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Original Article

Neutralization of interleukin-17 attenuates high fat diet-induced non-alcoholic fatty liver disease in mice

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Non-alcoholic fatty liver disease (NAFLD) is hepatic manifestation of a metabolic syndrome and includes a spectrum of hepatic steatosis, steatohepatitis, and fibrosis. Interleukin-17 (IL-17) has been reported to play a critical role in inflammatory progression of some liver diseases. The present study was designed to investigate the role of IL-17 on high fat diet-induced NAFLD in C57BL/6 mice. IL-17 blockade with anti-IL-17mAb significantly improved liver function, attenuated hepatic lipid accumulation, suppressed Kuffer cells activation, and decreased pro-inflammatory cytokines levels, which were associated with inhibition of NF-κB signaling cascades activation. Our data suggested that IL-17 was related to disease progression in NAFLD mouse model and blocking IL-17 may be a promising novel therapeutic approach for patients with NAFLD.

Keywords non-alcoholic fatty liver disease; IL-17mAb; NF-κB

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most frequent cause of chronic liver impairment in developed countries and includes a spectrum ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and liver fibrosis [1,2]. Although NAFLD is a general benign disease, almost 10–20% of NAFLD patients have NASH which will develop into liver fibrosis and increase the risk of liver carcinoma [3]. However, the natural history of NAFLD is still poorly defined. 'Two-hit theory', which is the most widely accepted theory, is presented for NAFLD progression. The first hit is accumulation of lipids in liver caused by excess free fatty acids (FFA), which make the liver vulnerable to aggressive factors of the second hit, such as inflammatory cytokines and oxidative stress [4]. Proinflammatory cytokines are abundantly expressed in liver and best known for their ability to recruit and activate inflammatory cells in the liver, which cause hepatocellular injury and contribute to NAFLD [5]. The expression of interleukin-6 (IL-6) in the liver is correlated positively with severity of inflammation and the degree of fibrosis observed in NAFLD patients, and is considered a key proinflammatory cytokine in NAFLD development [6]. Nuclear factor-kappa B (NF-κB), the key pro-inflammatory signaling pathway, is generally existing in human NAFLD [7] and in all animal models used for study [8,9]. FFAs accumulation in hepatocytes activate NF-κB-dependent inflammatory cytokines expression [tumor necrosis factor-α (TNF-α) and IL-6], which are considered to be the potential pathogenic factors causing second hit to lead to liver injury, inflammation, and fibrosis [10].

The liver is not only exerting the best-known metabolic functions, but also serves as an important immunological organ in homeostasis. In the liver, unbalanced Th1/Th2 T-cell responses are a vital characteristic of hepatic inflammation and subsequent fatty liver disease. The widely researched Th17 cells, a $CD4^+$ T helper cell subtype generating IL-17 (also known as IL-17A), IL-17F, IL-21, and IL-22 and so on, have initially been related to autoimmunity and to host defense against infections [11]. Th17 cells not only play vital roles in mediating pathogen clearance, but also contribute to regulation of tissue inflammatory responses progression [12]. Interleukin-17 receptor (IL-17R) expression has been detected in all types of liver cells, such as hepatocytes, Kupffer cells, stellate cells, biliary epithelial cells, and sinusoidal endothelial cells [13]. IL-17 and IL-6 are closely correlated with each other. IL-17 triggers a significant increase in IL-6 levels in the liver, while IL-6 is necessary to differentiate naive CD4 T cells into the Th17 lineage [14]. IL-17 activates common downstream inflammatory signaling, including NF-κB. Although NF-κB exists

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in multiple isoforms, gel shift analysis has shown that IL-17 activated NF-κB isoform through canonical pathway components, p65 [15]. In fact, IL-17 accelerates inflammatory progression in various liver diseases, such as alcoholic liver disease (ALD), autoimmune liver disease, viral hepatitis, and so on [16].

