

## Original Article



## Effect of Spleen Lymphocytes on the Splenomegaly in Hepatocellular Carcinoma-bearing Mice\*

FANG Jing Jing, ZHU Zhen Yuan, DONG Hui,  
ZHENG Guo Qiang, TENG An Guo, and LIU An Jun<sup>#</sup>

Key Laboratory of Food Nutrition and Safety, Ministry of Education, Tianjin University of Science and Technology, Tianjin 300457, China

### Abstract

**Objective** To study the effect of spleen lymphocytes on the splenomegaly by hepatocellular carcinoma-bearing mouse model.

**Methods** Cell counts, cell cycle distribution, the percentage of lymphocytes subsets and the levels of IL-2 were measured, and two-dimensional gel electrophoresis (2-DE) was used to investigate the relationship between spleen lymphocytes and splenomegaly in hepatocellular carcinoma-bearing mice.

**Results** Compared with the normal group, the thymus was obviously atrophied and the spleen was significantly enlarged in the tumor-bearing group. Correlation study showed that the number of whole spleen cells was positively correlated with the splenic index. The cell diameter and cell-cycle phase distribution of splenocytes in the tumor-bearing group showed no significant difference compared to the normal group. The percentage of CD3<sup>+</sup> T lymphocytes and CD8<sup>+</sup> T lymphocytes in spleen and peripheral blood of tumor-bearing mice were substantially higher than that in the normal mice. Meanwhile, the IL-2 level was also higher in the tumor-bearing group than in the normal group. Furthermore, two dysregulated protein,  $\beta$ -actin and S100-A9 were identified in spleen lymphocytes from H<sub>22</sub>-bearing mice, which were closely related to cellular motility.

**Conclusion** It is suggested that dysregulated  $\beta$ -actin and S100-A9 can result in recirculating T lymphocytes trapped in the spleen, which may explain the underlying cause of splenomegaly in H<sub>22</sub>-bearing mice.

**Key words:** Hepatocellular carcinoma; Splenomegaly; Lymphocytes;  $\beta$ -actin; S100-A9

*Biomed Environ Sci*, 2014; 27(1): 17-26

doi: 10.3967/bes2014.012

ISSN: 0895-3988

[www.besjournal.com](http://www.besjournal.com) (fulltext)

CN: 11-2816/Q

Copyright ©2014 by China CDC

### INTRODUCTION

As the largest immune organ, the spleen would play an important role in pathogenesis and development of many diseases because of the close relationship between the spleen and other organs, which even could contribute to the important functions of other

organs. The unique architecture of the spleen allows for interactions among the circulatory, reticuloendothelial, and immune systems. Based on these interactions in conjunction with its microanatomy, the spleen is able to maintain the integrity of the blood and respond to circulating antigens<sup>[1]</sup>.

Hepatocellular carcinoma (HCC) is a major worldwide health problem, causing almost one

\*This work was financially supported by program for Changjiang Scholars and Innovative Research Team in University (IRT1166), National Natural Science Foundation of China (No. 31271975).

<sup>#</sup>Correspondence should be addressed to LIU An Jun, Tel: 86-22-60601437, Fax: 86-22-60601437, E-mail: anjunliu666@hotmail.com

Biographical note of the first author: FANG Jing Jing, female, born in 1985, PhD candidate with Tianjin University of Science and Technology, majoring in cellular molecular biology.

Received: May 31, 2013;

Accepted: August 12, 2013

million deaths annually. It is one of the most common neoplasms in Asia, Africa, and the Mediterranean basin, as well as the leading cause of death due to malignancy in some parts of Asia<sup>[2]</sup>. Most HCCs develop on the basis of liver cirrhosis, and in China, 15% of the HCC patients die of G1 bleeding secondary to portal hypertension<sup>[3]</sup>. Previous studies have shown that splenomegaly is frequently encountered in patients with chronic liver diseases such as liver fibrosis and cirrhosis. Severe enlargement of the spleen can potentiate the sequestration and destruction of blood components, likely leading to hypersplenism manifested as anemia, thrombocytopenia and leucopenia. There are several therapeutic approaches available for secondary hypersplenism, including splenectomy, partial splenic embolization, microwave ablation, and radiofrequency ablation<sup>[4]</sup>.

Splenectomy has been widely used for treating splenomegaly in patients with hepatocellular carcinoma, however, Kyaw et al. have found that classic splenectomy is often associated with loss of splenic immune function and high morbidity and mortality rates<sup>[5]</sup>. It is well known that the spleen plays a critical role in immune surveillance and response since it contains lymphoid tissue and macrophages and is highly vascularized moreover the spleen lymphocyte plays a crucial role in splenic immune function<sup>[1]</sup>. According to our previous studies, the spleen lymphocyte counts significantly increased in H<sub>22</sub>-bearing mice compared with normal mice<sup>[6]</sup>, and we hypothesized that changes in the number of spleen lymphocytes could affect spleen size of H<sub>22</sub>-bearing mice. The aim of the present study was to test this hypothesis using H<sub>22</sub>-bearing mouse model (as it reproduces human HCC forms)<sup>[7-9]</sup>.

## MATERIALS AND METHODS

### Reagents

All chemicals were of highest grade purity. RPMI 1 640 medium was purchased from Gibco. Fetal calf serum was purchased from Sijiqing Biological Engineering Material (Hangzhou, China). CD3-FITC/CD19-PE reagent and CD4-PE/CD8-PerCP reagent were purchased from BD Biosciences. ELISA kit was purchased from Dakewe Biotech Company Limited (Beijing, China). All reagents for two-dimensional polyacrylamide gel electrophoresis and mass spectrometry protein-identification were from Amersham Pharmacia Biotech. Hanks' balanced salt solution, red blood cell lysis buffer, propidium iodide, RNase A and all other chemicals were purchased from Sigma-Aldrich, USA.

