Original Article

Effects of Interleukin-18 and Anti-interleukin-18-mAb on Experimental Immunological Liver Fibrosis Induced by Repeatedly Administered Concanavalin A and Its Mechanism

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Objective To explore the prevention of IL-18 or anti-IL-18-mAb to the immune liver fibrosis model induced by repeated injection of concanavalin A in BALB/c mice and its mechanism.

Methods Total of 120 BALB/c mice were divided into four groups, control group mice (Ga) were injected weekly with normal saline, concanavalin A group was divided into Gb, Gc, Gd. All mice were injected with concanavalin A (15 mg/kg) once a week. Moreover, Gc, Gd mice were injected weekly with IL-18 (7.5 mg/kg) and anti-IL-18-mAb (10 mg/kg) 2 hours before treatment with concanavalin A, respectively. Twenty-four hours after concanavalin A challenge at 1, 5, 12 and 20 weeks, 3 mice were killed by vena orbitalis, respectively. The sera were stored at 4°C for detecting of up TNF- α and IFN- γ by ELISA. The liver of mice in different groups were excised and fixed in 10% formalin for HE staining and Masson staining or frozen in liquid nitrogen for immunohistochemical staining for α -SMA. After extracting of total RNA from liver tissue, MMP-2 and TIMP-1 messenger RNA were amplified by reverse transcription polymerase chain reaction (PCR). Products were electrophoresed on agarose gel containing ethidium bromide and visualized under ultraviolet light. Densitometric RT-PCR data were standardized with β -actin signals.

Results After experiment, the number of dead mice of Ga, Gb, Gc and Gd were 0, 6, 15 and 3, respectively. There were significant difference on each group ($P < 0.05$). At the fifth week of experiment, hepatocellular necrosis in IL-18 administered group mice had become widespread throughout the lobule. Evidence of liver fibrosis was observed during this period. However, at the twelfth week of experiment, bridging fibrosis and large fibrosis strip in the parenchyma with hepatocellular necrosis was detectable in Gb, but at twentieth week, only the small fibrosis strip had been found in anti-IL-18-mAb administered group mice by HE staining and Masson staining. The serum levels of TNF- α and IFN- γ in IL-18 administered group were higher than that in concanavalin A group and anti-IL-18 administered groups ($P < 0.05$). Moreover, immunohistochemical staining for α -SMA indicated that the semi-quantu scores in IL-18 administered group were more than concanavalin A group and anti-IL-18-mAb administered groups ($P < 0.05$). MMP-2-mRNA, TIMP-1- mRNA expression levels increased significantly compared with concanavalin A group and anti-IL-18-mAb administered group ($P < 0.05$).

Conclusions The immune liver fibrosis model induced by repeated injection of concanavalin A in BALB/c mice could be worsened by IL-18 administration and block by anti-IL-18 mAb administration.

Key words: Concanavalin A; Mouse; Liver fibrosis; IL-18; Model

Liver fibrosis is a common feature of chronic hepatitis and chronic liver injury leading to liver cirrhosis. Regardless of the causes, the fibrosis is characterized by an increase in extracellular

matrix constituents that collectively form liver scars. But the immunological mechanisms for liver fibrosis are not well defined. For prevention of fibrosis, an understanding of such mechanisms is essential. Con A is a potent T cell mitogen with tropism for the liver. In mice, Con A induced acute hepatitis is a model of T lymphocyte mediated liver injury.² Kimura

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et al have found that weekly i.v. administration of concanavalin A (Con A) in BALB/c mice brought about a striking alanine aminotransferase increase, resulting in piecemeal necrosis with bridging fibrosis in the parenchyma.³ Using this fibrosis model method, immunological liver fibrosis model on BALB/c mice was established.

