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**Original Paper**

# Isoliquiritigenin Inhibits Interferon-γ-Inducible Genes Expression in Hepatocytes through Down-Regulating Activation of JAK1/STAT1, IRF3/MyD88, ERK/MAPK, JNK/MAPK and PI3K/Akt Signaling Pathways

Shanshan Wu[a] Jihua Xue[b] Ying Yang[a] Haihong Zhu[a] Feng Chen[a] Jing Wang[a] Guohua Lou[a] Yanning Liu[a] Yixian Shi[a] Ye Yu[a] Caixia Xia[a] Ying Hu[a] Zhi Chen[a]

[a]State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University. Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou, Zhejiang, [b]Department of Infectious Disease, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China

**Key Words**
Isoliquiritigenin • Interferon-γ-inducible genes • CXCR3 • HepG2 • L02 • Chemokine

**Abstract**
***Background & Aims:*** The high expression levels of interferon-γ (IFN-γ)-inducible genes correlate positively with liver diseases. The present study aimed to explore the effect of isoliquiritigenin (ISL) on the expression of genes induced by IFN-γ *in vitro*, and to elucidate the underlying molecular mechanisms. ***Methods:*** HepG2 and L02 cells were divided into control, ISL, IFN-γ, and IFN-γ plus ISL groups. The cytotoxicity of compounds to cells was evaluated by Cell Counting Kit 8 (CCK8) assay; the expression levels of chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10, CXCL11, and interleukin-6 (IL-6) in cells and supernatant were measured by quantitative real time polymerase chain reaction (qRT-PCR) and ELISA, respectively. Moreover, western blot was used to examine the phosphorylated levels of janus kinase (JAK)/signal transducer and activator of transcription 1 (STAT1), nuclear factor (NF)-κB, interferon regulatory factor 3 (IRF3)/myeloid differentiation factor 88 (MyD88), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Protein Kinase B (Akt) in HepG2 and L02 cells exposed to ISL, IFN-γ and IFN-γ plus ISL. ***Results:*** The results showed that IFN-γ treatment induced the expression of CXCL9, CXCL10, CXCL11, and IL-6 in HepG2 and LO2 cells, which could be significantly and dose-dependently inhibited by ISL treatment ($P < 0.05$ or $P < 0.01$), but the inhibitory effect of ISL on IL-6 expression was not so good as on CXCL9, CXCL10, and CXCL11 expression. Furthermore, ISL treatment dose-dependently

Dr. Zhi Chen

The First Affiliated Hospital,School of Medicine, Zhejiang University, Qingchun Road 79, Hangzhou 310003, Zhejiang, (China)
Tel. +86-0571-87236579, Fax +86-0571-87068731, E-Mail zju.zhichen@gmail.com

inhibited the activation of JAK1/STAT1, IRF3/MyD88, extracellular signal-regulated kinase (ERK)/MAPK, c-Jun N-terminal kinase (JNK)/MAPK, and PI3K/Akt signaling pathways ($P < 0.05$), but had no effect on the activation of JAK2/STAT1, NF-κB and p38/MAPK signaling pathways. ***Conclusion:*** We demonstrate that ISL inhibits IFN-γ-induced inflammation in hepatocytes via influencing the activation of JAK1/STAT1, IRF3/MyD88, ERK/MAPK, JNK/MAPK, and PI3K/Akt signaling pathways.



## Introduction

Viral hepatitis is a major type of chronic liver disease and one of the major diseases that threaten human health in China. Millions of people die from viral hepatitis-related cirrhosis, liver failure and hepatocellular carcinoma (HCC) [1, 2]. The progression of chronic liver diseases is resulted from the persistent hepatic inflammation and the secretion of cytokines, and chemokines released by liver cells and induced by cytokines are also important determinants [2]. Interferon gamma (IFN-γ) is a T-helper 1(Th1) proinflammatory cytokine that plays a critical role in defencing against viral infection, Th1 lymphocytes recruited to the liver during inflammation may be responsible for the enhanced IFN-γ and tumor necrosis factor (TNF)-α production, which in turn stimulates chemokines secretion from a variety of cells, therefore creating an amplification feedback loop [3].

The expression of chemokines during inflammation is regulated by both type I and type II IFNs [4]. Chemokine (C-X-C motif) receptor 3 (CXCR3) ligands have been extensively investigated in humans with liver disease [1, 5-7]. CXCL9, CXCL10 and CXCL11 can together be referred to as the CXCR3 ligands, They are also named monokine induced by IFN-γ(Mig), interferon (IFN)-γ-induced protein 10 (IP-10) and IFN-inducible T-cell α chemoattractant (I-TAC),respectively. And CXCL10 is the most thoroughly investigated CXCR3 ligand. Three chemokines sharing similar structure can be induced by IFN-γ [8] and are considered appropriately as a distinct subfamily. They are indeed differentially expressed by various cells and exert their effects through binding their corresponding receptor CXCR3 or its splice variant [9]. These receptors are found expressed at high levels on infiltrating lymphocytes in liver diseases [10]. CXCR3 and its ligands are undoubtedly an inflammatory chemokine system resulting in the establishment of chronic low-grade inflammation and an impaired viral clearance in liver diseases.

IFN-γ inducible chemokines have an established link with viral hepatitis and are mostly associated with advanced inflammation [1, 7, 11]. They are placed at the front line of the host defense against different infectious diseases, including liver diseases. Clinical studies have been showed that the levels of the IFN-γ inducible chemokines CXCL9-11 are significantly up-regulated in the serum and livers of patients with various liver disease [1, 12-14]. The high levels of CXCL9-11 have been found to be independently related to the development of clinically hepatic disease [15, 16]. According to some studies, elevated serum and intrahepatic CXCL9 and CXCL10 expression levels have been reported as important prognostic and predictive biomarkers of progressive liver injury [17-20], CXCL11, which is also expressed by hepatocytes, involves in pro-inflammatory T cells recruitment and differentiation [7]. Therefore, IFN-γ inducible chemokines have been reported as targets in anti-inflammatory therapy [21]. As well as demonstrated in various animal studies, inhibition of these chemokines reduces the inflammation [22], ameliorates experimentally-induced liver injury [23, 24], accelerates liver regeneration [24], and improves the outcome [25]. These observations suggest that CXCL9-11 help coordinate the persistent hepatic inflammatory response in various liver diseases and CXCR3 and its ligands lead to the development of new therapeutic strategies for persistent hepatic infection.

