Dynamics of the spleen and its significance in a murine H22 orthotopic hepatoma model

Baohua Li^{1,2}, Shu Zhang^{1,3}, Na Huang¹, Haiyan Chen¹, Peijun Wang³, Jun Li¹, Yansong Pu³, Jun Yang⁴ and Zongfang Li^{1,3}

¹National & Local Joint Engineering Research Center of Biodiagnosis and Biotherapy, The Second Affiliated Hospital, College of Medicine, Jiaotong University, Xi'an, Shaanxi 710004, China; ²Core Research Laboratory, The Second Affiliated Hospital, College of Medicine, Jiaotong University, Xi'an, Shaanxi 710004, China; ³Department of General Surgery, The Second Affiliated Hospital, College of Medicine, Jiaotong University, Xi'an, Shaanxi 710004, China; ⁴Department of Pathology, The Second Affiliated Hospital, College of Medicine, Jiaotong University, Xi'an, Shaanxi 710004, China

Corresponding author: Zongfang Li. Email: lzf2568@gmail.com

Abstract

The dynamics of the spleen during tumor progression remains incompletely understood. In this study, we established a murine H22 orthotopic hepatoma model and dynamically detected alterations in the percentages of immunocytes in the spleen. We observed a prominent myeloid-derived suppressor cell (MDSC) accumulation during the early response which persisted through all the stages of tumor growth. In addition, the percentage of regulatory T cells (Tregs) increased by week 2. Although the percentage of CD3⁺CD49b⁺ natural killer T (NKT) cells increased by day 3, and that of CD3⁺CD4⁺ T cells slightly increased by week 1, they decreased to either normal or lower levels compared with those of normal mice. The percentages of total CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T cells decreased by week 2, and that of NK cells decreased by week 3. The activation of non-Treg CD4⁺ T cells was scarce. Moreover, splenic MDSCs of tumor-bearing mice suppressed the activation of splenocytes. Therefore, a negative immune response gradually prevailed over a positive immune response during tumor growth. In addition, splenectomy was performed at the time of tumor inoculation, and we found that splenectomy could prolong the survival time, reduce the tumor weights, decrease the ascites volumes, and ameliorate the immune status of the tumor-bearing mice. Splenectomy also decreased the percentage of MDSCs and increased the percentages of CD8⁺ T cells, NK, and NKT cells in tumor tissues. Additionally, splenectomy decreased the percentage of MDSCs and increased that of CD8⁺ T cells in peripheral blood. Overall, our findings suggest that immune-negative cells are dominant in the spleen during tumor progression. Splenectomy could be helpful to improve the immune responses of tumor-bearing hosts.

Keywords: Spleen, hepatoma, H22, immunocytes, splenectomy

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Introduction

The spleen is the most important immune organ in the body; it consists of innate and adaptive immune cells and plays a critical role in the anti-tumor immune response. It contains both tumor-suppressive cells, including natural killer (NK) cells and activated T cells, and tumor-promoting cells, such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs). The spleen can eliminate nascent tumor cells at the early stages and lead to tumor immune tolerance at later stages of tumor progression. Therefore, it has a paradoxical role in tumor immunology.^{1–4}

The spleen is usually considered to be an indispensable part of the anti-tumor immune response. Splenic NK cells,

as the first line of defense in the initial immune response, may kill mutant cells during tumor onset.⁵ The transfection of IL-2 and/or IL-12 genes into the spleen has been reported for the treatment of liver cancer in rats. The mechanism relies on the enhancement of NK cell activation and production of cytokines.⁶ In an SC42 hepatoma murine model, during the early liver tumor stages, splenic NK cell activity has been shown to be increased, which contributes to the suppression of pulmonary metastasis.⁷ Splenic T cells are the major effector cells for cytotoxicity against tumor cells.⁸ Splenectomy has been reported to decrease T cell percentages and to promote W256 carcinosarcoma growth.⁹ Moreover, splenectomy performed on day 4 in colon 26 tumor-bearing mice has been reported to enhance liver