IL-18 was discovered as a factor that induces IFN- γ production from Th1 cells.^{4,5} But IL-18, without help of IL-12, induces Th2 cells by induction of IL-4 production from naïve T cells. Thus, IL-18 has the potential to stimulate both Th1 and Th2 responses. IL-18 is unique because it produces completely opposite effects depending on the presence or absence of IL-12. The molecular mechanism of the synergistic induction of IFN- γ by IL-12 and IL-18 as well as IL-18-mediated IL-4 production needs to be elucidated. Such elucidation will provide us a novel and powerful tool to control the Th1/Th2 balance at the transcriptional level. Because IL-18 is synthesized as a precursor molecule that becomes active after cleavage with caspase-1 or caspase-1 like enzymes, these converting enzymes might be useful therapeutic targets. IL-18 was discovered five years ago and the results of IL-18 research suggested the importance of IL-18 not only in the immune system but also in other systems.^{4,6}

In this study, the effects of IL-18 and anti-IL-18 on experimental liver fibrosis was determined, and the possible mechanism in vivo was explored.

MATERIALS AND METHODS

Animal experiments

Total of 120 adult BALB/c mice, weighing 20-30 g (provided by Experimental Animal Center of Chinese Academy, Shanghai, China), were included in the experiments. Con A (Sigma, St. Louis, MO) was dissolved in pyrogen-free PBS (Sigma) at a concentration of 1 mg/ml and i.v. injected into BALB/c mice at a dose of 15 mg/kg once a week. And 30 mice were used as controls. The mice were randomly divided into four groups: group A (normal group, n = 30), group B (Con A group, n = 30), group C (Con A + IL-18 group, n = 30), animals were

pretreated 30 min before the Con A challenge with 7.5 mg/kg of IL-18 (Sigma), and group D (Con A + anti-IL-18-mAb group, n = 30), animals were pretreated 2 hours before the Con A challenge with 10 mg/kg of anti-IL-18-mAb (Sigma). All animals were treated for 20 weeks, and at the 1st, 5th, 12th and 20th week 3 mice in each group were killed under narcosis. Serum samples from individual mice were obtained from the fundus oculi vein at indicated time points 24 h after each Con A injection and their right liver lobes were immediately excised and specimens were fixed in formaldehyde for the preparation of serial paraffin T sections at 5 μ m thickness. Some specimens were frozen in liquid nitrogen for RT-PCR.

Pathological observation and reticular collagen staining

Liver sections were stained with hematoxylin and eosin (H and E) for the assessment of liver cell degeneration and necrosis, infiltration of inflammatory cells, hyperplasia of the lattice fibers and formation of collagen bundles and pseudobulbs. Other sections were stained with Masson special staining for the assessment of fiber structure of the liver and analysis of hyperplasia of liver fiber tissues by computer image analysis system.

Expression of α -SMA

α -SMA expression was determined by the immunohistochemical ABC method, the paraffin sections were deparaffinized and rehydrated after being immersed in 0.3% peroxide-ethanol solution at room temperature for 10 min and digested with microwave for 10 min. After incubation with the mouse anti-rabbit α -SMA monoclonal antibody (working concentration 1:100, Long-island Antibody Diagnosis Company, Shanghai, China) at 37°C for 60 min and at 4°C overnight, the sections were incubated with the rabbit anti-rat antibody (working concentration 1:100, Long-island Antibody Diagnosis Company) at 37°C for 60 min, and finally incubated with DAB to develop color for microscopic examination and photography.

Serum levels of IFN- γ and TNF- α

The serum levels of IFN- γ and TNF- α were monitored

using commercially available ELISA kits (Sigma). The detectable lower limits of sensitivity of the kits were 125 and 35 pg/ml, respectively. To evaluate the role of IFN- γ and TNF- α in the fibrosis process, Serum samples from individual mice were obtained from the funds oculi vein at indicated time points 24 h after each Con A injection.

