Original Article

Individualized Immunosuppressive Protocol of Liver Transplant Recipient Should be Made Based on Splenic Function Status

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Abstract

Background: Lymphocyte subsets play important roles in rejection in liver transplant recipients, and the effect of splenic function on these roles remains unknown. The aim of this study was to explore the feasibility to adjust immunosuppressive agents based on splenic function status through detecting the lymphocyte subsets in liver transplant recipients.

Methods: The lymphocyte subsets of 49 liver transplant recipients were assessed in the 309th Hospital of Chinese People's Liberation Army between June 2014 and August 2015. The patients were divided into splenectomy group ($n = 9$), normal splenic function group ($n = 24$), and hypersplenism group ($n = 16$). The percentages and counts of CD4+ T, CD8+ T, natural killer (NK) cell, B-cell, regulatory B-cell (Breg), and regulatory T-cell (Treg) were detected by flow cytometer. In addition, the immunosuppressive agents, histories of rejection and infection, and postoperative time of the patients were compared among the three groups.

Results: There was no significant difference of clinical characteristics among the three groups. The percentage of CD19+CD24+CD38+ Breg was significantly higher in hypersplenism group than normal splenic function group and splenectomy group (3.29 ± 0.97% vs. 2.12 ± 1.08% and 1.90 ± 0.99%, $P = 0.001$). The same result was found in CD4+CD25+$FoxP_3$+ Treg percentage (0.97 ± 0.39% vs. 0.54 ± 0.31% and 0.56 ± 0.28%, $P = 0.001$). The counts of CD8+ T-cell, CD4+ T-cell, and NK cell were significantly lower in hypersplenism group than normal splenic function group (254.25 ± 149.08 vs. 476.96 ± 225.52, $P = 0.002$; 301.69 ± 154.39 vs. 532.50 ± 194.42, $P = 0.000$; and 88.56 ± 63.15 vs. 188.33 ± 134.51, $P = 0.048$). Moreover, the counts of CD4+ T-cell and NK cell were significantly lower in hypersplenism group than splenectomy group (301.69 ± 154.39 vs. 491.89 ± 132.31, $P = 0.033$; and 88.56 ± 63.15 vs. 226.00 ± 168.85, $P = 0.032$).

Conclusion: Splenic function status might affect the immunity of liver transplant recipients, that should be considered when we make immunosuppressive protocols.

Key words: Hypersplenism; Liver Transplantation; Lymphocyte Subsets; Sirolimus

Introduction

A goal in organ transplantation is the induction of specific immune tolerance or "almost tolerance." Immunosuppressive agents play a vital role in stabilizing recipient immunity to accomplish this goal. However, excessive immunosuppressive therapy increases the incidence of adverse effects such as infection and neoplasm.[1,2] The dosage of immunosuppressive agent is adjusted based on the drug concentration and the weight and age of the patient. Individualized therapy based on specific immune status is generally not included in these considerations. The basis of rejection in organ transplant recipients is the activity of lymphocytes[3] and immunosuppressive agents mainly act on patient's lymphatic system.[4,5] Therefore, the patient's lymphocyte subset status is considered an efficient parameter to reflect the possibility of rejection and the demand of immunosuppressive agents in

