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, Jihua Xue, Ying Yang, Haihong Zhu, Feng Chen, Jing Wang, Guohua Lou, Yanning Liu, Yixian Shi, Ye Yu, Caixia Xia, Ying Hu, Zhi Chen

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, 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 L02 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
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 defending 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% CO₂, 37°C. 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 instructions. 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 10⁴) 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°C. 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 PrimeScript™ RT Reagent Kit with the gDNA Eraser (Code no. RR047A, Takara). The gene expression analysis was performed by qRT-PCR with SYBR Premix Ex Taq™ 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 Taq™ 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.

Table 1. Primers used for real-time quantitative PCR

| Gene name | Accession number | Direction | Sequence |
|-----------|------------------|-----------|----------|
| CXCL9_F1  | NM_002416.2      | Forward   | GAGGGAAGGCGACACAGTTAT |
| CXCL9_R1  | NM_002416.2      | Reverse   | TGGAGTACGCCGAAAGAGCC |
| CXCL10_F1 | NM_001565.3      | Forward   | GCTGTACCCTGACATCGACATT |
| CXCL10_R1 | NM_001565.3      | Reverse   | ATGGCCTTCGATTCTTGGATT |
| CXCL11_F1 | NM_001302123.1   | Forward   | AAGCTGAGAACGACAGACAGCA |
| CXCL11_R1 | NM_001302123.1   | Reverse   | ATGCAAAAGACGCGTCCCTCT |
| β-actin_F1| NM_001101.3      | Forward   | GTGGCCGAGGCTTTGATTG |
| β-actin_R1| NM_001101.3      | Reverse   | AGTGGGTTGGCCTTATGATG |

Fig. 1. The chemical structure of ISL.
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-1TAG, 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 microtiter plate 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 (#H826), STAT1 (#9172), p-JNK (#4683p), JNK (#9258p), p-ERK 1/2 (#4370p), ERK 1/2 (#4695p), p-38 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...
Wu et al.: Isoliquiritigenin Inhibits Interferon-γ-Inducible Genes Expression in Hepatocytes and its Mechanism

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-
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
Wu et al.: Isoliquiritigenin Inhibits Interferon-γ-Inducible Genes Expression in Hepatocytes and its Mechanism

**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
Wu et al.: Isoliquiritigenin Inhibits Interferon-γ-Inducible Genes Expression in Hepatocytes and its Mechanism

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-treated 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 or P < 0.01).
Wu et al.: Isoliquiritigenin Inhibits Interferon-γ-Inducible Genes Expression in Hepatocytes and its Mechanism

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 inhibiting the activation 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/STAT1 but 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 CXCL110 [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 demonstrate 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-cell chemoattractant (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)

**Acknowledgements**

This work was supported by the States S&T Projects of 12th Five Year (2012ZX10002007) and the National Natural Science Foundation of China (81272679).
Disclosure Statement

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

References

1. Zeremski M, Petrovic LM, Chiriboga L, Brown QB, Yee HT, Kinkhabwala M, Jacobson IM, Dimova R, Markatou M, Talal AH: Intrahepatic levels of cxcr3-associated chemokines correlate with liver inflammation and fibrosis in chronic hepatitis c. Hepatology 2008;48:1440-1450.

2. Wasmuth HE, Tacke F, Trautwein C: Chemokines in liver inflammation and fibrosis. Semin Liver Dis 2010;30:215-225.

3. Campbell JD, Gangur V, Simons FE, HayGlass KT: Allergic humans are hyporesponsive to a cxcr3 ligand-mediated th1 immunity-promoting loop. FASEB J 2004;18:329-331.

4. Indraccolo S, Pfeffer U, Minuzzo S, Esposito G, Roni V, Mandruzzato S, Ferrari N, Anfosso L, Dell’Eva R, Noonan DM, Chieco-Bianchi L, Albin A, Amadori A: Identification of genes selectively regulated by ifns in endothelial cells. J Immunol 2007;178:1122-1135.

5. Manousou P, Kolios G, Drygiannakis I, Koulentaki M, Pyrovolaki A, Notas G, Bourikas L, Papadaki HA, Koursiantalis E: Cxcr3 axis in patients with primary biliary cirrhosis: A possible novel mechanism of the effect of ursodeoxycholic acid. Clin Exp Immunol 2013;172:9-15.

