Effect and mechanism of immune surveillance activated by autotransfusion on reducing liver cancer metastasis based on HLA-DQB1/PDCD1

Laiwei You[1,2], Yu Bai[3], Ying-Hui Cui[3], Jin-Huo Wang[4], Na Yao[2], Xiao-Fang Zhou[4], Xiao-Fei Chen[5], Jian-Rong Guo[2,4]

[1]Ningxia Medical University, Ningxia, China
[2]Postgraduate Training Base in Shanghai Gongli Hospital, Ningxia Medical University, Shanghai, China
[3]Graduate School of Wannan Medical College, Anhui, China
[4]Department of Anesthesiology, Shanghai Gongli Hospital, Naval Military Medical University, Shanghai, China
[5]Department of Anesthesiology, Lihuili Hospital, Medical School of Ningbo University, Zhejiang, China

Submitted: 23 March 2022; **Accepted:** 18 Jun 2022
Online publication: 18 July 2022

Arch Med Sci
DOI: https://doi.org/10.5114/aoms/151106

Corresponding authors:
Dr. Jian-Rong Guo
Shanghai Gongli Hospital
Naval Military Medical University
No. 219, Miaopu Road
Pudong New Area
Shanghai 200135, China
E-mail: guojianrong_sh2021@163.com

Xiao-Fei Chen
Department of Anesthesiology
Lihuili Hospital
Medical School of Ningbo University
Zhejiang, 315040, China
Phone: 86-58858730-5620
E-mail: gob73793@163.com

Abstract

Introduction: Liver cancer is a common tumor of the digestive system. Hepatectomy sometimes results in massive hemorrhage, and intraoperative autotransfusion is often required.
Material and methods: In this basic research, a liver cancer mouse model was established, the visible liver cancer tissue was surgically removed, and the blood of the mice was recovered.
Results: The results showed that the model group had the highest recurrence and metastasis of liver cancer, and the lowest was the autotransfusion group. Flow cytometry showed that the number of HLA-DQB1 positive cells, the content of CD4+CD25+T cells, and the proliferation of CD4+T cells were the highest in the autotransfusion group, followed by the model group, and the lowest in the auto+HLA-DQB1 group, which showed no difference from the control group. Immunohistochemistry and western blot analysis showed that the lowest expression of PDCD1 protein was in the autotransfusion group, which showed no significant difference from the control group, and the highest expression was in auto-HLA-DQB1, followed by the model group.
Conclusions: The study elucidates the molecular mechanism whereby autotransfusion will activate up-regulated expression of the signal molecule HLA-DQB1, enhance the immune surveillance of CD4+T cells, reduce the expression of PDCD1 on the surface molecule of liver cancer cells, and inhibit liver cancer metastasis.

Key words: liver cancer, autotransfusion, HLA-DQB1, PDCD1, immune surveillance.

Introduction

Liver cancer is a common tumor of the digestive system, and whether hepatectomy can be performed largely determines its prognosis. According to statistics, the 5-year survival rate of liver cancer patients undergoing hepatectomy can reach more than 70% [1]. However, the liver is rich in

blood supply and complicated in anatomy, and the tumor often invades the large intrahepatic vessels. Therefore, the implementation of standard hepatectomy often encounters the problem of massive hemorrhage during the surgery. It has been reported that the incidence of massive hemorrhage in patients undergoing hepatectomy is as high as 30–40% [2]. Due to the continuous progress of surgical techniques and instruments, the incidence of massive hemorrhage in hepatectomy is lower than before. But for liver cancer surgery with extensive vascular invasion or a large tumor, the risk of massive hemorrhage is high. Therefore, blood transfusion is still a commonly used method to maintain the stability of intraoperative vital signs [3].

At present, the most common methods of blood transfusion include allogeneic transfusion and autotransfusion. Allogeneic transfusion may cause allergic and rejection reactions, as well as immunosuppression and tumor proliferation [4, 5]. Autotransfusion refers to the infusion of a patient's own blood that has been stored in advance or recovered from blood loss when needed. Autotransfusion includes storage autotransfusion, dilution autotransfusion, and intra-operative cell salvage [6, 7]. Autotransfusion has been paid more and more attention because it can effectively avoid the occurrence of infectious diseases and adverse reactions to transfusion caused by allogeneic transfusion. Autotransfusion has been widely used in major surgical operations at home and abroad.

