

## Changes in count and function of splenic lymphocytes from patients with portal hypertension

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### Abstract

**AIM:** To investigate changes in numbers and proliferative function of splenic lymphocytes in patients with hypersplenism due to portal hypertension (PH), to provide evidence for further study of immune status of the spleen during PH.

**METHODS:** Twelve spleens from patients with hypersplenism due to PH served as the PH group, and four spleens from cases of traumatic spleen rupture were regarded as the control group. After weighing the spleen, lymphocytes were separated and counted using a cell counting plate to calculate the lymphocyte count per gram of spleen tissue (relative quantity) and total lymphocyte count in whole spleen (absolute quantity). The immunohistochemical SP method was used to observe the density and distribution of lymphocytes in the spleen. The MTT method was used to observe changes in lymphocyte proliferative function.

**RESULTS:** As compared to the control group, the splenic lymphocytes in the PH group showed that: (1) There was no difference in distribution but a significant decrease

in density; (2) the number of lymphocytes per gram of spleen (relative quantity) decreased significantly [ $(0.822 \pm 0.157) \times 10^8$  vs  $(1.174 \pm 0.254) \times 10^8$ ,  $P < 0.01$ ]; (3) with the significant increase in the weight of the PH spleen ( $832.6 \pm 278.2$  g vs  $211.7 \pm 85.6$  g,  $P < 0.01$ ), the total quantity of lymphocytes (absolute quantity) increased significantly [ $(0.685 \pm 0.072) \times 10^{11}$  vs  $(0.366 \pm 0.057) \times 10^{11}$ ,  $P < 0.01$ ]; and (4) the proliferative function of lymphocytes was enhanced: T lymphocytes,  $(0.022 \pm 0.005$  vs  $0.015 \pm 0.003$ ,  $P < 0.05$ ), and B lymphocytes  $(0.034 \pm 0.006$  vs  $0.023 \pm 0.001$ ,  $P < 0.01$ ).

**CONCLUSION:** Although lymphocyte density in the spleen decreased in patients with PH, the total quantity of lymphocytes increased because spleen weight increased greatly, along with the proliferating function. With respect to changes in lymphocytes, PH spleens may still have immune function, although it may be disordered. However, complete evaluation of the immune function of the spleen in PH requires more research.

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**Key words:** Portal hypertension; Spleen; Lymphocyte; Immune function

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### INTRODUCTION

Lymphocytes reside in different organs in the human body. They circulate through the primary lymphoid organs (thymus and bone marrow), secondary lymphoid organs (spleen, lymph nodes, tonsils and Peyer's patches), as well as non-lymphoid organs such as blood, lung and liver. Especially in lymphoid organs, lymphocyte subsets migrate and home to different compartments. About 15%-20% of the blood volume circulates through the spleen at any one time and about 15% of the lymphocytes reside in this organ<sup>[1,2]</sup>.

Therefore, lymphocytes are the immunocytes that have the highest count in the spleen. Their functional status directly influences the immune function of the spleen<sup>[3-5]</sup>.

Currently, there is still some dispute on the immune status of the spleen in patients with portal hypertension (PH)<sup>[6,7]</sup>. We have isolated splenic macrophages and demonstrated that their phagocytosis is enhanced in PH spleens<sup>[8-10]</sup>, but there is little compelling experimental evidence on the distribution, count and function of lymphocytes in the PH spleen. In this study, we isolated and cultured splenic lymphocytes from PH spleen, and observed changes in their density, distribution, count, and proliferative function using the immunohistochemical SP method and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT).

## MATERIALS AND METHODS

### Patients

Two groups of patients were studied. Twelve patients (median age 46.8 years, range 27-62 years; eight male and four female), with hypersplenism due to PH, in our hospital from September 2005 to March 2006, were included as the PH group. All patients underwent pericardial devascularization with splenectomy. Supporting evidence for hypersplenism due to PH and cirrhosis included clinical features, abnormal laboratory tests, and postoperative pathological examination. Four patients (median age 33.5 years, range 18-38 years; three male and one female) with traumatic rupture of the spleen were enrolled into the control group. Hepatitis, cirrhosis, history of hypersplenism, and abnormalities in postoperative laboratory findings and pathological examinations were absent in the controls. All patients provided written informed consent, and the protocol was approved by the ethics committee of our hospital.

