Fuzi-lizhong Decoction Alleviate Nonalcoholic Fatty Liver Disease by Blocking TLR4/MyD88/TRAF6 Signaling Pathway

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Research

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Abstract

Background Fuzi-lizhong decoction (FLD) derives from an ancient Chinese Pharmacopoeia and has been used for the clinical treatment for years. The present study aimed to investigate the activities and underlying mechanisms of FLD against non-alcoholic fatty liver disease (NAFLD).

Methods *In vivo* NAFLD in rats was induced by high-fat diet, and *in vitro* studies were performed on HL-7702 cells treated with oleic and linoleic. Serum levels of total cholesterol (TC), triglyceride (TG) and blood glucose (Glu) were detected using an automatic biochemical analyzer. Expression of IL-2, IL-6 and TNFα were detected by ELISA. Using the Western blot (WB) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to measure the levels of TLR4, MyD88 and TRAF6.

Results FLD significantly attenuated inflammation and improved collagen accumulation through down-regulating the levels of IL-2, IL-6, TNFα, NF-κB p65 by inhibited the activation of TLR4/MyD88/TRAF6 signaling pathway both *in vivo* and *in vitro*. TLR 4 overexpression in NAFLD was decreased by FLD, leading to the markedly down-regulated levels of myeloid differentiation factor 88 (MyD88) and TNF receptor associated factor 6 (TRAF6). In addition, the significant increased levels of total cholesterol (TC), triglyceride (TG) and blood glucose (Glu) in serum and free fatty acid (FFA) in liver were significant reduced by FLD treatment. **Conclusions** FLD exhibited potent protective effect against NAFLD via TLR4/MyD88/TRAF6 signaling pathway, which might provide a novel insight into the mechanisms of this compound as an anti-inflammatory candidate for the treatment of ALF in the future.

Background

Recently, high fat diet due to the improvement of living standards have resulted in an increase of the morbidity of non-alcoholic fatty liver disease (NAFLD), particularly in younger patients in countries such as China (1–3). NAFLD is a medical condition characterized by a series of hepatic pathological changes including simple steatosis, non-alcoholic steatohepatitis and cirrhosis (4, 5). The pathogenesis of NAFLD is an orchestrated multistep process in response to hepatic lipid accumulation and oxidative stress. It has been demonstrated that approximately 15% case of NAFLD could resulted in cirrhosis and even hepatocellular cancer (6, 7). In affluent regions of China, the incidence of NAFLD is approximately 15% (8). Although an appropriate nutritional intake and exercise are recommended to prevent excessive body weight, this health problem caused by overnutrition has not yet been conquered. Therefore, new strategies are required to reduce the risk of NAFLD.

The innate immune system is closely related with the progresses of NAFLD. Several studies described a close association between NAFLD development and many pathogenic events, such as activation of the innate immune system, hepatic macrophage recruitment changes in lipid homeostasis (9, 10). Toll-like receptors (TLRs) are pattern recognition receptors that recognise pathogen-associated molecular patterns and allow the host to detect microbial infection. (11). TLRs can involve NAFLD by regulate innate and adaptive immune response (12). TLR4 is the receptor for lipopolysaccharide (LPS), can trigger two

different signaling pathways, in which one is a myeloid differentiation factor 88 (MyD88)-dependent pathway and results to the activation of nuclear factor κB (NF-κB) and TNF receptor associated factor 6 (TRAF6), and the other is an MyD88-independent pathway requiring the Toll/interleukin-1 receptor (TIR)-containing adaptor molecule (13). The activation of TLR4 pathways can stimulate downstream signal cascades, resulting the production of proinflammatory cytokines, chemokines, and type I interferon. Moreover, the activation of TLR4 signaling pathway might perturbed at multiple steps during the initiation and progression of NAFLD (14).

Fuzi-lizhong decoction (FLD) is a Chinese herbal concoction consisting of *Panax ginseng* C.A.Mey., *Aconitum carmichaeli Debx.*, *Glycyrrhiza inflata* Bat., *Atractylodes macrocephala* Koidz and *Zingiber officinale* Rosc. FLD have significant therapeutic effects on dyspnea caused by chronic obstructive lung disease, pulmonary oedema provoked by heart failure and inflammation of the viscera, clearly relieving various respiratory and myocardium symptoms (15–17). However, the effect of FLD on liver disease is rarely reported. In the present study, we used a high-fat and high-fructose diet to establish a suitable animal model of NAFLD and explored the effect and potential mechanisms of FLD on NAFLD.