### Animals and Cell Lines

Female ICR mice (18-22 g, 6-weeks-old) were purchased from the Department of Experimental Animals, Academy of Military Medical Science, Beijing, and acclimatized for 1 week prior to use. The mice were fed on a standard diet ad libitum and housed at a temperature of 20-25 °C under a 12-h light/dark cycle throughout the experiment. All animals received humane care, and all animal handling procedures were performed in strict accordance with the PR China legislation of the use and care of laboratory animals and were approved by the college animal experiment committee. The mouse H<sub>22</sub> hepatocarcinoma cells were obtained from Immunological Laboratory of Tianjin Medical University and maintained *in vivo* by weekly intraperitoneal passage of 3×10<sup>6</sup> cells in female ICR mice.

### Animal Treatment and Experimental Design

H<sub>22</sub> cells were transferred into the abdominal cavity of ICR mouse. The ascites were taken from the mouse and diluted with physiological saline to 1×10<sup>7</sup> cells/mL. 5×10<sup>6</sup> H<sub>22</sub> cells were inoculated through subcutaneous injection at the right armpit of mouse to establish a solid tumor model<sup>[7]</sup>. At 24 h after H<sub>22</sub> tumor cells inoculation, thirty ICR mice were randomly divided into 3 groups (*n*=10 mice/group). The female tumor-bearing mice were weighed and killed at 7, 14, and 21 d post-inoculation (p.i.) respectively for 3 groups. The normal mice (*n*=10) were used as control, weighed and killed on the very day of inoculation. Blood samples from the animals were collected and processed to obtain serum, the spleen and thymus were excised from the animal and weighed immediately. The thymus and spleen indices were calculated according to the following formula<sup>[10]</sup>:

$$\text{Thymus or spleen index (mg/g)} = \frac{\text{Weight of thymus or spleen}}{\text{Body weight}}$$

### Cell Count and Cell Morphology

At different time points after inoculation, spleens from female tumor-bearing mice were aseptically removed for preparation of splenocytes. Splenocytes were prepared as described by Bahram Bibak et al.<sup>[11]</sup>. Briefly, removed spleens were homogenized in sterile Hanks' Balanced Salt Solution (HBSS). Homogenized spleen tissues passed through the mesh were washed twice with HBSS. The erythrocytes were lysed with red blood cell (RBC)

lysis buffer, while the remaining cells were suspended in RPMI 1 640 medium with 10% fetal calf serum (FCS). Cells were counted with a haemocytometer by trypan blue dye exclusion technique<sup>[12]</sup>. Cell viability exceeded 95%. For morphological observation, the cell were examined and photographed by the inverted phase contrast microscope (Nikon, Tokyo, Japan).

#### ***Analysis of Cell Cycle Distribution by Flow Cytometry***

The cell cycle distribution was analyzed through the flow cytometry (FCM) detection method<sup>[9]</sup>. Splenic cells in each group were isolated and then fixed in 70% ethanol at 4 °C overnight. After washing twice with phosphate- buffered saline (PBS), the cells were resuspended in cold propidium iodide (PI; 50 µg/mL) containing RNase A (0.1 mg/mL) in PBS (pH 7.4) for 30 min in the dark. Cell cycle distribution was assessed by DNA content detected by flow cytometry (BD FACScalibur Flow Cytometer, BD Biosciences). Ten thousand events were acquired for each sample and analyzed.

#### ***Lymphocyte Subpopulation Analysis***

Peripheral blood was collected from the orbital venous plexus, and spleen cell suspensions were produced by forcing the spleen through a wire-mesh screen. The direct fluorescence labeling method and flow cytometry were used to test the membrane surfaces CD3, CD19, CD4, and CD8 of peripheral blood in EDTA and splenocytes<sup>[13-14]</sup>. Briefly, each sample was divided into two groups: one containing CD3-FITC/CD19-PE reagent and the other containing CD4-PE/CD8-PerCP reagent. The tubes were then placed on an oscillator for thorough mixing, followed by 15 min on the bench, away from light. The cells were then incubated in RBC lysis buffer for 15 min to lyse red blood cells, washed twice with 1×PBS, resuspended in 1×PBS, and tested by FACScalibur flow cytometer (BD Biosciences), experimental data were collected and analyzed with CELLQuest software (BD Biosciences).

#### ***ELISA for the Detection of IL-2 Expression***

Interleukin-2 (IL-2) is a growth factor for variety of cell types and plays key roles in T cell growth and differentiation, as well as functional B cell development<sup>[15]</sup>. Peripheral blood was collected from the orbital venous plexus, and the serum samples were prepared by centrifuging the whole blood at 2 000 × g at 4 °C for 10 min. Levels of serum IL-2 were analyzed using a commercially available ELISA kit according to the manufacturers' instructions.

#### ***Two-dimensional Gel Electrophoresis***

Electrophoresis was performed as described by HE Yu Jun et al.<sup>[16]</sup>. Splenocytes were harvested and washed with ice-cold PBS, and suspended in lysis buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% (w/v) CHAPS, 0.5% IPG buffer pH 3-10, and 2.5% dithiothreitol (DTT) after PBS was drained. Lysed cells were subjected to centrifugation at 14 000 g at 4 °C for 20 min and the supernatant was collected as the cell extract. Protein concentration was determined by Bradford assay. Proteins (900 µg) were applied to isoelectric focusing gels (IEF). The gels (nonlinear gradient pH 3-10, 17 cm, Bio-Rad) were hydrated overnight in 8 mol/L urea, 4% CHAPS, 60 mmol/L DTT, and 0.5% IPG buffer. IEF was initiated at 200 V for 1 h, 500 V for 1 h, 1 000 V for 1 h, and then gradually increased to 10 000 V for 5-6 h. Focusing was carried out for 60 000 Vh. After IEF, IPG strips were equilibrated in 50 mmol/L Tris-HCl (pH 8.8), 6 mol/L urea, 20% glycerol, 2% SDS, and 10 mmol/L DTT for 15 min, washed with 50 mmol/L Tris-HCl (pH 8.8), 6 mol/L urea, 20% glycerol, 2% SDS, and 200 mmol/L iodoacetamide for another 15 min. Then the IPG stripe was carefully transferred to the top of the prepared 12% PAGE gel and embedded in low melting-point agarose. The electrophoresis ran at a constant current of 20 mA per gel. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250.