6. Friedman BH, Wolf JH, Wang L, Putt ME, Shaked A, Christie JD, Hancock WW, Olthoff KM: Serum cytokine profiles associated with early allograft dysfunction in patients undergoing liver transplantation. Liver Transpl 2012;18:166-176.

7. Helbig KJ, Ruszkiewicz A, Semendric L, Harley HA, McColl SR, Beard MR: Expression of the cxcr3 ligand i-tac by hepatocytes in chronic hepatitis c and its correlation with hepatic inflammation. Hepatology 2004;39:1220-1229.

8. Clark-Lewis I, Mattioli I, Gong JH, Loetscher P: Structure-function relationship between the human chemokine receptor cxcr3 and its ligands. J Biol Chem 2003;278:289-295.

9. Lasagni L, Francalanci M, Annunziato F, Lazzari E, Giannini S, Cosmi L, Sagninati C, Mazzinghi B, Orlando C, Maggi E, Marra F, Romagnani S, Serio M, Romagnani P: An alternatively spliced variant of cxcr3 mediates the inhibition of endothelial cell growth induced by ip-10, mig, and i-tac, and acts as functional receptor for platelet factor 4. J Exp Med 2003;197:1537-1549.

10. Shields PL, Morland CM, Salmon M, Qiu S, Hubscher SG, Adams DH: Chemokine and chemokine receptor interactions provide a mechanism for selective t cell recruitment to specific liver compartments within hepatitis c-infected liver. J Immunol 1999;163:6236-6243.

11. Fahey S, Dempsey E, Long A: The role of chemokines in acute and chronic hepatitis c infection. Cell Mol Immunol 2014;11:25-40.

12. Chuang YH, Lian ZX, Cheng CM, Lan RY, Yang GX, Moritoki Y, Chiang BL, Ansari AA, TsuneYama K, Coppel RL, Gershwin ME: Increased levels of chemokine receptor cxcr3 and chemokines ip-10 and mig in patients with primary biliary cirrhosis and their first degree relatives. J Autoimmun 2005;25:126-132.

13. Apolinario A, Majano PL, Alvarez-Perez E, Saez A, Lozano C, Vargas J, Garcia-Monzon C: Increased expression of t cell chemokines and their receptors in chronic hepatitis c: Relationship with the histological activity of liver disease. Am J Gastroenterol 2002;97:2861-2870.

14. Zhai Y, Shen XD, Gao E, Zhao A, Freitas MC, Lassman C, Luster AD, Busuttil RW, Kupiec-Weglinski JW: Cxcl10 regulates liver innate immune response against ischemia and reperfusion injury. Hepatology 2008;47:207-214.

15. Zeremski M, Dimova R, Astemborski J, Thomas DL, Talal AH: Cxcl9 and cxcl10 chemokines as predictors of liver fibrosis in a cohort of primarily african-american injection drug users with chronic hepatitis c. J Infect Dis 2011;204:832-836.

16. Tacke F, Zimmermann HW, Berer ML, Trautwein C, Wasmuth HE: Serum chemokine receptor cxcr3 ligands are associated with progression, organ dysfunction and complications of chronic liver diseases. Liver Int 2011;31:840-849.
Berres ML, Asmacher S, Lehmann J, Jansen C, Gortzen J, Klein S, Meyer C, Strunk HM, Fimmers R, Tacke F, Strassburg CP, Trautwein C, Sauerbruch T, Wasmuth HE, Trebiczka J: Cxcl9 is a prognostic marker in patients with liver cirrhosis receiving transjugular intrahepatic portosystemic shunt. J Hepatol 2015;62:332-339.

Harvey CE, Post J, Palladini P, Freeman AJ, Firench RA, Kumar RK, Marinos G, Lloyd AR: Expression of the chemokine ip-10 (cxcl10) by hepatocytes in chronic hepatitis c virus infection correlates with histological severity and lobular inflammation. J Leukoc Biol 2003;74:360-369.

Zhang X, Shen J, Man K, Chu ES, Yau TO, Sung JC, Go MY, Deng J, Lu L, Wong VW, Sung JJ, Farrell G, Yu J: Cxcl10 plays a key role as an inflammatory mediator and a non-invasive biomarker of non-alcoholic steatohepatitis. J Hepatol 2014;61:1365-1375.