Methods

Regents and materials. FLD is a classical Chinese herbal formulae and their ingredients are listed in Table 1. FLD is composed of Radix Codonopsis (15 g), Rhizoma Atractylodis Macrocephalae (9 g), Radix Glycyrrhizae (6 g), Rhizoma Zingiberis (9 g), and Radix Aconiti Lateralis Preparata (9 g). Herbs were purchased from Hubei Tianji Chinese Herbal Sliced Medicine Co., Ltd.. Decoction according to the method of Yang Xin (18). Polyene phosphatidylcholine was purchased from Sanofi Beijing pharmaceuticals Co., Ltd (cat No. 5JD065B). IL-2 (RA20132), IL-6 (RA20607) and TNFα (RA20035) ELISA kit were purchased from Bioswamp (Wuhan, China). Penicillin, streptomycin and antimycotic were obtained from Sigma-Aldrich (USA). TLR4 (ab13867, 1:1000 dilution), NFκB p65 (ab16502, 1:2000 dilution), p-NFκB (ab86299, 1:2000 dilution), MyD88 (ab2064, 1:1000 dilution) and TRAF6 (ab33915, 1:5000 dilution) antibodies were purchased from Abcam (USA). Anti-GAPDH antibody (2118, 1:10000 dilution) was purchased from CST (USA).

Animals and experimental groups. Forty-eight male Wistar rats were obtained from Hubei Provincial Academy of Preventive Medicine (certification No. 42000600013948). Rats were housed in a specific pathogen-free facility (SPF) at Wuhan First Hospital. Following an acclimatization period, rats were randomly divided into the control group (CON), NAFLD model group (MOD), positive control group (PC) and FLD with high dose group (HIG), middle dose group (MID), and low dose group (LOW), n = 8 animals per group. The rats in control group received standard laboratory diet, the model group received high-fat diet (standard laboratory diet + 2% cholesterol + 10% lard + 2.5% vegetable oil), the rats in various FLD group received 5, 10, and 20 g/kg/d FLD treatment, and the rats in positive group received 30 mg/kg/d polyene phosphatidylcholine. Rats were maintained under a 12 h light-dark cycle at 23 ± 2^{o}C. After the experiment, the mice were euthanized by intraperitoneal injection of 200 mg/kg pentobarbital, packaged

and eventually burned. All experimental procedures were approved by the Animal Ethics Committee of Wuhan Integrated TCM and Western Medicine Hospital (certificate no. 42000600013948).

Cell culture and treatment. Human liver HL-7702 cell line was obtained from Procell (CL-0111). HL-7702 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (ThermoFisher, Waltham, USA), 100 U/mL penicillin, 100 mg/ml streptomycin, and 100 mg/ml antimycotic. The cells were grown in an incubator with a humidified atmosphere (95% air/5% CO_2 v/v) at 37^{o}C for 48 h until 80% confluence, then washed, and exposed for 48 h to FLD at different concentrations (0.5, 1.0, and 1.5 g/ml in serum-free DMEM) or polyene phosphatidylcholine (5 μmol/l). In vitro steatosis was induced by incubating the hepatocytes with 6 mmol/l of a 1:1 v/v mixture of oleic (18:1) and linoleic acid (18:2).

Histological and serological examination. Liver tissue were fixed in 4% paraformaldehyde for 30 min and stained by 0.5% Oil Red O. The stained sections were observed under a microscope (Olympus CX31-LV320, Tokyo, Japan). Serum levels of total cholesterol (TC), triglyceride (TG) and blood glucose (Glu) were detected using an automatic biochemical analyser (model 7180, Tokyo, Japan). The free fatty acid (FFA) level was detected by Nonesterified Free Fatty Acids Assay Kit (A042-1, Nanjing Jiancheng, Nanjing, China).

ELISA assay. IL-2, IL-6 and TNFα levels in the serum or cell culture supernate were evaluated by ELISA kits and the assay was performed in accordance with the manufacturer's protocols.

Total RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from liver tissue or HL-7702 cells by using TRIzol reagent (Takara Bio Inc., Dalian, China) and assessed using an ultraviolet spectrophotometer and 1% agarose electrophoresis. For each sample, 1 μg RNA was reverse transcribed to obtain first-strand cDNA using the PrimeScript® RT reagent kit with gDNA Eraser (Takara Bio, Inc.) following the manufacturer's instructions. The reaction mixture (20 μl total volume) contained 10 μl 2 X SYBR Premix Ex Taq™ (Takara Bio, Inc.), 0.5 μl each primer and 0.2 ± 0.02 μg cDNA template. The following three-step qPCR reaction was performed: Pre-denaturation at 95^{o}C for 30 sec, followed by 40 cycles, including denaturation at 95^{o}C for 3 min and annealing at 60^{o}C for 20 sec and elongation at 72^{o}C for 20 sec. The primes used were shown in Table 1. Levels of gene expression were then calculated using the $2^{-\Delta\Delta Cq}$ method. For each group, three samples were measured and three technical replicates of each measurement were obtained.