A better understanding of the mechanisms involved in hepatic inflammation and its progression into NAFLD is important to develop efficient drugs to mitigate these liver diseases. IL-17 is an autoimmune cytokine with proinflammatory function and is involved in various inflammatory reactions in liver diseases, but its contribution to NAFLD remains to be established. The anti-inflammatory and immune regulation properties of anti-IL-17mAb make it a potential therapeutic target for treatment of many complications associated with NAFLD. Based on these data, we tested the hypothesis that extra injection of anti-IL-17mAb would decrease liver inflammation and injury responses implicated in the progression of a mouse model of NAFLD induced by high fat diet.

Materials and Methods

Reagents

Antibodies used in this study were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). All other chemicals and reagents were from Sigma-Aldrich (St Louis, USA) unless otherwise specified.

Animals and administration of anti-IL-17mAb

All experiments were performed according to the *National Institutes of Health Guidelines* for the care and use of animals and were approved by the Animal Care and Use Center of Tongji Medical College, Huazhong University of Science and Technology. Seven-week-old male C57BL/6 mice were purchased from HFK Bio-Technology Company (Beijing, China). The animals were housed on 12 h light/12 h dark cycling. Mice were fed with standard chow and *ad libitum* plus water. Mice (8-week-old) were randomly divided into high fat diet group ($n = 24$) and control group ($n = 8$). Mice in the high-fat diet group were fed with a high fat diet (60% fat; Beijing HFK Bio-Technology, Beijing, China) for 16 weeks and further randomly divided into three groups: high fat diet control group (HFD group) ($n = 8$) which were continually fed high fat diet; high fat diet isotype control group (HFD+Isotype control group) which were fed with high fat diet and treated with immunoglobulin (Ig)G Ab (100 μg/per mouse, R&D systems, Minneapolis, USA) for 14 days every other day through intraperitoneal injection; high fat diet treatment group (HFD+anti-IL-17 group) ($n = 8$) which were fed with high fat diet and treated with anti-IL-17mAb (100 μg/per mouse, R&D systems) for 14 days every other day through intraperitoneal injection. After 2 weeks, mice were fasted overnight and sacrificed. Blood was obtained from orbital canthus and serum was separated. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the serum were measured following a colorimetric procedure, using commercially available detection kits (Jiancheng Institute of Biotechnology, Nanjing, China). Liver samples were harvested, and then fixed in 10% formalin or snap frozen in liquid nitrogen for histological analysis or stored at −80°C for further analysis.

Histopathological and immunohistochemical analysis

Paraffin-embedded samples were stained with hematoxylin–eosin (H&E) and optimal cutting temperature (OCT) compound-embedded sections were stained with Oil-red O to detect hepatic lipid accumulation. Steatosis evaluations were blindly performed by an expert pathologist using a scoring system proposed by Kleiner *et al.* [17]. For immunohistochemical analysis, anti-F4/80 antibody was used to assess Kuffer cells activation and infiltration in hepatic tissue, as previously described [18]. Briefly, hepatic tissue sections were incubated with primary anti-F4/80 antibody overnight at 4°C, and then with biotinylated secondary antibody for 1 h at 37°C. The nuclei were counterstained with hematoxylin. Images were captured with an Eclipse TE2000 inverted microscope system (Nikon Instruments, Melville, USA) and analyzed with HAIPS Pathological Imagic Analysis System (Tongjiqianping Image Company, Wuhan, China).

Triglyceride content assay

Total lipids were extracted from frozen hepatic tissue (100 mg) homogenates with chloroform/methanol/glacial acetic acid (66:33:1, v/v), and phase separation was realized by addition of water. Dried lipids were redissolved in 1% (v/v) Triton X-100, and triglyceride content was measured using triglyceride quantitative assay kits (DiaSys, Holzheim, Germany). Hepatic triglyceride content was defined as mg of triglyceride per gram of the liver.

Enzyme-linked immunosorbent assay analysis

Serum IL-17, IL-6, TNF-α and IFN-γ levels were measured by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions. ELISA kits for IL-17, IL-6, and TNF-α were purchased from Boster Biotech, (Wuhan, China). IFN-γ ELISA kit was from R&D Systems. Intra-assay and inter-assay coefficients of variation for all ELISA were <5 and $<10\%$, respectively.

