model group, five rats were housed in one cage, and for the control group, six in one cage.

Reagents: Carbon tetrachloride (analytical reagent) was purchased from Guangdong Guanghua Sci-Tech Co., Ltd. (China) (batch number: 20140410). Commercially available Xiangmanyuan Brand peanut oil and Qianmen Brand Erguotou (alcohol content: 56% vol) were used. Pentobarbital sodium and deionized water used to prepare the anesthetic were provided by Hainan Drug Safety Evaluation Center.

Rabbit polyclonal antibodies for M-CSF, TNF-β, IFN-γ, and IL-10 were purchased from Santa Cruz Biotechnology, Inc. The Universal kit and DAB kit were purchased from Beijing Zhongshan Jinqiao Biological Technology Co., Ltd. (China). The enhanced chemiluminescence kit, developing and fixing kit, RIPA lysis buffer (medium), total RNA extraction kit, universal reverse transcription kit, and ComSYBRqPCR Mix (with ROX) were obtained from Shanghai Novland Co., Ltd. (China).

Experimental methods

Development of cirrhosis and hypersplenism: Rats were raised in the new environment for seven days after purchase for adaption, with an adequate supply of food and water. Rats in the control group were maintained on normal diet, without any treatment. Rats in the model group were gavaged a 40% CCL4/peanut oil solution at a dose of 3.0 ml/kg twice a week in accordance with the methods of Ye [9]. Gavage dose was adjusted according to weight gain in the previous week. The dose was not changed when the body weight increased or decreased by less than 5%, but was increased by 0.5 ml/kg with a weight gain of more than 5%, decreased by 0.5 ml/kg with a weight reduction of more than 5%, and discontinued with a weight reduction of more than 10%. After gavage administration, the water was replaced with a mixture of 10% liquor and deionized water in the first two weeks and with a mixture of 15% liquor and deionized water thereafter. At week 7, one randomly selected rat from the model group was anesthetized and sacrificed. The right liver lobe and spleen tissues were collected, then fixed in methanol, embedded in paraffin, and stained with hematoxylin-eosin (HE) to observe the pathological changes in the liver and spleen tissues under the microscope, in order to confirm the development of cirrhosis and hypersplenism. At week 8, when the development of cirrhosis and hypersplenism had been confirmed, 2 ml of orbital venous blood was collected from the rats in both groups for liver function and routine blood tests. After blood collection, all rats were sacrificed by cervical dislocation, and appropriate amounts of the right liver lobe and spleen tissues were collected, then fixed and preserved. The expression of M-CSF, TNF-β, IFN-γ, and IL-10 in the enlarged spleen of rats with cirrhosis was measured by immunohistochemistry. Another appropriate amount of spleen tissue was preserved at -80°C for later Western blotting and RT-PCR.

Criteria for development of cirrhosis and hypersplenism: a. monolineage or multilineage cytopenias (WBC, PLT, and RBC) as revealed by routine blood tests; b. abnormal liver function indexes; c. liver: visual observation of cirrhotic nodules and microscopic observation of pseudolobules; and d. spleen: splenomegaly compared with the control group, a significantly increased spleen index, and microscopic observation of red pulp expansion.

Immunohistochemistry: The fixed liver and spleen tissues were embedded in paraffin, and cut into 4 μm serial sections. One liver tissue section was stained with HE. One spleen tissue section was stained with HE, one was incubated with PBS in replacement of the primary antibodies and used as a negative control, and four were stained by immunohistochemical staining for M-CSF, TNF-β, IFN-γ, and IL-10, respectively. HE staining results were reviewed by two physicians from Hainan Provincial People's Hospital to confirm the diagnosis of cirrhosis and splenomegaly.