Western blot. Protein expression levels were analyzed by Western blot analysis and conducted using standard methods with modification. Liver tissue samples were homogenizing in RIPA lysis buffer containing protease inhibitor at 4^{o}C. For *in vitro* study, cells washed twice with phosphate-buffered saline (PBS) and lysed with RIPA buffer (Beyotime, China) containing protease inhibitor at 4^{o}C. Both cell lysate and tissue lysate were centrifuged at 12000×g for 15 min and supernatants were collected. Protein concentration was detected using a BCA kit (Bio-Swamp Life Science). Equal amounts of protein (30 μg) were separated by 10% SDS-polyacrylamide gel and then transferred onto a PVDF membrane (EMD

Millipore, Billerica, MA, USA). Membranes were blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline (20 mmol/l Tris, 500 mmol/l NaCl and 0.05% Tween 20). Subsequently, the membrane was incubated with primary antibodies. GAPDH was used as an internal reference. Membranes were subsequently washed with Tris-buffered saline and incubated with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (cat. no. W4011; dilution, 1:3,000; Promega Corporation, Madison, WI, USA) for 2 h at room temperature. Immunoreactivity was visualized via a colorimetric reaction using enhanced chemiluminescent substrate buffer (EMD Millipore). Membranes were analyzed using a Gel Doc EZ imager and bans were quantified using Quantity One 5.0 (Bio-Rad Laboratories, Hercules, CA, USA).

Experimental outcomes. We took the effect of FLD on TLR4/MyD88/TRAF6 signaling pathway in rat liver and the HL-7702 cells as the primary experimental outcomes. The inflammation factors and hepatic lipid contents were the secondary experimental outcomes.

Statistical analysis. The statistical differences of the experimental data were evaluated by analysis of variance (ANOVA) using SPSS 19.0 software package. Differences were considered as statistically significant at $P < 0.05$. All results were expressed as mean ± SD.

Results

Effects of FLD on serological examination and hepatic lipid contents of rats with NAFLD. All animals in this study were healthy before and after treatment, and without death and adverse events. To evaluate the treatment effect of FLD on NAFLD, lipid accumulation, serum levels of TC, TG and Glu, and the level of FFA in liver tissue were compared in each group. Compared to normal control group, the levels of TC, TG, Glu and FFA in NAFLD rats increased significantly. Compared to model group, FLD treatment significantly decreased the levels of TC, TG, Glu and FFA (Fig. 1A). Analysis of the hepatic lipid contents by Oil Red O staining revealed that hepatic lipid accumulation increased significantly in NAFLD rats, but this lipid accumulation was reversed by FLD treatment (Fig. 1B).

FLD treatment attenuates inflammation factors in serum and liver of rats. As shown in Fig. 2, the serum and liver levels of IL-2, IL-6 and TNF-α in model group were markedly increased compared with normal rats, which were significantly decreased by FLD administration.

FLD treatment attenuates the activation of TLR4/MyD88/TRAF6 signaling pathway in rat liver. As shown in Fig. 3A, the mRNA expression levels of TLR4, MyD88, TRAF6 and NF-κB p65 in model group were conspicuous increased compared with normal control rats, which were all significantly down-regulated by FLD treatment. The livers from the rats in the model group exhibited drastically increased hepatic protein levels of TLR4, MyD88 and TRAF6, which were all significantly decreased by FLD treatment (Fig. 3B). in addition, the activation of NFκB p65 in model group rat's liver was significantly inhibited by FLD treatment (Fig. 3C). Together, these data indicated that FLD attenuated NAFLD via blocking TLR4/MyD88/TRAF6 signaling pathway.

FLD attenuates the levels of IL-2, IL-6 and TNFα in HL-7702 cells. To verified the treatment effect of FLD on NAFLD, HL-7702 cells were used to induced NAFLD cell model and exposed to different dose of FLD. The IL-2, IL-6 and TNFα levels in cell supernatant were shown in Fig. 4. Compare with normal control group, the levels of IL-2, IL-6 and TNFα in model group increased significantly, which were all significantly decreased by FLD treatment.

FLD inhibits the activation of TLR4/MyD88/TRAF6 signaling pathway in HL-7702 cells. The protein and mRNA expression levels of TLR4, MyD88 and TRAF6 in HL-7702 cells were used to evaluated the treated effect of FLD on NAFLD cell, and the results were shown in Fig. 5. The protein and mRNA expression levels of TLR4, MyD88 and TRAF6 in model cells were remarkedly increased compared with normal control rats, which were all significantly down-regulated by FLD treatment.

Discussion

NAFLD is a passive and irreversible pathological process induced by the necrosis of liver parenchymal cells, and recent evidence has shown that even advanced fibrosis is reversible (19). Therefore, development novel and effective treatment strategies to reverse NAFLD is critical importance. In the present study, FLD showed significant effects against NAFLD as evidenced by decreased TC, TG, Glu and FFA levels, and the alleviation of histopathological changes. Furthermore, FLD was effective in suppressing the TLR4/MyD88/TRAF6 signaling pathway to inhibit the release of inflammatory factors IL-2, IL-6 and TNFα *in vivo* and in *vitro*.