metastasis by increasing Foxp3 mRNA expression in the liver.¹⁰

However, it has also been reported that the spleen plays an immunosuppressive role in tumor-bearing individuals. In patients with gastric cancer, CD62L⁺ cells accumulate in the spleen and produce transforming growth factor beta (TGF- β), which induces the production of Tregs in the spleen; both of these effects contribute to gastric cancer-associated immunosuppression.¹¹ In a genetically defined murine model of spontaneous pancreatic cancer, suppressive cells, including Tregs, MDSCs, and TAMs, in the spleen dominate the early response, and the anti-tumor immune response of the spleen is undermined from the start.¹² The spleen is fundamentally important for tumor-induced tolerance. Splenic CD11b⁺Gr-1^{int}Ly6C^{hi} cells are in close contact with memory CD8⁺ T cells and cause their tolerization. Splenectomy restores lymphocyte function and induces tumor regression when coupled with immunotherapy.¹³

The mechanism of the discrete function of the spleen in tumor immunity has not been completely elucidated. Because studies of the dynamic changes of immune cells in the spleen during tumor progression are sparse, we established a murine H22 orthotopically implanted liver tumor model and dynamically detected immunocytes in the spleen. We found that the percentage of MDSCs increased from the start of tumor growth, and the percentages of CD4⁺ T cells and natural killer T (NKT) cells increased within one week after tumor inoculation. By week 2, the percentage of Tregs was elevated and those of CD4⁺ and CD8⁺ T cells and NK cells were all decreased, suggesting that immune-negative cells gradually prevailed over immune-positive cells; the spleen was in an immunosuppressive state and deteriorated during tumor progression. Then, we performed splenectomy and detected its effect on tumor growth. It was observed that splenectomy could decrease tumor weights and ascites, prolong the survival of tumor-bearing mice, and improve the immune status of the peripheral blood and the tumor microenvironment, thereby delaying tumor growth.

Materials and methods

Mice

Female BALB/c mice (6–8 weeks old) were purchased from the animal center of Xi'an Jiaotong University. The mice were housed under specific pathogen-free conditions and were provided with water and a standard laboratory diet for at least one week before use. This research protocol complied with the Guide for the Care and Use of Laboratory Animal (NIH Publication, 1996) and was approved by the Institutional Animal Care and Use Committee of our institution.

Cell culture

H22 murine hepatoma cells were obtained from the China Center for Type Culture Collection (CCTCC) and were cultured in RPMI 1640 medium (Hyclone, Thermo Fisher Scientific, USA) supplemented with 10% fetal calf serum

(Hyclone) and 1% penicillin/streptomycin. The cells were cultured at 37°C in a humidified 5% CO₂ atmosphere.

Orthotopically implanted liver tumor model

Female BALB/c mice (6–8 weeks old) were administered 0.1 mL of H22 cells (10⁷/mL) via intraperitoneal injection. After 12 days, ascites were collected and washed twice with saline; subsequently, 20 μ L of ascites (0.2 $\times$ 10⁷/mL) was injected orthotopically under the capsule of the left lobe of the liver. After four days, a white tumor nodule was formed in the liver, indicating successful generation of the orthotopically implanted liver tumor model. Splenectomy was performed simultaneously after tumor inoculation. The survival times of the splenectomy group and control group mice were compared. The tumor weights and ascites volumes were recorded. The spleens and tumor tissues, as well as peripheral blood, were collected at the indicated time points for further testing. The spleen index (SI) was calculated according to the following formula: SI = spleen weight (mg)/body weight (g).