The same immunohistochemical procedure was performed for the four cytokines: Paraffin sections were dewaxed, dehydrated in gradient alcohol, placed in distilled water, transferred into pH-6.0 sodium citrate solution, and boiled at high pressure until 2 minutes and 30 seconds after valve rotation. The autoclave was then placed into a pot of cold water, with running water slowly rushing the surface. After cooling, the sections were placed into 3% H_2O_2 for 5 minutes, then into distilled water for 1 minute, PBS I for 1 minute, and then PBS II, III, and IV, respectively, for 3 minutes each. After the diluted primary antibodies were added dropwise, the sections were incubated at 37°C for 1 hour, and then rinsed with PBS II, III, and IV, respectively, for 3 minutes each. After the immunohistochemistry solution was added dropwise, the sections were incubated at 37°Cfor 1 hour, and then rinsed with PBS V, VI and VII, respectively, for 3 minutes each. Afterwards, the sections were developed with freshly prepared diaminobenzidine (DAB) solution for 3-5 minutes under the microscope, and then rinsed with running water. Finally, the sections were counterstained, dehydrated in gradient alcohol, cleared with xylene, and mounted with gum.

Result judgment: M-CSF, TNF-β, IFN-γ, and IL-10-positive cells were stained mainly in the cytoplasm and membrane, which turned brownish yellow or brown. Five high-power (×400) fields were selected to calculate the percentage of positive cells and expression intensity. The positive expression intensity was scored as follows: no staining was scored as 0; faint yellow staining as 1; yellow staining as 2; and brown staining as 3. The percentage of positive cells rate was scored as follows: 5% was scored as 0; 5-25% as 1; 26-50% as 2; 51 -75% as 3; and >75% as 4. The final score was obtained by summing the above two scores: A score of 0 indicated negative expression (-); a score of 1 and 2 indicated weak positive expression (+);a score of 3-5 indicated medium positive expression (+); and a score of 6 and 7 indicated strong positive expression (+++).

Western blotting: Each tissue sample was treated with 300 μl of cell lysis buffer, and mixed with a pipette until complete lysis was observed. The lysate was transferred to a new centrifuge tube. Next, 10 μl of sample was mixed with 10 μl of 2×SDS-PAGE loading buffer, heated at 100°C for 5 minutes, cooled on ice, and centrifuged at 12000 g for 5 minutes. Insoluble precipitates were removed. The samples were separated by 10% SDS-PAGE at a volume of 20 μl per well. After electrophoresis, the PVDF membranes were immersed in methanol for 1 minute. Next, the gel, filter papers, and the PVDF membranes that had been immersed in methanol were immersed in transfer buffer at 4°C for 10 minutes. Transfer sandwiches were then prepared (Note: Semi-dry electrophoretic transfer was performed using a semi-dry cell at 30 mA for 60 min). The PVDF membranes were blocked with blocking buffer at 4°C overnight, washed for 15 minutes three times with 1 × TBST the next day, and incubated with diluted primary antibodies at 37°C for 2 hours. Then, the membranes were washed with 1 × TBST for 10 minutes four times, incubated with diluted secondary antibodies at 37°C for 2 hours and finally washed with 1 × TBST for 10 minutes four times. After chemiluminescent detection using the hypersensitive luminescence solution, the membranes were exposed to x-ray films. The films were developed, fixed, dried, and photographed

by a gel imaging system. The images were analyzed by Gel-Pro Analyzer software.

RT-PCR: Total RNA was extracted according to the Trizol total RNA extraction kit instructions, and reversely transcribed according to the reverse transcription kit instructions. Primer sequences were as follows: M-CSF (126bp) upstream primer: 5'-GACTTGGCTTGGGATGATTCT-3', downstream primer: 5'-GAGGGTCTGGCAGGTACTC-3'; TNF-β (241bp) upstream primer: 5'-CCACCTCCTGAGGGTGCTTA-3', downstream primer: 5 '-ATGTCGGAGAAAGGCGCG-3'; INF-γ (108bp) upstream primer: 5'-GTCATCGAATCGCACCTGA-3', downstream primer: 5'-GTGCTGGATCTGTGGGTTG-3'; IL-10 (105bp) upstream primer: 5'-GCTCTTACTGGCTGGAGTGAG-3', downstream primer: 5'-CTCAGCTCTCGGAGCATGTG-3'; internal reference GAPDH (115bp) upstream primer : 5'-AAGGTCGGTGTGAACGGATTTG-3 ', downstream primer: 5'-TGTAGTTGAGGTCAATGAAGGGGTC-3'. All primers were synthesized by Shanghai Novland Co., Ltd. (China). Reverse transcription was performed for 30 min at 42°C, and 10 min at 85°C. Cycling parameters used for quantitative PCR were: denaturation at 95°C for 3 min, 95°C for 12 seconds, and 62°C for 40 seconds for 40 cycles. Fluorescent signal was collected at the extension phase of each cycle. After amplification was completed, fluorescent signal in cycles 3-15 was set as baseline, a threshold fluorescence value was established where all PCR amplifications were within the exponential phase, and GAPDH was set as an internal reference gene to calculate Ct values for each sample and each gene. Relative quantitation (RQ) of gene expression was determined by the 2-Ct method.