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organ transplant recipients. Lymphocyte subsets play various roles in immune responses to transplants.[6,7] CD8+ T-cells, CD4+ Th1 cells, NK cells, and effector B-cells participate in graft rejection; by contrast, CD4+ Th2, in particular, Treg and Breg play important roles in immune tolerance.[8-11] Many studies evaluated the immunity of organ transplant recipients through investigating lymphocyte subsets.[12-14] Spleen, the largest lymphoid organ in the human body, induces and regulates immune responses.[15-19] Liver cirrhosis and its associated hypersplenism are common among liver transplant recipients.[20-23] Macrophages in the hypertrophied spleen can destroy circulating blood cells,[24] including leukocytes, red blood cells, and platelets, resulting in pancytopenia. Liver transplantation can reduce portal vein pressure and alleviate hypersplenism. However, severe hypersplenism is not always completely eliminated after transplantation. Therefore, cirrhosis-associated hypersplenism is an important factor affecting the total number and subsets of peripheral blood lymphocytes.[17,25,26] Previous studies have indicated that peripheral lymphocytes in both viral hepatitis-derived and immune-mediated cirrhosis patients play important roles in virus activation[27] and the immune response.[28,29] The influence of viral hepatitis or autoimmune hepatitis on lymphocytes subsets disappears with the clearance of hepatitis virus and the alleviation of autoimmune reactions following liver transplantation. Immunosuppressive agents and hypersplenism critically affect lymphocyte subsets postoperatively. Which subsets does hypersplenism primarily affect, and to what degree? Does this contribute to graft rejection? Can adjusting immunosuppressive agent balance the effect of hypersplenism to lymphocyte subsets? These challenging questions warrant attention and evaluation in a clinical setting. This study attempted to determine the role of hypersplenism in the possibility of rejection in patients undergoing liver transplantation through assessing peripheral blood lymphocyte subsets. It also clinically investigated whether lymphocyte subset changes could influence graft rejection and whether immunosuppressive agents should be adjusted based on splenic function.

Methods

Patients and ethics statement

The blood samples were obtained between June 2014 and August 2015 from 49 liver transplant recipients; those were selected from 98 patients who underwent liver transplantation between October 2009 and December 2014 in the 309th Hospital of Chinese People's Liberation Army. The exclusion criteria were as follows: Postoperative time <6 months or >60 months (n = 18); hepatitis B or C recurrence (n = 4); hepatocellular carcinoma (HCC) recurrence or new malignant tumor (n = 8); severe diabetes (n = 6); gout or other metabolic diseases (n = 2); rejection diagnosed within 2 months prior to blood test (n = 4); or biliary complications (n = 7). All liver transplant operations of the included cases were in accordance with the *Declaration of Helsinki* and abided by the relevant laws and regulations of the People's Republic of China. Donor livers for all included cases were harvested in accordance with the current regulations of the Chinese government and the guidelines of the *Declaration of Helsinki*. All liver transplant operations and the present study were approved by the Ethics Committee of the 309th Hospital of Chinese People's Liberation Army. Written informed consent was obtained from each patient at the time of surgery, and there was no risk of breaching confidentiality.

Grouping criteria

A total of 49 cases were classified into three groups according to splenic function: a splenectomy group, a normal splenic function group (control group), and a hypersplenism group. The hypersplenism criteria were as follows: (1) platelet count $<75 \times 10^9$/L in three consecutive examinations and (2) spleen volume (based on computed tomography) >400 cm^3.[30] Patients who met at least one of the above criteria were classified into the hypersplenism group (n = 16). Patients who had undergone splenectomy were classified into the splenectomy group (n = 9). Patients who did not meet the above criteria and without splenectomy were classified into the normal splenic function group (n = 24).[31-33] The clinical data of subjects in the three groups are shown in Table 1. The postoperative time refers the time from transplantation to sampling time-point. The lymphocyte subsets proportions and counts were investigated in the three groups. Acute rejection was diagnosed in two splenectomy cases, four normal splenic function cases, and one hypersplenism case at least 2 months prior to the test date. One splenectomy case, one normal splenic function case, and four hypersplenism cases experienced infection after liver transplantation. The lymphocyte subsets were compared according to previous rejection or not and infection or not, too.

Immunosuppressive protocol

Tacrolimus, mycophenolate mofetil, and steroids were used in all cases. Sirolimus was used in patients with HCC or renal insufficiency. The tacrolimus dosage was halved when sirolimus was administered. Tacrolimus and sirolimus were administered orally with the dose titrated based on the combined blood concentration to achieve target ranges of 8–12 ng/ml during the first 3 months, 5–8 ng/ml from the 4th to the 12th month, and 3–5 ng/ml thereafter in all patients. A total of 1000 mg/d mycophenolate mofetil was orally administered, progressively tapered, and finally withdrawn at the end of the 6th month. Intravenous methylprednisolone (500 mg) was intraoperatively administered. Then, 20 mg/d prednisone was administered orally, progressively tapered, and finally withdrawn at the end of the 1st month. Finally, sirolimus was administered in 25 cases of the enrolled 49 patients. The lymphocyte subsets were also analyzed according to sirolimus application or not.

