) (Fig. 6E) were significantly decreased but lipolytic genes (*Ppara* and *Cyp4a14*) (Fig. 6E) were increased in p38α$^{fl/fl}$ hepatocytes co-cultured with BMDMs p38α$^{\Delta M\Phi}$ macrophages compared to those co-cultured with p38α$^{fl/fl}$ macrophages. In addition, p38α was

Fig. 5. Macrophage p38α deficiency causes M2 macrophage polarization. (A) Frequencies of total macrophages (CD45$^+$F4/80$^+$CD11b$^+$), M1 (CD45$^+$F4/80$^+$CD11b$^+$MHCII$^+$CD206$^-$) and M2 (CD45$^+$F4/80$^+$CD11b$^+$MHCII$^-$CD206$^+$) macrophages in the liver tissues of p38α$^{fl/fl}$ and p38α$^{\Delta M\Phi}$ mice fed with MCD; (B) mRNA expression of *Cd11c* and *Tlr4* and (C) arginase activity in the liver tissues of HFHC-fed p38α$^{fl/fl}$ and p38α$^{\Delta M\Phi}$ mice; (D) M2 macrophages (F4/80$^+$CD11b$^+$CD206$^+$) in both BMDMs and peritoneal macrophages isolated from p38α$^{\Delta M\Phi}$ and p38α$^{fl/fl}$ mice with *in vitro* stimulation of IL-4 by flow cytometry; (E) Lipid accumulation by Oil Red O staining, (F) cytokines by cDNA expression array, and *Tnfa* and *Il6* mRNA levels in isolated hepatocytes of p38α$^{\Delta M\Phi}$ and p38α$^{fl/fl}$ mice fed with NC or HFHC; (G) Schematic diagram for the changes of hepatocytes and macrophages in p38α$^{\Delta M\Phi}$ mice. Data are expressed as mean ± SD, n = 5–8 per group. **p* <0.05, ***p* <0.01, ****p* <0.0001 (unpaired *t* test or ANOVA). BMDMs, bone marrow-derived macrophages; HFHC, high-fat/high-cholesterol diet; NC, normal chow.

