Splenic CD4+ and CD8+ T-cells highly expressed PD-1 and Tim-3 in cirrhotic patients with HCV infection and portal hypertension

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Abstract

Introduction: The spleen plays an important role in regulating the immune response to infectious pathogens. T-cells dysfunction and exhaustion have been reported in patients with hepatitis B/C virus (HBV/HCV) infection, which contributes to persistent virus infection. The aims of this study were to investigate spleen-related evidence of immunosuppression and immune tolerance in HCV cirrhotic patients with portal hypertension (PH).

Methods: The expression of programmed cell death 1 (PD-1), T-cell immunoglobulin domain and mucin domain-containing molecule-3 (Tim-3) and its ligand PD-L1/2, and Galectin-9 in the spleens and livers of HCV cirrhotic patients (n = 15) was analyzed using real-time PCR and immunohistochemistry. Flow cytometry was used to evaluate the expression of PD-1 and Tim-3 on splenic T-cells and the peripheral blood T-cells before and after splenectomy (n = 8).

Results: Spleens from patients with PH showed significantly increased mRNA levels of PD-L2, Tim-3, Galectin-9, CD80, and CD86, and decreased levels of CD28 compared to control spleens (spleens removed due to traumatic injury) (all p < 0.05). Additionally, protein expression of inhibitory signaling molecules was significantly increased in both the spleens and livers of cirrhotic patients compared with controls (all p < 0.05). Peripheral blood and splenic CD4+ and CD8+ T-cells also expressed higher protein levels of PD-1, Tim-3, and CTLA-4 in cirrhotic patients as compared with healthy controls (all p < 0.05). The proportion of PD-1+CD4+ T lymphocytes (26.2% ± 7.12% vs. 21.0% ± 9.14%, p = 0.0293) and Tim-3+CD8+ T lymphocytes (9.4% ± 3.04% vs. 6.0% ± 2.24%, p = 0.0175) in peripheral blood decreased followed splenectomy.

Conclusion: The CD4+ and CD8+ T-cells in spleen and peripheral blood highly expressed PD-1 and Tim-3 in HCV-infected and cirrhotic patients with portal hypertension.
Highly expressed PD-1 and Tim-3 in peripheral blood T-lymphocytes can be partly reversed following splenectomy.

Keywords
hepatitis C virus, portal hypertension, spleen, programmed cell death-1, Tim-3, splenectomy

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Introduction

Hepatitis C virus (HCV) has infected up to 70 million people worldwide, and it is the primary cause of cirrhosis, hepatocellular carcinoma (HCC), and liver-related deaths.1 Innate and adaptive immune responses play a critical role in the outcome of HCV infection, with HCV-mediated immunocyte dysfunction preventing 50%-80% of infected individuals from spontaneously clearing the infection, leading to the development of chronic hepatitis C (CHC).1,2 Direct-acting antivirals (DAAs) can successfully treat more than 95% of chronic HCV infections. However, about one-third of HCV infected patients present with cirrhosis, although there is a lack of data indicating the rate of portal hypertension (PH) in cirrhotic patients with HCV infection prior to therapy.3,4 In addition, the elimination of HCV by DAAs cannot completely reverse the immunocyte dysfunction, especially in patients with advance liver fibrosis.5-10

One of the major mechanisms driving the persistence of HCV infection is T-cell exhaustion, which results in weak antigen-specific T-cell responses. Persistent HCV antigen stimulation leads to exhaustion of virus-specific T-cells, characterized by changes in the expression of multiple co-signaling molecules, including programmed cell death 1 (PD-1), T-cell immunoglobulin domain, mucin domain-containing molecule-3 (Tim-3), and cytotoxic T-lymphocyte antigen-4 (CTLA-4), which limit proliferative capacity, impair cytokine secretion, and reduce the elimination of HCV.7,11,12 Studies on the modulation of HCV infection mainly focus on the liver and peripheral blood. The spleen, as the largest peripheral lymphoid organ, is often ignored despite its important role in regulation of the immune response to infectious pathogen.13 However, studies have shown that the spleen can modulate cytokine expression and lead to peripheral tolerance and immunosuppression in cirrhotic patients with hepatitis B virus (HBV) infection and PH.14,15