Laboratorial methods

Fluorochrome-conjugated human monoclonal antibodies (CD3-fluorescein isothiocyanate [FITC]/CD4-allophycocyanin [APC]/CD8-phycoerythrin [PE]/CD45-Percp, CD3-FITC/CD16+CD56-PE/CD19-APC/CD45-Percp, CD4-FITC, CD25-PE, CD19-FITC, CD24-PE, CD38-APC) were obtained from BD PharMingen (San

Table 1: Characteristics of subjects undergoing liver transplantation in three groups

Variables	Splenectomy group (n = 9)	Normal splenic function group (control group) (n = 24)	Hypersplenism group (n=16)	Statistics	P
Gender (male/female)	8/1	23/1	12/4	0.250*	0.083
Age (years)	53.44 ± 4.67	50.88 ± 6.82	49.63 ± 5.02	1.195†	0.312
Postoperative time (months)	34.67 ± 16.19	28.25 ± 16.14	19.56 ± 15.54	2.834†	0.069
WBC count ($\times10^9$/L)	6.55 ± 1.75	5.12 ± 1.18	3.00 ± 1.06	25.548†	0.000
PLT count ($\times10^9$/L)	248.56 ± 94.37	153.54 ± 50.57	76.06 ± 41.63	25.685†	0.000
Spleen volume (cm^3)	–	304.56 ± 69.66	579.43 ± 136.35	8.402‡	0.000

Values are expressed as n or mean ± SD. *χ^2 values; †F values; ‡t values. –: Not applicable; WBC: White blood cell; PLT: Platelet; SD: Standard deviation.

Diego, CA, USA). Fluorochrome-conjugated $FoxP_3$-PE and its affiliated staining buffer set were purchased from eBioscience (San Diego, CA, USA). The percentages and counts of CD4+ T, CD8+ T, natural killer (NK) cell, B-cell, regulatory B-cell (Breg), and regulatory T-cell (Treg) were detected. The detection methods of CD4+ T, CD8+ T, NK, and B-cells were as follows: 50-μl peripheral whole blood was incubated with 5-μl CD3-FITC/CD4-APC/CD8-PE/CD45-Percp and 5-μl CD3-FITC/CD16+CD56-PE/CD19-APC/CD45-Percp, respectively, for 15 min at room temperature in the dark. The 500-μl FACS lysing solution (BD Biosciences, San Jose, CA, USA) was added to each tube for lysing the red blood cells. After a 10-min lysis period, the sample was analyzed by flow cytometer (BD FACSCalibur, San Jose, CA, USA). The 20-μl CD19-FITC, 5-μl CD24-PE, and 20-μl CD38-APC were used to mark Bregs. The detection procedure of Bregs was carried out following the above. Then Tregs, 100-μl peripheral blood was incubated with 5-μl CD4-FITC and CD25-PE for 15 min at room temperature in the dark. Red cells were then eliminated by incubation with lysing buffer for 50 min. After centrifugation, the sample was continued being incubated with 2-μl $FoxP_3$-PE for 30 min at room temperature in the dark, then washed by permeabilization buffer and assessed by flow cytometer. The results were analyzed using CellQuest™ Pro software (BD, San Jose, CA, USA). CD3 expression was regarded as the characteristic of T lymphocytes. Double staining of CD3+CD4+ or CD3+CD8+ were considered as CD4+ or CD8+ T-cells, respectively. B and NK cells were marked as CD19+CD3− and CD16+CD56+CD3−, respectively. Tregs were characterized by CD4+CD25+$FoxP_3$+ and Bregs were characterized by CD19+CD24+CD38+. The absolute values for each lymphocyte subset were obtained by multiplying the percentage of lymphocyte subsets and the absolute lymphocyte count.