Isoliquiritigenin (4,2',4'-trihydroxychalcone, ISL, Fig.1) is an isoflavone compound. It's one of the active ingredient of anti-inflammatory activity of licorice. Studies have shown that it has extensive pharmacological activity, such as antioxidant [26], anti-inflammation [27],

anti-platelet aggregation [28], and cancer-preventing properties [29]. However, the effects of ISL on the IFN-γ induced expression of CXCL9-11 are not fully characterized especially about its molecular mechanism. The present study proposes a possibility of the therapeutic application and an intracellular anti-inflammatory mechanism of ISL in the treatment of liver diseases. The results show that ISL significantly attenuates the expression of IFN-γ-inducible genes in hepatocytes, which might be, at least in part, via inhibiting IFN-γ-induced activation of JAK1/STAT1, IRF3/MyD88, ERK/MAPK, JNK/MAPK, and PI3K/Akt signaling pathways.

## Material and Methods

*Cell culture and treatment*

The human hepatoma cell line HepG2 and hepatic L02 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). They were cultured in DMEM (Gibco BRI, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin and maintained in a humidified atmosphere with 5% $CO_2$ at 37℃. ISL (catalog no. Y-008-1111002, Chengdu Herbpurify CO, LTD) was prepared in DMSO at 10 mg/ml stock solutions. IFN-γ (catalog no. 300-02; PEPROTECH) was prepared according to the manufacturer's instruction. HepG2 and L02 cells were divided into control, ISL (5 μg/ml), IFN-γ (10 ng/ml), and IFN-γ (10 ng/ml) plus ISL (0, 1.25, 2.5, 5 μg/ml) groups. Cells were pretreated with various concentrations of ISL for 1 h and then stimulated with 10 ng/ml of IFN-γ for an extra 48 h.

*Cell viability assayment*

The cytotoxicity of IFN-γ or ISL to cells was assessed by the Cell Counting Kit 8 (CCK-8) (catalog no. WBCK04-500T; Dojindo Molecular Technology Inc., Kumamoto, Japan) according to the manufacturer's instructions. HepG2 and L02 cells (1 x $10^4$) were seeded into 96-well culture plates and treated with various concentrations of IFN-γ (0, 2.5, 5, 10, 20, 40ng/ml) or exposed to IFN-γ (10 ng/ml) plus various concentrations of ISL (1.25, 2.5, 5 μg/ml) for 48 h. Then, the treated cells were incubated with CCK-8 solution (1/10 vol/vol in serum-free media) for another 3 h at 37 ℃. The absorbance was determined at 450nm using a microtiterplate reader (BioRad, Hercules).

*CXCL9, CXCL10, and CXCL11 mRNA quantification*

Total RNA was extracted from cells using TRIZOL reagent (Invitrogen Corp., Carlsbad, CA) following the manufacturer's instructions. For the analysis, the total RNA (1 μg) was reverse-transcribed using the PrimeScriptTM RT Reagent Kit with the gDNA Eraser (Code no. RR047A, Takara). The gene expression analysis was performed by qRT-PCR with SYBR Premix EX TaqTM II (Code no. RR820A, Takara) using the ABI PRISM 7900 sequence detector (Applied Biosystems, Foster City, CA, USA). The total amplification reaction volume of 20 μL contained 2× SYBR® Premix Ex TaqTM Ⅱ , 0.8 μmol/L primers, and 1 μL of template cDNA. Thermal cycling was carried out for 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, and 30 s at 60 °C. Each PCR assay was performed in triplicate, and the changes in mRNA levels were normalized by the levels of the control gene mRNA (β-actin). The primers purchased from Sangon Biotech (Shanghai) are listed in Table1.

**Fig. 1.** The chemical structure of ISL.

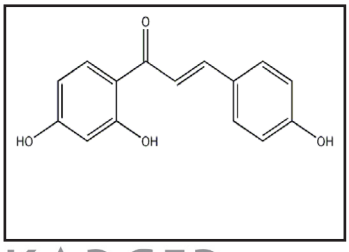


**Table 1.** Primers used for real-time quantitative PCR

| Gene name | Accession number | Direction | Sequence |
|---|---|---|---|
| CXCL9_F1 | NM_002416.2 | Forword | GAGGGCAAGAGCCACAGTAT |
| CXCL9_R1 | NM_002416.2 | Reverse | TGGAGTAGCCAGGAAAGAGC |
| CXCL10_F1 | NM_001565.3 | Forword | GCTGTACCTGCATCAGCATT |
| CXCL10_R1 | NM_001565.3 | Reverse | ATGGCCTTCGATTCTGGATT |
| CXCL11_F1 | NM_001302123.1 | Forword | AAGCTGAAGTAGCAGCAGCA |
| CXCL11_R1 | NM_001302123.1 | Reverse | ATGCAAAGACAGCGTCCTCT |
| β-actin_F1 | NM_001101.3 | Forword | GTGGCCGAGGACTTTGATTG |
| β-actin_R1 | NM_001101.3 | Reverse | AGTGGGGTGGCTTTTAGGATG |

*Detecting CXCL9, CXCL10, CXCL11, and IL-6 levels in culture supernatant*

The expression of CXCL9 (catalog no. ELH-MIG, RayBio, USA), CXCL10 (catalog no. ELH-IP10, RayBio, USA), CXCL11 (catalog no. ELH-1TAC, RayBio, USA), and IL-6 (catalog no. 317297, R&D System, Minneapolis, MN) in supernatant were analyzed using ELISA kits according to the manufacturer's recommendations. The absorbance was read at 450 nm using a microtiterplate reader (BioRad, Hercules, CA). Assays were performed in duplicate. Background activity in the negative control wells was subtracted from the experimental wells.