Real-time reverse transcriptase-polymerase chain reaction

Total RNA extraction from frozen liver tissue samples was performed using Trizol Reagent Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. cDNA

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was obtained using 1 mg of total RNA for reverse transcription using a reverse transcription kit (Takara, Dalian, China). Real-time quantitative polymerase chain reaction (PCR) was carried out on a StepOnePlus™ Real-time PCR System (Applied Biosystems, Foster City, USA). Primer sequences used specially for mouse samples were shown in **Table 1**. Each amplification reaction was completed as follows: 2 min at 50°C (1 cycle), 10 min at 95°C (1cycle), 15 s at 95°C, and 60 s at 60°C (40 cycles). The relative values in gene expression by real-time reverse transcriptase (RT)-PCR were calculated after adjusting for β-actin using the formula $2^{-\Delta\Delta CT}$.

Western blot analysis

Proteins were obtained from the liver by extraction with lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM Na_3VO_4, 1% Triton X-100, and 50 mM NaF) supplemented with protease and phosphatase inhibitors. Protein concentrations were estimated using a BCA-100 protein quantitative analysis kit (Pierce, Rockford, USA). Equal amounts of protein samples were separated by electrophoresis on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 with 5% skimmed milk for 2 h at room temperature and then incubated with primary antibodies against p-IκBα, IκBα, NF-κB(p65), Lamin B, and β-actin overnight at 4°C. The membranes were visualized by enhanced chemiluminescence using horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, Soham, UK) and quantified by densitometry analysis using Bio Image System (Syngene, Cambridge, UK).

Statistical analysis

All values were expressed as mean ± SEM ($n = 8$ per group). Statistical comparisons were performed using Student's unpaired *t*-tests (for two groups) or analysis of variance (for more than two groups). $P < 0.05$ was considered significant difference.

Results

Effects of anti-IL-17 mAb on liver function

Figure 1 showed the biochemical parameters of mice in both normal diet and high fat diet-fed groups. Compared with normal control diet group, mice fed with high fat diet had significantly increased serum ALT, AST levels, markers of liver injury ($P < 0.05$). However, the increase of serum ALT and AST levels was partially reversed by treatment with anti-IL-17 mAb ($P < 0.05$).

Treatment with anti-IL-17 mAb attenuated HFD-induced hepatic steatosis

Oil red O staining displayed that lipid accumulation was increased by high fat diet, which was remarkably reduced in anti-IL-17 mAb treated-mice [**Fig. 2(A)**]. Hepatic H&E staining showed that the high fat diet increased hepatocellular vacuolation as a consequence of cellular lipid accumulation, extended hepatocytes, moved nuclei toward the plasma membrane, and narrowed sinusoidal channels. Interestingly, these pathological changes were attenuated by anti-IL-17 mAb treatment [**Fig. 2(B)**]. Steatosis was scored on H&E-stained sections according to Kleiner's criteria. Anti-IL-17 mAb treatment attenuated increases in hepatic steatosis that were caused by high fat diet and lowered steatosis score [**Fig. 2(C)**]. These results were further confirmed by a significant decrease of hepatic triglyceride content in anti-IL-17 mAb-treated mice [**Fig. 2(D)**]. In conclusion, treatment with IL-17 mAb attenuated hepatic steatosis in high fat diet-induced mice.

Treatment with anti-IL-17 mAb reduced upregulation of inflammatory cytokines levels in serum and hepatic tissue induced by HFD

To investigate effects of anti-IL-17 mAb treatment on inflammatory cytokines produced by high fat diet, ELISA and real time RT-PCR analysis were carried out. As shown in **Fig. 3**, anti-IL-17 mAb treatment significantly inhibited the increase of serum IL-17, IL-6, TNF-α, and IFN-γ cytokines production induced by high fat diet. Similarly, as illustrated in **Fig. 4**, mRNA levels of inflammatory cytokines IL-17 and IL-6 in hepatic tissue significantly increased in high fat diet-fed mice, interestingly, the increase expression levels of these genes were reduced in anti-IL-17 mAb-treated mice.