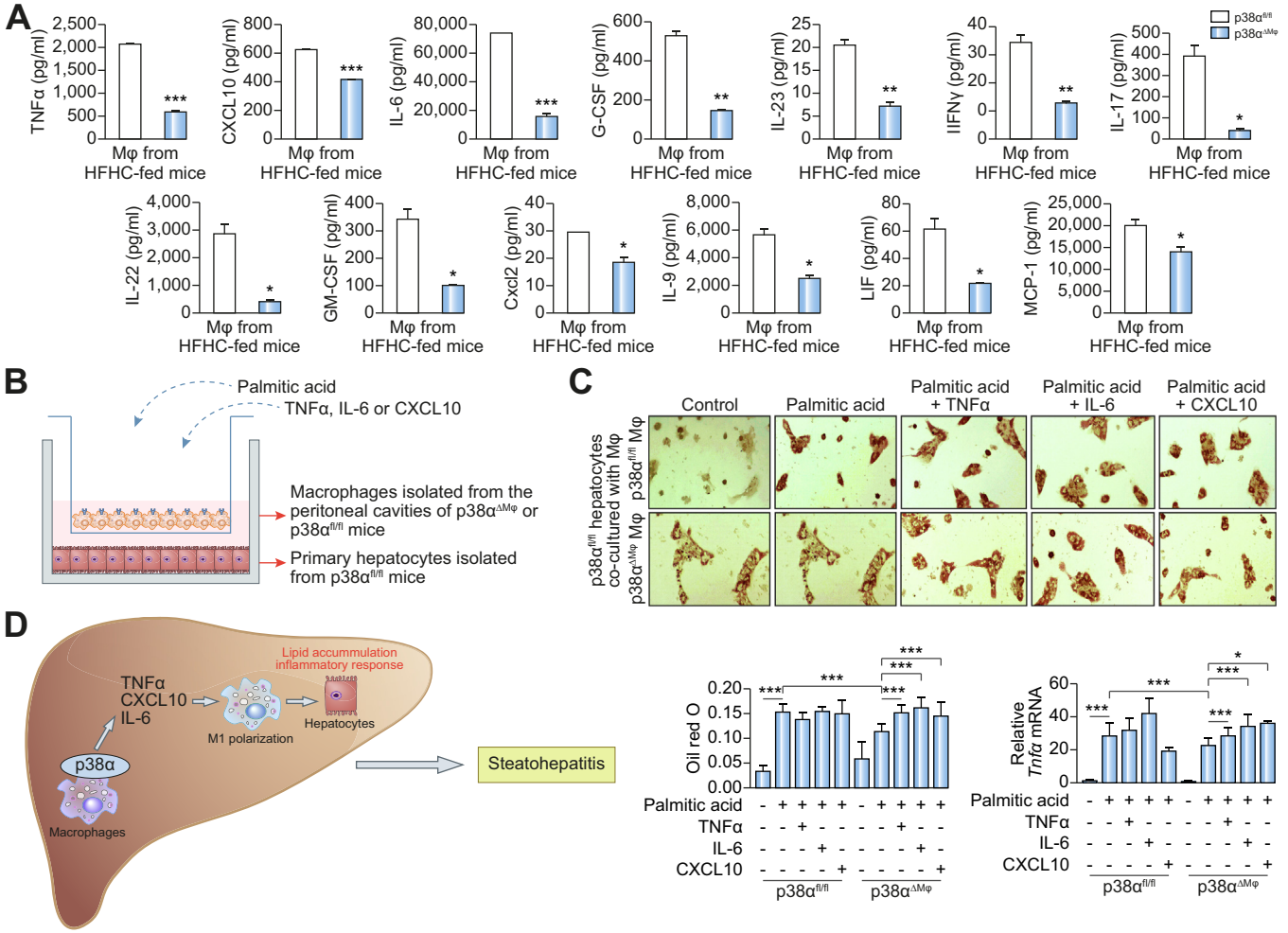


Fig. 7. Macrophage p38α causes M1 macrophage polarization and hepatocyte steatohepatitic changes through the induction of cytokines. (A) Cytokine profiles of culture medium from primary macrophages isolated from HFHC-fed p38α$^{\Delta M\Phi}$ or p38α$^{fl/fl}$ mice; (B) Schematic showing that primary p38α$^{fl/fl}$ hepatocytes with p38α$^{\Delta M\Phi}$ macrophages or p38α$^{fl/fl}$ macrophages, in the presence or absence of TNF-α, CXCL10 or IL-6 with palmitic acid; (C) Lipid accumulation by Oil Red O staining and *Tnfa* mRNA in primary p38α$^{fl/fl}$ hepatocytes co-cultured with p38α$^{\Delta M\Phi}$ and p38α$^{fl/fl}$ macrophages under palmitic acid medium; (D) Schematic diagram showing the effect of macrophage p38 on co-culture system of primary hepatocytes. Data are expressed as mean ± SD. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.0001 (unpaired *t* test or ANOVA). HFHC, high-fat/high-cholesterol diet.

activated in hepatocytes co-cultured with p38α$^{\Delta M\Phi}$ peritoneal macrophages (Fig. S3), suggesting that p38α deletion in macrophages could increase hepatocyte p38α activity, reinforcing the steatohepatitic changes of hepatocytes.

On the other hand, primary p38α$^{fl/fl}$ macrophages co-cultured with p38α$^{\Delta hep}$ primary hepatocytes showed enhanced mRNA expression of the M1 macrophage marker *Mcp1* (*Ccl2*) and decreased mRNA expression of the M2 macrophage marker *Cd206* (*Mrc1*) compared to those co-cultured with p38α$^{fl/fl}$ primary hepatocytes (Fig. S4). These results indicate that hepatocyte p38α deletion promotes macrophage polarization to M1 phenotype during steatohepatitis development.