*Western blot assay*

The cells were lysed in RIPA lysis buffer (catalog no. C1053, Applygen Technologies Inc.) with protease and phosphatase inhibitor cocktail (catalog no.78440, Pierce, USA) for 10 min on ice. Lysates were cleared by centrifugation at 12,000 g for 10 min at 4 °C. BCA Protein Quantification Kit (Product No. 23225, Thermo Scientific, Rockford, USA) was used to quantify the protein concentrations. Western blot analysis was performed as previously described [30]. Equivalent protein amounts (40 μg) were loaded onto 10 % or 12 % SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), which were subsequently blocked in TBST containing 1% BSA and target proteins were detected with the following primary antibodies: p-JAK1 (#3331), JAK1 (#3332), p-JAK2 (#3776s), JAK2 (#3230s), p-STAT1 (#8826), STAT1 (#9172), p-JNK (#4668p), JNK (#9258p), p-ERK 1/2 (#4370p), ERK 1/2 (#4695p), p-p38 MAPK (#4511), p38 MAPK (#9212p), p-PI3K (#4228s), PI3K (#4257), p-Akt (#4060), Akt (#9272); IRF3 (#4302s), MyD88 (#4283s), GAPDH (#5174), p-NF-κB p65 (#3036), NF-κB p65 (#ab7970); p-IκBα (#2859S) and IκBα (#4812). NF-κB p65 were obtained from Abcam, and the remaining antibodies were purchased from Cell Signaling Technology. After incubating at 4°C overnight, the membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit immunoglobulin G (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) diluted in TBST with 5 % non-fat milk for 1 h at room temperature. Then the blots were visualized and detected with enhanced chemiluminescence (Thermo Fisher Scientific Inc., Rockford) following exposure to X-ray films. The protein levels were normalized by the GAPDH proteins. The densitometric analysis was performed using Quantity One v4.62 (Bio-Rad, Inc., Berkeley, CA, USA).

*Statistical analysis*

Statistical analyses were performed using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL, USA) or GraphPad Prism 5.0 software (GraphPad Software Inc,USA). Analysis of variance (ANOVA) and LSD tests were used for comparisons among the groups and between the paired data, respectively. When the data were not normally distributed, the Mann-Whitney U test and the one-way non-parametric ANOVA (Kruskal-Wallis test) were used to compare quantitative variables between two groups and among more than two groups, respectively. Data were expressed as the mean ± SD and P value < 0.05 was considered to reveal a significant difference.

## Results

*Maximal chemokines induction in IFN-γ-stimulated HepG2 and L02 cells without an influence on the viability of hepatocytes*

Fig. 2A showed that IFN-γ at 20 ng/ml or 40 ng/ml inhibited the growth and viability of HepG2 cells significantly ($P < 0.05$), whereas all concentrations of IFN-γ did not affect the viability of L02 cells (Fig. 2B). As shown in Fig. 3, the levels of IFN-γ inducible chemokines CXCL9, CXCL10, CXCL11 and inflammatory cytokine IL-6 (data not shown) were very low in unstimulated cells, but they were up-regulated in a dose- and time-dependent fashion in cells exposed to IFN-γ ($P < 0.05$ or $P < 0.01$). However, the induction effect of IFN-γ on CXCL11 was not so obvious in HepG2 cells. The levels of all these chemokines reached the peak when exposed to IFN-γ at 10 ng/ml for 48 h. Therefore, the stimulating cells with IFN-γ at 10 ng/ml for 48 h would be used in our next experiments.

*ISL attenuates chemokines transcription in IFN-γ-stimulated HepG2 and L02 cells*

The mRNA levels of CXCL9, CXCL10, and CXCL11 were analysed using qRT-PCR to examine the effect of ISL on the production of these chemokines. Fig. 4 showed that the mRNA expression levels of CXCL9, CXCL10, and CXCL11 in the IFN-γ-induced cells were

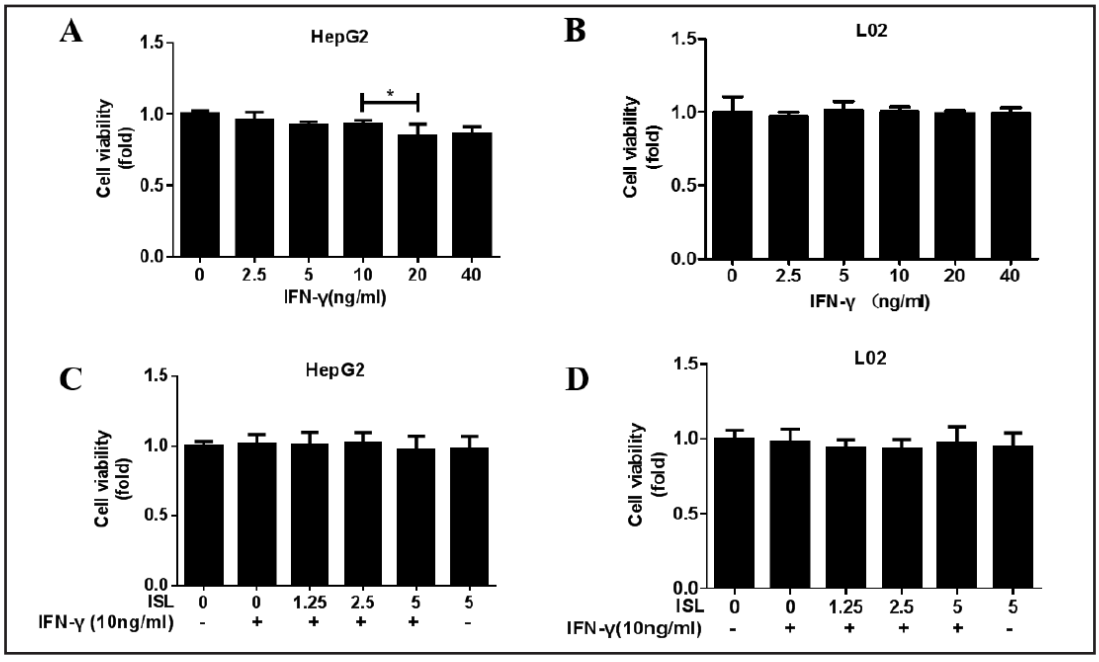


**Fig. 2.** The effects of IFN-γ and ISL on cells viability. (A-B) HepG2 and L02 cells were seeded in 96-well plates in triplicate. After 16 h, cells were treated with various doses of IFN-γ for 48 h, cell viability was determined by CCK8 assay. (C-D) HepG2 and L02 cells were treated with IFN-γ (10 ng/ml) in the presence and absence of ISL (1.25, 2.5, 5 μg/ml) for 48 h, and control group was treated with 0.2 % DMSO. After treatment, cell viability was evaluated as described in the materials and methods. Each bar represented mean ± SD from 3 independent experiments. *$P$ < 0.05, IFN-γ (20 ng/ml) group vs. IFN-γ (10 ng/ml) group.

significantly higher than those in control group (P < 0.05 or P < 0.01). However, ISL pretreatment drastically restored CXCL9, CXCL10 or CXCL11 mRNA expression levels in response to IFN-γ in a dose-dependent manner (P < 0.05 or P < 0.01). Treatment of ISL at 5 μg/ml alone did not affect the basal level of CXCL9, CXCL10, and CXCL11 production. And as shown in Fig. 2C and Fig. 2D, ISL at a concentration of up to 5 μg/ml did not affect the viability of two cell lines.