Treatment with anti-IL-17 mAb lowered HFD-induced macrophage infiltration in liver

Immunostaining against the macrophage surface marker F4/80 demonstrated that numbers of F4/80-positive cells were increased by high fat diet feeding and were markedly reduced by anti-IL-17 mAb treatment [**Fig. 5(A,B)**]. In accordance with the decreased inflammation observed histologically, the abundance of macrophage-specific surface protein F4/80 mRNA was consistently lower in IL-17mAb-treated mice compared with in age-matched high fat diet-fed group [**Fig. 5(C)**]. Together, all these data suggested that hepatic macrophage activation and infiltration induced by high fat diet were significantly inhibited by anti-IL-17mAb treatment.

Effects of anti-IL-17 mAb on NF-κB signaling pathway

To investigate the mechanism of anti-IL-17 mAb treatment on NAFLD mice, we assayed proteins involved in NF-κB signaling pathway, which is an important mediator in inflammatory response. The degradation and phosphorylation of

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Table 1 Primer sequences for real-time RT-PCR

Gene	Forward	Reverse
IL-17	5′-TCAGCGTGTCCAAACACTGAG-3′	5′-CGCCAAGGGAGTTAAAGACTT-3′
IL-6	5′-CTGCAAGAGACTTCCATCCAG-3′	5′-AGTGGTATAGACAGGTCTGTTGG-3′
F4/80	5′-CTGCACCTGTAAACGAGGCTT-3′	5′-GCAGACTGAGTTAGGACCACAA-3′
β-Actin	5′-GGCTGTATTCCCCTCCATCG-3′	5′-CCAGTTGGTAACAATGCCATGT-3′

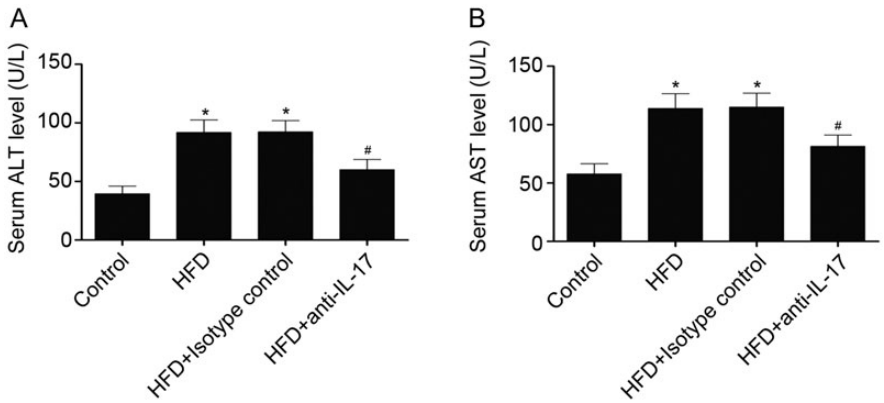

Figure 1 Serum ALT and AST levels. Anti-IL-17 mAb treatment decreased elevation of serum (A) ALT and (B) AST levels induced by high fat diet in mice. $^{*}P < 0.05$ vs. WT control mice; $^{\#}P < 0.05$ vs. HFD mice.

IκBα are necessary to translocate NF-κB p65 into the nucleus. As shown in **Fig. 6**, western blot demonstrated that high fat diet increased the degradation and phosphorylation of IκBα and nuclear NF-κB p65 subunit levels, which were reversed by anti-IL-17 mAb treatment. These results displayed that anti-IL-17mAb treatment could obviously decrease the high fat diet-induced IκBα phosphorylation and degradation, inhibit NF-κB p65 nuclear translocation, and reduce inflammatory response in liver.

Discussion

This study was undertaken to determine whether blocking IL-17 with anti-IL-17 mAb affected the progression of hepatic steatosis and inflammation in a high fat diet-induced NAFLD mouse model. Our results showed that treatment with anti-IL-17mAb markedly improved liver function, reduced the degree of hepatic steatosis, suppressed Kuffer cells activation and decreased proinflammatory mediators' levels in serum and injured liver of NAFLD mice. To explore the mechanism of anti-IL-17mAb treatment on high fat diet-induced NAFLD, we determined several proteins which played important roles in the signal transduction of NF-κB signaling pathway, and the results showed that the protective roles of anti-IL-17mAb treatment on NAFLD was mediated through the inhibition of IκB-α phosphorylation, degradation, and NF-κB p65 subunit nuclear translocation. Taken together, we have demonstrated that high fat diet induced significant liver impairment, hepatic steatosis, inflammatory cell activation and infiltration, and signaling pathway activation in mice, which were markedly attenuated by treatment with anti-IL-17mAb. These results provided strong evidence that IL-17 at least partially mediated inflammatory progression in NAFLD, and blocking IL-17 may be a promising novel therapeutic approach for patients with NAFLD.