Spleen hematoxylin and eosin staining

Paraformaldehyde-fixed, paraffin-embedded spleens were cut with a microtome (RM2235, Leica Microsystems Inc., Germany) into 5- μ m thick sections. The sections were dewaxed and hydrated, stained with hematoxylin for 10 min, washed with distilled water for 3 min, differentiated in 1% acid alcohol, and rinsed in tap water. Subsequently, the slides were stained with eosin for 1 min, dehydrated, cleared, mounted in neutral resin, and observed under a light microscope (E100, Nikon Corporation, Japan).

Generation of single-cell suspensions of spleen and hepatoma tissues

Spleen and hepatoma tissues were collected on day 0, day 1, day 3, week 1, week 2, and week 3 after tumor inoculation. They were dissociated into single-cell suspensions using a gentle MACS Dissociator (Miltenyi Biotech, Bergisch Gladbach, Germany). To dissociate each spleen and tumor tissue sample, a C tube was loaded on the machine with a volume of buffer (0.01 mol/L phosphate-buffered saline [PBS], 0.5% bovine serum albumin [BSA], and 2 mmol/L ethylenediaminetetraacetic acid [EDTA]), and an installed program was selected ("m_spleen_01" or "m_imp Tumor_03", respectively). After completion of the program, the whole-cell suspensions were centrifuged at 300 g at room temperature for 30 s. Then, the suspensions were collected and filtered through a 70- μ m-pore-size nylon cell strainer to remove clumps and generate single-cell suspensions.

Cytofluorometric analysis

Erythrocytes of the prepared single-cell suspensions and peripheral blood samples were lysed with ammonium-chloride-potassium (ACK) lysis buffer (0.15 mol/L NH₄Cl, 1 mmol/L KHCO₃, and 0.1 mmol/L EDTA, pH 7.2) and washed twice with PBS. Cells (10⁶) were blocked with anti-CD16/CD32 and incubated for 30 min with the

following antibodies: FITC anti-Ly-6G/Ly-6C (Gr-1) (clone RB6-8C5), FITC anti-CD3 (clone 17A2), PE anti-CD4 (clone GK1.5), APC anti-CD8a (clone 53-6.7), and PerCP/Cy5.5 anti-CD49b (clone DX5). The above antibodies were all purchased from BioLegend (San Diego, CA). PE anti-CD11b (clone M1/70) was obtained from eBioscience (San Diego, CA). The corresponding isotype controls were also stained. PerCP anti-CD45 and APC anti-CD45 monoclonal antibodies (BioLegend) were added to the tumor tissues to gate the population of white blood cells (WBCs). To quantitate regulatory T cells (Tregs), a mouse Treg Flow™ Kit (clone 150D) was used. Splenocytes were stained with APC-CD4 and PE-CD25. The cells were then fixed and permeabilized for intracellular staining with Alexa Fluor 488-conjugated anti-Foxp3, according to the manufacturer's protocol (BioLegend). After 30 min, the cells were detected by flow cytometry (Canto II, BD BioSciences, USA) and analyzed using Diva 7.0 software.

MDSC suppression assay

MDSCs of tumor-bearing mice at week 1 were sorted using a myeloid-derived suppressor cell isolation kit (Miltenyi Biotech), according to the manufacturer's protocol. The MDSC purity was >95% in all samples. To verify the suppressive activity of the sorted MDSCs, MDSCs (10^5 cells) were added at a 1:4 ratio to splenocytes (4×10^5 cells) from normal BALB/c mice in a 96-well plate and were then stimulated with $0.5 \mu\text{g/mL}$ anti-CD3 and $5 \mu\text{g/mL}$ anti-CD28 (eBioscience) for 48 h. The levels of interferon- γ (IFN- γ) in the cell culture supernatants were detected by mouse IFN- γ platinum ELISA (eBioscience). Each sample, blank, and standard were assayed in duplicate. Absorbance was measured using a microplate reader (PowerWave XS2, BioTek Instruments Inc., USA) at a primary wavelength of 450 nm and a reference wavelength of 620 nm.