The effect of blood transfusion on the immune system is known as transfusion-related immunomodulation, which involves both an enhanced and suppressed immune response. In recent years, most studies believe that the immunomodulation induced by blood transfusion is mainly suppression, mainly manifested as non-specific immunosuppression and specific immunosuppression [8]. The mechanism of immunosuppression induced by blood transfusion is complex, and the exact mechanism has not been confirmed yet. Some studies have suggested that the protein PDCD1 has a regulatory effect on T cell proliferation, which is closely related to the immune surveillance function.

PDCD1 is an immunoglobulin that is mainly expressed on the surface of activated CD4+/CD8+T cells, B lymphocytes and myeloid cells [9]. So far, more and more evidence indicates that PDCD1 is also widely expressed in tumor cells, and it can help tumor cells evade immune surveillance, thus improving the migration and invasion ability of tumor cells [10, 11]. Previous studies have shown that inhibition of PDCD1 can inhibit the proliferation and metastasis of gastric cancer cells [12].

Human leukocyte antigen (HLA)-DQB1 allele polymorphism has been confirmed to be involved in human tumors [13]. Human leukocyte antigen (HLA) is associated with almost all immune-related diseases to varying degrees, especially autoimmune diseases, tumors and infectious diseases [14]. HLA-DQB1 expression is associated with tumor immunity. HLA-DQB1 is an HLA class II β chain analogue that is essential for immune response. Class II molecules are expressed by antigen-presenting cells and play a central role in the immune system by presenting peptides from extracellular proteins. Previous studies on HLA-DQB1 mainly focused on polymorphism and tumor susceptibility [15, 16]. However, the relationship between HLA-DQB1 expression level and tumor prognosis in lung cancer has rarely been reported. It is noteworthy that in our study, HLA-DQB1 protein is expressed on tumor cells and affects anti-tumor immunity issues. Tumors with higher HLA-DQB1 levels show greater infiltration of CD4+ and CD8+ positive T lymphocytes [17].

In addition, through the protein expression profile database, it was found that the signaling molecule HLA-DQB1 had a regulatory axis relationship with the protein PDCD1, as shown in Figure 1A. This study proposed the hypothesis that "the expression of the signal molecule HLA-DQB1 is upregulated to enhance the immune surveillance of CD4+T cells, reduce the expression of PDCD1 on the surface of liver cancer cells, and inhibit the migration and invasion of tumor cells", as shown in Figure 1B. Meanwhile, the relevant molecular mechanism of autotransfusion to activate immune surveillance and reduce the metastasis of liver cancer was explored.

Material and methods

Material

HepG2 cell lines were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences; DMEM medium, FBS and trypsin were purchased from Sigma. CFDA-SE (C-1157) was purchased from Molecular Probes. CD4-PE antibody and CD25-FITC antibody were purchased from Sigma. FITC-CD4 antibody, PE-CD25 antibody, PDCD1, ECM1, and E-cadherin antibody were purchased from proteintech. Diaminobenzidine (DAB) was purchased from Innochem. An ELISA Kit was purchased from Beyotime Biotechnology. A domestic self-2000 blood recovery machine (BW-8100A) was purchased from Beijing Wandong Medical Equipment Co., Ltd. Western blotting instruments (Eppendorf); Flow meter BD; Ultra-thin slicer (LKB-V; JEOL Co., Japan). Healthy SD mice, grade SPF, 7 weeks, 30–40 g, were purchased from Beijing Weitonglihua Experimental Animal Technology Co., Ltd., Quality certificate No.: SCXK (Beijing) 2016-0006. All mice were kept in an animal house