RNA preparation

Frozen liver tissue was mechanically pulverized and total hepatic RNA was isolated for the analysis of cytokine mRNA expression at the indicated time points after Con A injection. Total RNA was isolated by the guanidinium isothiocyanate-phenol-chloroform method using RNeasy (Qiagen, Crawley, UK). In brief, tissue samples were homogenized with RNeasy and 0.2 ml chloroform was added to 2 ml homogenate. The samples were shaken vigorously for 15 s and incubated for 5 min on ice, and then centrifuged at 12 000 g for 15 min. The aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol and placed for 15 min on ice. After centrifugation at 12 000 g for 15 min, the RNA pellet was washed once with ice-cold 75% ethanol with vortexing, centrifuged at 7 500 g for 5 min, dried for 10 min and dissolved in diethylpyrocarbonate (Sigma)-treated RNase-free solution. Total RNA contents of the samples were estimated spectrophotometrically by absorbance at 260 nm. Purity of RNA was determined from the 260/280 nm absorbance ratio.

RT-PCR analysis

Total RNA (1 μ g) was reversely transcribed into cDNA using AMV reverse transcriptase (Boehringer, Mannheim, Germany) and the cDNA was amplified by PCR. Briefly, 1 μ g of total RNA was denatured by heating for 10 min at 70°C and added to a mixture containing 2 μ l of oligo-dT (0.5 mg/ml) (Boehringer), 6 μ l of 5-fold concentrated reverse transcriptase buffer, 2 μ l of DTT (Boehringer; 0.1 mol/l), 1 μ l of dNTP (Boehringer; 10 mmol/l) and 1 μ l of reverse transcriptase. Each cDNA synthesis was performed for 1 h at 42°C and stopped by incubation for 10 min at 98°C. PCR was performed in a volume of 50 μ l

containing 400 pmol/L of each primer, 2 μ g of cDNA, Tris-HCl (10 mmol/L, pH 8.3), KCl (50 mmol/L), MgCl₂ (1.5 mmol/L), dNTP (0.2 mmol/L) and 2 U of Taq DNA polymerase (Boehringer). Thermal cycle conditions were 94°C for 45 s for denaturing, 60°C for 45 s for annealing and 72°C for 2 min for extension. The number of amplification cycles was 28 for each set of primers. For each set of primers, dilutions of cDNA were amplified for 20, 23, 25, 28, 30, 32, 34, 36 and 38 cycles to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength. After the last cycle of amplification, the samples were incubated for 7 min at 72°C. The PCR products were electrophoresed on 1.2% agarose gels and stained with ethidium bromide. To ensure that contaminating DNA had not been amplified, PCR without reverse transcriptase was simultaneously demonstrated. Sense and anti-sense primers were based on Clontech (Palo Alto, CA) Amplimer sets for mouse TIMP-1 and MMP-2. The sense and antisense primers were mouse TIMP-1 Sense 5'-CCTTTGCATCTCTGGCATCTGGCA-3'; Antisense 5'-CCAACAGCCAGCACTATAG GTGTT-33', 437 bp. Mouse-MMP-2, Sense 5'-ACAAGTGGTCCGCGTAAAGT-3'; Antisense 5'-CGGTCATCATCGTAGGTT-3' 501 bp. β -actin, 265 bp, sense 5'-AGGCCGGCTTCGCGGGCG-3'; antisense 5'-CTCGGGAGCCACACGCAG-3'. PCR products were electrophoresed on agarose containing ethidium bromide and visualized under ultraviolet light. Densitometric RT-PCR data were standardized with β actin signals.

Statistical analysis

A one-way ANOVA with Student-Newman-Keuls post-hoc comparisons was conducted using SPSS 12.0 for Windows (Cambridge, MA).