Statistical analysis

Results were presented as the mean $\pm$ standard deviation (SD). Comparisons between two groups were performed using Student's *t*-test and PRISM 5 software (GraphPad software Inc., La Jolla, CA). The Pearson correlation test was applied to assess the relationship between MDSCs and CD4⁺ T cells, CD8⁺ T cells, NK cells, and NKT cells. Linear regression analysis was also performed to confirm the association. The ascites volumes of the two groups were compared using the Kruskal-Wallis test. *P* < 0.05 was considered statistically significant.

Results

Dynamics of the spleen during H22 hepatoma progression

Spleens were collected and weighed during tumor progression; spleen indices were calculated for the tumor-bearing and normal mice. The spleen weights were observed to decrease by day 1 after tumor inoculation, but they subsequently increased and were significantly higher by week 2 (Figure 1(a)). The spleen indices of the tumor-bearing mice increased by weeks 1 and 2 compared with those of the

normal mice, but the indices did not significantly differ at other time points (Figure 1(b)). Spleen hematoxylin and eosin (H&E) staining showed no obvious changes in the tumor-bearing mice compared with the normal mice by week 1. However, by weeks 2 and 3 after implantation, the architecture of the spleen was disrupted, and no clear boundary between the white pulp and red pulp could be observed (Figure 1(c)).

In addition to the histological changes, we also detected cellular changes in the spleens of the tumor-bearing mice by flow cytometry. We found that the percentage of MDSCs increased from the beginning after tumor inoculation and reached up to $43.70 \pm 11.33\%$ of the total splenocytes by week 2. The percentage of CD4⁺CD25⁺Foxp3⁺ spleen Treg cells decreased by week 1 ($7.53 \pm 0.46\%$) and increased by week 2 ($16.50 \pm 2.62\%$) compared with those in normal spleen samples ($12.08 \pm 1.28\%$). The percentage of total CD3⁺ T cells increased by week 1 and decreased by week 2. The percentage of CD3⁺CD4⁺ T cells slightly increased by week 1; however, the percentages of both CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells were then notably reduced by week 2 ($13.35 \pm 4.03\%$ and $5.13 \pm 1.86\%$, respectively) compared with those in the normal mice ($23.68 \pm 4.06\%$ and $13.23 \pm 2.39\%$, respectively). The percentage of NK cells decreased by week 3 ($1.88 \pm 0.51\%$) compared with that in the normal mice ($5.29 \pm 1.39\%$). The percentage of NKT cells was elevated on day 3 and then declined to a normal level (Figure 2(a) and (b)). The above results showed that although the percentages of NKT and CD4⁺ T cells increased within one week after tumor inoculation, they decreased to normal levels or lower thereafter. MDSCs dominated during all stages of tumor growth, while CD4⁺ T, CD8⁺ T, NK, and NKT cells were all inhibited during tumor progression, indicating that immune-negative cells prevailed over immune-positive cells in the spleen during the progression of orthotopic hepatocellular carcinoma.

Minimal activation of non-Treg CD4⁺ T cells in the spleen during tumor progression

In addition to the evaluation of T cell percentages in the tumor-bearing mice, T cell activation was also examined. Because CD25 can be used as a marker of T cell activation, the expression of CD25 was also evaluated in non-Treg populations (i.e. CD4⁺Foxp3⁻ T cells) in the spleen. Over 76.10% of CD4⁺Foxp3⁺ T cells were CD25 positive; nevertheless, only fewer than 7.47% of CD4⁺Foxp3⁻ T cells (i.e. non-Treg CD4 population) on average expressed CD25 (Figure 3(a) and (b)). These results indicated the minimal activation of non-Treg CD4⁺ T cells in the spleen, which further confirmed the negative immune status of the spleen during tumor progression.