### Lymphocyte count in the spleen

Spleens were weighed after removal from patients. The splenic tissue samples were cut from the upper pole, lower pole, and hilum, and were transferred to the cell culture room, and kept in sealed aseptic bottles filled with 4°C precooled PBS. Further preparations were made: Weighing 5 g tissue with an electronic balance, using a 200-mesh screen to grind the tissue sample into cell suspension, and purifying the lymphocytes with lymphocyte separating medium by gradient centrifugation. After preparation, lymphocytes were counted using a cell counting plate. The lymphocyte count per gram of spleen tissue (relative quantity) was then calculated and multiplied by the weight of the spleen to derive the total lymphocyte count in the spleen (absolute quantity).

### Density and distribution of lymphocytes

The splenic tissue samples were cut from the upper pole, lower pole, and hilum, fixed in phosphate buffer (pH 7.2) containing 4% paraformaldehyde, embedded in paraffin wax, and sectioned at 5 µm. CD3 and CD20 SP method staining was adopted to show T and B lymphocytes, respectively. Periarterial lymphatic sheath (PALS), splenic corpuscle (F), red pulp (RP), and marginal zone (MZ) of spleen tissue were

observed. Five fields of vision were also randomly observed in each part. Positive cells were counted respectively. For the negative control, the primary antibody was replaced by PBS.

### Proliferative function of lymphocytes

Lymphocytes with RPMI1640 culture solution containing 10% fetal calf serum were placed in 96-well flat-bottomed microplates in triplicate at  $2 \times 10^5$  cells/well, then concanavalin A (Con A) or lipopolysaccharide (LPS; both from Sigma, St. Louis, MO, USA) was added to the wells at a final concentration of 10 µg/L and 20 µg/L, respectively. The cells were then incubated in a total volume of 200 µL/well. Serum-free RPMI-1640 medium was used as a control. Cell proliferation was measured by MTT assay 44 h after culture. MTT (Sigma) solution of 20 µL (5 g/L) was added to each well. After 4 h incubation, the cells were lysed and the purple formazan crystals were solubilized. We then measured  $A_{570}$  of each well on an enzyme labeling instrument, and the proliferation level was calculated. Proliferation level = experimental group  $A$  (ConA or LPS) - negative control group  $A$ .

### Statistical analysis

$P$  values were calculated using the independent sample  $t$  test and considered significant at  $P < 0.05$ . All the results were represented by mean  $\pm$  SD.

## RESULTS

### Change in density and distribution of lymphocytes

The distribution of T and B lymphocytes was almost the same in PH spleen as in normal spleen (Figures 1 and 2). Cell counts in single fields of view were significantly less in PH spleen than in normal spleen (Table 1, Figures 3 and 4).

### Change in lymphocyte count

Lymphocyte count was significantly less in PH spleen (relative quantity) than in normal spleen. However, with the increase in spleen weight, lymphocyte count of whole spleen (absolute quantity) was significantly greater in PH spleen than in normal spleen (Table 2).

### Change in lymphocyte proliferative function

The proliferative function of T and B lymphocytes was significantly higher in PH spleen than in normal spleen (Table 3).