IL-17 is a pro-inflammatory cytokine that prompts macrophages, fibroblasts, and epithelial cells to produce and release other cytokines and chemokines which mediate tissue infiltration and destruction [11]. Th17 cells have been carried out in inflammatory pathogenesis of some liver diseases. Liver infiltration with IL-17 secreting cells is an important feature of alcoholic hepatitis [19]. Serum Th17-related cytokines and IL-17^{+} lymphocytic infiltration in liver tissues from patients with primary biliary cirrhosis were significantly increased [20]. Th 17 cells are also found to be highly enriched in both peripheral blood and livers of CHB patients, and may aggravate liver damage during chronic HBV infection [21]. Chronic excessive macronutrient composition of the diet intake, many metabolic changes, including excessive visceral adipose accumulation, body weight gain, insulin resistance, and system inflammation, may promote lipogenesis in liver. IL-17 has been found to exacerbate hepatocyte steatosis in HepG2 cells *in vitro* [22], while lipid composition in the atherosclerotic lesions are significantly diminished in IL-17A$^{-/-}$ mice fed high fat diet [23].

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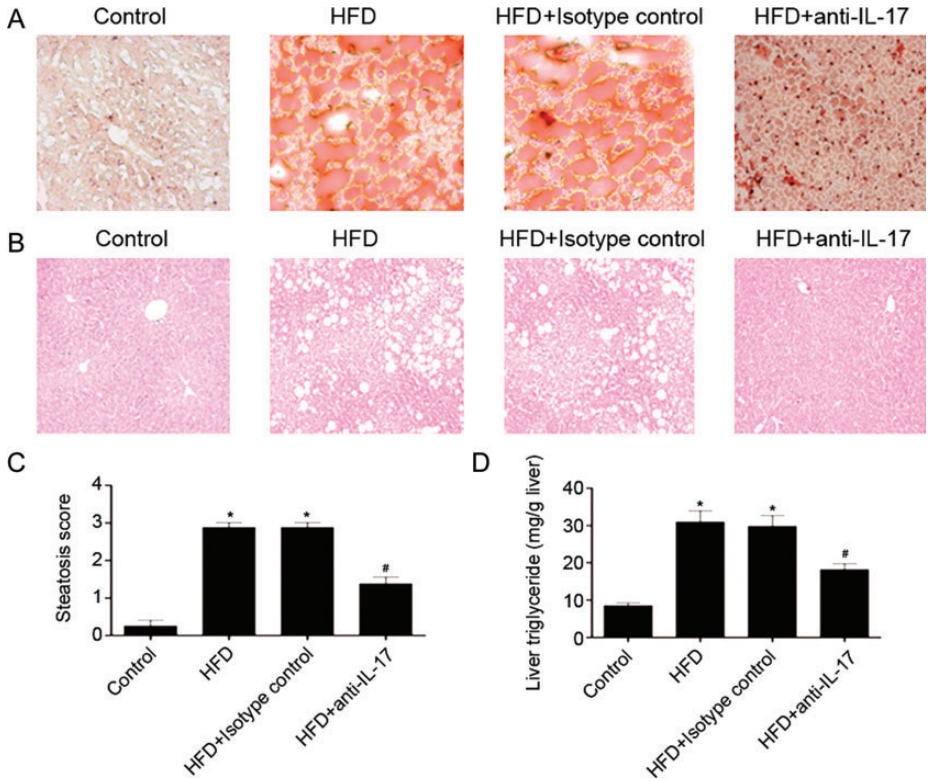

Figure 2 Hepatic histology and triglyceride content in mice. (A) Oil Red O or (B) H&E staining of hepatic sections from representative mice from each group ($\times 100$). (C) Steatosis evaluations were performed using a scoring system. (D) Liver triglycerides were quantified with commercial kits. $^{*}P < 0.05$ vs. WT control mice; $^{\#}P < 0.05$ vs. HFD mice.