Splenectomy, a common surgical treatment for cirrhotic patients with PH and hypersplenism, can effectively reduce portal pressure and the risk of variceal hemorrhage and improve pancytopenia and liver function, as well as facilitate the interferon-based therapy for HCV-related cirrhotic patients with PH before the era of DAAs.16-19 Studies also demonstrated that peripheral tolerance in HCV-related cirrhosis is promoted by the pathologic spleen,20 and splenectomy can induce some beneficial immunological changes20,21 and decrease the viral burden in cirrhotic patients with HCV.22 Nevertheless, patients with PH in the spleen have immunosuppression and peripheral immune tolerance that need to be further explored. The aim of our study was to evaluate the immune function of the spleen in HCV-related cirrhosis with PH and to determine possible mechanisms.

Materials and methods

Patients and clinical data collection

A total of 15 HCV-related cirrhosis patients with PH and hypersplenism who underwent splenectomy were enrolled at the Second Affiliated Hospital of Xi’an Jiaotong University, Xi’an, China. This group is designated as the PH group. Fifteen patients with CHC and 15 sex- and age-matched healthy individuals who underwent routine health checkups in our hospital were enrolled as controls. The spleens of 5 patients who underwent splenectomy for trauma (trauma-spleen group) and the liver tissue of 3 patients who underwent hepatectomy for liver hemangiomia were selected as histopathological controls. The exclusion criteria included the following: (1) co-infection with HBV or human immunodeficiency virus; (2) autoimmune diseases; (3) connective tissue diseases; (4) liver and/or extraphepatic tumors; and (5) use of significant immunosuppression agents within 3 months of the collection of specimens. Data on clinical characteristics, including age, sex, blood cell counts, and liver function, were collected from the hospital’s electronic medical record database and reviewed retrospectively. The baseline characteristics of patients and controls are summarized in Table 1.

All subjects provided written informed consent. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Xi’an Jiaotong University in Xi’an City, Shaanxi Province, China (No. 2018-059).

Quantitative PCR

Total RNA was isolated from spleen tissue using the Trizol reagent (Invitrogen, Camarillo, CA, USA) and was quantified using NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized using a PrimeScript™ RT reagent kit with gDNA Eraser (Code No. RR047A; Takara, Shiga, Japan), following the manufacturer’s protocol. Quantitative real-time PCR (qPCR) was performed using TB green™ Premix Ex Taq™ II (Code No. RR820A; Takara) according to the
manufacturer’s instructions. Primers were designed using the mRNA sequence of target genes from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and Primer 5.0 (http://www.premierbiosoft.com/primerdesign/). PCR primer sequences for these reactions are listed in Table 2. The expression of each respective gene was normalized to β-actin RNA as an internal control. The primers were synthesized by GENEWIZ (South Plainfield, NJ, USA). Data analysis was performed by using the 2-ΔΔCt method; β-actin served as an internal control.

Immunohistochemical staining of spleen and liver specimens

A total of 15 spleen and liver specimens from PH group patients who underwent splenectomy were paraformaldehyde-fixed, paraffin-embedded, and sliced into 4-μm thick sections by microtome (RM2235; Leica Microsystems Inc., Wetzlar, Germany). Deparaffinized and rehydrated sections were subjected to immunostaining for the molecules PD-1 (EPR4877(2), 1:250), PD-L1 (EPR1161(2), 1:100), Tim-3 (ab185703, 1:100), Galectin-9 (ab69630, 4 μg/mL), CD28 (ab243228, 1:500), CD80 (ab134120, 1:200) (all from Abcam, Cambridge, MA, USA), PD-L2 (GTX85449, 2.5 μg/mL; GeneTex, Inc., Irvine, CA, USA), CTLA4 (SC-376016, 1:100; Santa Cruz Biotechnology, Dallas, TX, USA), and CD86 (13395-1-AP; Proteintech, Rosemont, IL, USA). We measured the same molecules in 5 spleens that had been removed due to traumatic injury, serving as controls (trauma-spleen group). The sections underwent heat-mediated antigen retrieval with citrate buffer, pH 6, or Tris-EDTA buffer, pH 9, followed by incubation with primary antibody overnight at 4 °C. Then, the sections were washed and incubated sequentially with biotinylated antibody and peroxidase-labeled streptavidin, in accordance with the manufacturers’ instructions. The sections were visualized with diaminobenzidine-chromogen (DAB) and observed under a light microscope (E100; Nikon Corp., Tokyo, Japan). Image analysis was employed for semiquantitative assessment of the immunostained specimens. Each processed specimen was evaluated in a blinded fashion. Ten fields per section were selected randomly and then quantified using Image-Pro Plus 6.0 software (Media Cybernetics Inc, Rockville, MD, USA) to determine the mean density of the positive stained area for the respective PD-1, PD-L1, PD-L2, CTLA4, CD28, CD80, CD86, Tim-3, and Galectin-9. Mean density of the total fields examined in each specimen was equal to their integrated optical density (IOD) divided by the area of the positive stained distribution area (mean density = IOD/area).