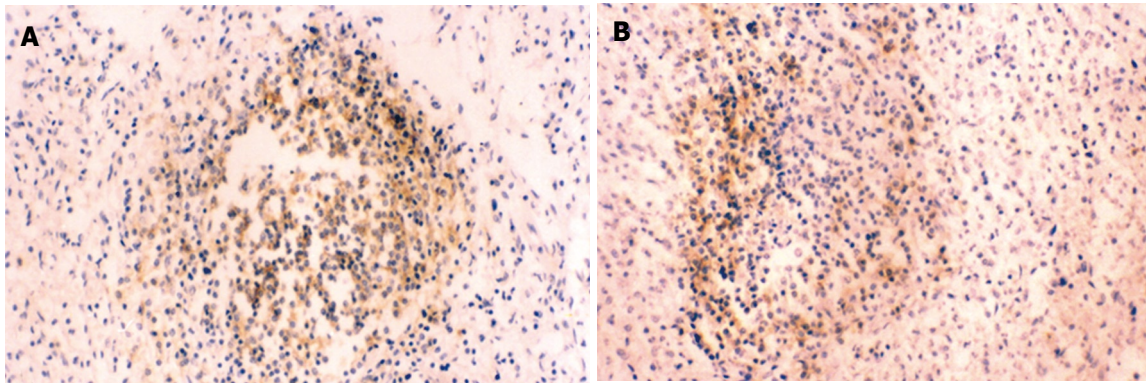
## DISCUSSION

At present, the immune function of the PH spleen is still the subject of some dispute<sup>[7,11,12]</sup>. It is unclear if the preservation of splenic tissue with splenomegaly is beneficial to patients with PH and cirrhosis<sup>[13-15]</sup>. The lymphocytes are important immunocytes in the spleen. The spleen can participate in specific immunity through T-cell-mediated cellular immunity and B-cell-mediated humoral immunity<sup>[16-18]</sup>. Therefore, the evaluation of changes in lymphocyte count and functions in PH spleen is extremely important to an in-depth study of the immune status of the spleen in PH.