RESULTS

Hepatic pathological findings

In the normal control group, the structure of liver

lobules was normal, central vein was clear and the size and shape of hepatic cells were normal and distributed centrifugally (Figure 1A). In the Con A group, at the fifth week of experiment, the normal structure of lobules was destroyed, and small fibrosis stripes were formed. Hepatic cells swelled. At twelfth week of experiment, the more fibrosis strips were found (Figure 1B), hyperplasia of fibers were observed surrounding the lobules on the 20th week. At the fifth week of experiment in IL-18 group mice, hepatocellular necrosis had become widespread throughout the lobule. Evidence of liver fibrosis was observed at this period. However, at the twelfth week of experiment, bridging fibrosis and large fibrosis strip in the parenchyma with hepatocellular necrosis was detectable in IL-18 group (Figure 1C), at the twentieth week, the hepatic lobules were encysted and separated by collagen bundles, with pseudolobule formation, and associated with bile duct 'proliferation', infiltration of inflammatory cells, bordering the portal areas. Abundant fiber tissues were present, extended outwards in stellar shape. But at twentieth week, only the small fibrosis strip had been found in anti-IL-18 group mice by HE staining and Masson staining. However, these changes were significantly milder compared with Con A group and IL-18 group (Figure 1D).

Expression of liver reticular collagen

In the control groups, there were few fiber tissues seen in portal areas (Figure 2A). In the Con A group, at the twelfth week of experiment, the normal structure of lobules was destroyed, and fibrosis strips were formed (Figure 2B). However, in Con A + IL-18 group, the pseudolobules were surrounded by strong, multi-layer reticular fibers, which netted into pseudolobules (Figure 2C). There was significant difference between Con A group and Con A + IL-18 group. In Con A + anti-IL-18-mAb group, there were only a few fiber tissues seen in portal areas (Figure 2D). However, there was a significant decrease in reticular fibers in this group, compared with Con A + IL-18 group at the twelfth week of experiment. Image pattern analysis showed that the expression of collagen in the experimental groups (Con A group, Con A +

IL-18 and Con A + anti-IL-18 group) was much stronger than that in the control groups. Moreover, the expression of collagen in Con A + IL-18 group was significantly stronger than that in Con A group and Con A + anti-IL-18 group ($F = 31.89$, $P < 0.05$) (Figure 4).

At the twentieth week of experiment, α -SMA antigen in control mice liver was little detected in myofibroblasts and vascular endothelial cells. The expression of α -SMA in experimental groups was significantly stronger than that in control groups (Figures 3A-3D). The expression of α -SMA in Con A + IL-18 group was significantly stronger than that in Con A group and Con A + anti-IL-18 group ($F = 31.89$, $P < 0.05$) (Figure 5).

Role of IFN- γ and TNF- α

Since it has been reported that both IFN- γ and TNF- α play critical roles in Con A induced liver injury,⁶⁻⁹ changes in serum levels of IFN- γ and TNF- α at several time points after Con A injection were investigated. In order to reveal the effects of IL-18 on IFN- γ or TNF- α in liver fibrosis, we investigated the protective effects of passive immunization with anti-IL-18 mAb in this experiment model. As shown in Figures 6 and 7, the serum IFN- γ or TNF- α levels were markedly triggered or suppressed by the administration of either IL-18 or anti-IL-18 mAb after every Con A injection. Histopathological examination similarly revealed worsened or inhibitory hepatocellular injury and liver fibrosis (Figures 1C, 1D, 2C and 2D) at each period. To evaluate whether the aggravated or inhibitory effects were due to IFN- γ or TNF- α , the serum levels of IFN- γ or TNF- α were monitored after Con A injection with IL-18 or anti-IL-18 mAbs. The results were shown in Figures 6 and 7 (Con A group and Con A + IL-18 group), the levels of IFN- γ showed a 64% increase in the peak after Con A injection with IL-18, as compared with the Con A injection alone. Similarly, an 87% increase in the peak of TNF- α was observed after Con A injection with IL-18, as compared with the Con A injection alone; moreover, the levels of IFN- γ showed a 64% reduction in the peak after Con A injection with anti-IL-18 mAb, as compared with the Con A injection

Figure 1. Pathological observation of liver sections.
Note: stained with hematoxylin and eosin (H and E) 200 ×

Figure 2. Expression of liver reticular collagen.
Note: stained with hematoxylin and eosin (H and E) 200 ×

Figure 3. Expression of α -SMA in the liver.