Routine blood tests, hepatic and renal function tests, and tests of immunosuppressive agent concentrations in the blood were also performed.

Statistical analysis

Continuous data were tested for normal distribution and were expressed as the mean ± standard deviation (SD). Data from multiple groups were analyzed using one-way analysis of variance (ANOVA) with *post hoc* Bonferroni's correction. Categorical data were analyzed using Pearson's Chi-square test. Statistical analyses were performed using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA). The data were graphed using GraphPad Prism™ 5 software (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Influence of immunosuppressive agents and previous complications on lymphocyte subsets

The use of immunosuppressive agents is presented in Table 2. There were no significant differences (all groups) in the total dosages and concentrations of tacrolimus and sirolimus among the three groups. We then compared the proportion and count of the lymphocyte subsets according to the use of sirolimus. The Breg percentage was higher in sirolimus application patients than nonsirolimus application patients (2.80 ± 1.13% vs. 2.07 ± 1.10%, $P = 0.028$). The CD8+ T-cell count was lower in sirolimus application patients than nonsirolimus application patients (325.38 ± 161.31 vs. 449.09 ± 239.22, $P = 0.037$). However, further analysis revealed that the total drug concentration was higher in sirolimus application patients than nonsirolimus application patients (7.85 ± 3.13 vs. 4.40 ± 2.15, $P = 0.000$). Linear correlation analysis revealed a negative correlation between the CD8+ T-cell count and the immunosuppressive agent concentration ($P = 0.014$). The lymphocyte subsets were compared between previous rejection cases and nonrejection cases. CD8+ T-cell count was higher in previous rejection patients than nonrejection patients (560.29 ± 283.07 vs. 353.98 ± 182.13, $P = 0.014$). On the contrary, both the percentage and count of Treg were lower in previous rejection patients than nonrejection patients (0.32 ± 0.24% vs. 0.74 ± 0.37%, $P = 0.009$ and 4.03 ± 1.89 vs. 8.31 ± 4.90, $P = 0.030$), and both the percentage and count of Breg were also lower in previous rejection patients than nonrejection patients (1.20 ± 0.77% vs. 2.67 ± 1.09%, $P = 0.001$ and 16.36 ± 12.79 vs. 30.10 ± 16.71, $P = 0.044$). There was no statistical difference in the infection complication incidence among the three groups (4/16 vs. 1/24 vs. 1/9, $P = 0.832$). Although some lymphocyte subsets were related to sirolimus application, previous rejection, there were no significant differences in the above factors among different splenic function status groups.

Table 2: Comparisons of immunosuppressive agents and previous complications among the three groups

Variables	Splenectomy group (n = 9)	Normal splenic function group (control group) (n = 24)	Hypersplenism group (n = 16)	Statistics	P
Cases using SRL	6	11	8	0.042*	0.776
Tacrolimus concentration (ng/ml)	3.700 ± 2.258	3.375 ± 1.569	4.669 ± 2.494	1.971†	0.151
Total concentration of tacrolimus and SRL (ng/ml)	6.500 ± 4.247	5.525 ± 2.744	7.131 ± 3.149	1.262†	0.293
Total dosage of tacrolimus and SRL (mg)	0.029 ± 0.015	0.030 ± 0.017	0.043 ± 0.022	2.826†	0.070
Cases with previous rejections	2	4	1	0.167*	0.252
Cases with previous infection	1	1	4	0.191*	0.190

Values are expressed as n or mean ± SD. *χ^2 values; †F values. SRL: Sirolimus; SD: Standard deviation.