*Effects of ISL on IFN-γ-inducible genes and IL-6 protein levels in IFN-γ-stimulated HepG2 and L02 cells*

To investigate IFN-γ-dependent inflammatory responses, the CXCL9, CXCL10, CXCL11, and IL-6 levels in the supernatant were measured by ELISA. As shown in Fig. 5, the significant increased CXCL9, CXCL10, CXCL11, and IL-6 levels were observed at 48 h after IFN-γ administration (P < 0.05 or P < 0.01), which could be reversed by ISL treatment (P < 0.05 or P < 0.01). However, upon exposure to IFN-γ, the level of CXCL11 was increased least among these chemokines. Moreover, compared with the IFN-γ induced cells, less than 50 % reduction in IL-6 level was shown when ISL was given at a dose of 5 μg/ml in the two cells (Fig.5D). These results indicate that ISL could exhibit stronger inhibitory effects on the expression of IFN-γ inducible chemokines than those of cytokines in IFN-γ-induced hepatocytes.

*Effects of ISL on the activation of IFN-γ/JAKs/STAT1 and NF-κB signaling pathways in IFN-γ-stimulated HepG2 and L02 cells*

The activation of IFN-γ/JAKs/STAT1 and NF-κB signaling pathways are determined to explore the mechanisms underlying the suppression effect of ISL on IFN-γ-stimulated genes. Fig. 6 showed that although there were no significant differences in unphosphorylated JAKs/ STAT1 and NF-κB protein levels between control and IFN-γ-induced groups, the phosphorylated levels of JAKs/STAT1 and NF-κB were increased significantly in IFN-γ-

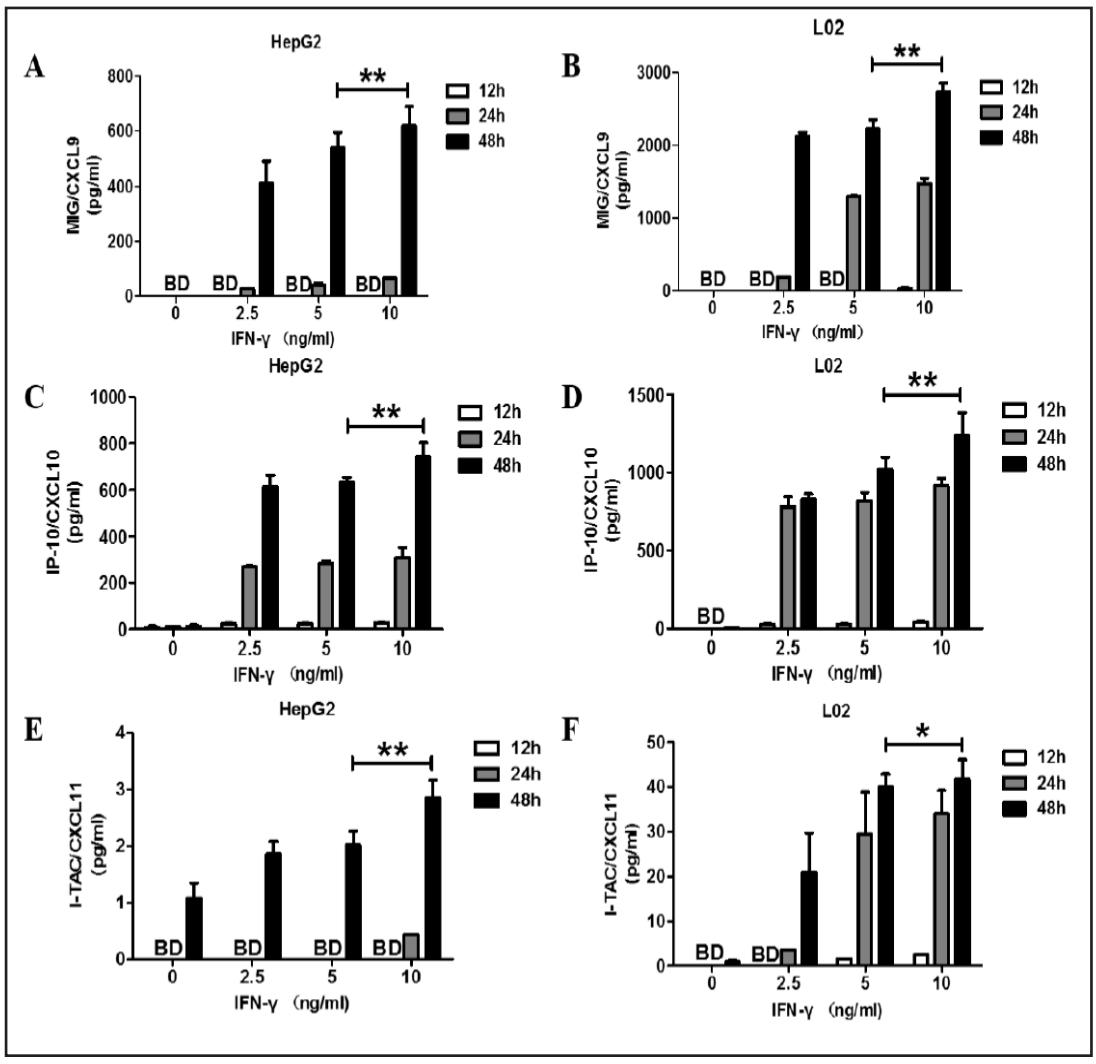


**Fig. 3.** IFN-γ stimulation induces the expression of IFN-γ- inducible genes in human hepatocytes. Cells were treated as illustrated in materials and methods, and the supernatant was harvested after incubation for 12, 24, 48 h and subjected to the ELISA analysis to detect the levels of CXCL9, CXCL10, and CXCL11. The fold change expression of the indicated genes relative to their expression in unstimulated cultures is shown. Each bar represented mean ± SD from 3 independent experiments. (A-F) The levels of CXCL9, CXCL10, and CXCL11 in the supernatant of HepG2 and L02 cells, respectively. *$P < 0.05$ and **$P < 0.01$, IFN-γ (10 ng/ml) group vs. IFN-γ (5 ng/ml) group; BD, below detection limit.

induced cells ($P < 0.01$), as compared with control group. However, ISL pretreatment could not reverse the IFN-γ-induced increase in phosphorylated JAK2 and NF-κB but not JAK1/STAT1 protein levels ($P < 0.05$). As shown in Fig. 6A, the phosphorylated levels of JAK1/STAT1 increased markedly in response to IFN-γ, and pretreating with ISL at 1.25-5 μg/ml upon exposure to IFN-γ inhibited the phosphorylated levels of JAK1/STAT1 in a dose-dependent manner in both HepG2 and L02 cells ($P < 0.05$ or $P < 0.01$). Suggesting that ISL is effective for inhibiting JAK1/STAT1 signaling pathway to affect IFN-γ-inducible genes production.