Macrophage p38α causes M1 macrophage polarization and hepatocyte steatohepatitic changes through the induction of cytokines

To identify the cytokines secreted by macrophages responsible for inducing M1 macrophage polarization and hepatocyte steatohepatitic changes, the cytokine profiles of the culture medium of primary peritoneal macrophages isolated from HFHC-fed p38α$^{\Delta M\Phi}$ and p38α$^{fl/fl}$ mice were analyzed. As shown in Fig. 7A, 13 cytokines (TNF-α, CXCL10, IL-6, G-CSF, IL-23, IFN-γ, IL-17, IL-22, GM-CSF, CXCL2, IL-9, LIF and MCP-1) were significantly decreased in the culture medium of macrophages from HFHC-fed p38α$^{\Delta M\Phi}$ mice compared to medium from p38α$^{fl/fl}$ mice. Among them, TNF-α, CXCL10 and IL-6 were the most significantly altered cytokines (p <0.001). We evaluated the effect of macrophage-derived TNF-α, CXCL10 and IL-6 cytokines at inducing steatohepatitis using the co-culture system of p38α$^{\Delta M\Phi}$ macrophages or p38α$^{fl/fl}$ macrophages with primary p38α$^{fl/fl}$ hepatocytes (Fig. 7B). Restoration of TNF-α, CXCL10 or IL-6 induced lipid accumulation and inflammatory responses in p38α$^{fl/fl}$ hepatocytes co-cultured with p38α$^{\Delta M\Phi}$ macrophages (Fig. 7C).

Collectively, these results suggest that macrophage p38α induces the secretion of pro-inflammatory cytokines, leading to M1 macrophage polarization and aggravated steatohepatitic changes of hepatocytes (Fig. 7D).

Pharmacological p38 MAPK inhibitors prevent HFHC-induced steatohepatitis in mice

We examined the effects of p38 inhibition on the development of steatohepatitis. Two selective p38 MAPK inhibitors SB203580 and BIRB796[14,21] were administered for 10 days to mice that