Splenic MDSCs played an immunosuppressive role in murine H22 transplantable hepatoma

MDSCs and Tregs are negative regulators of the immune response. The percentage of Tregs out of total splenocytes in the hepatoma-bearing mice was less than 3%; however, the MDSC percentage reached as high as 40%. Therefore,

Figure 1 Changes of spleen weight and histology during tumor progression. Spleens were collected from tumor-bearing mice ($n=6$) and normal mice ($n=6$) at the indicated time points for analysis of (a) spleen weight, (b) spleen index (SI = spleen weight (mg)/body weight (g)) and (c) spleen H&E staining. Magnification for all images is $\times 100$. (The bars indicate $\pm$ SD, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with the normal group.) (A color version of this figure is available in the online journal.)

Figure 2 The spleen gradually became immune negative during tumor growth. Splenic single-cell suspensions were prepared and stained for Gr-1⁺CD11b⁺ MDSCs, CD4⁺CD25⁺Foxp3⁺ Tregs, total CD3⁺ T cells, CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, CD3⁺CD49b⁺ NK cells, and CD3⁺CD49b⁺ NKT cells, then detected by Canto II flow cytometry and analyzed using Diva software. (a) Percentages of immunocytes in the spleen. (b) Representative flow cytometry plots of one mouse spleen from each group. ** $P < 0.01$ and *** $P < 0.001$ compared with the normal group

Figure 3 Minimal activation of non-Treg CD4⁺ T cells in the spleens of tumor-bearing mice. Splenic single-cell suspensions were prepared and stained using a mouse Treg flow kit, according to the manufacturer's protocol. CD25 expression in CD4⁺Foxp3⁺ T (Treg) cells and in CD4⁺Foxp3⁻ T (non-Treg CD4⁺ T) cells was detected by flow cytometry. (a) The expression of CD25 in Treg cells and non-Treg CD4⁺ T cells. (b) Representative flow cytometry plots of CD25 expression in one mouse from each group. ** $P < 0.01$ and *** $P < 0.001$ compared with the normal group

the MDSCs were the main negative immune cells rather than Tregs in the murine H22 transplantable liver tumors. We then assessed the correlation between MDSCs and the other splenic immune cells. The CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, and CD3⁺CD49b⁺ NKT cells were all negatively correlated with the MDSCs, with Pearson r values of -0.7392 , -0.7908 , and -0.4559 , respectively (Figure 4(a)). It is possible that the splenic MDSCs had inhibitory effects on the T and NKT cells in the hepatoma mice. To ascertain whether splenic MDSCs have a suppressive effect in this murine hepatoma model, splenic MDSCs at one week after tumor inoculation were magnetically sorted using a mouse myeloid-derived suppressor cell isolation kit. The purity of the sorted MDSCs was $>95\%$ (Figure 4(b)). When the MDSCs were co-cultured with splenocytes stimulated with anti-CD3 and anti-CD28, the level

of IFN- γ notably decreased compared with that in splenocytes cultured alone (Figure 4(c)).

Splenectomy improved survival time and reduced tumor weight and ascites volume of hepatoma-bearing mice

Because the spleen was found to play an immunosuppressive role in tumor progression, the effect of splenectomy on tumor growth was assessed. The survival time of the tumor-bearing mice in the splenectomy group (24 days) was significantly prolonged compared with that of the mice in the control group (21 days) (Figure 5(a)). Splenectomy significantly reduced the tumor weight by week 2 (Figure 5(b)). There were no ascites by week 1 in either group. By week 2, the ascites volume in the splenectomy group was