*Effects of ISL on the activation of IRF3/ MyD88, MAPKs, PI3K/Akt signaling pathways in IFN-γ-stimulated HepG2 and L02 cells*

We next investigated the activation of IRF3/MyD88, MAPKs, and PI3K/Akt signaling pathways in cells. Fig. 7 showed that although there were no significant differences in basic IRF3/MyD88, ERK1/2, JNK and PI3K/Akt protein levels between the control and

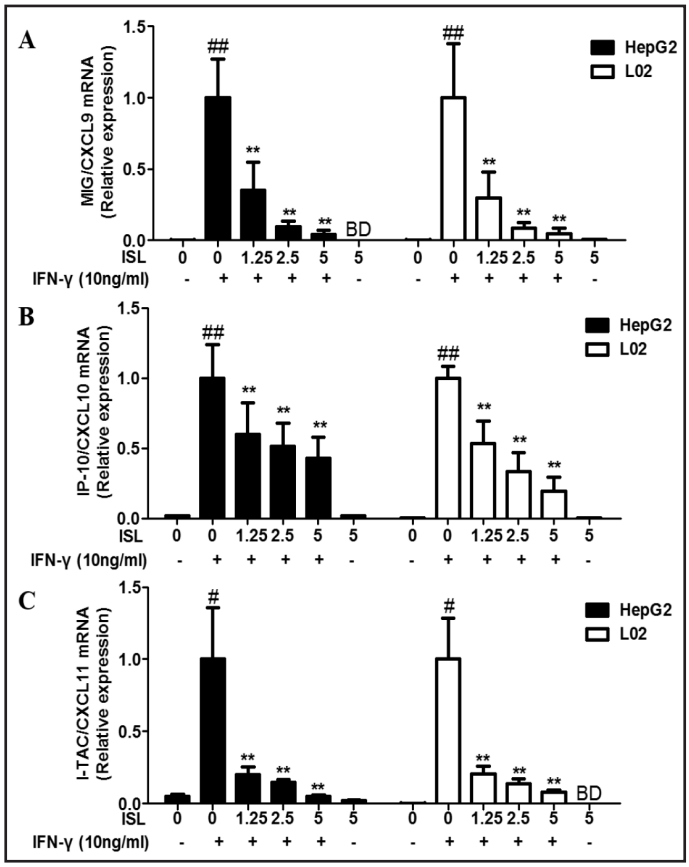


**Fig. 4.** ISL treatment suppress the mRNA levels of CXCL9, CXCL10, and CXCL11 in HepG2 and L02 cells. HepG2 and L02 cells were incubated in the presence and absence of incremental doses of ISL (1.25, 2.5, 5 μg/ml) for 1 h and subsequently stimulated with IFN-γ (10ng/ml) for 48 h. Cells were collected for RNA isolation, and then qRT-PCR analyses for mRNA levels of CXCL9, CXCL10, and CXCL11 were performed as described in materials and methods. Similar results were obtained in 3 independent experiments. The expression levels of CXCL9, CXCL10, and CXCL11 in IFN-γ-treated group were arbitrarily assigned a value of 1, and the data are presented as percentage reductions. (A-C) Relative mRNA levels of CXCL9, CXCL10, and CXCL11 in the HepG2 and L02 cells, respectively. Data value are presented as mean ± SD from 3 independent experiments. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with the untreated control group; $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the IFN-γ-treated group; BD, below detection limit.

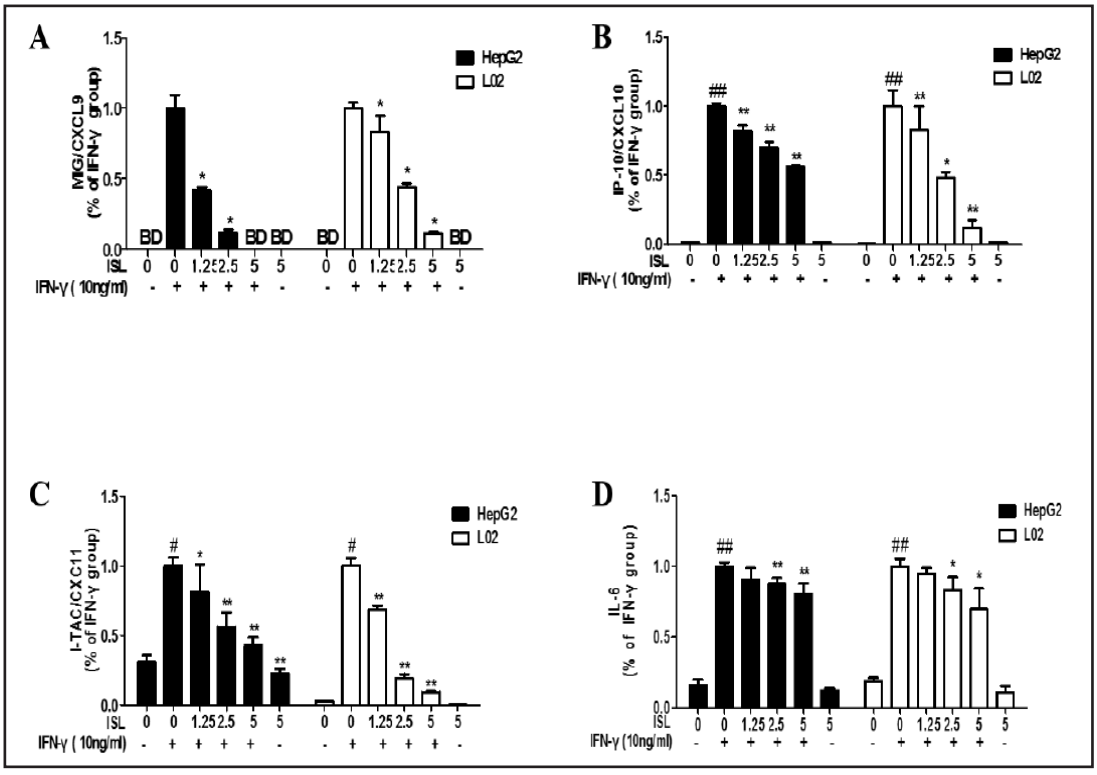


**Fig. 5.** ISL attenuates the expression levels of IFN-γ-inducible genes and IL-6 in HepG2 and L02 cells. HepG2 and L02 cells were cultured in 96-well plates and divided into control (0.2 % DMSO), ISL (5 μg/ml), IFN-γ