Isolation of mononuclear cells

Peripheral blood (before and 2-weeks after splenectomy) and spleen samples from 8 out of 15 HCV-related cirrhotic patients with PH, as well as 5 trauma-spleen samples, were collected for the isolation of mononuclear cells. Peripheral blood samples from 15 CHC patients and 15 healthy subjects were also collected for the control groups. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (GE Healthcare, Helsinki, Finland) gradient centrifugation. Single-cell suspensions of freshly obtained spleen tissues were prepared using a gentle MACS tissue dissociator (Miltenyibiotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Dissociated cells were filtered through 100 μm nylon mesh and separated by Ficoll-Hypaque gradient centrifugation to obtain spleen mononuclear cells (SMCs). All cells were washed, cryopreserved in fetal calf serum containing 10% dimethylsulphoxide (commonly referred to as DMSO), and stored in liquid nitrogen until use.

Flow cytometry

This study used the following human antibodies for flow cytometry to evaluate the expression of PD-1, CTLA-4, and Tim-3 on splenic T-cells and the peripheral blood T-cells: FITC-anti-CD4, FITC-anti-CD8, PerCP-Cy5.5-anti-PD-1 (CD279), PE-anti-CTLA4 (CD154), and PE-Cy7-anti-
CD3, all from BD Biosciences (Franklin Lakes, NJ, USA), and APC-anti-Tim-3 from eBioscience (San Diego, CA, USA). The control cells were stained with corresponding isotype-matched control antibodies. Cryopreserved PBMCs (0.5–1 × 10⁶ cells) were stained with the above surface antigen antibodies at 4 °C in the dark for 30 min, washed twice in 2 mL phosphate-buffered saline (commonly referred to as PBS) containing 1% bovine serum albumin, and fixed in 400 μL of 1% paraformaldehyde. The stained cells were detected using the FACSAria II Flow Cytometer (BD Biosciences) and analyzed using the accompanying FACSDiva 7.0 and FlowJo 7.6 software.

Statistical analysis
All data were analyzed with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA) and presented as mean ± standard deviation. Differences between the expression of mRNA and protein in spleen and liver specimens were analyzed using the Mann–Whitney U test. Differences between the expression of PD-1 and Tim-3 in patients’ peripheral blood and spleen, and peripheral blood pre- and post-surgery, were analyzed using the paired t-test or Mann–Whitney U test. A two-tailed p < 0.05 was considered statistically significant.

Results
Upregulated inhibitory signaling molecules mRNA in the spleens of patients with HCV-related cirrhosis with PH compared with trauma spleen

Real-time PCR demonstrated an increase in mRNA expression levels in spleens from patients with HCV-related cirrhosis with PH (PH group) as compared to those that experienced traumatic injury (trauma-spleen group) for PD-1 (3.87 ± 2.96 vs 1.51 ± 0.72, p = 0.027), PD-L2 (3.09 ± 1.48 vs 1.02 ± 0.32, p = 0.012), CD80 (3.98 ± 2.58 vs 1.9 ± 0.89, p = 0.038), CD86 (5.47 ± 3.08 vs 3.07 ± 1.36, p = 0.015), and Tim-3 (2.37 ± 1.95 vs 1.25 ± 0.42, p = 0.038) (Figure 1(a)–(c)), while the mRNA expression of the co-stimulatory molecule CD28 in the spleens from patients in the PH group was significantly lower than that found in the spleens of the trauma-spleen group (0.99 ± 0.31 vs 1.59 ± 0.71, p = 0.035) (Figure 1(c)). However, the mRNA expression of PD-L1 (2.89 ± 1.76 vs 1.79 ± 0.85, p = 0.222), CTLA-4 (1.3 ± 0.75 vs 1.51 ± 0.51, p = 0.575), and Galectin-9 (1.15 ± 0.48 vs 1.05 ± 0.20, p = 0.356) was not different between the two groups (Figure 1(a)–(c)).