Note: stained with hematoxylin and eosin (H and E) 200 $\times$

Figure 4. Contents of collagen in livers of rats in different groups.

Figure 5. Expression of α -smooth muscle actin in the 20th week mice group (ratio of positive areas).

Figure 6. The expression of TNF- α .

Figure 7. The expression of IFN- γ .

Figure 8. The expression of TIMP-1 and MMP-2.

Notes: Data are the mean $\pm$ standard of three independent experiments different superscripts denote statistically distinguishable means ($P < 0.05$)

alone. Similarly, an 87% reduction in the peak of TNF- α was observed after Con A injection with anti-IL-18 mAb, as compared with the Con A injection alone, as shown in Figure 4 (Con A group and Con A + anti-IL-18-mAb group).

MMP-2 and TIMP-1 expression

After extracting total RNA from liver tissue, MMP-2 and TIMP-1 messenger RNAs were amplified by reverse transcription polymerase chain reaction (PCR). Products were electrophoresed on agarose gel containing ethidium bromide and visualized under ultraviolet light. Densitometric RT-PCR data were standardized with β -actin signals. MMP-2 mRNA, TIMP-1 mRNA expression levels increased significantly compared with concanavalin A group and anti-IL-18-mAb group ($P < 0.05$). Significant differences between normal control and experimental groups for the ratios MMP-2/TIMP-1 (5.7 ± 1.1 vs 3.1 ± 1.6 ; $P < 0.0001$), the ratio of MMP-2/TIMP-1 in Con A + IL-18 group was higher than that of Con A and Con A + anti-IL-18 groups.

DISCUSSION

Fibrosis is an important component of advanced chronic inflammatory liver disease. Although the changes in hepatic structure that occur in liver cirrhosis have been described in detail,⁷ the immunopathogenesis of fibrosis in this process is largely unknown. In order to elucidate the underlying mechanisms, an appropriate animal model with essential similarities to patients with chronic hepatitis should be established. Ideally, the animal model should have the following features: (i) it should be based upon immunological mechanisms, (ii) liver fibrosis should be induced following continuous liver injury and (iii) the inducer of liver fibrosis should not be a direct hepatotoxin. However, no animal model that satisfies these criteria has been established.⁸ Con A is a potent T cell mitogen with tropism for the liver. In mice, Con A induced acute hepatitis is a model of T lymphocyte-mediated liver injury.⁹ Previous studies have shown that Con A-induced hepatitis requires

Figure 9. The ratio of TIMP-1 mRNA and MMP-2 mRNA to actin by RT-PCR.

TNF- α and is attenuated by TNF- α inhibitors.¹⁰

It has been recently described that Con A-induced experimental liver injury in mice is mediated by activated T cells,^{11, 12} Con A is a lectin from jack bean and is known as a T lymphocyte mitogen *in vitro*. Tiegs et al reported that acute liver injury was induced within 8 h after i.v. injection of Con A in BALB/c mice. The organ specificity was attributed to the preferential binding of Con A in the liver. Because immunodeficient mice (SCID or athymic mice) or mice treated with immunosuppressive drugs such as cyclosporine, FK506 and corticosteroids do not develop hepatitis after Con A injection, it is accepted that the model requires T cell activation. Cell depletion experiments identified CD4⁺ T cells as the effector cells.¹³ Furthermore, neutralization experiments revealed that TNF- α and IFN- γ play critical roles in the development of Con A-induced hepatitis.^{14, 15} Since these results indicate that Con A-induced hepatitis is based upon immunological mechanisms and has similarities to chronic inflammatory liver disease in man, Con A-induced hepatitis model was thought to be one of the potent models to induce liver fibrosis mediated by hepatocellular injury.