Hepatic inflammatory is tightly related to liver disease. Chronic activation of the inflammatory pathways have been shown to promote hepatocarc-inogenesis (20). During the progression of NAFLD, TLR4 signaling pathway can bind with its ligand, involving in liver injury and repair related functions (21). The present study illustrated that TLR4/MyD88/TRAF6 signaling pathway was significantly activation in NAFLD rats. It was reported that activation of TLR4 pathway under the induction of choline-deficient L-amino acid in nonalcoholic hepatitis (NASH), resulting up-regulation of TNFα expression, suggesting that TLR4 might further induce liver injury (22). In this study, the activation of TLR4/MyD88/TRAF6 signaling pathway in NAFLD rats were blocked by FLD administration. Pro-inflammatory cytokines including IL-1, IL-6 and TNFα are released from inflammatory cells, and their levels are strictly regulated by pro-inflammatory and anti-inflammatory responses (23). In these processes, NF-κB plays a critical role in the regulation of inflammatory responses by affect the production of various pro-inflammatory cytokines such as IL-1, IL-2, IL-6 and TNFα (24, 25). In this study, the anti-inflammatory capability of FLD mainly resulted from decreased the levels of IL-2, IL-6, TNFα and NF-κB p65 via the reduction of TLR4/MyD88/TRAF6 signaling pathway *in vivo* and in *vitro*.

FLD have been used in clinical treatment of some disease for hundreds of years, such as pulmonary and heart disease. Previous studies have been reported that 21 constituents were identified in Fuzi Lizhong by using ultra-performance liquid chromatography with time-of-flight mass spectrome, including ginsenoside Rb1, mesaconitine, aconitine, salsolinol, Glycyrrhizic acid et al (26). Ginsenoside was

determined to be the most compound of *P. ginseng*, showing anti-inflammatory effect by inhibition of the activation of NF-κB (26). In addition, aconitine have been demonstrated anti-inflammatory properties by suppress TNFα and NF-κB activation (27, 28). Furthermore, ginsenoside can improve inflammatory disease by inhibiting IRAK-1 activation via TLR-4 and MAPK signaling pathway (29). In the present study, the expression of TNFα and NF-κB were significant decreased by FLD in NAFLD rats, indicating that FLD can attenuates NAFLD by inhibiting of inflammatory response.

In conclusion, based on our observations, a simplified pathway to describe the possible involvement of TLR4/MyD88/TRAF6 signaling pathway in the treatment of NAFLD by FLD *in vivo* and *in vitro* was found. FLD inhibited the activation of TLR4/MyD88/TRAF6 pathway via down-regulating of TLR4, MyD88 and TRAF6 mRNA and protein levels. Inhibition of NF-κB leaded to the inhibition of inflammatory response. Notably, he underlying mechanisms are certainly more complex than what is described here. In addition, our results do not exclude the possible involvement of other signaling pathways and mechanisms caused by FLD to suppress NAFLD. These findings provide novel insights into the mechanisms of FLD as a potent anti-inflammatory agent that may be used to treat NAFLD. However, at the present time, the clinical application and data to support the present findings of this traditional Chinses medicine are necessary in the future.

Conclusions

FLD exhibited potent protective effect against NAFLD via TLR4/MyD88/TRAF6 signaling pathway, which might provide a novel insight into the mechanisms of this compound as an anti-inflammatory candidate for the treatment of ALF in the future.

Abbreviations

Name	Abbreviations
non-alcoholic fatty liver disease	NAFLD
Toll-like receptors	TLRs
Lipopolysaccharide	LPS
Myeloid differentiation factor 88	MyD88
activation of nuclear factor κB	NF-κB
TNF receptor associated factor 6	TRAF6
Fuzi-lizhong decoction	FLD
Dulbecco's modified Eagle's medium	DMEM
Total cholesterol	TC
Triglyceride	TG
Blood glucose	Glu
Reverse transcription-quantitative polymerase chain reaction	RT-qPCR
Enzyme-linked immuno sorbent assay	ELISA

Declarations

Ethics approval and consent to participate

Approval

Consent for publication

Approval

Availability of data and material

Available

Competing interests

The authors confirm that they have no competing interests.

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Authors' contributions

JYYand HFY were responsible for the paper writing and experimental operation. YL, LS, SZ, CYL, LA and NLD were in charge of literature review and experimental operation. ZHSandWM were responsible for the design of experiment, and all authors have read and approved the manuscript.

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Tables

Table 1 is not available with this version.