Influence of splenic function status on distributions of lymphocyte subsets

The distributions of the peripheral blood lymphocyte subsets in the three groups are summarized in Figure 1. When the three groups were compared, the proportion of Breg and Treg differed significantly among the three groups (1.90 ± 0.99% vs. 2.12 ± 1.08% vs. 3.29 ± 0.97%, P = 0.001 and 0.56 ± 0.28% vs. 0.54 ± 0.31% vs. 0.97 ± 0.39%, P = 0.001). When in-deep pairwise comparison was performed between each two groups, the percentage of Breg was significantly higher in the hypersplenism group than the normal splenic function group (3.29 ± 0.97% vs. 2.12 ± 1.08%, P = 0.003), and splenectomy group (3.29 ± 0.97% vs. 1.90 ± 0.99%, P = 0.007), and the percentage of Treg was also higher in the hypersplenism group than the normal splenic function group (0.97 ± 0.39% vs. 0.54 ± 0.31%, P = 0.001) and splenectomy group (0.97 ± 0.39% vs. 0.56 ± 0.28%, P = 0.015). There were no significant differences in the percentages of CD8+ T-cell, CD4+ T-cell, NK cell, or B-cell between any two groups.

Influence of splenic function status on counts of lymphocyte subsets

The count data for the peripheral blood lymphocyte subsets in the three groups are presented in Figure 2. These data differed from the distribution data above. CD8+ T-cell, CD4+ T-cell, and NK cell counts differed significantly among the three groups (363.78 ± 118.65 vs. 476.96 ± 225.52 vs. 254.25 ± 149.08, P = 0.003; 491.89 ± 132.30 vs. 532.50 ± 194.42 vs. 301.69 ± 154.39, P = 0.001; and 226.00 ± 168.85 vs. 188.33 ± 134.51 vs. 88.56 ± 63.15, P = 0.016), particularly CD8+ T-cell and CD4+ T-cell. An in-deep pairwise comparison was performed concurrently among the three groups. CD8+ T-cell, CD4+ T-cell, and NK cell counts were significantly lower in the hypersplenism group than those in the normal splenic function group (254.25 ± 149.08 vs. 476.96 ± 225.52, P = 0.002; 301.69 ± 154.39 vs. 532.50 ± 194.42, P = 0.000; and 88.56 ± 63.15 vs. 188.33 ± 134.51, P = 0.048). In addition, CD4+ T-cell and NK cell counts were significantly lower in the hypersplenism group than the splenectomy group (301.69 ± 154.39 vs. 491.89 ± 132.31, P = 0.033 and 88.56 ± 63.15 vs. 226.00 ± 168.85, P = 0.032). There were no significant differences in the lymphocyte subset counts between the splenectomy group and the normal splenic function group. There were also no significant differences in B-cell, Breg, or Treg counts between any two groups.

Discussion

Immunosuppressive agents are still necessary for organ transplant recipients. It is beneficial for patient's long-term survival to minimize the immunosuppressive agents in consideration of their adverse effects. The basis of rejection or tolerance in organ transplant recipient is the activity of lymphocytes, different subsets of which play different roles in rejection and tolerance.[3,7] Immunosuppressive agents mainly act on patients' lymphatic system. Changes in peripheral blood lymphocyte subsets might reflect the possibility of rejection in patients undergoing liver transplantation. Determining these changes in lymphocyte subsets would facilitate the development of appropriate immunosuppressive protocols. Different preoperative primary diseases lead to variations of lymphocyte subset levels.[34,35] Lymphocyte subsets in the different stages of a disease are not stable, even in the same patient.[36] Lymphocyte subsets in liver transplant recipients gradually stabilize with the disappearance of primary disease and the improvement of the physical state following the 6th postoperative month.[30,37] Immunosuppressive agents were simplified gradually in this period, and the dosages had not been adjusted based on the splenic function. Determining the influence of splenic function on lymphocyte subsets is helpful because the lymphocyte subsets have specific roles following organ transplantation. CD8+ T-cells are effector T-cells, which are directly involved in cellular rejection.[38,39] B-cells are involved in humoral rejection, and NK cells are an important component of innate immunity. CD4+ T-cells include types Th1 and Th2. The Th1 subset can play positive regulatory roles and induce cellular and humoral rejection.[7,40] Treg belongs to the Th2 subset.[41] The cells in this subset play negative regulatory roles and participate in inducing tolerance. Breg, a small part of B-cells, also plays a negative regulatory role and even induces immune tolerance by secretion of interleukin-10 and transforming growth factor beta.[10,11]