Statistical analysis: Test data were analyzed using the SPSS 19.0 software package. Normally distributed measurement data were expressed as $\bar{x}\pm S$. The results of routine blood and liver function tests, Western blotting, and RT-PCR were compared using the independent samples t-test. Immunohistochemical positive expression was compared using the chi-square test. P<0.05 was considered statistically significant.

Results

General conditions

Twenty six (43.3%) rats in the model group died from week 5 onward, and nine of them died instantly due to poor gavage technique. The 34 surviving rats had matted fur, severe fur loss, poor appetite, weight loss, and low activity. These rats were in poor spirits, relatively weak, insensitive to external stimuli, and had a black tongue, which was an obvious sign of intoxication. In contrast, no deaths occurred in the control group, and the rats had glossy fur and good appetite, and were in good spirits, sensitive to external stimuli, and lively.

Laboratory tests

Routine blood tests: WBC count (×109/L) was significantly higher in the model group than in the control group (25.81 ± 11.50 *vs.* 6.16 ± 2.42, t value=5.67, P<0.05). RBC count ($\times10^{12}$/L) and PLT count ($\times10^{9}$/L) were significantly lower in the model group than in the control group (6.08 ± 1.76 *vs.* 7.96 ± 0.62, t value=-2.51, P<0.05; 355.25 ± 286.06 *vs.* 1109.17 ± 161.30, t-value=-7.14, P<0.01) (Table 1).

Blood Cells	Model Group (n=28)	Control Group (n=12)	t Value	P Value
WBC ($\times10^{9}$/L)	25.81 ± 11.50	6.16 ± 2.42	5.67	<0.05
RBC ($\times10^{12}$/L)	6.08 ± 1.76	7.96 ± 0.62	-2.51	<0.05
PLT ($\times10^{9}$/L)	355.25 ± 286.06	1109.17 ± 161.30	-7.14	<0.01

Table 1: Comparison of routine blood test results between the two groups.

Liver function: ALT (U/L), AST (U/L), and TBIL (μ mol/L) levels were significantly higher in the model group than in the control group (264.14 ± 111.05 *vs.* 26.50 ± 7.89, t value=5.65, P<0.05; 687.43 ± 298.64 vs. 123.83 ± 20.38, t value=4.98, P<0.05; 20.07 ± 17.75 *vs.* 1.38 ± 0.39, t value=2.58, P<0.05). TP (g/L) and ALB (g/L) levels were significantly lower in the model group than in the control group (54.44 ± 7.59 *vs.* 64.87 ± 2.96, t value=-3.35, P<0.05; 24.53 ± 4.36 *vs.* 30.25 ± 1.65, t-value=-3.02, P<0.05) (Table 2).

Liver Function	Model Group (n=28)	Control Group (n=12)	t Value	P Value
ALT (U/L)	264.14 ± 111.05	26.50 ± 7.89	5.65	<0.05
AST (U/L)	687.43 ± 298.64	123.83 ± 20.38	4.98	<0.05
TBIL (μ mol/L)	20.07 ± 17.75	1.38 ± 0.39	2.58	<0.05
TP (g/L)	54.44 ± 7.59	64.87 ± 2.96	-3.35	<0.05
ALB (g/L)	24.53 ± 4.36	30.25 ± 1.65	-3.02	<0.05

Table 2: Comparison of liver function between the two groups.