Upregulated expression and distribution of co-signaling molecules and their ligands in spleens of patients with HCV-related cirrhosis compared with trauma spleen

Evaluation of the spleens from patients in the PH group had fewer splenic follicles, expanded white pulp and marginal zone areas, and significantly reduced numbers of CD4, CD8, and HLA-DR cells as compared to the trauma-spleen group (Figure S1). The spleens from patients in the PH group also had significantly higher expression of PD-1, PD-L2, CD80, CD86, Tim-3, and Galectin-9 but significantly lower expression of CD28 than those in the traumaspleens (all p < 0.05) (Figure 2(a) and (b)). PD-L1 and

Table 2. Primer sequences of co-signaling molecules and their ligands used for qRT-PCR.

| Gene      | Genbank accession | Primer sequences | PCR product size, bp |
|-----------|-------------------|------------------|----------------------|
| PD-1      | NM_005018         | F: 5’- AAGGGCCAGATCAAGAGAGGAC-3’ | 124 |
| PD-L1     | NM_014143         | F: 5’- TGCCGACTCAGGAATTCTAGCTG-3’ | 150 |
| PD-L2     | NM_025239         | F: 5’- CTGTTGGTCCAGATCTGGTGGTC-3’ | 149 |
| CTLA4     | NM_005214         | F: 5’- ACGGAACTCTACATTCTGGCAAG-3’ | 121 |
| CD28      | NM_006139         | F: 5’- GAGAAGAGCAATGGAACCATTATG-3’ | 122 |
| CD80      | NM_005191         | F: 5’- CTCTTGXTGCTGGTGTTCCT-3’ | 136 |
| CD86      | NM_175862         | F: 5’- CCATCAGCTTGTCGTGTTCC3’ | 154 |
| Tim-3     | NM_032782         | F: 5’- GACTCTAGCAACGACTGAGGATC-3’ | 163 |
| Galectin-9| NM_009587         | F: 5’- ACACCCAGATCGAACAATCCTG-3’ | 144 |
| β-actin   | NM_001101         | F: 5’- CACATTTGCCGAATGAGCGGTC3’ | 135 |
CTLA-4 expression levels were not significantly different between the two groups (Figure 2(a) and (b)).

As shown in Figure 2(a), PD-1 was primarily found in the marginal and red pulp zones of the spleen, and predominantly localized to the cellular membrane rather than in the cytoplasm. PD-L1 and PD-L2 were primarily found in T-cell zone dendritic cells, the red pulp, the capillary endothelium of periarterial lymphatic sheath (known as PALS), and the arteriolar and sinusoidal endothelium, and mainly localized within cellular membranes and the endomembrane system. Tim-3 was mainly found in the cellular membranes within the red pulp zone of the spleen, while Galectin-9 was mainly expressed in cytoplasm of the red pulp zone and marginal zone but less so in the white pulp (Figure 2(a)).

In the spleens of the trauma-spleen group, CD28 was present to a greater extent in the white pulp than in the red pulp, and mainly localized in the cellular membrane and cytoplasm. However, only a few CD28-positive cells were seen in the PH-spleen. CTLA-4 and CD80 were widely distributed in the cellular membrane and cytoplasm of the spleen, while CD86 was mainly localized in the cellular membrane of red pulp but absent in the white pulp (Figure 2(b)).