Figures

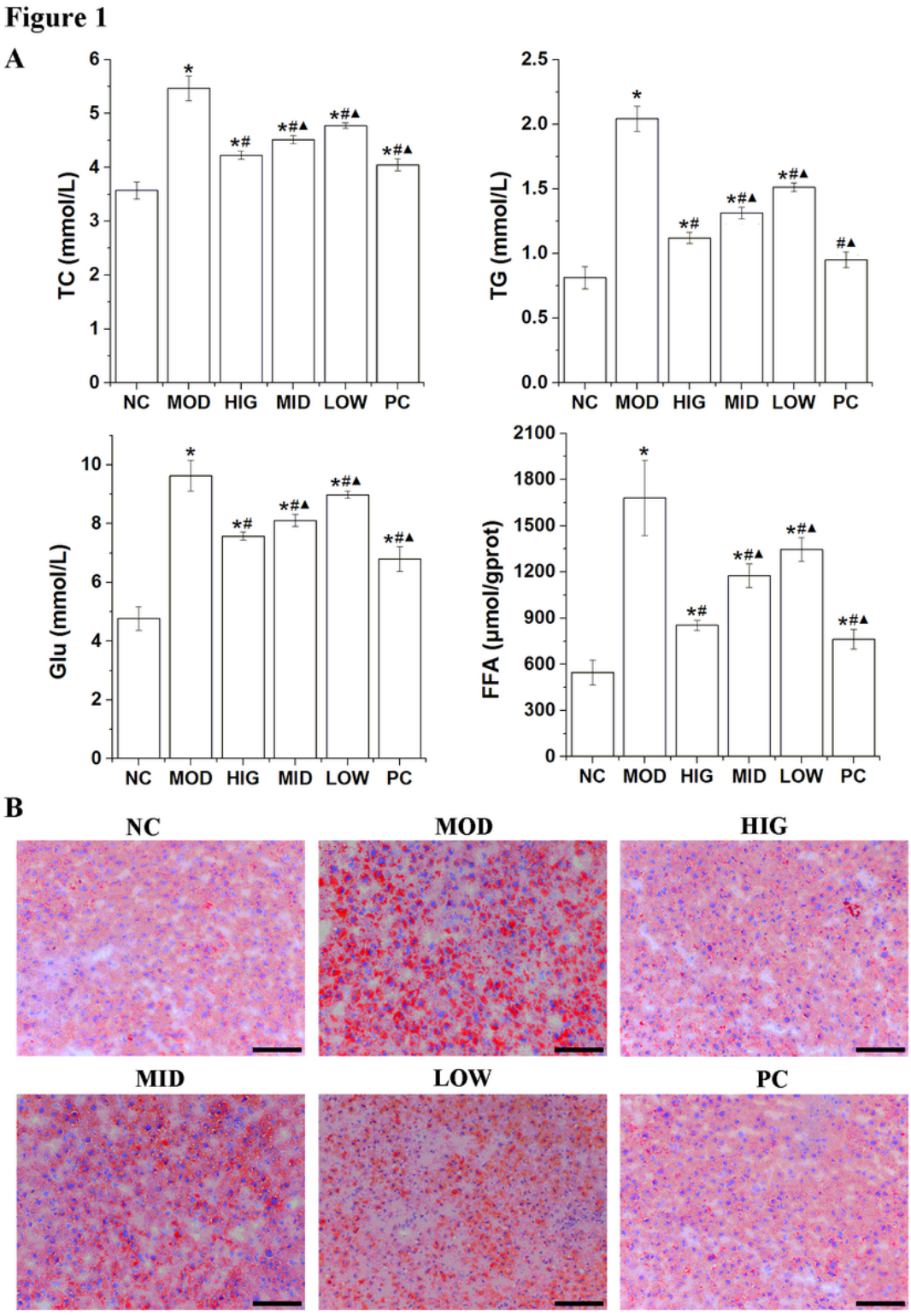

Figure 1

FLD improve the symptoms of NAFLD. A: The serum levels of TC, TG, Glu and liver levels of FFA were detected by ELISA kits. B: Representative images of Oil Red O-stained liver sections from each group (bars 100 μm). All values are expressed as the mean ± S.D. (n=3). *P<0.05 versus NC group; #P<0.05 versus MOD group; ▲P<0.01 versus HIG group.