Changes in the spleen index

Spleen index=spleen weight (mg)/body weight (g). The spleen index was 3.14 ± 0.98 for the model group, which was significantly different from the 2.33 ± 1.03 for the control group (t=-2.351, P<0.05).

Immunohistochemistry

In the model group, 61.76% (21/34, including 10 rats with a medium or higher level of positive expression) of the rats were M-CSF positive (a score of ≥ 2), which was significantly higher than the 25.00% (3/12) in the control group (X2=4.29, P<0.05). Furthermore, 79.41% (27/34, including 12 rats with a medium or higher level of positive expression) of the rats were TNF-β positive, which was significantly higher than the 33.33% (4/12) in the control group (X2=5.70, P<0.01).

In addition, 64.70% (22/34, including 11 rats with a medium or higher level of positive expression) of the rats were IFN-γ positive, which was significantly higher than the 16.67% (2/12) in the control group (X2=7.62, P<0.01). Finally, 88.24% (30/34, including 13 rats with a medium or higher level of positive expression) of the rats were IL-10 positive, which was significantly higher than the 50.00% (6/12) in the control group (X2=3.97, P<0.05) (Figures 1 and 2).

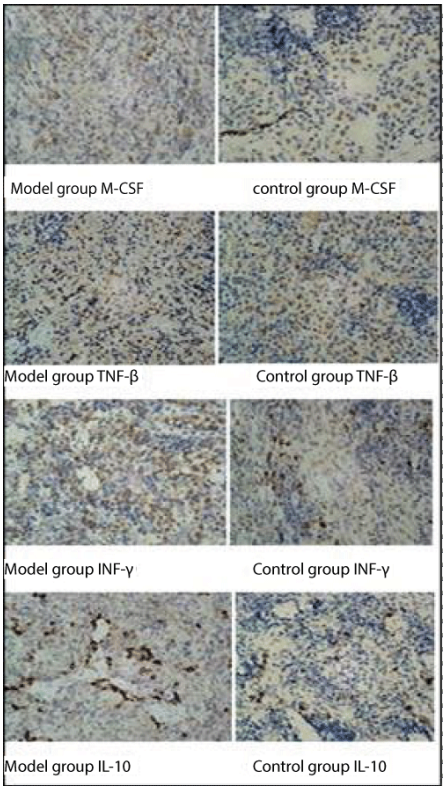

Figure 1: Positive expression of M-CSF, TNF-β, IFN-γ, and IL-10 in the spleen in the two groups (400×).

Western blotting

As revealed by Western blotting (with GAPDH as a control), the relative protein expression intensities of M-CSF, TNF-β, IFN-γ, and IL-10 in the spleen were 0.63 ± 0.58, 1.06 ± 0.49, 0.99 ± 0.38, and 1.12 ± 0.42, respectively, in the 34 rats with liver cirrhosis and hypersplenism in the model group. These values were significantly higher than the 0.18 ± 0.12, 0.52 ± 0.27, 0.38 ± 0.28, and 0.60 ± 0.32 in the 12 healthy rats in the control group (all $P<0.05$) (Figures 3 and 4).

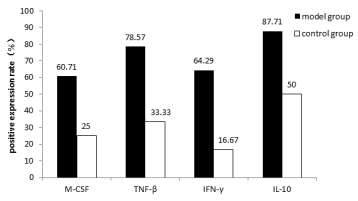

Figure 2: Positive expression rates of M-CSF, TNF-β, IFN-γ, and IL-10 in the spleen in the two groups determined by immunohistochemistry.

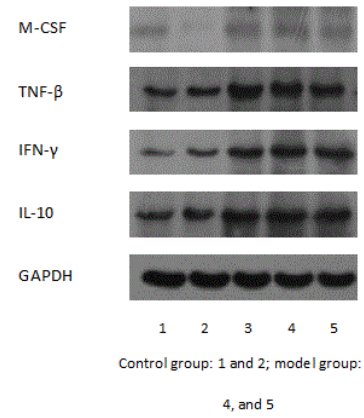

Figure 3: Comparison of M-CSF, TNF-β, IFN-γ, and IL-10 protein levels in the spleen between the two groups.