#### ***Mass Spectrometry Protein-identification***

Protein spots from Coomassie stained preparative 2-DE gels were selected and excised manually using a clean scalpel or razor blade, then put into 1.5 mL Eppendorf tubes, covered with water and stored in the freezer until digestion. The samples were prepared for MALDI-TOF-MS analysis using a slightly modified published procedure<sup>[16-17]</sup>. Briefly, spots were excised from the stained gel and destained with 25 mmol/L ammonium bicarbonate/50% acetonitrile, and then dried with a SpeedVac plus SC1 10. The dried gel was rehydrated in trypsin solution at 37 °C overnight. After rehydration, peptides were first eluted with 5% trifluoro-acetic acid (TFA) at 40 °C for 1 h, then with 2.5% TFA/50% acetonitrile at 30 °C for 1 h. Acetonitrile was removed by centrifugation in a vacuum centrifuge. Analysis was performed primarily using the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Burker Company, German). The peptide mass fingerprint (PMF) map was used to search for the matched protein based on NCBI-NR Metazoa database with Matrix Science software.

### Statistical Analysis

Data from the present study were presented as mean±SD from at least three independent experiments with different batches of mice, and each one was performed in duplicate or triplicate. All statistical comparisons were carried out using one-way ANOVA test followed by *t* test. *P*-values less than 0.05 were considered statistically significant while *P*-values less than 0.01 were considered extremely significant.

## RESULTS

### Spleen and Thymus Indices in H22-bearing Mice

At different time points after inoculation, the spleen and thymus from female ICR mice were aseptically removed. The spleen index in the tumor-bearing group was significantly greater than that in the normal group ( $P<0.01$ ), as seen in Table 1. Although the thymus index was decreased in the tumor-bearing group after 7 and 14 d of inoculation, the decrease was not statistically significant, as compared to the normal group (Table 1). The thymus index, however, showed a significant decrease in the tumor-bearing mice after 21 d of inoculation in comparison with the normal control group ( $1.48\pm 0.14$  vs.  $2.09\pm 0.34$ ,  $P<0.01$ ; Table 1).

### Macroscopic Observation of Spleen Cell

The cell diameter of spleen lymphocytes in the tumor-bearing group showed no significant change compared to the normal group (Figure 1A). Conversely, whole spleen cell number analyses performed in the tumor-bearing group showed a significant increase compared to the normal group and had statistically significant positive correlation with splenic index ( $r=0.999$ ,  $P<0.01$ ; Figure 1B).

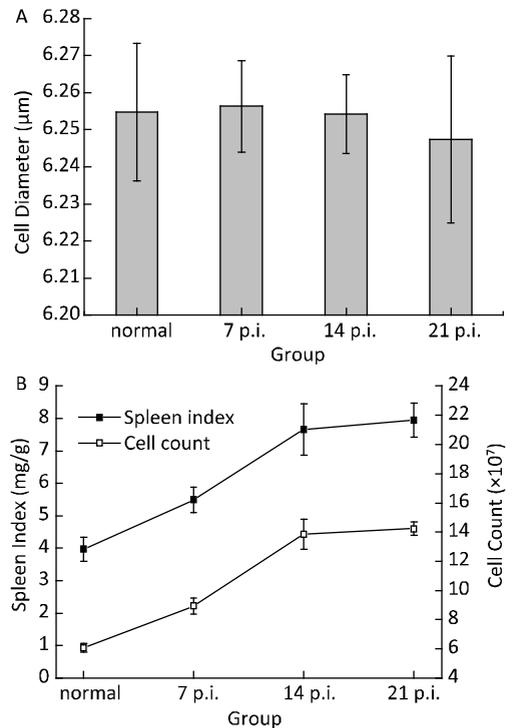
**Table 1.** Spleen and Thymus Indexes of the Mice in Different Groups ( $n=10$  for each group)

Groups	Spleen Index (mg/g)	Thymus Index (mg/g)
Normal group	$3.98\pm 0.37$	$2.09\pm 0.34$
7 p.i. Tumor-bearing Group	$5.49\pm 0.39^{**}$	$1.90\pm 0.25$
14 p.i. Tumor-bearing Group	$7.66\pm 0.80^{**}$	$1.86\pm 0.12$
21 p.i. Tumor-bearing group	$7.95\pm 0.52^{**}$	$1.48\pm 0.14^{**}$

**Note.** Results are presented as mean±SD.  $^{**}P<0.01$  versus normal group.

### Analysis of Cell-cycle Phase Distribution

Cell-cycle phase distribution of splenic cells was analyzed by flow cytometry with PI staining. As was shown in Table 2, there was no significant difference in cell-cycle phase distribution between the tumor-bearing group and the normal group, and cell cycle arrest at  $G_0/G_1$  phase in each group.



**Figure 1.** Macroscopic observation of spleen cells. (A) Diameters of spleen lymphocytes in different groups. (B) Correlation of spleen index and cell count ( $\times 10^7$ ). Results are presented as mean±SD ( $n=10$  for each group).