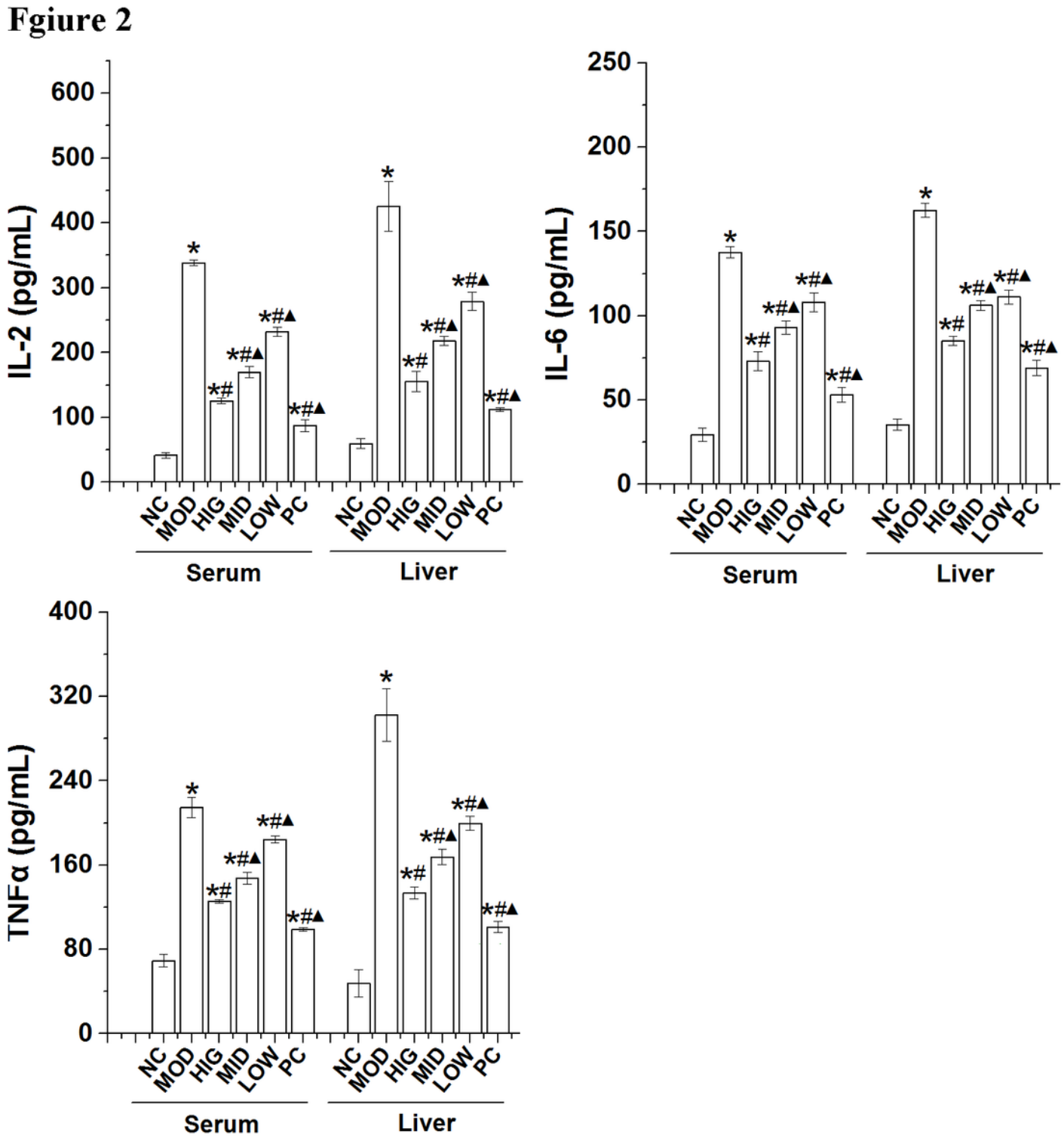

Figure 2

FLD inhibited the production of IL-2, IL-6 and TNFα in NAFLD rat serum and liver tissue. All values are expressed as the mean ± S.D. (n=3). *P<0.05 versus NC group; #P<0.05 versus MOD group; ▲P<0.01 versus HIG group.