In the present study, we established a liver fibrosis model with repeated administrations of Con A (Figure 2). Hepatocellular injury was consistently observed in terms of sALT elevation 24 h after each Con A injection (Figures 1A and 1B) following an

increase in serum levels of both IFN- γ and TNF- α (Figure 2A). Cytokine involvement could be proven in this model.

Although IL-18 is primarily considered as an IFN- γ inducing factor, this cytokine possesses other activities, including induction of proinflammatory cytokines. It has been recently described that IL-18 plays an important role in Con A-induced experimental liver injury in mice.^{16,17} In our study, the serum IFN- γ or TNF- α levels were markedly triggered or suppressed by the administration of either IL-18 or anti-IL-18 mAb after every Con A injection. Histopathological examination similarly revealed worse or inhibition of hepatocellular injury and liver fibrosis (Figures 3B and 3C) at each period. To evaluate whether the aggravation or inhibitory effects were due to IFN- γ or TNF- α , the serum levels of IFN- γ or TNF- α were monitored after Con A injection with IL-18 or anti-IL-18 mAb. As shown in Figure 4 (Con A group and Con A + IL-18 group), the levels of IFN- γ showed a 64% increase in the peak after Con A injection with IL-18, as compared with the Con A injection alone. Similarly, an 87% increase in the peak of TNF- α was observed after Con A injection with IL-18, as compared with the Con A injection alone; moreover, the levels of IFN- γ showed a 64% reduction in the peak after Con A injection with anti-IL-18 mAb, as compared with the Con A injection alone. Similarly, an 87% reduction in the peak of TNF- α was observed after Con A injection with anti-IL-18 mAb, as compared with the Con A injection alone, as shown in Figure 4 (Con A group and Con A + anti-IL-18 mAb group)

IL-18 is primarily considered an IFN- γ inducing factor. This cytokine possesses other activities, including induction of proinflammatory cytokines. In the present study, these data are confirmed *in vivo*. In fact, ob/ob mice exhibited reduced IL-18, but not IFN- γ levels. More importantly, neutralization of IL-18 activity reduced Con A-induced liver damage without significantly altering IFN- γ levels.¹⁸ Therefore, IL-18 can mediate liver toxicity independently of IFN- γ . In our study, challenged with IL-18, the serum of TNF- γ and IFN- γ of experiment mice increased significantly, neutralization of anti-

IL-18-mAb, the serum of TNF- α and IFN- γ of experiment mice all reduced, but the serum of TNF- α were more markedly triggered or suppressed by IL-18 or anti-IL-18-mAb than the serum of IFN- γ . Both TNF- α , IFN- γ and IL-18 appeared to be essential mediators of T cell mediated liver injury. These results demonstrated that TNF- α may play a more important role on Con A-induced liver fibrosis.

Studies on the pathogenesis of liver fibrosis have shown that HSCs within the space of Disse experience a transform, in the wake of liver injuries by various causes. In normal liver, HSCs store vitamin A and show minimal proliferation and collagen synthesis. However, in an injured liver, HSCs lose vitamin A and transform into myofibroblasts (MFB), called activated HSCs, which express α -SMA and have the function of contractibility, proliferation and fibrogenesis.^{19,20} So, the expression of α -SMA was regarded as signs of the proliferation of HSC, the results of immunohistochemical staining showed that the expression of α -SMA in Con A + IL-18 group was significantly stronger than that in Con A group. In contrast, the expression of α -SMA in Con A + anti-IL-18 group was lower than that of Con A group. The expression of α -SMA is sign of HSC proliferation. It is presumed that IL-18 does have a effect on the activation of HSCs.