Upregulated expression and distribution of co-signaling molecules and their ligands in the liver compared with the control liver

Liver specimens from patients in the PH group had significantly more cells that expressed PD-1, PD-L1, PD-L2, Tim-3, Galectin-9, CTLA-4, CD28, CD86, and CD80 compared with control livers (all \( p < 0.05 \)) (Figure 3(a) and (b)). Increased PD-1-, PD-L1-, and PD-L2-positive cells in the livers of HCV-related cirrhosis patients with PH were mainly distributed in liver-infiltrating lymphocytes centered on portal tracts and the periportal area or extending to hepatic parenchyma, and the expression intensity of PD-L2 was higher than that of PD-L1. A few scattered PD-1-, PD-L1-, and PD-L2-positive cells could be observed in normal livers. Tim-3-, Galectin-9-, CTLA-4-, and CD80-positive
cells were also increased in the livers of HCV-related cirrhosis patients with portal hypertension, and they were mainly distributed in the liver parenchymal cells and infiltrating inflammatory cells. These positive cells were mainly located in hepatic lobules and portal area (Figure 3(a) and (b)). Similarly, Tim-3-, Galectin-9-, CTLA-4-, and CD80-positive cells could be observed in normal control livers, albeit in fewer numbers. CD28- and CD86-positive cells were rarely seen in control liver parenchymal cells, but primarily found in infiltrating inflammatory cells of portal area (Figure 3(b)).

Figure 2. Immunohistochemical staining for the expression and distribution of co-signaling molecules and their ligands in the spleens of HCV-related cirrhotic patients with portal hypertension. (a) The PH-spleen had significantly higher expression of PD-1, PD L2, Tim-3, and Galectin-9; (b) The PH-spleen had higher expression of CD80 and CD86 but lower expression of CD28. *p < 0.05; **p < 0.01; n.s., not significant (p > 0.05). Trauma-spleen (n = 5); PH-spleen, portal hypertension-spleen (n = 15).
Upregulated inhibitory signaling molecules in the PH-splenic T-cells compared with trauma spleen

As seen in Figure 4, compared with the peripheral blood, the frequency of splenic CD8+T-cells that also expressed PD-1 (PD-1+CD8+T-cell: spleen 20.7% ± 3.27% vs. PBMC 15.6% ± 4.19%, \( p = 0.0084 \)) and Tim-3 (Tim-3+CD8+T-cell: spleen 13.5% ± 2.44% vs. PBMC 9.4% ± 3.04%, \( p = 0.0289 \)) was significantly higher in cirrhosis patients. However, the frequency of spleen and peripheral blood CD4+T-cells that expressed PD-1 (PD-1+CD4+T-cell: spleen 30.3% ± 9.77% vs. PBMC 26.2% ± 7.12%, \( p = 0.14 \)) and Tim-3 (Tim-3+CD4+T-cell: spleen 7.5% ± 1.52% vs. PBMC 8.4% ± 4.56%, \( p = 0.77 \)) is not different in cirrhosis patients (Figure 4).

The expression of PD-1 in splenic CD4+T-cells (PD-1+CD4+T-cell: 30.3% ± 9.77% vs. 12.9% ± 4.54%, \( p = 0.0031 \)) and CD8+T-cells (PD-1+CD8+T-cell: 20.7% ± 3.27% vs. 13.4% ± 2.89%, \( p = 0.0016 \)) was significantly higher in spleens from patients in the pH group as compared with trauma-spleen group. Similarly, the splenic CD4+T-cells (Tim-3+CD4+T-cell: 7.5% ± 1.52% vs. 4.9% ± 1.04%, \( p = 0.0186 \)) and CD8+T-cells (Tim-3+CD8+T-cell: 13.5% ± 2.44% vs. 6.4% ± 2.36%, \( p = 0.0016 \)) in cirrhotic patients with HCV infection have a higher expression of Tim-3 compared with those from patients with a traumatic spleen injury (Figure 4).

Expression of inhibitory signaling molecules in peripheral blood T-lymphocytes before and after splenectomy
As seen in Figure 5(a), the proportion of PD-1+CD4+ T-lymphocytes was significantly higher in patients in the PH group than that in either the CHC group (26.2% ± 7.12% vs. 15.0% ± 5.09%, \( p = 0.0027 \)) or the healthy control group (26.2% ± 7.12% vs. 9.4% ± 4.45%, \( p = 0.0003 \)) in peripheral blood. Patients in the PH also showed significantly higher proportion of PD-1+CD8+ T-lymphocytes than that either the CHC group (15.6% ± 4.19% vs. 9.3% ± 2.86%, \( p = 0.0014 \)) or the healthy control group (15.6% ± 4.19% vs. 6.6% ± 2.73%, \( p = 0.0003 \)) in peripheral blood.
The proportion of PD-1⁺CD4⁺ T-lymphocytes in peripheral blood decreased following splenectomy (21.0% ± 9.14% vs. 26.2% ± 7.12%, p = 0.0293); however, the percentage of PD-1⁺CD8⁺ T-cells was not statistically different in patients in the PH group before and after having a splenectomy (p > 0.05).