**Table 2.** Cell Cycle Analysis of Splenic Cells in Different Groups (%) ( $n=10$  for each group)

Groups	$G_0/G_1$ (%)	S (%)	$G_2/M$ (%)
Normal group	$90.20\pm 1.49$	$7.53\pm 0.35$	$1.48\pm 0.11$
7 p.i. Tumor-bearing group	$91.17\pm 1.34$	$7.84\pm 0.26$	$1.59\pm 0.18$
14 p.i. Tumor-Bearing group	$90.91\pm 1.52$	$8.06\pm 0.48$	$1.38\pm 0.27$
21 p.i. Tumor-bearing group	$92.01\pm 1.50$	$6.63\pm 0.84$	$1.55\pm 0.15$

**Note.** Results are presented as mean±SD.

### Changes in the Percentage of Spleen and Peripheral Blood Lymphocytes

The lymphoid cell populations within spleens and peripheral blood of tumor-bearing mice and normal mice were analyzed by flow cytometry. As shown in Table 3, tumor-bearing mice had higher numbers of circulating T cells in the spleens and peripheral blood than normal mice, and CD8<sup>+</sup> T cells were the most affected subset ( $P<0.01$ ). Thus, the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> T cell was lower for tumor-bearing mice. No statistically significant differences between the tumor-bearing group and the normal group were found when comparing the percentage of B lymphocytes in spleens, but the proportion of B cells in the peripheral blood of tumor-bearing mice was higher than that of the normal control mice ( $P<0.01$ ).

### Determination of IL-2 Levels in Peripheral Blood

The blood from the mice was centrifuged at 2000 × g for 10 min and the serum was collected for the detection of IL-2 levels with an enzyme-linked immunosorbent assay according to the indication of the manufacturer. As shown in Figure 2, the expression of IL-2 level in tumor-bearing mice was significantly higher than that in normal mice ( $P<0.01$ ).

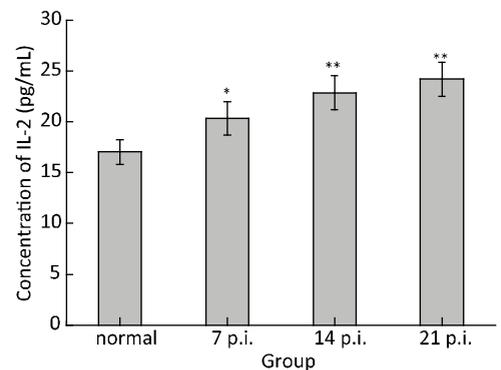
### 2-DE Analysis of Proteins in Spleen Lymphocytes from H<sub>22</sub>-bearing Mice after 21 D of Inoculation and Normal Mice

2-DE was employed to separate the proteins in spleen lymphocytes obtained from H<sub>22</sub>-bearing mice after 21 d of inoculation and normal mice using the standard procedure. A comparative post- extraction

proteomic analysis in spleen lymphocytes of H<sub>22</sub>-bearing mice after 21 d of inoculation and normal mice revealed that 30 spots were significantly higher in the H<sub>22</sub>-bearing group and 14 spots were significantly lower in the H<sub>22</sub>-bearing group as compared to the normal group (data not shown). After the evaluation of one paired gels, we selected two proteins (spot 1 and spot 2, Figure 3) whose levels in the H<sub>22</sub>-bearing group were at least 10-fold higher or lower than in the paired normal group. These proteins were identified by mass-spectrometry.

### Identification of Protein Spots by MALDI-TOF Mass Spectrometry

The protein spots that differed prominently in spleen lymphocytes between H<sub>22</sub>-bearing mice after 21 d of inoculation and normal mice were excised from the Coomassie blue staining gel, then subjected to in-gel trypsin digestion and identified by MALDI-TOF mass spectrometry (MS). The spot was

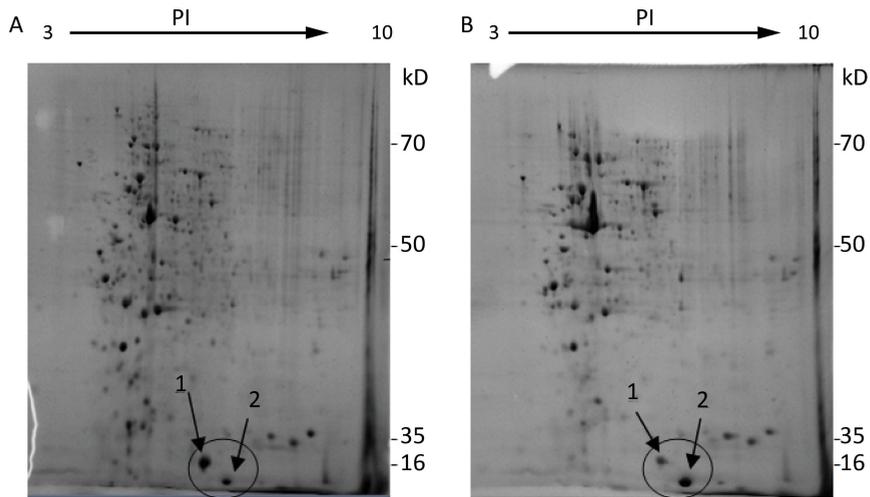


**Figure 2.** Concentration of IL-2 in different groups. \* $P<0.05$  versus normal group, \*\* $P<0.01$  versus normal group.