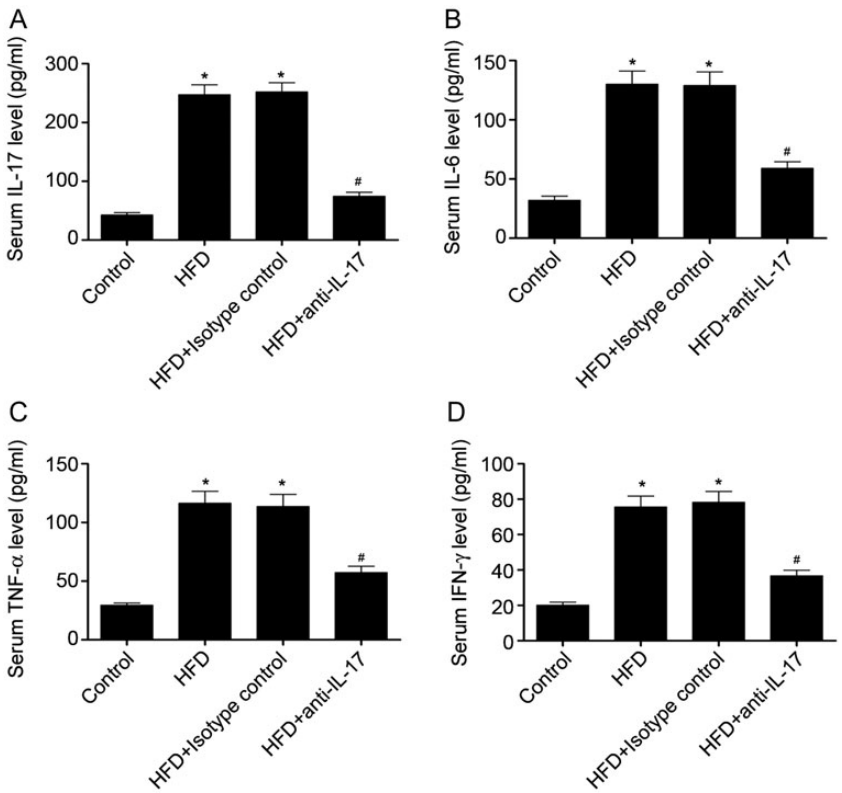

Figure 3 Serum inflammatory cytokines levels. Lower levels of pro-inflammatory cytokines (A) IL-17, (B) IL-6, (C) TNF-α, and (D) IFN-γ in serum of anti-IL-17 mAb treatment mice fed a high fat diet. $^{*}P < 0.05$ vs. WT control mice; $^{\#}P < 0.05$ vs. HFD mice.

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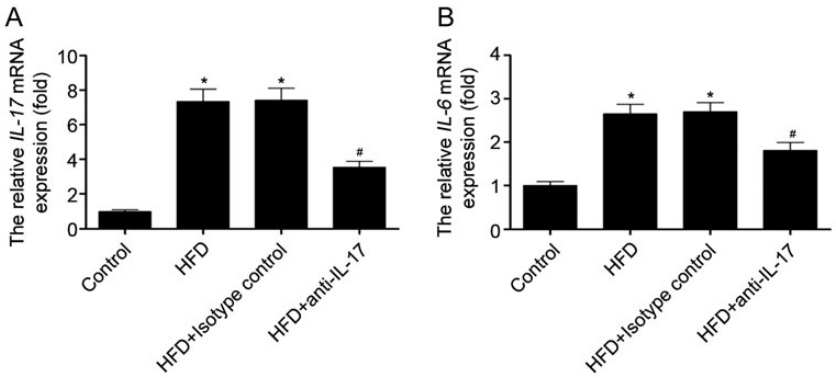

Figure 4 *IL-17* and *IL-6* mRNA expression level in hepatic tissue. Hepatic pro-inflammatory cytokines (A) *IL-17* and (B) *IL-6* mRNA expression levels were significantly suppressed by anti-IL-17 mAb treatment in high fat diet-fed mice, as shown by real-time RT-PCR. $^{*}P < 0.05$ vs. WT control mice; $^{\#}P < 0.05$ vs. HFD mice.