Patients in the PH group had a significantly higher proportion of Tim-3⁺CD4⁺ T-lymphocytes than either the CHC group (8.4% ± 4.56% vs. 4.9% ± 2.0%, p = 0.049) or the healthy control group (8.4% ± 4.56% vs. 3.7% ± 1.26%, p = 0.0074) in peripheral blood (Figure 5(b)). Patients in the PH group also showed significantly higher proportion of Tim-3⁺CD8⁺ T-lymphocytes than either the CHC group (9.4% ± 3.04% vs. 6.9% ± 2.16%, p = 0.042) or the healthy control group (9.4% ± 3.04% vs. 6.0% ± 1.88%, p = 0.0089) in peripheral blood. The proportion of Tim-3⁺CD8⁺ T-lymphocytes in peripheral blood decreased following splenectomy (9.4% ± 3.04% vs. 6.0% ± 2.24%, p = 0.0175). However, the percentage of Tim-3⁺CD4⁺ T-cells was not different pre- and post-splenectomy in HCV-related cirrhotic patients (p > 0.05) (Figure 5(b)).

**Expression of PD-1⁺ Tim-3⁺ T-cells in HCV-related cirrhotic patients**

As seen in Figure 6(a), patients in the PH group had a significantly higher proportion of PD-1⁺Tim-3⁺CD4⁺ T-lymphocytes in the spleen (6.88% ± 1.34% vs. 3.52% ± 0.87%, p = 0.0016) and in their peripheral blood CD4⁺ T-lymphocytes (6.88 ± 1.34% vs. 4.11 ± 1.87%, p = 0.01) than in the patients in the trauma-spleen group. The proportion of PD-1/Tim-3-double-positive CD4⁺ T-lymphocytes
in peripheral blood decreased following splenectomy (before vs. after: 4.11% ± 1.87% vs. 3.11% ± 1.82%, \( p = 0.0264 \)). Similarly, patients in the PH group had a significantly higher proportion of PD-1\(^+\)Tim-3\(^+\)CD8\(^+\) T-lymphocytes in their spleens (6.04% ± 3.1% vs. 2.58% ± 1.2%, \( p = 0.019 \)) and peripheral blood CD8\(^+\)T-lymphocytes (6.04% ± 3.1% vs. 3.18% ± 1.82%, \( p = 0.046 \)) than the trauma-spleen group. The proportion of PD-1/Tim-3-double-positive CD8\(^+\)T-lymphocytes in peripheral blood decreased following splenectomy (before vs. after: 3.12% ± 1.82% vs. 2.28% ± 1.31%, \( p = 0.013 \)) (Figure 6(b)).

**Discussion**

The spleen is a secondary lymphatic organ of sophisticated histological structure and multifunction that is mainly engaged in immune surveillance of the blood.\(^{13}\) The present study was therefore aimed to clarify the relationship between spleens from patients with PH and T-cell exhaustion in HCV-related cirrhosis. Our results demonstrate that higher expression of inhibitory signaling molecule markers in the liver, spleen, and PBMCs, especially the splenic CD4\(^+\) and CD8\(^+\) T-cells, have higher levels expression of PD-1 and Tim-3, and PH-spleens have higher levels of PD-L2 and Galectin-9-expressing cells. Of note, T-cell function improves following splenectomy, as indicated by decreased expression of PD-1 and/or Tim-3 in peripheral blood CD4\(^+\) and CD8\(^+\)T-cells. We speculate that the spleen acts as a reservoir of exhausted T-cells, and splenectomy may reduce the efflux of exhausted T-cells.