**Table 3.** Comparison of Spleen and Peripheral Blood Lymphocyte Subsets in Different Groups (%) ( $n=5$  for each group)

Groups	B Cells (%)	T Cells (%)	CD8 <sup>+</sup> T Cells (%)	CD4 <sup>+</sup> T Cells (%)	CD4 <sup>+</sup> /CD8 <sup>+</sup> T Cells
<b>Spleen lymphocyte subsets</b>					
Normal group	43.58±1.84	31.85±1.75	11.22±0.72	21.39±1.74	1.91±0.12
7 p.i. Tumor-bearing group	45.59±3.28	36.06±1.91*	12.33±0.42*	22.92±1.18	1.86±0.07
14 p.i. Tumor-bearing group	44.37±2.58	36.99±0.86**	12.91±0.50**	23.52±1.86	1.82±0.08
21 p.i. Tumor-bearing group	44.26±1.98	39.31±2.09**	15.09±0.39**	24.95±0.98*	1.65±0.03**
<b>Peripheral blood lymphocyte subsets</b>					
Normal group	6.73±0.24	53.08±1.20	14.79±0.10	38.29±1.10	2.59±0.06
7 p.i. Tumor-bearing group	8.77±0.44**	57.66±1.10**	16.91±0.20**	40.75±1.30	2.41±0.11
14 p.i. Tumor-bearing group	10.67±1.46**	62.18±1.10**	19.05±0.07**	43.13±1.17**	2.27±0.07**
21 p.i. Tumor-bearing group	13.32±0.92**	68.46±1.06**	23.77±1.27**	44.69±2.33**	1.88±0.20**

**Note.** Results are presented as means±SD. \* $P<0.05$  versus normal group, \*\* $P<0.01$  versus normal group.



**Figure 3.** Two-dimensional electrophoresis patterns of total proteins from spleens. (A) The pattern of total proteins in spleen lymphocyte obtained from normal mice. (B) The pattern of total proteins in spleen lymphocyte obtained from  $H_{22}$ -bearing mice after 21 days of inoculation.

selected for PMF that was used to search for the matched protein (Figure 4). As compared with the normal mice, the protein (spot 1) down-regulated in  $H_{22}$ -bearing mice after 21 d of inoculation was  $\beta$ -actin and the up-regulated protein (spot 2) was S100-A9. Peptide sequences from  $\beta$ -actin and S100-A9 matching with peaks obtained from MALDI-MS spectra are shown in Figure 4B and Figure 4D.

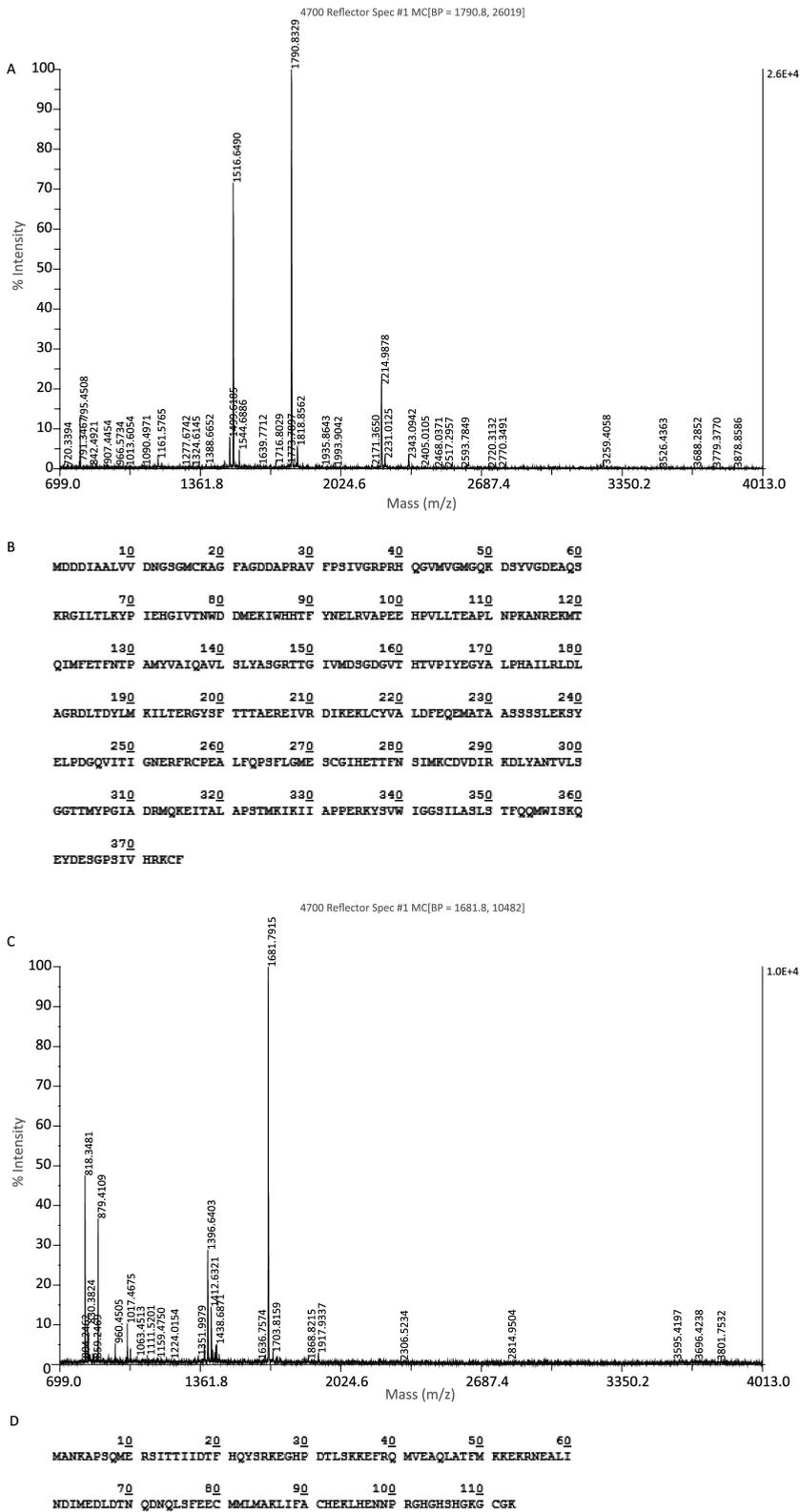
### DISCUSSION

Splenomegaly is a common complication of HCC, but its pathogenesis is not clear. To our knowledge, there is little information regarding the effect of spleen lymphocytes on the splenomegaly in patients with HCC. Our previous studies have shown that  $H_{22}$ -bearing mice had higher numbers of spleen lymphocytes than normal mice<sup>[6]</sup>. Given the important role of spleen lymphocytes in the immune system, further studies are needed to investigate the relationship between spleen lymphocytes and splenomegaly. Hence, in this study, we have established  $H_{22}$ -bearing mouse model and taken the spleen lymphocyte as a study object to investigate the cause of splenomegaly in  $H_{22}$ -bearing mice.