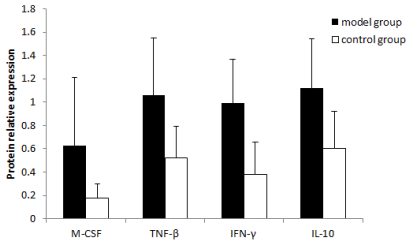

Figure 4: Expression of M-CSF, TNF-β, IFN-γ, and IL-10 in the spleen in the two groups determined by Western blotting.

Pathological changes

Liver pathological changes: The liver showed marked atrophy, a nodular surface, hyperplasia, and obvious periportal fibrosis, with pseudolobules of different sizes enveloping liver cells (Figure 5).

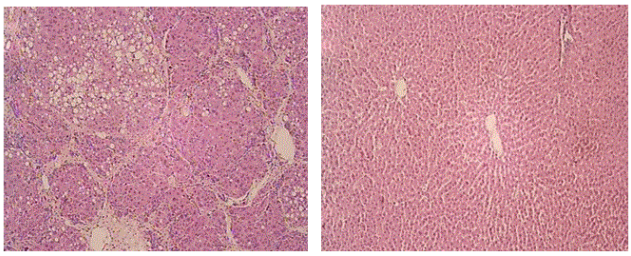

Figure 5: Pathological findings of the liver (200×). 1a: Model group, 1b: Control group.

Spleen pathological changes: The spleen was significantly enlarged. Thickened splenic capsule, dilated, congestive and fibrotic splenic sinus, expanded red pulp, decreased white pulp, and increased fibrous tissue were observed under the microscope (Figure 6).

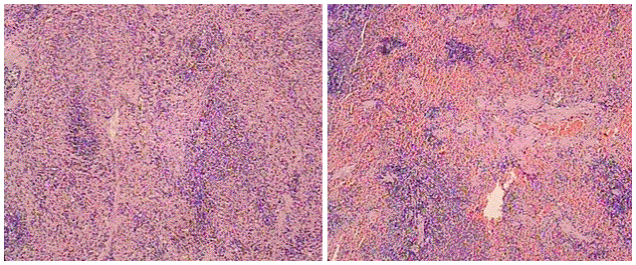

Figure 6: Pathological findings of the spleen (200×). 2a: Model group, 2b: Control group.

RT-PCR

As revealed by fluorescence quantitative RT-PCR, the relative mRNA expression levels of M-CSF, TNF-β, IFN-γ, and IL-10 in the spleen were 2.06 ± 0.11, 4.07 ± 0.19, 2.98 ± 0.11, and 7.94 ± 0.27, respectively, in the model group, which were significantly higher than the 1.01 ± 0.05, 1.06 ± 0.11, 1.00 ± 0.31, and 1.02 ± 0.08 in the control group (all P <0.05) (Figure 7).

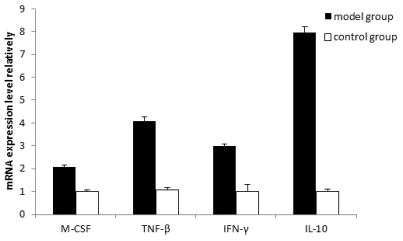

Figure 7: Relative mRNA expression levels of M-CSF, TNF-β, IFN-γ, and IL-10 in the spleen in the two groups determined by RT-PCR.