Figure 4 Splenic MDSCs played an immunosuppressive role in hepatoma-bearing mice. (a) Correlation analysis between MDSCs and CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, and CD3⁺CD49b⁺ NK cells in the spleens of tumor-bearing mice. (b) Splenic MDSCs of tumor-bearing mice by week 1 were sorted using a myeloid-derived suppressor cell isolation kit, and purity was confirmed by flow cytometry using Gr-1 and CD11b fluorescent antibodies. (c) Sorted splenic MDSCs were added to splenocytes from normal BALB/c mice in a 96-well plate and stimulated with anti-CD3 and anti-CD28 for 48 h. The levels of IFN-γ in the supernatants were detected using ELISA. *** $P < 0.001$

significantly decreased. By week 3, the ascites volume was reduced in the splenectomy group, but it did not significantly differ from that of the control group (Figure 5(c)). Moreover, the incidence of ascites was markedly reduced in the splenectomy group. By week 2, the incidence of ascites was 16.7% in the splenectomy group compared to 83.3% in the control group. By week 3, the incidence was 20% in the splenectomy group compared to 100% in the control group (Table 1).

Splenectomy ameliorated immune status of hepatoma-bearing mice

To determine whether splenectomy altered the immune status of the tumor-bearing mice, immune cells from tumor tissues and peripheral blood were analyzed by flow cytometry. In the tumor tissues (Figure 6(a)), the percentages of MDSCs and CD4⁺CD25⁺Foxp3⁺ Treg cells significantly increased during tumor progression. In the splenectomy group, the percentage of MDSCs was markedly reduced by week 3, and that of CD4⁺CD25⁺Foxp3⁺ Treg cells was elevated by week 2. The percentage of CD3⁺CD4⁺ T cells was unchanged during tumor progression and was comparable in the untreated and splenectomy groups. The percentage of CD3⁺CD8⁺ T cells was not significantly altered during tumor growth, while it increased in the splenectomy group by week 2. CD3⁺CD49b⁺ NK cells were reduced by week 3 after tumor inoculation, and splenectomy increased the percentages of NK and CD3⁺CD49b⁺

NKT cells by week 2 after tumor inoculation. Therefore, splenectomy reduced the percentage of MDSCs and elevated the percentages of CD3⁺CD8⁺ T cells, CD3⁺CD49b⁺ NK cells, and CD3⁺CD49b⁺ NKT cells in the tumor tissues.

In peripheral blood (Figure 6(b)), the percentage of MDSCs increased during tumor growth; by week 3, it reached up to $71.08 \pm 9.65\%$. One week after tumor inoculation, the percentage of MDSCs was elevated in the splenectomy group compared with the untreated group, but it was significantly reduced by week 2. Splenectomy did not influence the percentage of CD3⁺CD4⁺ T cells, but it significantly elevated the percentage of CD3⁺CD8⁺ T cells by weeks 1 and 3 of tumor growth. The percentage of CD3⁺CD49b⁺ NKT cells increased by week 1 and decreased by week 2 after tumor inoculation. Splenectomy had no effect on the percentage of either CD3⁺CD49b⁺ NK cells or CD3⁺CD49b⁺ NKT cells. Therefore, splenectomy decreased the percentage of MDSCs and increased the percentage of CD3⁺CD8⁺ T cells in the peripheral blood.

Discussion

The spleen is the largest immune organ, and it contains one-fourth of all of the immune cells in the body. Using color-coded imaging, McElroy *et al.* have observed that splenocytes traffic to tumor sites,¹⁴ suggesting that the spleen actively participates in tumor growth. They also have reported that the injection of tumor cells alone via the portal vein leads to tumor cell death. However,

Figure 5 Splenectomy improved survival time and reduced both tumor weight and ascites of hepatoma-bearing mice. The mice were divided into two groups, the TB group (■) and splenectomy (spx) group (▲). (a) The survival times of the mice in the untreated TB group and spx group were compared ($n = 9$ and 14 , respectively). (b) The tumor weights and (c) ascites volumes by weeks 1, 2, and 3 of each group were recorded and compared. * $P < 0.05$ and ** $P < 0.01$ compared with the TB group

Table 1 The incidence of ascites in splenectomy and untreated tumor-bearing mice

Group	1 week (%)	2 week (%)	3 week (%)
TB	0	83.3	100
TB + spx	0	16.7	20

TB, tumor bearing; spx, splenectomy.

the co-injection of tumor cells and splenocytes via the portal vein or the injection of tumor cells directly into the spleen leads to the liver metastasis of tumor cells.¹⁵ These results indicate that the spleen may facilitate tumor growth and metastasis.