It is difficult to investigate the state of immune system function in natural populations because intrinsic factors such as genetic background, sex, and age introduce intraspecies variation<sup>[18]</sup>. As the crucial lymphoid organ in the body, the spleen and thymus are involved in the capture and destruction of pathogens and the induction of immune responses.

Determination of thymus and spleen indices are the most frequently used approaches in evaluating the state of immune system function in animals<sup>[10,19]</sup>. Previous studies found that the spleen weight of mice with hepatoma was much higher than that of normal mice, on the other hand, the thymus weight of the tumor-bearing group was much lower than that of the normal group, and that the spleen performed no immunologic function with the progression of cancer, but rather a negative one<sup>[20-21]</sup>. In this study, the change trends of thymus and spleen indices in  $H_{22}$ -bearing mice were in agreement with those of the previous studies, demonstrating that a novel splenomegaly murine model was built successfully, which provided us a tool for analyzing the effect of spleen lymphocytes on the splenomegaly in patients with HCC.

To analyze the relationship between the spleen lymphocyte and splenomegaly in  $H_{22}$ -bearing mice, first cell diameter and spleen cell number were examined by microscope. Cell diameter of spleen lymphocyte in tumor-bearing group showed no significant change compared to the normal group, conversely, whole spleen cell number in the tumor-bearing group showed a significant increase compared to the normal group and had statistically significant positive correlation with splenic index. These results indicated that there was no significant correlation between splenomegaly and cell diameter, and enlarged spleen organs in the tumor-bearing group was caused by increased whole spleen cell number.



**Figure 4.** The specific protein spots in 2-DE gel were analyzed by mass spectrometry. The picture shows: (A) PMF map of the 1st protein spot, (B) the amino acid sequence of the 1st protein spot, (C) PMF map of the 2nd protein spot, (D) the amino acid sequence of the 2nd protein spot.

To clarify why the whole spleen cell number increased in H<sub>22</sub>-bearing mice, cell-cycle phase distribution was analyzed by flow cytometry with PI staining. Cell cycle is the important regulatory mechanisms of cell growth, development and differentiation. In mammals, the cell cycle comprises the G<sub>1</sub>, S, G<sub>2</sub>, and M phases<sup>[22]</sup>. In the present study, the finding of FCM assays revealed that there was no significant difference in cell-cycle phase distribution between the tumor-bearing group and the normal group, and the same to cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> phase in each group. This finding indicated that cell proliferation of splenocytes was inhibited in the tumor-bearing group, suggesting that there was no correlation between increased whole spleen cell number in H<sub>22</sub>-bearing mice and splenocyte proliferation.

In the immune system, cellular and humoral immunity, mediated by T and B lymphocytes, respectively, plays key roles against infection. CD3<sup>+</sup> cells are T lymphocytes, CD19<sup>+</sup> cells are B lymphocytes, and T lymphocyte subsets are primarily composed of CD4<sup>+</sup> T lymphocytes and CD8<sup>+</sup> T lymphocytes, which are differentiated according to the different antigens expressed on the surface of the cells. Changes in the number of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells and the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> T cells represent changes in immune function<sup>[13,23-24]</sup> and can further affect prognosis<sup>[25]</sup>. On the other hand, it has been indicated that the spleen plays a predominant role in lymphocyte recirculation and that lymphocyte subpopulations differ markedly in their circulation pattern through the spleen<sup>[21]</sup>. Based on these data, the present study revealed some changes in the subpopulation distribution by monitoring spleen and peripheral blood lymphocyte subsets from tumor-bearing mice and the normal mice. Significantly higher numbers of circulating T cells in the peripheral blood and spleens were found in tumor-bearing mice (CD8<sup>+</sup> T cells were the most affected subset), along with an increase in the IL-2 level in serum and a decrease in thymus index. It is well known that, after a primary differentiation in the bone marrow and thymus, lymphocytes undergo further functional maturation in the periphery and then migrate into the spleen<sup>[26-27]</sup>. Furthermore, IL-2 is a cytokine secreted by activated lymphocytes that plays an important role in the proliferation of T- and B-cells during inflammation. IL-2 is a pleiotropic cytokine that can act as a potent T cell growth factor during the initiation of an immune response<sup>[28]</sup>. Thus, these findings suggest that T lymphocytes in lymphocyte recirculation might be trapped in the

spleen, which could explain why the spleen was enlarged in tumor-bearing mice.

Proteins are responsible for the functional execution in a diversity of cells. Many regulatory processes and disease processes occur at the protein level, and most drug targets are found through protein studies. 2-DE is a highly versatile technique for separating proteins according to their charge and size. It has been used in comparative studies of protein expression levels between healthy and diseased states<sup>[29]</sup>. In this study, 2-DE was used to separate spleen lymphocyte proteins from H<sub>22</sub>-bearing mice after 21 d of inoculation and normal mice, which enabled the detection of two proteins whose level in H<sub>22</sub>-bearing group were at least 10-fold higher or lower than in the paired normal group. The proteins were identified by MALDI-TOF mass spectrometry, and the two protein,  $\beta$ -actin and S100-A9, were for the first time observed to be dysregulated in spleen lymphocytes from H<sub>22</sub>-bearing mice.