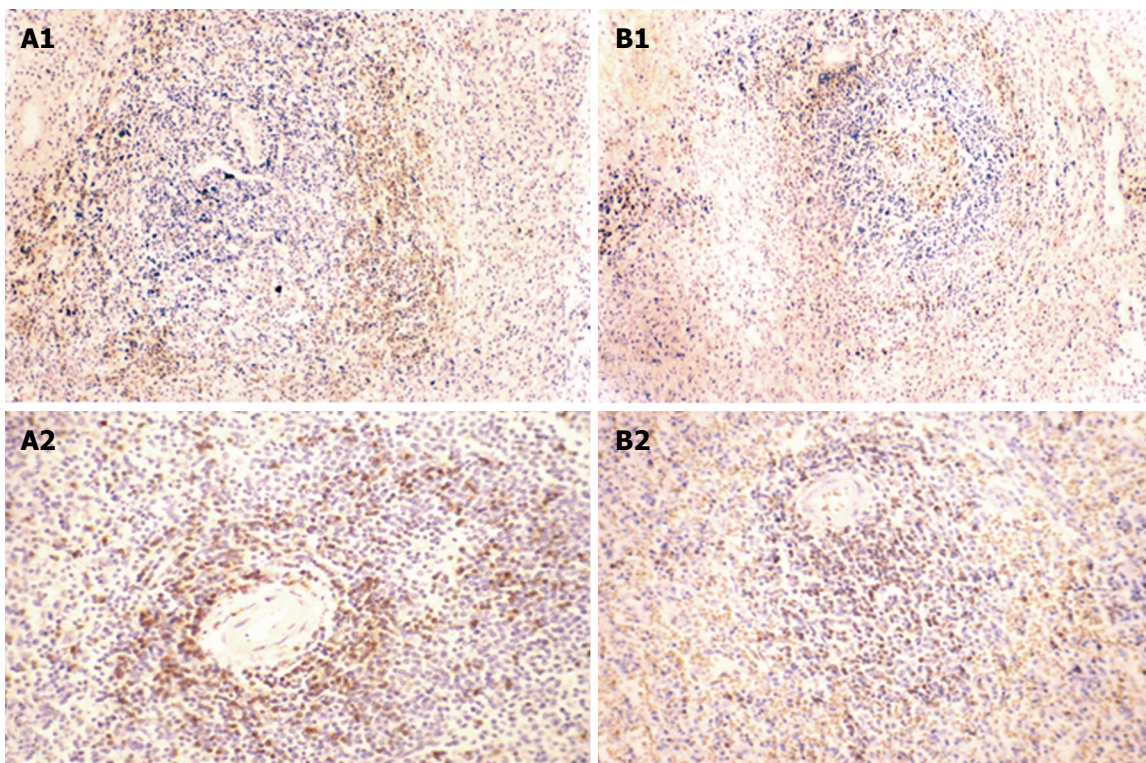


Table 1 Changes in density of lymphocyte in PH spleen

Group	T lymphocytes				B lymphocytes			
	F	MZ	PALS	RP	F	MZ	PALS	RP
PH	89.5 ± 14.7	120.0 ± 14.1	122.9 ± 12.1	12.2 ± 2.9	356.5 ± 31.2	138.0 ± 19.5	113.8 ± 21.6	7.4 ± 1.7
Control	126.5 ± 19.3	140.5 ± 11.6	137.0 ± 6.2	20.45 ± 4.5	418.3 ± 22.4	196.0 ± 22.0	153.8 ± 25.8	12.8 ± 4.6
P value	< 0.01	< 0.05	< 0.05	< 0.01	< 0.01	< 0.01	< 0.05	< 0.01



**Figure 1** CD20 immunostaining for distribution of B lymphocytes. There was no significant difference in distribution of B lymphocytes between PH and control groups; they were all mainly located in the splenic corpuscle. **A:** Control group; **B:** PH group ( $\times 100$ ).



**Figure 2** CD3 immunostaining for distribution of T lymphocytes. There was no significant difference in distribution of T lymphocytes between PH and control groups; they were all mainly located in the marginal zone and PALS. **A1:** Control group, marginal zone; **A2:** Control group, PALS; **B1:** PH group, marginal zone; **B2:** PH group, PALS ( $\times 100$ ).

Lymphocytes include T and B lymphocytes. CD3 and CD20 are important differentiation antigens on T- and B-cell membranes. CD3 and CD20 immunohistochemical staining is ideal for analyzing the distribution and count of T and B lymphocytes in tissue<sup>[19]</sup>. Wang *et al* have used the

method to observe lymphocytes in pathological sections of PH spleen. They believe that in PH splenomegaly, lymphocyte density in the spleen decreases, which results in a decrease in lymphocyte count<sup>[20]</sup>. We also found in our experiment that the distribution area of lymphocytes had