Figure 3

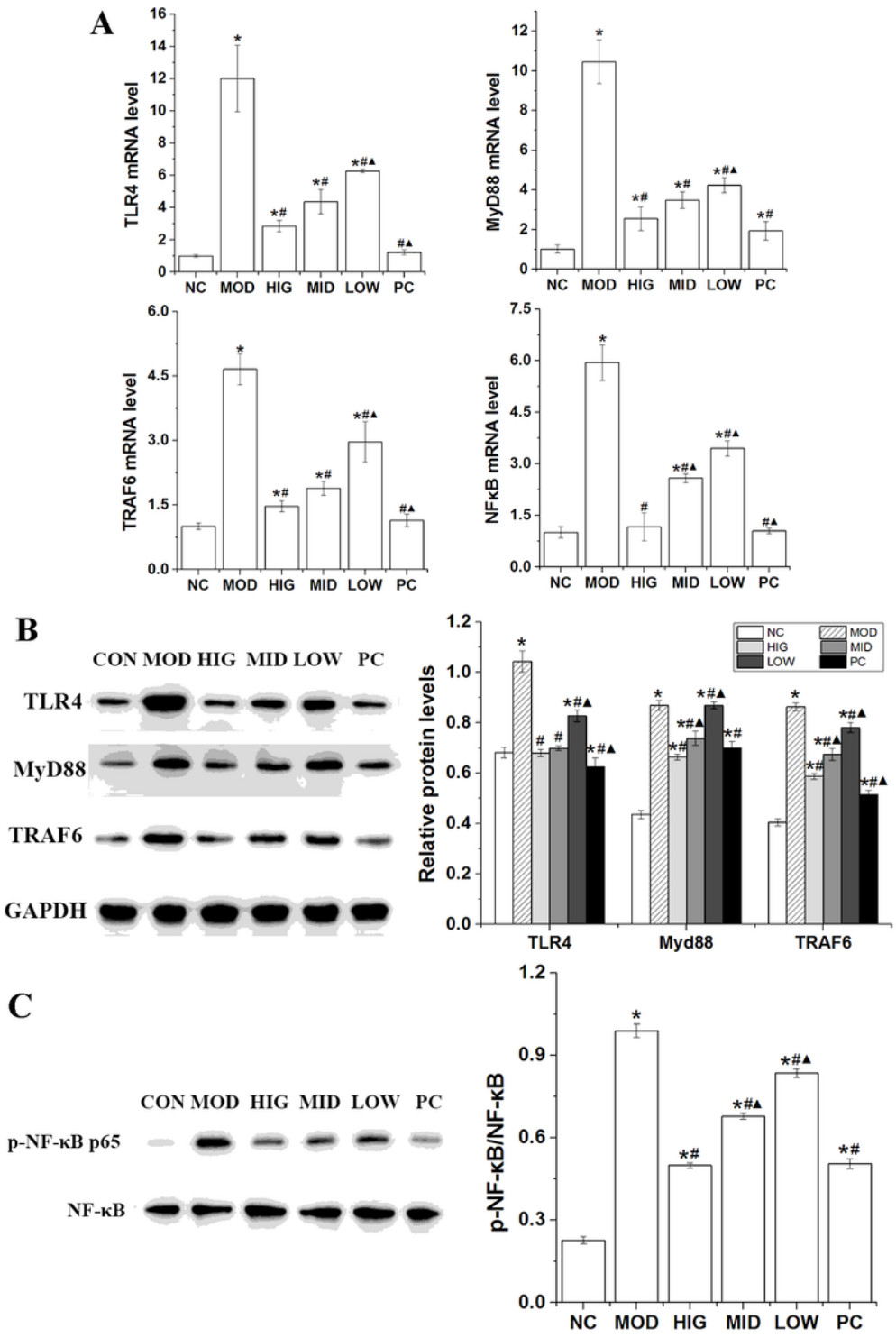

Figure 3

FLD inhibited the activation of TLR4/MyD88/TRAF6 signaling pathway in NAFLD rat. A: The mRNA expression levels of TLR4, MyD88, TRAF6 and NFκB p65 in rat liver tissue were detected by RT-qPCR. B:

The TLR4, MyD88 and TRAF6 protein levels in rat liver tissue were detected by western blot. C: The protein levels of NFκB p65 and p-NFκB in rat liver tissue were detected by western blot. All values are expressed as the mean ± S.D. (n=3). *P<0.05 versus NC group; #P<0.05 versus MOD group; ▲P<0.01 versus HIG group.

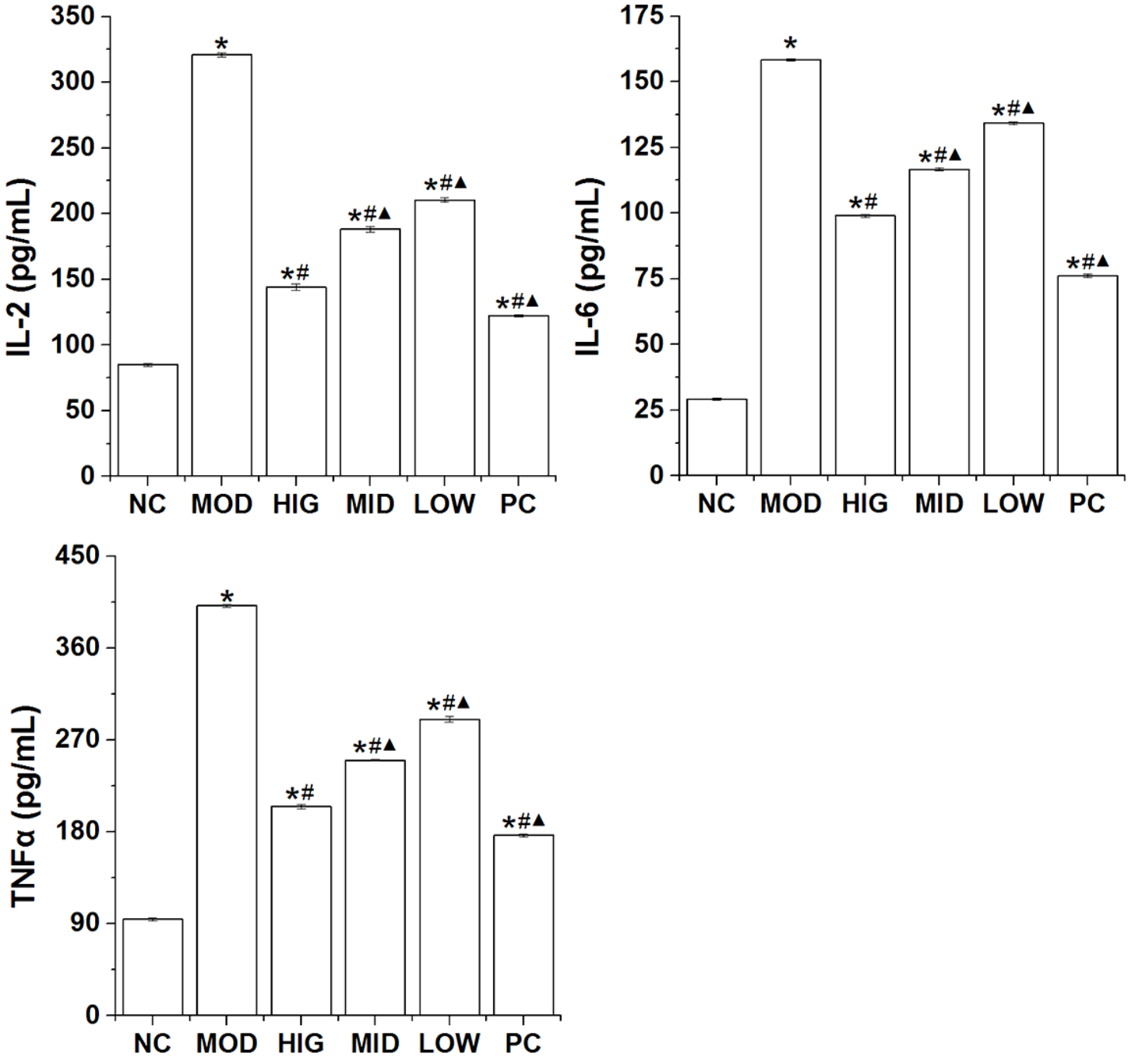

Figure 4

The production of IL-2, IL-6 and TNFα in HL-7702 cells were evaluated by ELISA kits. All values are expressed as the mean ± S.D. (n=3). *P<0.05 versus NC group; #P<0.05 versus MOD group; ▲P<0.01 versus HIG group.