$\beta$ -actin-containing molecular complexes are critical in cytoskeletal rearrangement upon cell shape modulating signals. A well-recognized function of  $\beta$ -actin is its participation in cellular contractility and motility. For example, in osteoclasts,  $\beta$ -actin is known to organize the podosomes during osteoclasts motility<sup>[30-31]</sup>. Consistent with this view, over-expression of  $\beta$ -actin is considered to increase cell speed by enlarging areas of protrusion and retraction<sup>[32]</sup>. S100-A9 protein, a member of the S100 calcium-binding protein family, was found to be over-expressed in preneoplastic and neoplastic colon mucosa<sup>[33]</sup>. The S100-A9 protein, formerly called calgranulin B, MRP 14, or LI heavy chain, is a protein of about 13 kD that can occur in three different charge isoforms depending on the level of its phosphorylation. This protein is found predominantly in the cytosol, but it can be also expressed on the cell surface or even secreted into the surroundings<sup>[34]</sup>. The S100-A9 protein plays critical roles in numerous cellular processes such as motility and danger signaling by interacting and modulating the activity of target proteins<sup>[35]</sup>. In this study, we found that as compared with the normal mice,  $\beta$ -actin (spot 1) was down-regulated in H<sub>22</sub>-bearing mice after 21 d of inoculation, whereas S100-A9 (spot 2) was up-regulated. Together, these findings indicate that in H<sub>22</sub>-bearing mice, expression levels of  $\beta$ -actin and S100-A9 are associated with the migration ability of recirculating T lymphocytes in spleen, and trapped T lymphocytes may be attributable to dysregulation of  $\beta$ -actin and S100-A9.

Based on several papers that have reported a strong correlation between spleen size and HCC, splenomegaly as a result of portal hypertension has been considered to be the most important determinant<sup>[4]</sup>. On the other hand, splenic metastasis of hepatocellular carcinoma<sup>[36]</sup>, aneurysm of the splenic artery<sup>[37]</sup>, miliary splenic tuberculosis<sup>[38]</sup>, or littoral cell angioma<sup>[39]</sup> may also cause splenomegaly in patients with hepatocellular carcinoma, suggesting that factors other than portal hypertension are also operative in increasing the spleen size of patients with hepatocellular carcinoma. Our results have demonstrated that dysregulated  $\beta$ -actin and S100-A9 can result in recirculating T lymphocytes trapped in spleen, which may explain the underlying cause of splenomegaly in H<sub>22</sub>-bearing mice. Although detailed studies are needed, this study provides fundamental information about the cause of splenomegaly in H<sub>22</sub>-bearing mouse that may promote an understanding of the relationship between pathogenesis and the clinical manifestation of splenomegaly commonly observed in HCC patients, and we believe that  $\beta$ -actin and S100-A9 proteins may be valuable as potential diagnostic biomarkers that may assist in the early detection and treatment of splenomegaly patients with HCC.

### CONTRIBUTION ANNOUNCEMENT

FANG Jing Jing has been drafting the article and has made substantial contributions to acquisition of data, analysis and interpretation of data; ZHU Zhen Yuan has been advising the article critically for important intellectual content and checking it for spelling; DONG Hui, ZHENG Guo Qiang, and TENG An Guo have made substantial contributions to acquisition of data; LIU An Jun has made substantial contributions to conception and design of data.

### REFERENCES

1. Chadburn A. The spleen: anatomy and anatomical function. *Semin Hematol*, 2000; 37, 13-21.
2. Barazani Y, Hiatt JR, Tong MJ, et al. Chronic viral hepatitis and hepatocellular carcinoma. *World J Surg*, 2007; 31, 1243-8.
3. Han MJ, Zhao HG, Ren K, et al. Partial splenic embolization for hypersplenism concomitant with or after arterial embolization of hepatocellular carcinoma in 30 patients. *Cardiovasc Intervent Radiol*, 1997; 20, 125-7.
4. Shi B, Zhu H, Liu YJ, et al. Experimental studies and clinical experiences on treatment of secondary hypersplenism with extracorporeal high-intensity focused ultrasound. *Ultrasound Med Biol*, 2012; 38, 1911-7.
5. Duan YQ, Gao YY, Ni XX, et al. Changes in peripheral lymphocyte subsets in patients after partial microwave