This study demonstrated that the Breg and Treg percentages were significantly higher in the hypersplenism group than the splenectomy and normal splenic function groups. However, there were no significant differences in the Breg and Treg counts among the three groups, possibly indicating that Breg and Treg had escaped macrophage phagocytosis. For the

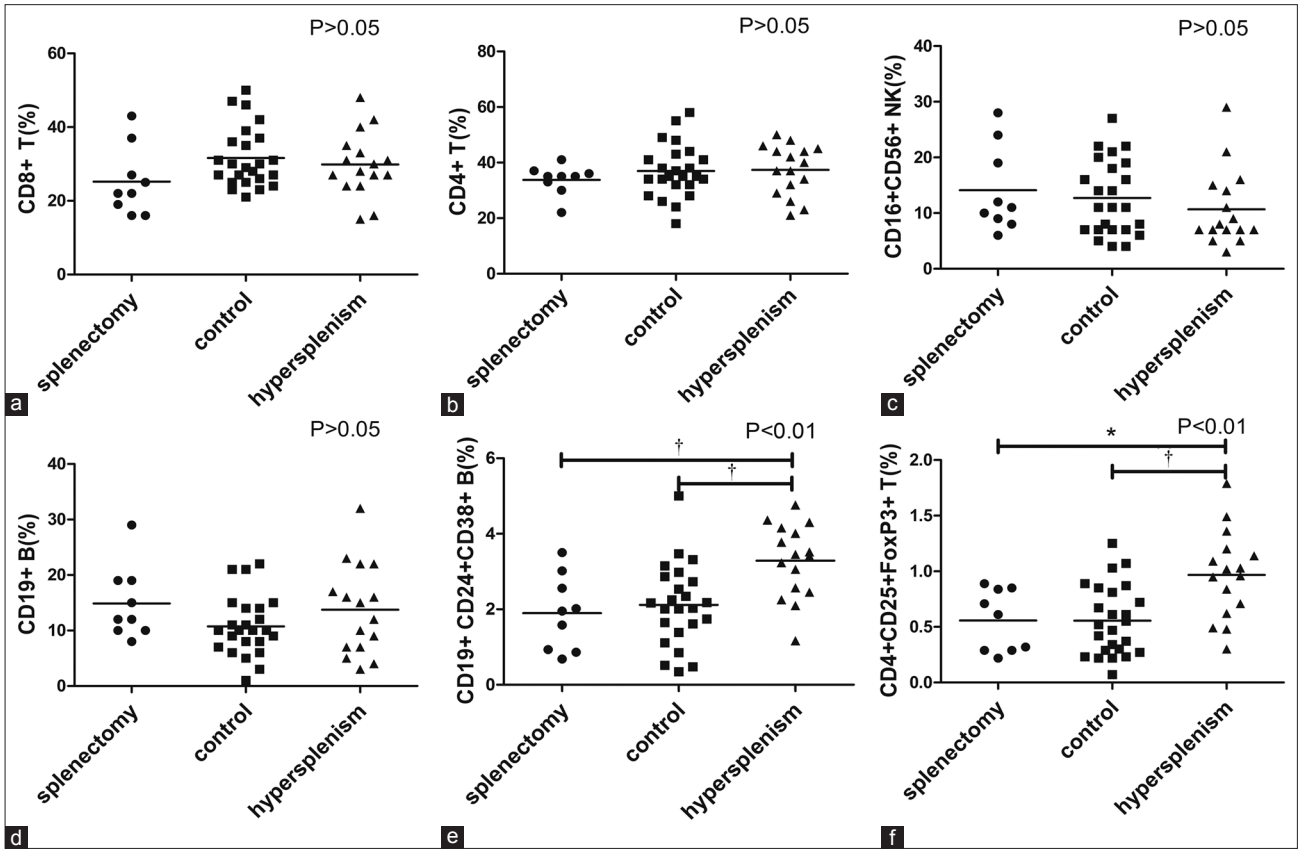

Figure 1: Comparison of the proportions of lymphocyte subsets among the three groups using one-way analysis of variance. (a) CD8+ T-cell percentage. (b) CD4+ T-cell percentage. (c) CD16+CD56+ NK cell percentage. (d) CD19+ B-cell percentage. (e) CD19+CD24+CD38+ regulatory B-cell percentage. (f) CD4+CD25+FoxP$_3$+ regulatory T-cell percentage. *P* values among the three groups are indicated in the top right corner. Positive *P* values between two groups are indicated by lines (*$P < 0.05$; †$P < 0.01$). NK: Natural killer.