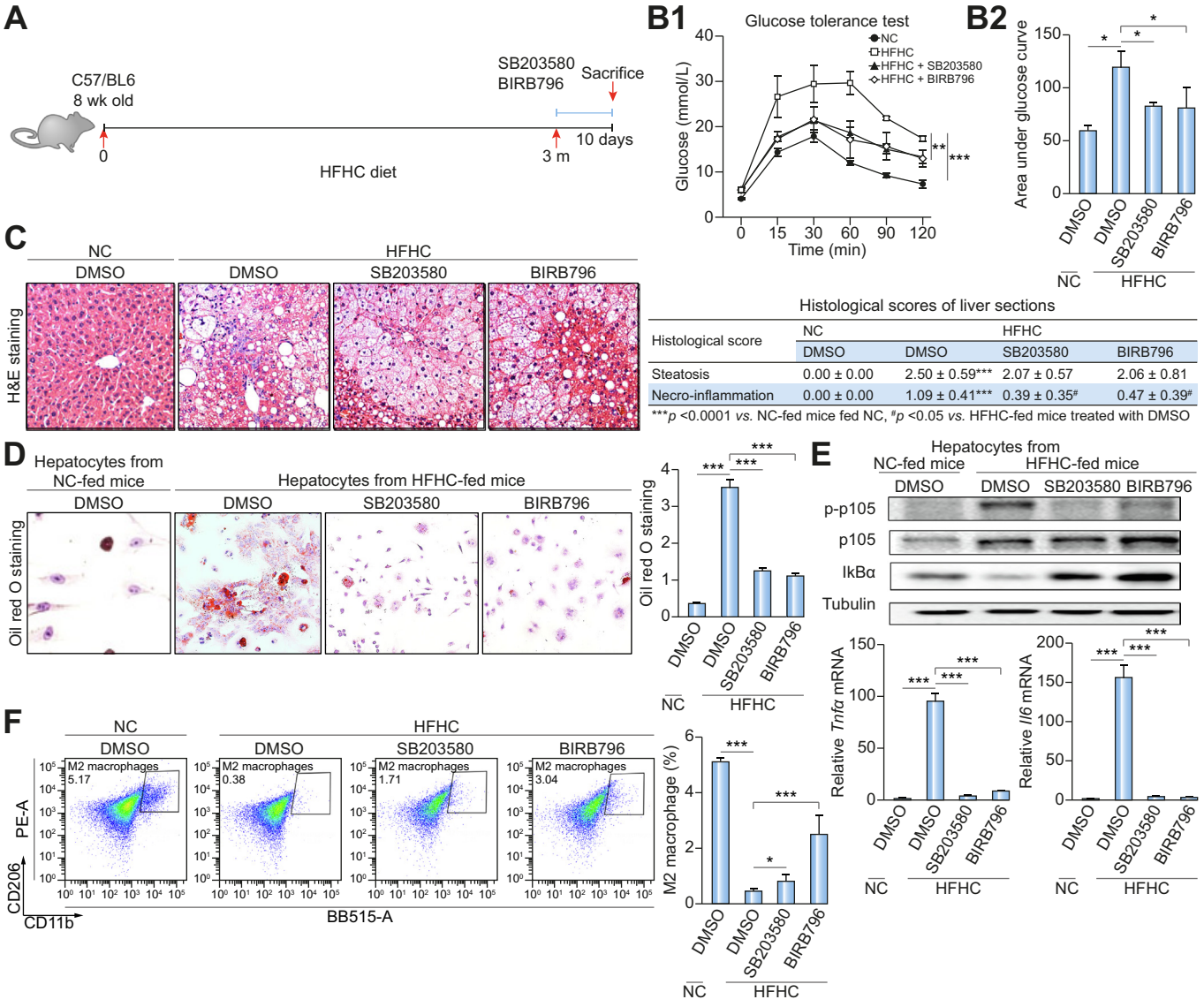


Fig. 8. Pharmacological p38 MAPK inhibitors prevent HFHC-induced steatohepatitis in mice. (A) Schematic illustration of treatment with SB203580 and BIRB796 in HFHC-fed WT mice; (B) Glucose tolerance test and area under curve in mice; (C) Representative H&E staining of liver sections from HFHC-fed mice treated with or without SB203580 and BIRB796. Histological scores of steatosis and inflammation were calculated in each mice (n = 6–8 per group); (D) Representative pictures and quantitation of Oil Red O staining, (E) phosphorylated NF-κB p105, p105 and IκBα protein levels and *Tnfa* and *Il6* mRNA levels in hepatocytes of HFHC-fed mice with or without treatment with SB203580 and BIRB796; (F) M2 macrophage polarization was restored by the administration of SB203580 or BIRB796. Data are expressed as mean ± SD. *p <0.05, **p <0.01, ***p <0.0001 (unpaired t test or ANOVA). H&E, hematoxylin & eosin; HFHC, high-fat/high-cholesterol diet; WT, wild-type.

received HFHC for 3 months (Fig. 8A). We found that p38α was inactivated in hepatocytes but activated in non-parenchymal liver cells in mice after 3 months of HFHC feeding (Fig. S5), indicating that macrophage p38α, not hepatocyte p38α, was predominant in steatohepatitis. After the administration of SB203580 or BIRB796, p-p38α was suppressed in non-parenchymal cells including macrophages (Fig. S5). Although the body weight, liver weight, liver-to-body weight ratio and serum ALT levels were not altered (Fig. S6), glucose tolerance was significantly improved by treatment with either SB203580 or BIRB796 compared to untreated mice fed with the same diet (Fig. 8B). Liver histology was also improved as evidenced by decreased inflammatory scores by both treatments (Fig. 8C) in mice fed with HFHC. Lipid accumulation was decreased in hepatocytes of HFHC-fed mice treated with either SB203580 or BIRB796 as shown by Oil Red O staining (Fig. 8D). Accordingly, treatment with p38α MAPK inhibitors suppressed NF-κB activation in hepatocytes as shown by decreased phospho-p105, increased NF-κB suppressor IκB protein levels, and reduced *Tnfa* and *Il6* mRNA expression (Fig. 8E). Notably, M2 anti-inflammatory macrophage polarization was restored by the administration of SB203580 or BIRB796 (Fig. 8F). These results suggest that pharmacological p38α MAPK inhibitors can prevent HFHC-induced steatohepatitis in mice by inducing p38α activation in hepatocytes and suppressing p38α activation in macrophages resulting in their M2 polarization.