- ablation of the spleen for secondary splenomegaly and hypersplenism: A preliminary study. *Int J Hyperthermia*, 2007; 23, 467-72.
6. Dong H, Zheng GQ, Teng AG, et al. Mechanism of splenic excess augmentation in tumor-bearing mice. *Cancer Research on Prevention and Treatment*, 2012; 39, 940-3. (In Chinese)
7. Fan YL, Wang WH, Song W, et al. Partial characterization and anti-tumor activity of an acidic polysaccharide from *Gracilaria lemaneiformis*. *Carbohydr Polym*, 2012; 88, 1313-8.
8. Pan ZQ, Fang ZQ, and Lu WL. Analysis of the dynamic changes of blood hormone levels in H<sub>22</sub> liver cancer mice of poisonous pathogenic factors syndromes to different degrees. *Chin J Integrated Traditional and Western Medicine*, 2012; 32, 1361-5. (In Chinese)
9. Li JT, Zhang JL, He H, et al. Apoptosis in human hepatoma HepG2 cells induced by corn peptides and its anti-tumor efficacy in H<sub>22</sub> tumor bearing mice. *Food Chem Toxicol*, 2013; 51, 297-305.
10. Chen JR, Yang ZQ, Hu TJ, et al. Immunomodulatory activity in vitro and in vivo of polysaccharide from *Potentilla anserina*. *Fitoterapia*, 2010; 81, 1117-24.
11. Bibak B, Gharib FG, Daneshmandi S, et al. The Immunomodulatory effects of abortion-prone mice decidual and serum soluble factors on macrophages and splenocytes. *Eur J Obstet Gynecol Reprod Biol*, 2012; 165, 331-6.
12. Alonso-Castro AJ, Juarez-Vazquez MD, Dominguez F, et al. The antitumor effect of the American mistletoe Phoradendron serotinum (Raf.) MC Johnst. (Viscaceae) is associated with the release of immunity-related cytokines. *J Ethnopharmacol*, 2012; 142, 857-64.
13. Chen X, Ye J, and Ye J. Analysis of peripheral blood lymphocyte subsets and prognosis in patients with septic shock. *Microbiol Immunol*, 2011; 55, 736-42.
14. Okazaki I, Hiai H, Kakazu N, et al. Constitutive expression of AID leads to tumorigenesis. *J Exp Med*, 2003; 197, 1173-81.
15. Merluzzi VJ, Welte K, Savage DM, et al. Expansion of cyclophosphamide-resistant cytotoxic precursors *in vitro* and *in vivo* by purified human interleukin 2. *J Immunol*, 1983; 131, 806-9.
16. He Y, Mou Z, Li W, et al. Identification of IMPDH2 as a tumor-associated antigen in colorectal cancer using immunoproteomics analysis. *Int J Colorectal Dis*, 2009; 24, 1271-9.
17. Encarnacion S, Guzman Y, Dunn MF, et al. Proteome analysis of aerobic and fermentative metabolism in *Rhizobium etli* CE3. *Proteomics*, 2003; 3, 1077-85.
18. Zuk M and Stoehr AM. Immune defense and host life history. *Am Nat*, 2002; 160, S9-S22.
19. Kataranovski M, Mirkov I, Zolotarevski L, et al. Basic indices of spleen immune activity in natural populations of norway rats (*rattus norvegicus* berkenhout, 1769) in serbia. *Arch Biol Sci*, 2009; 61, 723-32.
20. Sharp JG, Riches AC, Littlewood V, et al. The incidence, pathology and transplantation of hepatomas in CBA mice. *J Pathol*, 1976; 119, 211-20.
21. Toge T, Kuroi K, Kuninobu H, et al. Role of the spleen in immunosuppression of gastric cancer: predominance of suppressor precursor and suppressor inducer T cells in the recirculating spleen cells. *Clin Exp Immunol*, 1988; 74, 409-12.
22. Park KI, Park HS, Nagappan A, et al. Induction of the cell cycle arrest and apoptosis by flavonoids isolated from *Korean Citrus aurantium L.* in non-small-cell lung cancer cells. *Food Chem*, 2012; 135, 2728-35.
23. Zanetti M, Castiglioni P, Ingulli E. Principles of memory CD8 T-cells generation in relation to protective immunity. *Adv Exp Med Biol*, 2010; 684, 108-25.

24. Mu J, Jeyanathan M, Shaler CR, et al. Respiratory mucosal immunization with adenovirus gene transfer vector induces helper CD4 T cell-independent protective immunity. *J Gene Med*, 2010; 12, 693-704.
25. Marshall JC, Charbonney E, Gonzalez PD. The immune system in critical illness. *Clin Chest Med*, 2008; 29, 605-16.
26. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, et al. Longitudinal survey of lymphocyte subpopulations in the first year of life. *Pediatr Res*, 2000; 47, 528-37.
27. Villaescusa A, Garcia-Sancho M, Rodriguez-Franco F, et al. Early-life longitudinal survey of peripheral blood lymphocyte subsets in Beagle dogs. *Vet Immunol Immunopathol*, 2012; 149, 126-31.
28. Brunet M. Cytokines as predictive biomarkers of alloreactivity. *Clin Chim Acta*, 2012; 413, 1354-8.
29. Du XL, Hu H, Lin DC, et al. Proteomic profiling of proteins dysregulated in Chinese esophageal squamous cell carcinoma. *J Mol Med*, 2007; 85, 863-75.
30. Destaing O, Sanjay A, Itzstein C, et al. The tyrosine kinase activity of c-Src regulates actin dynamics and organization of podosomes in Osteoclasts. *Mol Biol Cell*, 2008; 19, 394-404.
31. Leavitt J, Bushar G, Kakunaga T, et al. Variations in expression of mutant beta actin accompanying incremental increases in human fibroblast tumorigenicity. *Cell*, 1982; 28, 259-68.
32. Tondeleir D, Lambrechts A, Mueller M, et al. Cells lacking beta-Actin are genetically reprogrammed and maintain conditional migratory capacity. *Mol Cell Proteomics*, 2012; 11, 255-71.
33. Stulik J, Kovarova H, Macela A, et al. Overexpression of calcium-binding protein calgranulin B in colonic mucosal diseases. *Clin Chim Acta*, 1997; 265, 41-55.
34. Stulik J, Osterreicher J, Koupilova K, et al. The analysis of S100A9 and S100A8 expression in matched sets of macroscopically normal colon mucosa and colorectal carcinoma: The S100A9 and S100A8 positive cells underlie and invade tumor mass. *Electrophoresis*, 1999; 20, 1047-54.
35. Vogl T, Gharibyan AL, and Morozova-Roche LA. Pro-inflammatory S100A8 and S100A9 proteins: self-assembly into multifunctional native and amyloid complexes. *Int J Mol Sci*, 2012; 13, 2893-917.
36. Yan ML, Wang YD, Lai ZD, et al. Pedunculated hepatocellular carcinoma and splenic metastasis. *World J Gastroenterol*, 2009; 15, 5239-41.
37. Urca. Aneurysm of the splenic artery associated with primary carcinoma of the liver and splenomegaly. *Angiology*, 1971; 22, 470-6.
38. Solsona Conillera J. Splenomegaly due to miliary splenic tuberculosis and hypertrophic alcoholic liver cirrhosis with jaundice (Esplenomegalia por tuberculosis miliar del bazo y cirrosis hepatica alcoholica hipertrofica con ictericia.). *Rev Cli*, 1954; Esp 52, 418-20.
39. Lin CH, Yu JC, Shih ML, et al. Littoral cell angioma of the spleen in a patient with hepatocellular carcinoma. *J Formos Med Assoc*, 2005; 104, 282-5.