higher Treg percentage in hypersplenism group, Guo *et al*. gave another explanation that hypersplenism stimulates an increase in Treg.[26] If macrophage could induce Tregs amplification as Guo *et al*., reported, besides Treg percentage, a higher Treg count would be found in hypersplenism group than the other two groups. The proportions of the lymphocyte subsets that upregulate immunity were similar among the three study groups. However, lymphocyte subset counts revealed a different pattern. The counts of CD8+ T-cell, CD4+ T-cell, and NK cell were lower in the hypersplenism group than the normal splenic function group. In addition, the CD4+ T-cell count was lower in the hypersplenism group than the splenectomy group, but there were no significant differences in lymphocyte subset counts between the splenectomy group and the normal splenic function group. These results indicate that the phagocytosis of macrophages in the hypertrophied spleen is primarily aimed at the immunoenhancement lymphocyte subsets. Thus, the immunity of a patient with hypersplenism might be lower than that of a patient with normal splenic function or splenectomy due to differences in the counts of lymphocyte subsets. Because of the differences of lymphocyte subsets between hypersplenism patients and nonhypersplenism patients, rejection occur less frequently in patients with hypersplenism following liver transplantation. However, these patients might be at high risk of infections and new tumors. Immunosuppressive agents could be administered on an individualized basis according to immune status.

Tacrolimus and sirolimus can all affect the lymphocyte subsets in different extent even though the immunosuppressive mechanisms of them are different.[42-44] Immunosuppressive agent use and previous rejections were also retrospectively analyzed in this study. There were no significant differences in the total dosages or concentrations of tacrolimus and sirolimus among the three groups. CD8+ T-cell counts were lower in patients using tacrolimus and sirolimus than in those using only tacrolimus. However, the total drug concentration in patients using tacrolimus and sirolimus was higher than the tacrolimus concentration in patients using only tacrolimus. Although the calculation of mixing tacrolimus and sirolimus concentrates together seemed not absolutely reasonable, this result was an indirect evidence of CD8+ T-cell suppression by immunosuppressive agents. The incidence of previous rejection was slightly but not significantly higher in the normal splenic function group and splenectomy group than in the hypersplenism group, and the statistic result of incidence of infection complication reversed among the three groups, these differences were likely due to the small number of patients included. Significantly higher CD8+ T-cell count has been reported in liver transplant recipients with acute rejection.[45] Seven patients with previous rejections in our study also had higher