In addition, HSCs synthesize a number of collagens and enzymes that inhibit degeneration of extracellular matrix (ECM), and some cytokines that promote fibrosis. Thus, the balance between the deposition and degeneration of ECM is broken, leading to the startup and development of liver fibrosis. Some studies have shown that TIMP is a very important promoting factor of liver fibrosis, and it inhibits matrix metal protease (MMP) to deposit ECM. Strong expression of TIMP-1 reflects the severity of liver fibrosis.^{21,22} We speculate the proliferation of HSC may be induced by TNF- α and IFN- γ . Combining the above quantitative data of reticular collagen, we noticed that the stronger the expression of TIMP-1 was the more reticular collagens were secreted, and vice versa. It is suggested that TIMP-1 plays an important role in the process of liver fibrogenesis induced by repeatedly

administered concanavalin A.

On the other hand, MMPs play an important role because their activity is largely responsible for matrix breakdown.²³ The results of previous study showed that circulating concentrations of the MMP-2 as well as TIMP-1 change in the course of liver fibrosis. MMP-2/TIMP-1 ratio has shown correlation to degree of fibrosis. Such changes have also been described by other groups.²⁴

Our study addresses potential alterations in matrix degradation contributing to enhanced matrix deposition. Therefore, we also calculated the ratios between the MMP-2 and TIMP-1 measured in this study, clear changes of the MMP-2/TIMP-1. The results were shown in Figures 8 and 9. Significant differences between normal control and experimental groups for the ratios MMP-2/TIMP-1 (5.7 ± 1.1 vs. 3.1 ± 1.6 ; $P < 0.0001$), the ratio of MMP-2/TIMP-1 in Con A + IL-18 group was higher than that of Con A and Con A + anti-IL-18 group. Therefore, it is possible that alterations of the expression of MMP-2/TIMP-1-mRNA in liver fibrosis model induced by repeatedly administered concanavalin A may contribute to the interference of IL-18 or anti-IL-18-mAb. Its mechanism may be induced by the change of TNF- α or IFN- γ .