Discussion

As a chemical hepatotoxicant, CCL4 has been widely used for its reliability to induce liver fibrosis and cirrhosis in experiments [10,11], despite its high mortality rate (43.3%). This study demonstrates that CCL4 can also be used to induce hypersplenism. In the model group, ALT, AST, and TBIL levels were significantly increased, while TP and ALB levels were significantly decreased, which indicated impaired liver function and was one of the criteria for diagnosis of liver fibrosis and cirrhosis [12,13]. For the model group, a nodular liver surface, an increased spleen index, and a significantly enlarged spleen were observed; pseudolobules in the liver tissue, and expanded red pulp, reduced white pulp, and increased fibrous tissue in the spleen were observed under the microscope, indicating the development of liver cirrhosis and hypersplenia in rats. Cirrhosis and hypersplenism often lead to peripheral cytopenias [14]. Compared with the control group, PLT and RBC counts were decreased significantly in the model group, which is similar to conditions in human cirrhosis and hypersplenism with peripheral cytopenias [15]. The significantly increased WBC count in the model group may be related to uncontrolled inflammation after modeling.

M-CSF is produced by activated macrophages, B cells, and T cells, and can promote transformation of monocytes to macrophages, increase the number and survival of macrophages [16], and stimulate macrophages to enhance phagocytosis, digestion, and cytotoxicity. It is also an important inflammatory cytokine that can induce monocytes to release tumor killers, such as IFN and TNF. TNF-β and IFN-γ are important proinflammatory cytokines generated by T cells after antigen or mitogen stimulation. They can promote macrophage activation through immunomodulation, and play important roles in strengthening and maintaining the activated state of macrophages [17]. IL-10, which is mainly secreted by Th2 cells and macrophages, is an anti-inflammatory cytokine secreted by a subset of regulatory T cells, and has an anti-hepatic fibrosis effect [18]. In addition to mediating immune response, it can also cause feedback inhibition of macrophages, and inhibit the expression of inflammatory cytokines, such as M-CSF, TNF-β, and IFN-γ [19].

As revealed by immunohistochemistry, the positive expression rates of M-CSF, TNF-β, IFN-γ, and IL-10 were significantly higher in the spleen of rats with liver cirrhosis and hypersplenism compared with normal spleens (P<0.05). Moreover, ≥ 43% of rats in the model group had medium or strong positive expression, while only partial medium expression was detected in the control group (no strong positive expression was detected). As determined by highly sensitive and specific Western blotting, the relative protein expression intensities of M-CSF, TNF-β, IFN-γ, and IL-10 were significantly higher in the model group than those in the control group (P<0.05). As demonstrated by RT-PCR, the relative mRNA expression levels of M-CSF, TNF-β, IFN-γ, and IL-10 were also significantly higher in the model group than those in the control group (P<0.05). The high expression of M-CSF, TNF-β, and IFN-γ in liver cirrhosis and hypersplenism can promote the transformation of blood monocytes to macrophages, increase and enhance macrophage activation [20], and enhance the phagocytosis and destruction of blood cells Eubank et al. [7], which may be an important cause of peripheral cytopenias. The number and activity of macrophages are known to be enhanced by the pro-inflammatory cytokines, M-CSF, TNF-β, and IFN-γ, and inhibited by the anti-inflammatory cytokine IL-10. These cytokines constitute a regulatory system to regulate the number and phagocytosis of macrophages. Although the level of the anti-inflammatory cytokine IL-10 was significantly increased in hypersplenism, it cannot antagonize the effects of pro-inflammatory cytokines and is not sufficient to control the progression of inflammation. The increased pro-inflammatory/anti-inflammatory cytokine ratio eventually leads to the occurrence and development of peripheral cytopenias in cirrhosis and hypersplenism. It is possible to recover the normal number and activity of macrophages and thereby treat peripheral cytopenias by treatment with drugs that inhibit M-CSF, TNF-β, and IFN-γ or promote the synthesis of IL-10.

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References

1. Bashour FN, Teran JC, Mullen KD (2000) Prevalence of peripheral blood cytopenias (hypersplenism) in patients with nonalcoholic liver disease. Am J Gastroenterol 95: 2936-2939.