(10 ng/ml), and IFN-γ plus ISL (10 ng/ml IFN-γ plus 1.25, 2.5, and 5 μg/ml ISL, respectively) groups. The concentrations of CXCL9, CXCL10, CXCL11, and IL-6 in the culture supernatant were determined by ELISA (n = 5). The expression levels of the indicated proteins in IFN-γ-treated group were arbitrarily assigned a value of 1, and the data are presented as percentage reductions. (A-D) The concentrations of CXCL9, CXCL10, CXCL11, and IL-6 in the culture supernatant of two hepatocytes. Results are presented as mean ± SD from 3 independent experiments. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with untreated control group; $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with IFN-γ-treated group; BD, below detection limit.

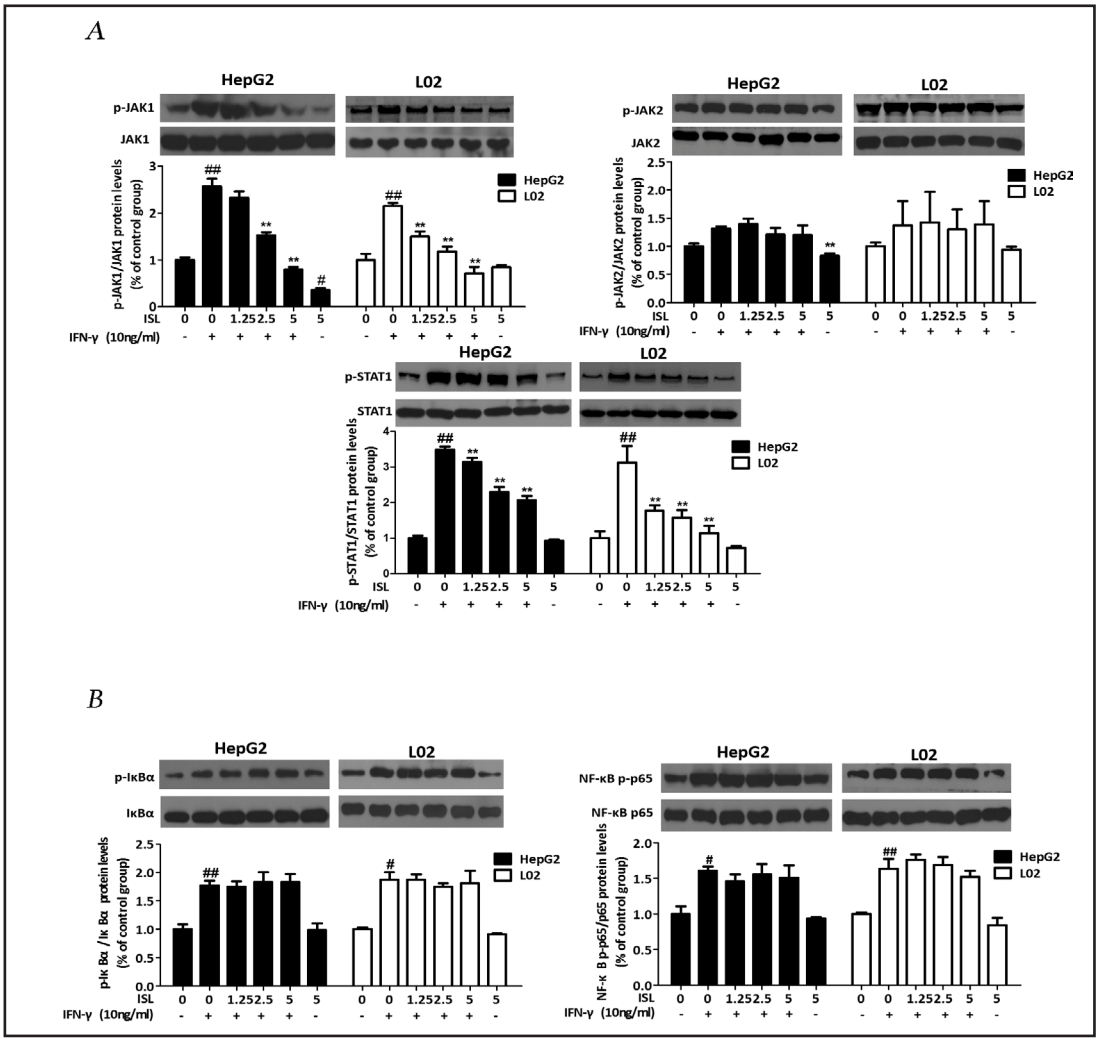


**Fig. 6.** ISL inhibits the activation of JAK/STAT and NF-κB signaling pathways. HepG2 and L02 cells cultured in 6-well plates were divided into groups as follows: control (0.2 % DMSO), ISL (5 μg/ml), IFN-γ (10 ng/ml), and IFN-γ plus ISL (10 ng/ml IFN-γ plus 1.25, 2.5, and 5 μg/ml ISL, respectively) groups. The phosphorylation of the JAK/STAT and NF-κB signaling pathways was analyzed by western blot analysis. (A) The phosphorylation and total JAKs/STAT1 levels in HepG2 and L02 cells. (B) The phosphorylation and total IκBα/NF-κB levels in HepG2 and L02 cells. Data are representative of 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with IFN-γ treatment group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with control group.