T-cell exhaustion contributes to the persistence of chronic virus infections, and is maintained by negative immune regulators, such as PD-1, Tim-3, and CTLA-4. The negative immune regulators on T-cells bind to their cognate ligand to deliver inhibitory signaling, which then inhibit T-cell reactions and cytokine production. Studies have reported that pathologic spleen may contribute to the failure to control HCV and promote peripheral tolerance.\(^{20,21}\)

Inhibitory signals through the PD-1 pathway regulate T-cell activation, T-cell tolerance, and T-cell exhaustion. Studies of PD-1 function which have focused primarily on effector T-cells showed PD-1 promotes immune exhaustion
Figure 5. Effect of splenectomy on the expression of inhibitory receptors (PD-1 and Tim-3) in peripheral blood T-lymphocytes. (a) The frequencies of PD-1-expressing CD4+ and CD8+ T-cells were significantly decreased in peripheral blood after splenectomy. (b) The frequencies of Tim-3-expressing CD8+ T-cells were significantly decreased in peripheral blood after splenectomy. H-control, healthy-control (n = 15); CHC, chronic hepatitis C (n = 15); Pre-surgery (n = 8); Post-surgery (n = 8). *p < 0.05; **p < 0.01; ***p < 0.001; n.s., not significant (p > 0.05).

Figure 6. Effect of splenectomy on the expression of PD-1+Tim-3+T-cells in HCV-related cirrhotic patients. (a) The frequencies of PD-1+Tim-3+T-cells-expressing CD4+ cells were significantly decreased in peripheral blood after splenectomy. (b) The frequencies of PD-1+Tim-3+T-cell-expressing CD8+ T-cells were significantly decreased in peripheral blood after splenectomy. Con-S, control-spleen (n = 5); LC-S, liver cirrhosis-spleen (n = 8); Pre-surgery (n = 8); Post-surgery (n = 8). *p < 0.05; ***p < 0.01; n.s., not significant (p > 0.05).
by inducing antiviral T-cell motility paralysis in the splenic marginal zone/red pulp zone, prolongs states of negative immune regulation, and directly inhibits T-cell receptor signaling by recruiting phosphatase SHP2. We found higher expression of PD-1 and its ligands, especially PD-L2, in the marginal zone and red pulp zone of spleens taken from patients with PH than that in the spleens from patients who had received trauma to the spleen. The frequency of PD-1 expression in splenic CD4+ and CD8+ T-cells was significantly higher in the PH-spleen than those in the trauma-spleen. In addition, the livers from HCV-related cirrhosis patients overexpress PD-1 in the liver-infiltrating lymphocytes, while the ligands, PD-L1 and PD-L2, were found to be overexpressed in Kupffer cells, liver sinusoidal endothelial cells, and leukocytes in our study. However, only a few scattered PD-1/PD-L1/PD-L2-positive cells were observed in control livers. These findings are consistent with previous reports. Moreover, expression of PD-1 was significantly upregulated in CD4+ and CD8+ T-cells in the peripheral blood of cirrhotic patients before splenectomy compared with those from healthy controls, but these levels were significantly reduced after splenectomy. These results support the possibility that activation of the PD-1 pathway induced by HCV infection in the PH-spleen contributes to peripheral immunotolerance in cirrhotic patients with HCV infection. However, splenectomy may partially improve T-cell impaired function and immune tolerance via reduction of exhaustion markers in our study.

The Tim-3/Galectin-9 pathway also contributes to T-cells exhaustion, playing important roles in persistent viral infection. Upregulation and immunomodulatory function of Tim-3 in viral specific CD4+ and CD8+ T-cells have been shown to contribute to viral persistence during chronic HBV/HCV infection, and the blockade of the Tim-3/Galectin-9 pathway restored T-cell proliferation and cytokine production in response to viral antigen stimulation. In line with these results, our data found significantly increased numbers of Tim-3/Galectin-9-double-positive cells in both the spleens and livers of patients with HCV-related cirrhosis, and the frequency of Tim-3-expressing CD4+ and CD8+ T-cells was increased in the PH-spleen compared to those in the trauma-spleen. These data suggest that upregulated Tim-3/Galectin-9 signaling in the PH-spleen is at least one of the probable causes of the prolonged immunosuppressive status of the spleen and may mediate T-cells immune exhaustion.