2. YunfuLv, Xiaoyu Han, Xiaoguang Gong,Qingyong Ma, Shunwu Chang, et al. (2015) Grading of peripheral cytopenias caused by nonalcoholic cirrhotic portal hypertension and its clinical significance. Cell Biochemistry and Biophysics 71: 1141-1145.
3. Li ZF, Zhang Y, Gao J (2004) Expression and significance of Toll-like receptor 4 of splenic macrophage in patients with hypersplenism due to portal hypertension. Zhonghuayixuezazhi 84: 1088-10891.
4. Li ZF, Zhang S (2009) Changes in splenic macrophage function of hypersplenism due to portal hypertension . Zhonghuawaikezazhi Chinese journal of surgery 47: 89-91.
5. Jiang A, Zhang S, Li Z (2011) miR-615-3p promotes the phagocytic capacity of splenic macrophages by targeting ligand-dependent nuclear receptor corepressor in cirrhosis-related portal hypertension. Exp Biol Med (Maywood) 236: 672-680.
6. Mantovani A, Sica A, Sozzani S (2004) The chemokine system in diverse forms ofmacrophage activation and polarization. Trends Immunol 25: 677-686.
7. Eubank TD, Galloway M, Montague CM, Waldman WJ, Marsh CB (2003) M-CSF induces vascular endothelial growth factor production and angiogenic activity from human monocytes. J Immunol 171: 2637-2643.
8. Ma J, Chen T, Mandelin J, Ceponis A, Miller NE, et al. (2003) Regulation of macrophage activation. Cell Mol Life Sci 60: 2334-2346.
9. Ye CH, Liu XY (2005) Study on method of inducing hepatic cirrhosis model in rats by carbon tetrachloride. Journal of Clinical Research 05: 619-622.
10. Zheng L, Chen X, Guo J, Sun H, Liu L, et al. (2012) Differential expression of PTEN in hepatic tissue and hepatic stellate cells during rat liver fibrosis and its reversal. Int J Mol Med 30: 1424-1430.
11. Zhang ZG, Lu XB, Xiao L, Tang L, Zhang LJ, et al. (2012) Antioxidant effects of the Uygur herb, FoeniculumVulgare Mill, in a rat model of hepatic fibrosis. ZhonghuaGanZang Bing ZaZhi 20: 221-226.
12. Li N, Cui XF, Zhang XL, Pei YH (2014) Liver function test results in patients with hepatitis B and fatty liver. Clinical Medicine of China 1: 51-53.
13. Li TM (2014) Clinical value of combined measurement of six liver function indexes in liver disease diagnosis. Guide of China Medicine 29: 272.
14. YunfuLv, Xiaoguang Gong, XianheXie, Baochun Wang, Yijun Yang, et al. (2014) Clinical study on the relationship between hematocytopenia and splenomegaly caused by cirrhotic portal hypertension. Cell Biochem Biophys 70: 355-360.
15. Zapatero MC, Pérez P, Vázquez MJ, Colmenarejo G, Frailes M, et al. (2016) Discovery of novel inhibitors of the tautomeraseactivity of macrophage migration inhibitory factor (MIF). J Biomol Screen.
16. Tran DD, Saran S, Dittrich-Breiholz O, Williamsonet AJK, Färber Sk, et al. (2013) Transcriptional regulation of immediate-early gene response by THOC5, a member of mRNA export complex, contributes to the M-CSF-induced macrophage differentiation. Cell Death Dis 4: e879.
17. Kurihara Y, Furue M (2013) Interferon-gamma enhances phorbolmyristate acetate-induced cell attachment and tumor necrosis factor production via the NF-kappaB pathway in THP-1 human monocytic cells. Mol Med Rep 7: 1739-1744.
18. Bouzgarrou N, Hassen E, Bahri O, Gabbouj S, Mami NB, et al. (2011) Combined effect of pro- and anti-inflammatory cytokine gene polymorphisms on susceptibility to liver cirrhosis in Tunisian HCV-infected patients. Hepatology International 5: 681-687.
19. Mittal SK, Cho KJ, Ishido S, Roche PA (2015) IL-10 mediated immunosuppression: March-I induction regulates antigen presentation by macrophages but not dendritic cells. J Biol Chem 290: 27158-27167.
20. Lu YF, Li XQ, Han XY, Gong XG, Chang SW, et al. (2013) Peripheral blood cell variations in cirrhotic portal hypertension patients with hypersplenism. Asian Pac J Trop Med 6: 663-666.