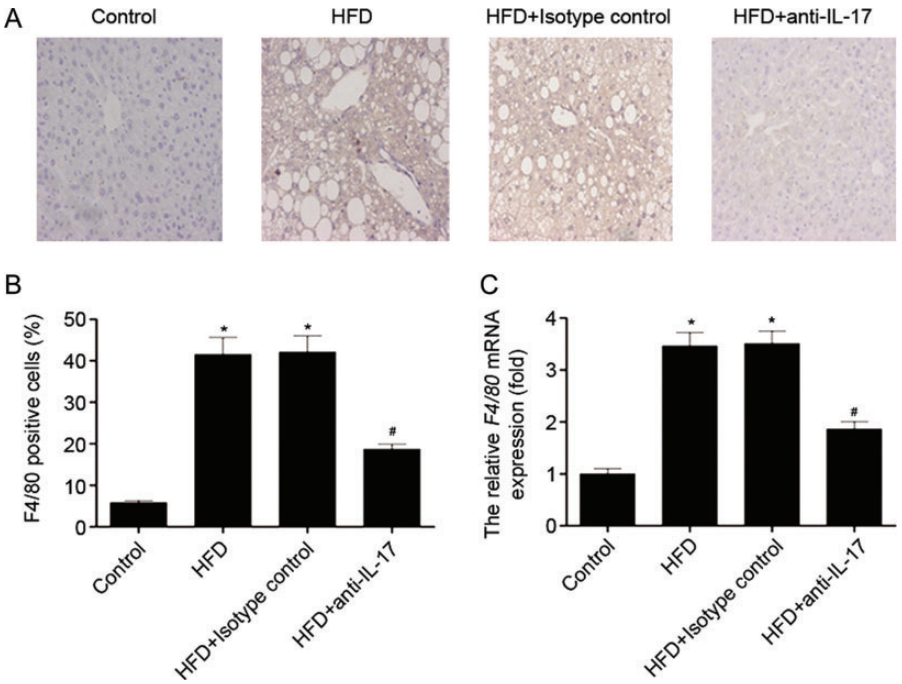

Figure 5 Anti-IL-17 mAb treatment lowered HFD-induced macrophage infiltration in liver. (A) Representative photomicrographs of immunohistochemical detection of F4/80 positive cells in livers from four groups (×200). (B) Quantification of F4/80 staining. (C) *F4/80* mRNA expression was determined by real-time RT-PCR and was normalized to *β-actin* expression. $^{*}P < 0.05$ vs. WT control mice; $^{\#}P < 0.05$ vs. HFD mice.

However, little is known about the roles of Th17 cells in the pathogenesis of NAFLD. Our results showed that IL-17 was closely related with NAFLD progression, and treatment with anti-IL-17mAb improved liver function and markedly reduced lipid accumulation and inflammatory cell infiltration in mice fed with high fat diet.

The pro-inflammatory effect of IL-17 is associated with an increased concentration of IL-6, and IL-6 is both a downstream cytokine of IL-17 pathway and a differentiation factor for Th17 [24]. Since liver is known to be an important source of IL-6, Th17 differentiation may be favored in the liver. IL-6 levels are elevated in the plasma and peripheral blood monocytes of patients with fatty liver diseases, such as ALD and NASH, and increase of IL-6 levels correlate with the progression and severity of liver disease [25,26]. The animal model show that genetic deletion of IL-6 markedly attenuates hepatic inflammation in NASH mice, which is observed both histologically and by analyzing hepatic genes expression that regulate the inflammatory response and promote liver fibrosis [27]. IL-17 increases the production of IL-6 by hepatocytes, which, in turn, further stimulates Th17 cells and provides a positive feedback loop between Th17 cells and hepatocytes exacerbating the inflammatory process [28]. In our study, anti-IL-17mAb treatment significantly decreased serum IL-17, IL-6, TNF-α, and IFN-γ levels, and reduced liver IL-17 and IL-6 mRNA expression.