Furthermore, studies showed interferon-free DAA therapy-mediated HCV clearance does not fully restore the exhausted phenotype of HCV-specific CD4+ and CD8+ T-cells during chronic HCV infection, and the reversal of immune exhaustion phenotype is absent in patients with cirrhosis and advanced liver fibrosis. Our data found that splenectomy decreased the expression of PD-1 and/or Tim-3 in peripheral blood CD4+ and CD8+ T-cells, suggesting the PH-spleen in cirrhotic patients with HCV infection may induce immune tolerance by upregulating the expression of the immunosuppressive molecules in splenic CD4+ and CD8+ T-cells.

Patients with advanced liver fibrosis have an increased risk of developing HCC, and this may be associated with the co-expression of multiple inhibitory receptors. How the increased expression of the negative regulators of immune activation in the liver and spleen of patients with PH, including PD-1/Tim-3 and their ligands, as well as CD80 and CD86, may affect susceptibility to HCC remains to be clarified. Our previous study showed that the modulation of cytokine expression following splenectomy may reduce the risk of HCC development in HBV-related cirrhotic patients with PH. In another study that included a large cohort of 2678 patients, PH and hypersplenism were found to be correlated with increased risk of HCC and splenectomy was identified as a protective factor in HCC development. Thus, splenectomy to partly restore T-cell exhaustion in HCV-related cirrhotic patients with PH may be a potential therapeutic strategy to reduce the risk of HCC and improve prognosis.

There are some limitations in our study. (1) This study had a small sample size (n = 15), and only 8 out of the 15 patients have complete spleen and peripheral blood samples for evaluating the expression of PD-1, CTLA-4, and Tim-3 on splenic T-cells and the peripheral blood T-cells in baseline and/or 2-weeks post-splenectomy. (2) Due to HCV-related cirrhosis patients without PH not requiring a splenectomy, this study lacked spleens from this group. To minimize potential bias from this important group, traumatic spleens and peripheral blood of CHC patients without cirrhosis and healthy people were included as controls. However, we have not designed to access the expression of immune tolerance molecules in peripheral CD4+ and CD8+ T-cells in these cirrhosis patients without PH, further study to access the peripheral T cells function is needed. (3) We did not detect other T-cell exhaustion markers, for example, LAG-3 and TIGIT, on T-cells. (4) We did not measure the phenotype change of B-cells, which play critical roles on regulating immune response to infectious pathogens. (5) We had not assessed the effect of T-cell phenotype change on the clinical outcome of HCV cirrhotic patients. (6) We did not investigate the possible role that the spleen may play in the regulation of the expression of PD-L1/2, Tim-3, and Galectin-9 on non-immune cells in the liver.

**Conclusions**

We performed a detailed investigation of T-cell phenotypes in the spleen, liver, and peripheral blood of HCV-related cirrhosis patients with PH, and identified markers of T-cell exhaustion in the spleen and liver in this population. The CD4+ and CD8+ T-cells in the spleen and peripheral blood highly expressed...
immune tolerance molecules, such as PD-1 and Tim-3, and these highly expressed immune tolerance molecules in peripheral blood T-lymphocytes can be partly reversed following splenectomy. Mechanisms underlying these phenotypes need further study to provide a better understanding of immune dysregulation and will lead to the development of targeted therapies to improve their immune function.

**Author contributions**

FP Ji and ZF Li participated in the design of the manuscript; N Huang, R Zhou, HY Chen, S Zhang, J Li, W Wei, J Sun, and BH Li performed the experiments; N Huang and R Zhou analyzed the data and drafted the manuscript; S Zhang, H Deng, J Yang, and FP Ji analyzed the data and participated the discussion on the manuscript. FP Ji and ZF Li revised the manuscript. All authors have read and approved the final version of the manuscript.

**Declaration of conflicting interests**

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**Ethics approval**

Ethical approval for this study was obtained from the Ethics Committee of the Second Affiliated Hospital of Xi’an Jiaotong University in Xi’an City, Shaanxi Province, China (No. 2018-059).

**Informed consent**

Written informed consent was obtained from all subjects before the study.

**Trial registration**

Not applicable.

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**Supplementary Material**

Supplementary material for this article is available online.

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