REFERENCES

1. Xie G, Wang X, Wang L, Atkinson RD, Kanel GC. Role of differentiation of liver sinusoidal endothelial cells in progression and regression of hepatic fibrosis in rats. *Gastroenterology* 2012;142(4):918-927.
2. Tomiyama C, Watanabe H, Izutsu Y, Watanabe M, Abo T. Suppressive role of hepatic dendritic cells in concanavalin A-induced hepatitis. *Clin Exp Immunol* 2011;166(2):258-268.
3. Kimura K, Ando K, Ohnishi H, Ishikawa T, Kakumu S, Takemura M, et al. Immunopathogenesis of hepatic fibrosis in chronic liver injury induced by repeatedly administered concanavalin A. *Int Immunol* 1999;11(9):1491-1500.
4. Munk RB, Sugiyama K, Ghosh P, Sasaki CY, Rezanka L, Banerjee K, et al. Antigen-independent IFN-gamma production by human naive CD4 T cells activated by IL-12 plus IL-18. *PLoS One* 2011;6(5):e18553.
5. Lee HR, Yoon SY, Song SB, Park Y, Kim TS, Kim S, et al. Interleukin-18-mediated interferon-gamma secretion is regulated by thymosin beta 4 in human NK cells. *Immunobiology* 2011; 216(10):1155-1162.
6. Tu Z, Hamalainen-Laanaya HK, Crispe IN, Orloff MS. Synergy between TLR3 and IL-18 promotes IFN-gamma dependent TRAIL expression in human liver NK cells. *Cell Immunol* 2011; 271(2):286-291.
7. Schuppan D. Liver fibrosis: pathogenesis, prevention and treatment. *Rev Gastroenterol Peru* 2000;20(2):164-168.
8. Kimura K, Ando K, Ohnishi H, Ishikawa T, Kakumu S, Takemura M, et al. Immunopathogenesis of hepatic fibrosis in chronic liver injury induced by repeatedly administered concanavalin A. *Int Immunol* 1999;11(9):1491-1500.
9. Ishiwata Y, Yokochi S, Hashimoto H, Ninomiya F, Suzuki T. Protection against concanavalin A-induced murine liver injury by the organic germanium compound, propagermanium. *Scand J Immunol* 1998;48(6):605-614.
10. Gantner F, Leist M, Lohse AW, Germann PG, Tiegs G. Concanavalin A-induced T-cell-mediated hepatic injury in mice: the role of tumor necrosis factor. *Hepatology* 1995;21(1):190-198.
11. Tiegs G, Gantner F. Immunotoxicology of T cell-dependent experimental liver injury. *Exp Toxicol Pathol* 1996;48(5):471-476.
12. Tiegs G, Hentschel J, Wendel A. AT cell-dependent experimental liver injury in mice inducible by concanavalin A. *J Clin Invest* 1992;90(1):196-203.
13. Bozza M, Bliss JL, Maylor R, Erickson J, Donnelly L, Bouchard P, et al. Interleukin-11 reduces T-cell-dependent experimental liver injury in mice. *Hepatology* 1999;30(6):1441-1447.
14. Ishiwata Y, Yokochi S, Hashimoto H, Ninomiya F, Suzuki T. Protection against concanavalin A-induced murine liver injury by the organic germanium compound, propagermanium. *Scand J Immunol* 1998;48(6):605-614.
15. Lee JH, Yoon JH, Lee YJ, Yang JI, Kwak MS, Cho EJ, et al. Mesalamine-induced B7-H1 expression on hepatic stellate cells attenuates autoimmune liver injury. *Hepatol Res* 2011;41(1):79-86.
16. Fujino M, Kawasaki M, Funesima N, Kitazawa Y, Kosuga M, Okabe K, et al. CrmA gene expression protects mice against concanavalin-A-induced hepatitis by inhibiting IL-18 secretion and hepatocyte apoptosis. *Gene Ther* 2003;10(20):1781-1790.
17. Ishiwata Y, Yokochi S, Hashimoto H, Ninomiya F, Suzuki T. Protection against concanavalin A-induced murine liver injury by the organic germanium compound, propagermanium. *Scand J Immunol* 1998;48(6):605-614.
18. Sennello JA, Fayad R, Morris AM, Eckel RH, Asilmaz E, Montez J, et al. Regulation of T cell-mediated hepatic inflammation by adiponectin and leptin. *Endocrinology* 2005; 146(5):2157-2164.
19. Maubach G, Lim MC, Chen J, Yang H, Zhuo L. miRNA studies in vitro and in vivo activated hepatic stellate cells. *World J Gastroenterol* 2011;17(22):2748-2773.
20. Lu P, Liu H, Yin H, Yang L. Expression of angiotensinogen during hepatic fibrogenesis and its effect on hepatic stellate cells. *Med Sci Monit* 2011;17(9):248-256.
21. Lütflögl M, Sakallıoğlu EE, Sakallıoğlu U, Gülbahar MY, Muğlalı M, Baş B, et al. Excessive fluoride intake alters the MMP-2, TIMP-1 and TGF-beta levels of periodontal soft tissues: an experimental study in rabbits. *Clin Oral Investig* 2012;16(6):1563-1570.
22. Alatas FS, Masumoto K, Matsuura T, Hayashida M, Saeki I,

- Kohashi K, et al. Synchronized expressions of hepatic stellate cells and their transactivation and liver regeneration during liver injury in an animal model of cholestasis. *J Pediatr Surg* 2011; 46(12):2284-2290.
23. Troeger JS, Schwabe RF. Neuropilin and liver fibrosis: Hitting three birds with one stone. *Hepatology* 2011;54(3):1091-1093.
24. Di Marco V, Bronte F, Calvaruso V, Capra M, Borsellino Z, Maggio A, et al. IL28B polymorphisms influence stage of the liver fibrosis and spontaneous or interferon-induced viral clearance in thalassemia patients with hepatitis C virus infection. *Haematologica* 2012; 97(5):679-686.