Considering the important role of the spleen in tumor progression, we dynamically detected the cellular changes of the spleen at each stage of tumor development. In this study, the percentage of $CD3^+CD49b^+$ NKT cells increased by day 3, and that of $CD3^+CD4^+$ T cells was slightly elevated by week 1 after tumor inoculation, which indicates that an anti-tumor immune response may occur in the spleen during the early stages. Meanwhile, the percentage of tumor-promoting MDSCs began increasing very early on day 1 after tumor inoculation. This result suggested that, in

the H22 orthotopically transplanted hepatoma model, the spleen responded quickly and initiated both anti-tumor and tumor-promoting immune reactions once tumor cells were inoculated in the liver. Two weeks later, the percentages of total $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD3^-CD49b^+$ NK cells all decreased, and that of MDSCs consistently increased throughout the tumor growth process. The percentage of Treg cells was also increased by week 2. The above results indicated that with tumor growth, the anti-tumor immune response was weakened, and the tumor-promoting immune response was strengthened. In addition, there was minimal activation of non-Treg $CD4^+$ T cells in the spleen, and splenic MDSCs played an inhibitory role in splenocyte activation, which further verified that the immune-negative cells predominated in the spleen during tumor growth.

The results of our current study are consistent with those previously reported by Hoffman *et al.* Both studies have shown that the spleen promotes tumor growth. The reason why immune-negative cells dominate in the spleen throughout the tumor growth process in this study is perhaps due to the transplantable tumor model we have used. This type of model does not mimic the native microenvironment of an endogenously growing tumor, and it cannot reflect the naturally occurring *in vivo* immune responses to

Figure 6 Splenectomy ameliorated the immune status of tumors and peripheral blood in tumor-bearing mice, as determined by flow cytometry. (a) Single-cell suspensions of hepatoma tissues from the TB and spx groups ($n = 6$ for each group) were stained with specific fluorescent antibodies to detect the percentages of MDSCs, Tregs, CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, CD3⁺CD49b⁺ NK cells, and CD3⁺CD49b⁺ NKT cells. (b) The percentages of these immune cells in the peripheral blood after splenectomy were compared with those of the untreated TB group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

tumor cells during the course of tumor progression. The injection of tumor cells into the liver, or directly into the spleen, probably causes similar effects; the host body receives a heavy tumor load, and the anti-tumor effect is quickly overwhelmed by the pro-tumor effect of the spleen. Therefore, the spleen facilitates tumor development.

Because immune-negative cells prevailed in the spleen during tumor progression in the orthotopic hepatoma model, we then assessed whether splenectomy could improve the immune responses of the tumor-bearing mice. Our findings showed that splenectomy decreased the percentage of MDSCs not only in peripheral blood but also in tumor tissues. Moreover, splenectomy increased the

percentages of CD3⁺CD8⁺ T cells in peripheral blood and CD3⁺CD8⁺ T, CD3⁺CD49b⁺ NK and CD3⁺CD49b⁺ NKT cells in tumor tissue. All of these changes ameliorated the immune status of the peripheral blood and the tumor microenvironment. Therefore, splenectomy improved the immune responses and prognoses of the tumor-bearing mice. In other words, removal of the immune-negative effect of the spleen in the orthotopic tumor model delayed tumor growth. These results are in line with those of a previous study reporting that splenectomy is an effective adjuvant therapy for hepatocellular carcinoma because it results in a decreased percentage of MDSCs in peripheral blood and decreased carcinoma growth and metastasis.¹⁶