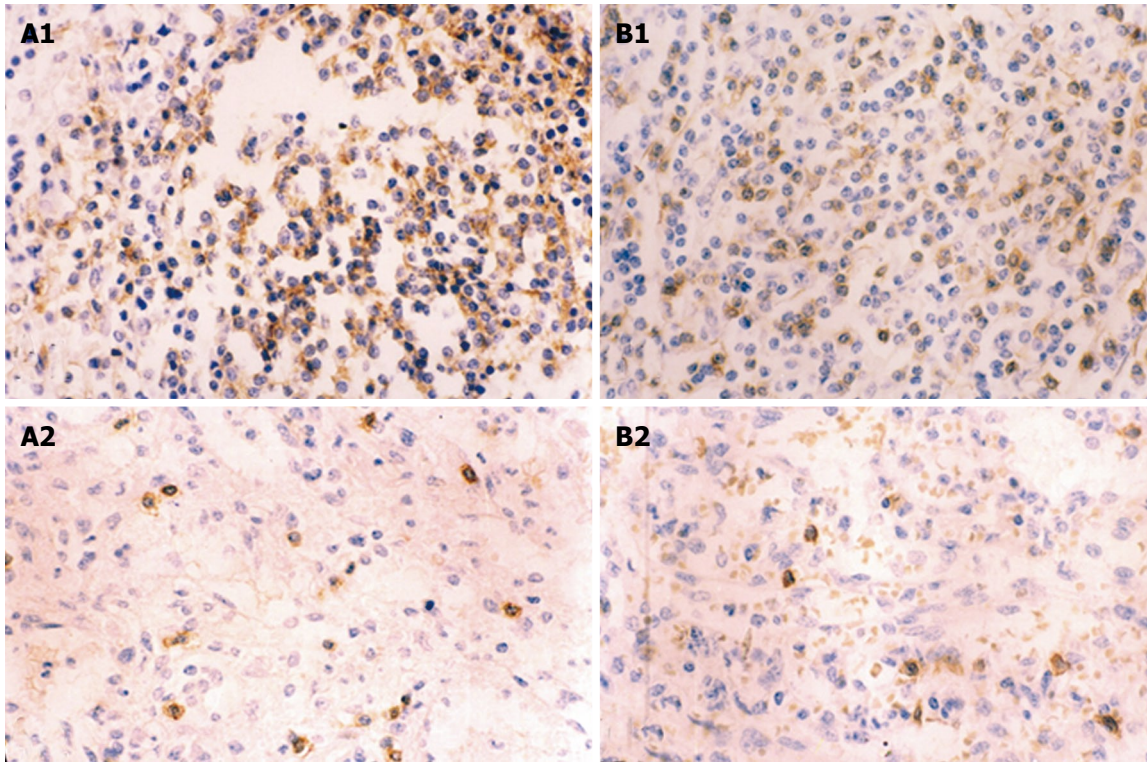


**Table 2** Changes in weight of spleen and lymphocyte count

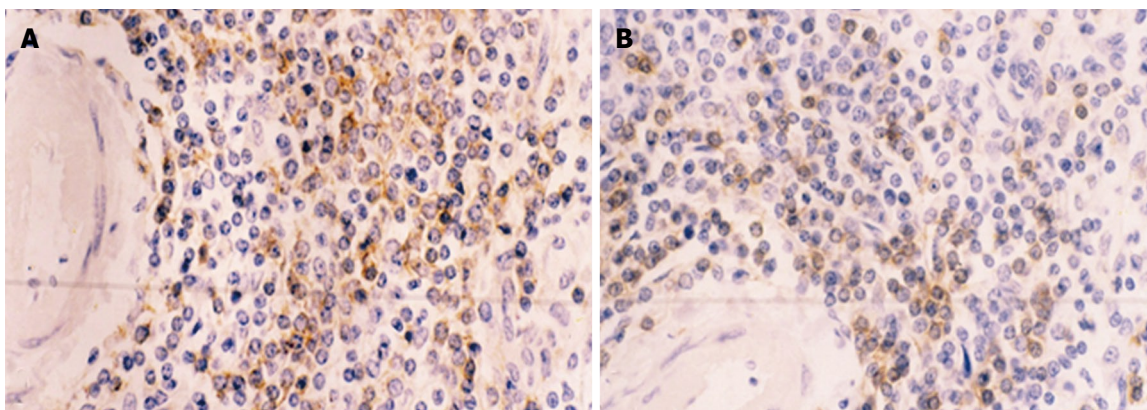
Group	Relative quantity ( $\times 10^8$ )	Weight of spleen (g)	Absolute quantity ( $\times 10^{11}$ )
PH	$0.822 \pm 0.157$	$832.6 \pm 278.2$	$0.685 \pm 0.072$
Control	$1.714 \pm 0.254$	$211.7 \pm 85.6$	$0.366 \pm 0.057$
P value	< 0.01	< 0.01	< 0.01

**Table 3** Changes in proliferative function of lymphocyte in PH spleen

Group	T lymphocyte (ConA $10 \mu\text{g/L}$ )	B lymphocyte (LPS $20 \mu\text{g/L}$ )
PH	$0.022 \pm 0.005$	$0.034 \pm 0.006$
Control	$0.015 \pm 0.003$	$0.023 \pm 0.004$
P value	< 0.05	< 0.01



**Figure 3** CD20 immunostaining for density of B lymphocytes. Compared to the control group, the density of B lymphocytes in the PH group decreased significantly in the splenic corpuscle and RP. **A1**: Control group, splenic corpuscle; **A2**: Control group, RP; **B1**: PH group, splenic corpuscle; **B2**: PH group, RP ( $\times 100$ ).