Figure 5

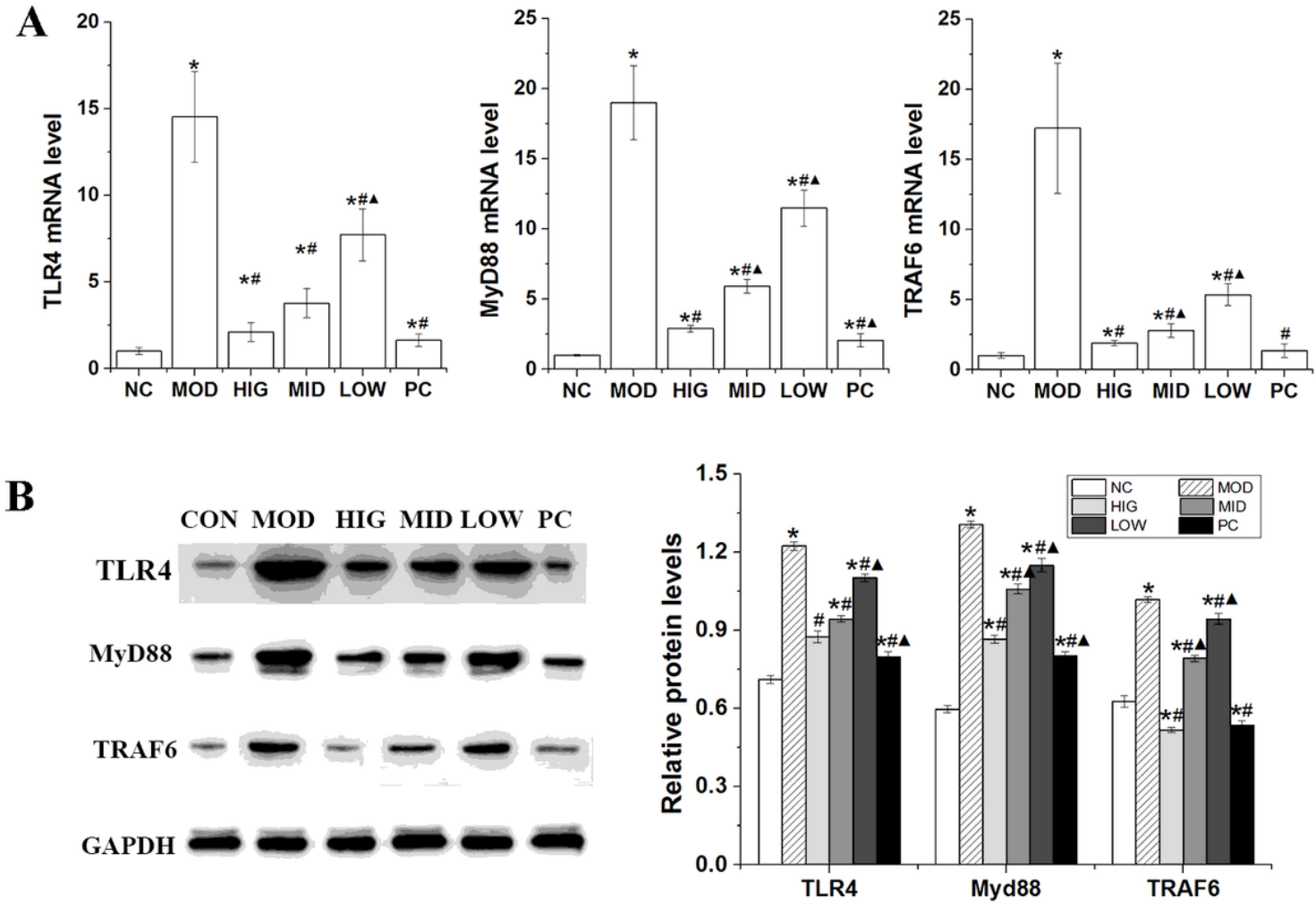

Figure 5

FLD blocked TLR4/MyD88/TRAF6 signaling pathway in HL-7702 cells. A: The mRNA expression levels of TLR4, MyD88 and TRAF6 in HL-7702were detected by RT-qPCR. B: The TLR4, MyD88 and TRAF6 protein levels in HL-7702 were detected by western blot. All values are expressed as the mean ± S.D. (n=3). *P<0.05 versus NC group; #P<0.05 versus MOD group; ▲P<0.01 versus HIG group.