In our current study, MDSCs were the most markedly changed cells in the spleen, peripheral blood, and tumor tissues before and after splenectomy. It has been reported that MDSCs induce Treg expansion.^{17–20} In our study, the percentage of MDSCs increased from the beginning of tumor inoculation, while that of Tregs increased by week 2 in the spleen. In the tumor tissues, the percentages of MDSCs and Tregs persistently increased throughout tumor growth. Whether MDSCs also induce Treg expansion in the H22 orthotopic hepatoma mouse model requires further investigation. MDSCs could inhibit the activation of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells,^{21,22} and suppress the cytotoxicity of NK cells.^{23,24} Therefore, the decreased percentages of CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, and NK cells in the spleen during tumor growth may be caused by MDSCs. The percentage of tumor MDSCs decreased, and those of tumor CD3⁺CD8⁺ T cells, CD3⁺CD49b⁺ NK cells, and CD3⁺CD49b⁺ NKT cells increased after splenectomy, which was possibly due to the reduced suppressive effects of the MDSCs on these immune-positive cells.

Our results showed that one week after splenectomy, the percentage of blood MDSCs increased, but that of tumor MDSCs did not increase accordingly compared with the untreated group. Thereafter, the percentages of both peripheral blood and tumor MDSCs decreased in the splenectomy group. These findings indicated that splenectomy changed the distribution of MDSCs in the peripheral blood and tumor microenvironment. The following reasons might explain these results. First, the spleen can probably act as an MDSC reservoir. When stressed, splenic MDSCs proliferate and quickly enter the bloodstream.^{25,26} Therefore, after splenectomy, the reservoir of MDSCs is depleted, and MDSCs mobilized from the bone marrow cannot reside in the spleen, and thus accumulate in the peripheral blood, causing an increased MDSC percentage in the blood one week after splenectomy. Second, the spleen might be the processing site for MDSCs and therefore constitute an indispensable route from the bone marrow to the tumor site. It is known that splenic MDSCs and tumor MDSCs exhibit distinct phenotypes and functions.^{17,27–30} Tumor MDSCs have higher arginase I, iNOS, CD80, PD-L1 expression, and lower ROS activity; thus they have a more potent suppressive activity than splenic MDSCs. It appears that bone marrow MDSCs entered the spleen and had an immature phenotype and a lower suppressive activity through primary processing in the spleen. Tumor-derived factors promoted the migration of splenic MDSCs to the tumor site. Under the influence of the tumor microenvironment, the tumor MDSCs became more mature after secondary processing and had an increased suppressive ability. After splenectomy, the primary processing of MDSCs in the spleen was lacking, and thus, the percentage of functional MDSCs was greatly reduced in the tumor tissues. Third, chemokines play an important role in the recruitment of MDSCs to tumors.^{31–34} We also found that chemokine expression in the spleen and chemokine receptors on the surfaces of MDSCs were dynamically altered at the different stages of tumor progression (unpublished data). We speculated that without the modification of the spleen, the sensitivity of MDSCs to the chemotaxis of tumor tissues

may be decreased. This decreased sensitivity might be another reason why the percentage of MDSCs in the peripheral blood increased one week after splenectomy, while that of tumor MDSCs did not increase proportionally.

In conclusion, immune-negative cells predominated in the spleen during tumor progression in the H22 orthotopic hepatoma model, and splenectomy delayed tumor growth. MDSCs with potent suppressive activity were the most strikingly changed cells in the current study. Splenectomy changed the distribution of MDSCs, and the mechanism of this effect requires further exploration.

Author's contributions: B-HL performed all experimental procedures and statistical analysis and prepared the first draft of the manuscript. NH, H-YC, P-JW, JL, and Y-SP assisted in the construction of the animal model and collection of the tissue specimens. SZ assisted in designing the study and writing the manuscript, JY and Z-FL contributed to the design and review of the study. All authors approved the final version of the manuscript for submission.

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DECLARATION OF CONFLICTING INTERESTS

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