Zeremski M, Dimova R, Brown Q, Jacobson IM, Markatou M, Talal AH: Peripheral cxcr3-associated chemokines as biomarkers of fibrosis in chronic hepatitis c virus infection. J Infect Dis 2009;200:1774-1780.

Zimmermann HW, Tacke F: Modification of chemokine pathways and immune cell infiltration as a novel therapeutic approach in liver inflammation and fibrosis. Inflamm Allergy Drug Targets 2011;10:509-536.

Hintermann E, Bayer M, Pfleischhifer JM, Luster AD, Christen U: Cxcl10 promotes liver fibrosis by prevention of nk cell mediated hepatic stellate cell inactivation. J Autoimmun 2010;35:424-435.

Sahin H, Borkham-Kamphorst E, Kuppe C, Zaidkwar MM, Grouls C, Al-samman M, Nellen A, Schmitz P, Heinrichs D, Berres ML, Doliesel D, Schoot D, Weiskirchen R, Moeller MJ, Kiessling E, Trautwein C, Wasmuth HE: Chemokine cxcl9 attenuates liver fibrosis-associated angiogenesis in mice. Hepatology 2012;55:1610-1619.

Yoneyama H, Kai Y, Koyama J, Suzuki K, Kawachi H, Narumi S, Ichida T: Neutralization of cxcl10 accelerates liver regeneration in carbon tetrachloride-induced acute liver injury. Med Mol Morphol 2007;40:191-197.

Hou FQ, Wu XJ, Wang Y, Chen J, Liu YZ, Ren YY, Song G, Ding YP, Yu M, Wang GQ: Rapid downregulation of programmed death-1 and interferon-gamma-inducible protein-10 expression is associated with favourable outcome during antiviral treatment of chronic hepatitis b. J Viral Hepat 2013;20:18-26.

Zhang X, Yeung ED, Wang J, Panzhinskiy EE, Tong C, Li W, Li J: Isoliquiritigenin, a natural anti-oxidant, selectively inhibits the proliferation of prostate cancer cells. Clin Exp Pharmacol Physiol 2010;37:841-847.

Kim YJ, Park SJ, Yun KJ, Cho YW, Park HJ, Lee KT: Isoliquiritigenin in isolated from the roots of glycyrrhiza uralensis inhibits lps-induced inos and cox-2 expression via the attenuation of nf-kappab in raw 264.7 macrophages. Eur J Pharmacol 2008;584:175-184.

Tawata M, Aida K, Noguchi T, Ozaki Y, Kume S, Sasaki H, Chin M, Onaya T: Anti-platelet action of isoliquiritigenin, an aldose reductase inhibitor in licorice. Eur J Pharmacol 1992;212:87-92.

Lee CK, Son SH, Park KK, Park JH, Lim SS, Chung WY: Isoliquiritigenin inhibits tumor growth and protects the kidney and liver against chemotherapy-induced toxicity in a mouse xenograft model of colon carcinoma. J Pharmacol Sci 2008;106:444-451.

Qin Q, Zuo Y, Yang X, Lu J, Zhan L, Xu L, Zhang C, Zhu H, Liu J, Liu Z, Tao G, Dai S, Zhang X, Ma J, Cai J, Sun X: Smac mimetic compound lcl161 sensitizes esophageal carcinoma cells to radiotherapy by inhibiting the expression of inhibitor of apoptosis protein. Tumour Biol 2014;35:2565-2574.

Nguyen N, de Esch C, Cameron B, Kumar RK, Zekry A, Lloyd AR: Positioning of leukocyte subsets in the portal and lobular compartments of hepatitis c virus-infected liver correlates with local chemokine expression. J Gastroenterol Hepatol 2014;29:860-869.

Apolinario A, Majano PL, Lorente R, Nunez O, Clemente G, Garcia-Monzon C: Gene expression profile of t-cell-specific chemokines in human hepatocyte-derived cells: Evidence for a synergistic inducer effect of cytokines and hepatitis c virus proteins. J Viral Hepat 2005;12:27-37.

Berres ML, Trautwein C, Schmeding M, Eurlich D, Tacke F, Bahra M, Neuhaus P, Neumann UP, Wasmuth HE: Serum chemokine cxcl10 predicts fibrosis progression after liver transplantation for hepatitis c infection. Hepatology 2011;53:596-603.