**Figure 4** CD3 immunostaining for density of T lymphocytes. Compare to the control group, the density of T lymphocytes in the PH group decreased significantly in PALS. **A**: Control group; **B**: PH group ( $\times 100$ ).

almost no differences between PH and normal spleens, while the lymphocyte density was significantly lower in the PH spleen. However, the lymphocyte density seen in a single microscopic field of view cannot represent the total lymphocyte count in the spleen. Therefore, in this

study, we purified and counted lymphocytes in the spleen, and then calculated lymphocyte count per gram of spleen tissue (relative quantity) and lymphocyte count in whole spleen (absolute quantity). This made the result more scientific and accurate. The results showed that although

the relative lymphocyte count per gram of spleen tissue was less in the PH spleen than that in normal spleen, as spleen weight increased greatly, the total lymphocyte count in whole spleen was significantly higher in the PH spleen than that in the normal spleen.

In addition, the proliferation of T and B lymphocytes is known as a response to stimulation induced by antigen or mitogens. The proliferative function is one of the important indices of lymphocyte immune function. Shi *et al* have reported that the expression of proliferating cell nuclear antigen (PCNA) in PH spleen is strongly positive in the lymphocyte aggregation area, which indirectly reflects the high proliferation status of lymphocytes<sup>[21]</sup>. PCNA is isolated as a protein with elevated levels during S-phase of the cell cycle. Its expression level may be a marker of the S-phase and represent the proliferative function of cells<sup>[22]</sup>. However, PCNA immunohistochemical staining cannot precisely distinguish S-phase and non-S-phase PCNA-positive cells under a light microscope<sup>[23]</sup>. The result has limited reliability in reflecting lymphocyte proliferation. Furthermore, sample fixation, immunostaining, and other laboratory procedures have certain influences on the demonstration of PCNA. While cellular multiplication induced by Con A is commonly used to detect T lymphocyte immunity *in vitro*, LPS-induced activation of B cells and subsequent immunoglobulin synthesis reflect B-lymphocyte immunity<sup>[24]</sup>. Therefore, the proliferative function of T and B lymphocytes was evaluated by MTT assay after being stimulated with LPS and Con A, respectively. The proliferative function of lymphocytes was also significantly higher in PH spleen than in the normal control group.

The total count and proliferative function of splenic lymphocytes increased in PH spleen. A possible reason is that long-term contact between noxious substances, such as endotoxin and hepatitis virus, and spleen tissue has promoted activation and hyperplasia of lymphocytes in the spleen<sup>[16,25]</sup>. Also, this contact has enhanced its function to maximize the elimination of toxins in the body and maintain body balance<sup>[26-29]</sup>. From this perspective, PH spleen has not completely lost immune function but does have some disorder. However, the immunological mechanism of the spleen is quite complicated. Hence, to confirm whether PH spleen has normal immune function<sup>[30]</sup> and to achieve precise evaluation of the immune function of the PH spleen, further research should be conducted.

## COMMENTS

### Background

A better understanding of the function of the spleen has been gained recently, owing to in-depth studies on its structure, cellular function, secretion and innervation. It is generally accepted the spleen is an important part of the regulatory network between the immune, nervous and endocrine systems. The spleen has many more functions besides blood filtering and storage, hematogenesis, and immunization, and its immune function has characteristics of both "two-way" and "phase". Present knowledge about the immune function of the spleen in patients with PH is still incomplete; it is unclear whether preservation of splenic tissue with splenomegaly is beneficial to patients with PH and cirrhosis. Lymphocytes play a key role in the immune function of the spleen. Studies on splenic lymphocytes will be helpful for precise evaluation of spleen function, especially in a pathological state.

### Research frontiers

It has previously been reported that phagocytosis of macrophages is enhanced

in the PH spleen, but there is little compelling experimental evidence on the distribution, count, and function of lymphocytes in the PH spleen.

### Innovations and breakthroughs

This study proved the total quantity and the proliferative function of lymphocytes were increased. It suggests that the PH spleen may still have immune function, although perhaps with some disorder.

### Applications

Although this was an initial study on the changes in lymphocytes in the spleen in PH, it may offer new evidence for complete evaluation of the immune function of the PH spleen.

### Peer review

The authors investigated changes in the number and proliferative function of splenic lymphocytes in patients with PH. This was a very interesting study.

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