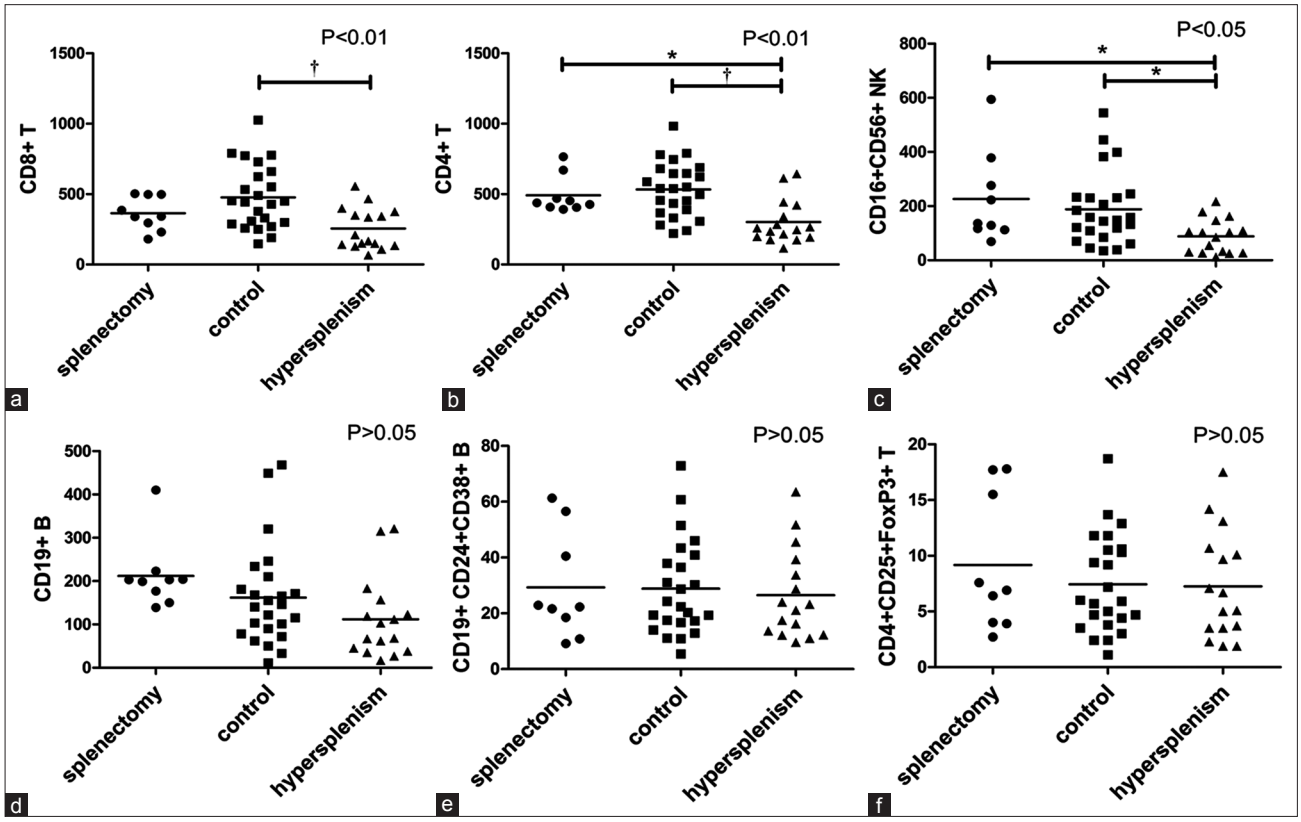

Figure 2: Comparison of the counts of lymphocyte subsets among the three groups using one-way analysis of variance. (a) CD8+ T-cell count. (b) CD4+ T-cell count. (c) CD16+CD56+ NK cell count. (d) CD19+ B-cell count. (e) CD19+CD24+CD38+ regulatory B-cell count. (f) CD4+CD25+FoxP$_3$+ regulatory T-cell count. *P* values among the three groups are indicated in the top right corner. Positive *P* values between two groups are indicated by lines (*$P < 0.05$; †$P < 0.01$). NK: Natural killer.

CD8+ T-cell counts, suggesting a close relationship between CD8+ T-cells and rejection. Both the proportion and count of Breg and Treg were lower in patients with previous rejection than in patients without previous rejection, indicating that immunosuppressive therapy should be enhanced in patients with previous rejection. Sirolimus might increase systemic Tregs, DCregs, and immunoregulatory proteogenomic signatures in liver transplant recipients.[46] The failure to observe similar increases with administration of sirolimus in the present study might be due to the combined use of tacrolimus and sirolimus. The effect of sirolimus on Tregs was not assessed in this study.

Actually, some further divisional lymphocyte subsets might influence the immunity of liver transplant recipient, such as Th17, γδ T-cells. This study only investigated the common six lymphocyte subsets. A more comprehensive investigation of lymphocyte subsets in liver recipient with different splenic status should be further performed. In summary, splenic function can affect the immunity of liver transplant recipients via lymphocyte subsets. The formulation of immunosuppressive protocols should be made based on splenic function.

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Conflicts of interest

There are no conflicts of interest.

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