Antonelli A, Ferri C, Ferrari SM, Colaci M, Fallahi P: Immunopathogenesis of hcv-related endocrine manifestations in chronic hepatitis and mixed cryoglobulinemia. Autoimmun Rev 2008;8:18-23.

Adianti M, Aoki C, Komoto M, Deng L, Shoji I, Wahyuni TS, Lusida MI, Soetjipto, Fuchino H, Kawahara N, Hotta H: Anti-hepatitis c virus compounds obtained from glycyrrhiza uralensis and other glycyrrhiza species. Microbiol Immunol 2014;58:180-187.
36. Clarke DL, Clifford RL, Jindarat S, Proud D, Pang L, Belvisi M, Knox AJ: Tnfalpha and ifngamma synergistically enhance transcriptional activation of cxcl10 in human airway smooth muscle cells via stat-1, nf-kappab, and the transcriptional coactivator creb-binding protein. J Biol Chem 2010;285:29101-29110.

37. Zhou Y, Wang S, Ma JW, Lei Z, Zhu H, Lei P, Yang ZS, Zheng B, Yao XX, Shi C, Sun LF, Wu XW, Ning Q, Shen GX, Huang B: Hepatitis b virus protein x-induced expression of the cxc chemokine ip-10 is mediated through activation of nf-kappab and increases migration of leukocytes. J Biol Chem 2010;285:12159-12168.

38. Brownell J, Bruckner K, Wagoner J, Thomas E, Loo YM, Gale M, Jr., Liang TJ, Polyak SJ: Direct, interferon-independent activation of the cxcl10 promoter by nf-kappab and interferon regulatory factor 3 during hepatitis c virus infection. J Virol 2014;88:1582-1590.

39. Sun D, Ding A: Myd88-mediated stabilization of interferon-gamma-induced cytokine and chemokine mRNA. Nat Immunol 2006;7:375-381.

40. Shen Q, Zhang R, Bhat NR: Map kinase regulation of ip10/cxcl10 chemokine gene expression in microglial cells. Brain Res 2006;1086:9-16.

41. Petrai I, Rombouts K, Lasagni L, Anunziato F, Cosmi L, Romanelli RG, Sagrinati C, Mazzinghi B, Pinzani M, Romagnani S, Romagnani P, Marra F: Activation of p38(mapk) mediates the angiostatic effect of the chemokine receptor cxc3-b. Int J Biochem Cell Biol 2008;40:1764-1774.

42. Nakamichi K, Inoue S, Takasaki T, Morimoto K, Kurane I: Rabies virus stimulates nitric oxide production and cxc chemokine ligand 10 expression in macrophages through activation of extracellular signal-regulated kinases 1 and 2. J Virol 2004;78:9376-9388.

43. Shahabuddin S, Ji R, Wang P, Brailoiu E, Dun N, Yang Y, Aksoy MO, Kelsen SG: Cxcr3 chemokine receptor-induced chemotaxis in human airway epithelial cells: Role of p38 mapk and pi3k signaling pathways. Am J Physiol Cell Physiol 2006;291:C34-39.

44. Mendez-Samperio P, Perez A, Rivera L: Mycobacterium bovis bacillus calmette-guerin (bcg)-induced activation of pi3k/akt and nf-kb signaling pathways regulates expression of cxcl10 in epithelial cells. Cell Immunol 2009;256:12-18.

45. Li Y, Zhao H, Wang Y, Zheng H, Yu W, Chai H, Zhang J, Falck JR, Guo AM, Yue J, Peng R, Yang J: Isoliquiritigenin induces growth inhibition and apoptosis through downregulating arachidonic acid metabolic network and the deactivation of pi3k/akt in human breast cancer. Toxicol Appl Pharmacol 2013;272:37-48.

46. Kang SW, Choi J, Choi YJ, Bae JY, Li J, Kim DS, Kim JL, Shin SY, Lee YJ, Kwon IS, Kang YH: Licorice isoliquiritigenin dampens angiogenic activity via inhibition of mapk-responsive signaling pathways leading to induction of matrix metalloproteinases. J Nutr Biochem 2010;21:55-65.