Discussion

In this study, we found the enhanced mRNA expression of p38α in liver tissues of patients with NAFLD. p-p38α protein was expressed in both hepatocytes and liver macrophages in human

tissues. p38α knockout in the hepatocytes of mice increased the histological severity of steatohepatitis, decreased lipolysis and increased insulin resistance, oxidative stress, ER stress signaling and production of pro-inflammatory cytokines (Fig. 2B). Hepatic fatty acid oxidation occurs via mitochondrial and peroxisomal β-oxidation and CYP4A-catalyzed ω-oxidation. ACO and LCAD are the key enzymes of fatty acid oxidation systems in the liver and are regulated by lipolytic transcription factor PPARα. We found downregulated PPARα, Cyp4a14, ACO and LCAD in MCD-fed p38α$^{\Delta hep}$ mice, suggesting that hepatocyte p38α inhibits lipid accumulation by inducing these lipolytic genes.

Following the observation of increased p38α in liver macrophages of humans with NAFLD (Fig. 1) and accumulated macrophages in p38α$^{\Delta hep}$ mice (Fig. 2), we investigated the role of macrophage p38α in the development of steatohepatitis. In contrast to p38α$^{\Delta hep}$ mice, p38α$^{\Delta M\Phi}$ mice fed with HFHC showed significantly ameliorated steatohepatitis along with decreased obesity, insulin resistance and oxidative stress compared to p38α$^{fl/fl}$ mice fed with the same diet (Fig. 3). The effect of macrophage-specific p38α deletion in repressing steatohepatitis was confirmed in p38α$^{\Delta M\Phi}$ mice fed with MCD. In keeping with the improved liver histology, pro-inflammatory factors, cytokines, chemokines, and their related signaling pathways including toll-like receptor, cytokine-cytokine interaction and immune response signaling (Fig. 4) were all significantly blunted by macrophage p38α deletion. These results suggest that macrophage p38α induces steatohepatitis, at least in part, through activation of pro-inflammatory factors and their related pathways.

Liver macrophages exhibit heterogeneity, especially under pathological conditions such as steatohepatitis.[22] Macrophage polarization helps to explain the different results observed after hepatocyte- and macrophage-specific p38α deletion. Macrophages are generally classified as M1 and M2 phenotypes.[23] The classical M1 macrophages promote a pro-inflammatory response and increased insulin resistance, whereas the alternative M2 macrophages inhibit inflammation, improve insulin sensitivity and induce tissue remodeling. In the liver, M1 macrophages contribute to the pathogenesis of hepatic steatosis and inflammation by secreting cytokines such as TNF-α and IL-1β.[24] In contrast, activation of the alternative M2 macrophages improves hepatic steatosis and insulin resistance in HFD-fed mice.[25] Given that loss of p38α in macrophages led to ameliorated steatohepatitis, we speculated that macrophage polarization was skewed toward the M2 phenotype in the absence of macrophage p38α. To test this speculation, we investigated the population of M1 and M2 macrophages from liver tissues of p38α$^{fl/fl}$ and p38α$^{\Delta M\Phi}$ mice. We demonstrated that M1 polarization was induced by macrophage p38α during the development of steatohepatitis as evidenced by decreased M1 macrophages and increased M2 macrophages in the livers of p38α$^{\Delta M\Phi}$ mice (Fig. 5). This was confirmed by the reduced hepatic M1 macrophage markers (*Cxcl2, Il-1b, Cxcl10, Il6, Cd11c, Tlr4*) seen in p38α$^{\Delta M\Phi}$ mice. Arginase activity, the hallmark M2 mouse macrophage marker,[26] was increased in HFHC-fed p38α$^{\Delta M\Phi}$ mice compared to p38α$^{fl/fl}$ mice. The above findings from these animal experiments were further confirmed by *in vitro* assays. The pro-inflammatory cytokines which are mainly secreted by M1 macrophages were decreased in primary peritoneal macrophages isolated from HFHC-fed p38α$^{\Delta M\Phi}$ mice compared to p38α$^{fl/fl}$ mice. Moreover, p38α$^{\Delta M\Phi}$ macrophages isolated from p38α$^{\Delta M\Phi}$ mice showed significantly more M2 macrophage polarization compared to p38α$^{fl/fl}$ macrophages isolated from p38α$^{fl/fl}$ mice under IL-4 stimulation. Taken together, these results suggest that macrophage p38α deletion ameliorates the pathogenesis of steatohepatitis at least in part due to M2 polarization.