IFN-γ-induced cells, the phosphorylated levels of all proteins except the P38/MAPK were increased dramatically in cells exposed to IFN-γ, as compared with control group (P <0.05 or P <0.01). However, treatment of ISL at 1.25-5 μg/ml could decrease the phosphorylated IRF3/MyD88, ERK1/2 and JNK, and PI3K/ Akt levels in a dose-dependent manner (P < 0.05

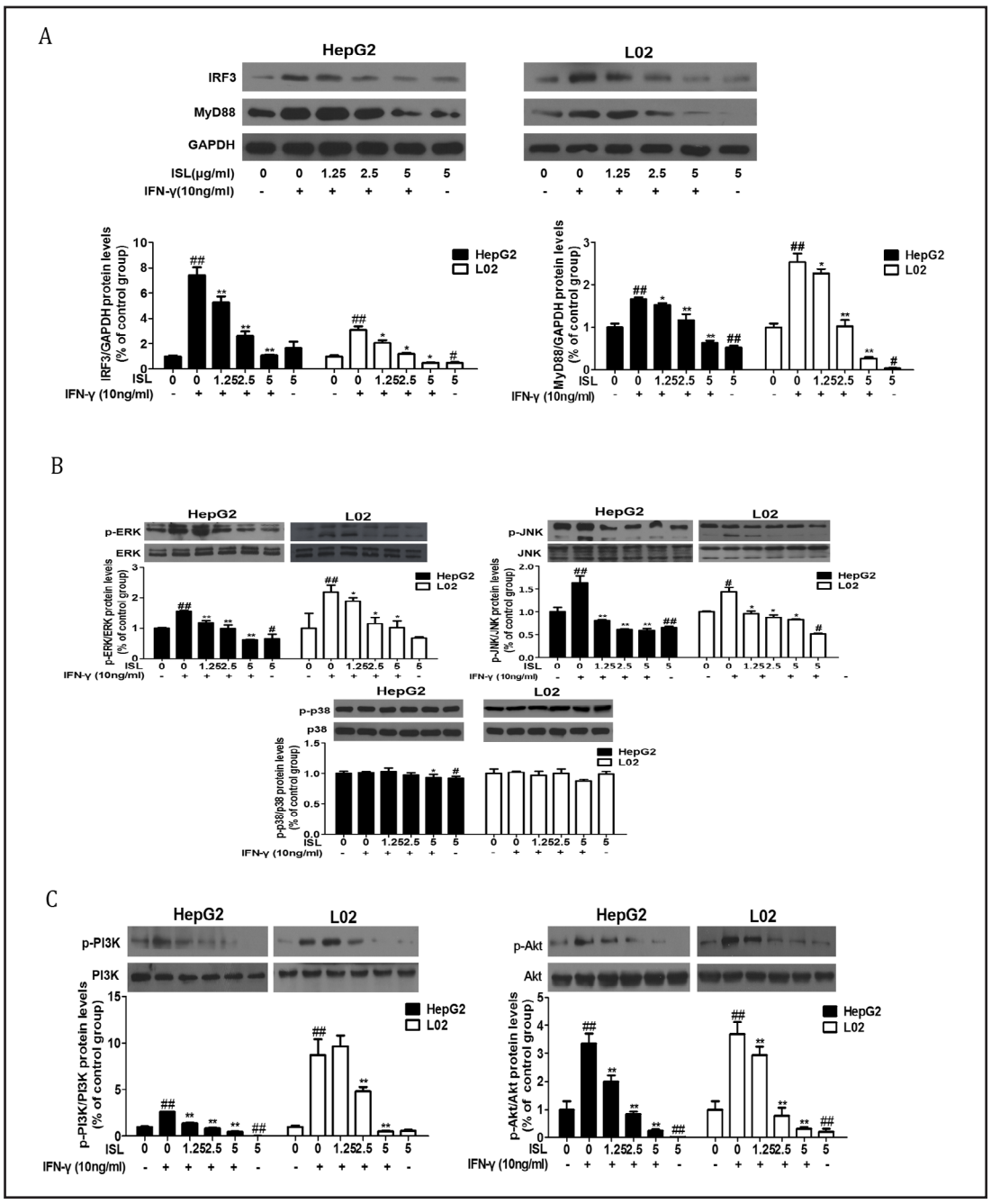


**Fig. 7.** ISL suppress the activation of IRF3/MYD88, MAPK and PI3K/Akt signaling pathways. HepG2 and L02 cells cultured in 6-well plates were divided into groups as follows: control (0.2 % DMSO), ISL (5 μg/ml), IFN-γ (10 ng/ml), and IFN-γ plus ISL (10 ng/ml IFN-γ plus 1.25, 2.5, and 5 μg/ml ISL, respectively) groups. The activation of the IRF3/MYD88, MAPK and PI3K/Akt signaling pathway was analyzed by western blot analysis. GAPDH was used as internal reference control. (A-C) The phosphorylation and total IRF3/MyD88, ERK, JNK, p38 and PI3K/Akt levels in the two kinds of cells. Data are representative of 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with IFN-γ treatment group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with control group.

or P < 0.01). These results clearly suggested that ISL suppressed IFN-γ inducible chemokines and cytokines expression, at least, in part through inhibting the actvation of IRF3/MyD88, ERK-MAPK, JNK-MAPK, and PI3K/Akt signaling pathways.

## Discussion

The present study aims to identify ISL as an anti-inflammatory candidate to be used in the treatment of liver diseases. Persistent hepatic inflammation is the main mechanism responsible for the progression of liver diseases. And the increased binding of the chemokine receptor CXCR3 to its ligands released from the cells especially parenchymal hepatic cells in patients with liver diseases may play an important role in perpetuating liver injury [7, 18]. CXCR3 and its ligands have a broad spectrum of biological activities, and they are strongly linked to Th1-type inflammation and the establishment of Th1 amplification loop mediated by IFN-γ *in vitro* and *in vivo* [3]. High levels of three chemokines (CXCL9-11) reflect an inflammatory syndrome and are associated with a worse prognosis in patients with liver diseases [16, 18]. CXCL9-11 were identified as IFN-γ inducible protein in many types of cells including hepatocyte [18, 31, 32]. IFN-γ-stimulated HepG2 and L02 cells, which are used as the experimental model of inflammation in the present study to study the anti-inflammation effect of ISL. IFN-γ receptors or CXCR3 are well expressed at high levels in the two hepatocytes. However, the expression of IFN-γ-induced inflammatory genes in these cells is rarely well characterized.