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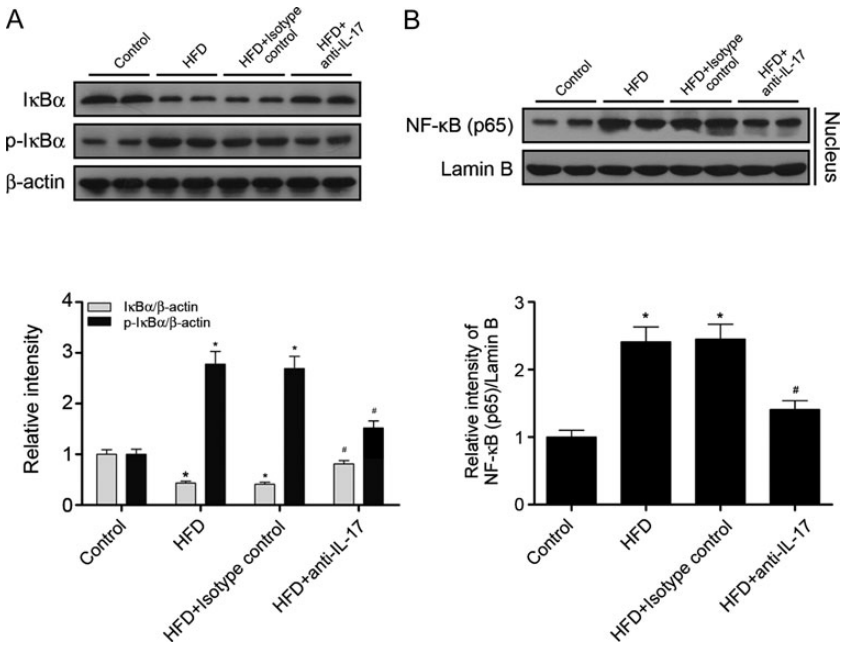

Figure 6 Anti-IL-17 mAb treatment inhibited NF-κB signaling pathway activation. The levels of (A) IκBα, p-IκBα, and (B) nuclear protein NF-κB (p65) from four treatment groups were detected by western blot analysis. $^{*}P < 0.05$ vs. WT control mice; $^{\#}P < 0.05$ vs. HFD mice.

F4/80, a marker of mature macrophages, was highly expressed on subpopulations of Kupffer cells in liver. And Kupffer cell activation played a central role in inflammatory actions and progression of NAFLD [29]. We found that F4/80 was highly expressed by subpopulations of resident Kupffer cells in liver of high fat diet-fed mice, which was reversed in anti-IL-17mAb-treated mice. Together, our data showed that the alleviation of inflammatory cytokines by anti-IL-17mAb treatment was accompanied with reduction of macrophage accumulation in injury livers.

NF-κB is a key transcription factor in control of molecular pathways related to inflammation [30]. NF-κB activation is uniformly found in human NAFLD and animal model that have been studied, which is essential for hepatic inflammatory recruitment in fatty liver disease [31,32]. In resting cells, NF-κB is sequestered in the cytoplasm by its interaction with members of the inhibitor of κB (IκB) family, which prevents NF-κB nuclear translocation and DNA-binding domains by masking the nuclear localization signal of at least one member of the NF-κB dimmers [33]. Classical NF-κB activation requests phosphorylation of IκBα, which is considered to be the major IκB protein that regulates the classical NF-κB signaling pathway, leads to its proteasomal degradation, and NF-κB nuclear translocation [34]. In our study, western blot results demonstrated that activation of NF-κB was involved in the development of NAFLD induced by high fat diet. After intervention with anti-IL-17mAb, increased phosphoration and degradation of IkBα, and nuclear NF-κB p65 subunit levels were significantly inhibited, hence, the NF-κB signaling pathway was dramatically suppressed.

In conclusion, anti-IL-17mAb treatment attenuated progression of hepatic steatosis and inflammation non-alcoholic fatty liver in high fat diet model. The effects of anti-IL-17mAb treatment may attribute to improve liver function, attenuate hepatic lipid accumulation, decrease pro-inflammatory cytokines levels, and inhibit NF-κB signaling pathway. Our findings support a potential beneficial role of anti-IL-17mAb for prevention and treatment of NAFLD in humans. As IL-17 is an important immune cytokine, more *in vivo* and *in vitro* studies are needed.

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