The failure of M1/M2 switching in macrophages may cause a defect in crosstalk between macrophages and hepatocytes during steatohepatitis progression.[22] To confirm this speculation, we co-cultured primary hepatocytes with primary p38α$^{\Delta M\Phi}$ macrophages and p38α$^{fl/fl}$ macrophages. p38α$^{\Delta M\Phi}$ macrophages but not p38α$^{fl/fl}$ macrophages exhibited decreased hepatocyte lipid accumulation and inflammatory injury induced by MCD or palmitic acid, suggesting that macrophage p38α inhibited hepatocyte steatohepatitic changes. Moreover, we found that macrophages with p38α deletion restored expression of lipolytic genes (*Ppara* and *Cyp4a14*) and phosphorylated p38α in hepatocytes (Fig. 6). Collectively, this suggests that macrophage p38α induces steatohepatitis by inducing a switch in macrophage polarization to the M1 phenotype and inhibiting hepatocyte lipolysis.

Having shown that macrophage p38α is a crucial mediator of macrophage M1 polarization in steatohepatitis, we looked for the effector(s) of macrophage p38α. Thirteen pro-inflammatory cytokines were significantly lower in the culture medium taken from primary macrophages of HFHC-fed p38α$^{\Delta M\Phi}$ mice compared to the culture medium of primary macrophages of p38α$^{fl/fl}$ mice fed with the same diet. Among them, TNF-α, CXCL10 and IL-6, which are key pro-inflammatory factors in the development of steatohepatitis,[12] were predominantly decreased. TNF-α can activate p38α after binding with TNF-α receptor-associated factor 2.[27] TNF-α is also known to mediate the alteration of M1/M2 macrophage polarization.[28] CXCL10 contributes to p38 activation when bound to its receptor CXCR3[29] and mediates hepatic macrophage M1 polarization.[30] *Il6* gene expression could be induced by p38α and NF-κB.[31] In our study, administration of TNF-α, CXCL10 and IL-6 restored the ameliorated steatohepatitic changes of hepatocytes produced by macrophage p38α knockout (Fig. 7). These results further confirm that macrophage p38α induces a macrophage polarity switch to the M1 phenotype and enhances pro-inflammatory factors, which contribute to the pathogenesis of nutritional steatohepatitis.

Pharmacological inhibition of p38α has proven to be effective in treating or alleviating various inflammatory conditions, including rheumatoid arthritis, Alzheimer's disease and inflammatory bowel disease.[14] However, the effect of p38 inhibitors in the treatment of liver inflammatory diseases including NASH is still not known. It is helpful to know how hepatic cells respond to p38 inhibitors in order to better understand how these targeted therapies might be applied in patients with NASH. We found that p38 inhibitors (SB203580 and BIRB796) could specifically inhibit p38α activity in hepatic non-parenchymal cells including macrophages (rather than hepatocytes), resulting in significantly ameliorated experimental steatohepatitis. This result provides a potential rationale for future clinical trials to investigate the use of targeted p38 inhibitors for the treatment of NASH.

In conclusion, this study identified a specific role of p38α in nutritional steatohepatitis. Macrophage p38α promotes the progression of steatohepatitis by inducing M1 macrophage polarization and the secretion of pro-inflammatory cytokines, whilst hepatocyte p38α, whose expression is low in

steatohepatitis, has the opposite effect. Specific p38α inhibition in macrophages by p38 inhibitors might provide a new approach for the treatment of steatohepatitis.

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Conflict of interest
The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions
XZ and LF were involved in study design, conducted the experiments and drafted the paper; JW, HX, WYL, KF, JW and KL performed the experiments; KM provided material support; XY, JH and JR commented the manuscript; JY designed, supervised the study and wrote the paper.

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Supplementary data
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Author names in bold designate shared co-first authorship

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