IFN-γ inducible chemokines have been extensively investigated and their relationship with liver diseases was also assessed. Up-regulation of these chemokines are known to predict a worse prognosis in patients with various chronic liver diseases [12-16], such as viral hepatitis[13, 18, 20, 31], alcoholic hepatitis or non-alcoholic steatohepatitis [19], liver transplantation [33], autoimmune hepatitis [34], liver cirrhosis [12, 17], and so on. These studies revealed that the serum and intrahepatic levels of these chemokines are increased during liver injury, and therefore contributing to the development of clinically hepatic disease [7, 12-20]. The experimental studies indicated that deficiency of CXCR3 or its ligands significantly impairs cell-mediated immunity in various disease models [14, 21, 23]. Therefore, CXCR3 activation may promote effective immune responses. Modulation of these chemokines on the basis of routine treatment might help to alleviate symptoms and improve the treatment efficiency [22-25]. Taken together, these findings indicate that the IFN-γ inducible chemokines system could be a candidate therapeutic target for the treatment of liver diseases that acts by attenuating active inflammation.

Results from our study showed that CXCL9-11 are undetectable in the supernatant of either cell cultured without IFN-γ or treated with ISL only. But the expression of CXCL9-11 were increased significantly in IFN-γ-stimulated HepG2 and L02 cells. The expression of CXCL9-11 could be reduced dramatically by ISL treatment at both the mRNA and protein levels without affecting the viability of the cells. We have also explored the effect of ISL on IFN-γ-induced inflammation in murine macrophage-like RAW264.7 cells (RAW264.7 cells) and the results showed the same (data not shown). Subsequently, the molecular mechanisms by which ISL decreasing CXCL9-11 expression in hepatocytes were investigated. To our knowledge, our data for the first time demonstrate that ISL suppress the increased CXCL9-11 expression in IFN-γ-induced HepG2 and L02 cells at least partly via inhibiting the activation of IFN-γ/JAK1/STAT1, IRF3/MyD88, ERK/MAPK, JNK/MAPK and P13K/Akt but not IFN-γ/JAK2, NF-κB, and p38/MAPK signaling pathways.

Herbal remedies have long been used in the treatment of different diseases including viral infections and liver diseases. Their high chemical diversity, drug-likeliness properties, and capacity of being absorbed and metabolized by the body with little or no toxicity than synthetic ones make plant derived compounds unique. Isoliquiritigenin is a flavonoid components isolated from licorice with the simple chalcone structure as glycyrrhizin which is widely used in the treatment of many diseases such as liver diseases. Many previous studies have demonstrated its variety of biological functions, such as anti-inflammatory [27], anti-oxidative [26], chemopreventive activities [29], and anti-platelet aggregation properties [28], and so on. Also, ISL has been reported to suppress replication of HCV in a dose-dependent manner [35].

IFN-γ/JAKs/ STAT1 pathway is the classical pathway to regulate IFN-inducible genes expression, and several studies showed that the expression of CXCL9-10 was induced via the activation of JAK1, JAK2/STAT1 [36]. Also, several other studies pointed out that the transcription activation of IFN-inducible genes (eg. CXCL10) is mediated by NF-κB signaling pathway [36-38]. Thus it seems that inhibition of CXCL9-11 expression in target cells by targeting the JAKs/STAT1 and NF-κB signaling pathways could exert anti-inflammatory effects. Therefore, the involvement of these signaling pathways in ISL-mediated suppression of IFN-γ-induced CXCL9-10 expression is determined in our study. The results show that ISL treatment inhibit IFN-γ-induced JAK1/STAT1but not JAK2 and NF-κB activation. Suggesting the existence of other mechanisms underlying ISL attenuating CXCL9-11 production in hepatocytes.

We subsequently explore other potential mechanisms underlying the suppression of ISL on CXCL9-11 in hepatocytes. Besides JAKs/STAT1, MyD88 involves in IFN-γ-mediated signaling. IFN-γ can activate p38 in a MyD88-dependent way in macrophages for IFN-γ-induced CXCL10 expression [39]. MyD88-independent signaling mediated by IRF3 is also crucial positive regulator of IFN-inducible genes express, such as CXCL10 [38]. Moreover, previous studies reported that the induction of IFN-inducible genes in human macrophages, Hela cells, cancer cells, microglia and epithelial in response to various stimuli was via ERK, JNK, p38, and PI3K/Akt signaling pathways [39-44]. Previous reports have also reported that ISL exerted a number of effects through down-regulation of MAPK or PI3K/Akt signaling pathways [45, 46]. Therefore, it will be interesting to examine the effect of ISL on these signaling pathways. And our results demonsrate that inhibition of CXCL9-11 by ISL may partly through down-regulating the IFN-γ-induced activation of IRF3/MyD88, ERK/MAPK, JNK/MAPK, and PI3K/Akt signaling pathways.

In conclusion, the study provides potential mechanisms underlying ISL regulating IFN-γ signaling and then controlling inflammation. Our study evaluate the possibility of the therapeutic application of ISL in the treatment of liver diseases. And our findings suggest a novel role of ISL in the suppression of the protein and mRNA levels of CXCL9-11 induced by IFN-γ. Which may partly through inhibiting the activation of JAK1/STAT1, IRF3/MyD88, ERK/MAPK, JNK/MAPK and PI3K/Akt signaling pathways. The above results provide preliminary evidence that ISL can represent a new generation of anti-inflammatory strategies in liver diseases. However, when applied in clinical practice, results from *in vitro* studies should be interpreted with caution.

## Abbreviation

Isoliquiritigenin (ISL); Interferon-γ (IFN-γ); Monokine induced by IFN-γ(Mig)/ Chemokine (C-X-C motif) ligand 9 (CXCL9); Interferon (IFN)-γ-induced protein 10 (IP-10)/ Chemokine (C-X-C motif) ligand 10 (CXCL10); IFN-inducible T-cellαchemoattractant (I-TAC)/ Chemokine (C-X-C motif) ligand 11 (CXCL11); Interleukin 6 (IL-6); Mitogen-activated protein kinase (MAPK); Janus kinase (JAK); Signal transducer and activator of transcription (STAT); Nuclear factor (NF)-κB; Interferon regulatory factor 3 (IRF3); Myeloid differentiation factor 88 (MyD88); Mitogen-activated protein kinase (MAPK); Phosphatidylinositol 3-kinase (PI3K); Protein Kinase B (Akt); Extracellular signal-regulated kinase (ERK); C-Jun N-terminal kinase (JNK); Lipopolysaccharide (LPS); Double-stranded RNA (dsRNA)

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## Disclosure Statement

All of the authors who have taken part in this study declare no conflict of interest to this manuscript.

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