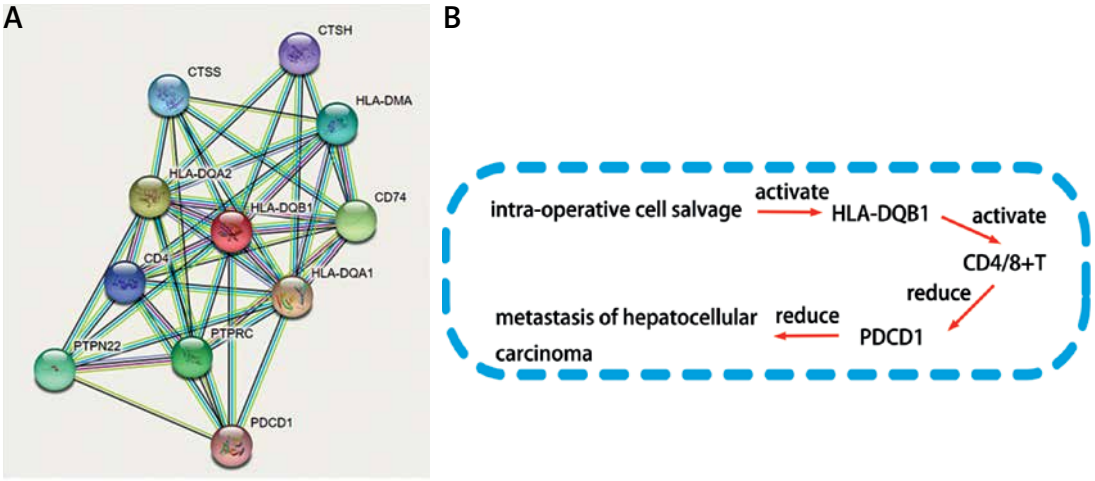

Figure 1. Hypothesis diagram. **A** – Protein expression profile database. **B** – Diagram of molecular mechanism hypothesis of autotransfusion to reduce liver cancer metastasis based on HLA-DQB1/PDCD1

with constant temperature and humidity, and were fed with clean grade special feed. All animal experiments have passed the review of ethics Committee of Shanghai Gongli Hospital, Naval Military Medical University (No. 20210224).

Establishment of liver cancer mouse model

Sixty SD mice were randomly divided into 4 groups. One group was the model group. HepG2 cells were injected subcutaneously into the other 3 groups. The tumorigenesis of mice was observed and recorded every 3 days. The mouse model of liver cancer was established for 42 days. The experimental groups were named as the normal group, liver cancer model group 1, liver cancer model group 2, and liver cancer model group 3.

Autotransfusion in mice

After the liver cancer tissue was resected in the model mice, the operative wound was sutured with surgical suture, and blood was recovered from the operative field of each mouse by the domestic autologous 2000 blood recovery machine. Normal mice and liver cancer model group 1 were intravenously injected with 1.0 ml of normal saline. Autologous blood red cells (1.0 ml) were collected from each mouse in model groups 2 and 3 by tail vein infusion. Model group 3 was injected with HLA-DQB1 antibody for 8 weeks. The experimental group was reassigned as control, model, autotransfusion, and autotransfusion + HLA-DQB1 (auto+HLA-DQB1).

Tumor recurrence and metastasis rates in mice

After 8 weeks of feeding, the mice were anesthetized by chloral hydrate and sacrificed by neck amputation. The liver tissues of the mice in each group were collected, and the recurrence and metastasis of the mice in each group were observed and recorded. The recurrence rate was calculated by the number of animals with tumor recurrence. Tumor metastasis rate: distant metastasis rate was calculated by counting tumor nodules on the surface of liver tissue. Liver tissue was fixed with Bouin solution, and liver tissue was observed for yellow tumor metastases and white after 24–48 h. The number of tumor metastases was calculated by the naked eye.

Number of HLA-DQB1 positive tumor cells

CFDA-SE was dissolved with DMSO (5 mmol/l) and stored at –20°C, then adjusted to room temperature before use. 2.5 µmol of CFDA-SE was thoroughly mixed with HLA-DQB1 protein. The number of HLA-DQB1 positive cells in the blood of each group was detected by flow cytometry.

Content of CD4+CD25+T cells

Abdominal aorta blood and peripheral blood mononuclear cells were collected from each group, then PBS was added and the cell density was adjusted to 1×10^6/l. The cells were incubated with FITC-CD4 antibody and PE-CD25 antibody. The content of CD4+CD25+T cells was detected by flow cytometry.

Proliferation of CD4+T cells

Abdominal aorta blood of mice in each group was collected, cells were centrifuged, and CD4+T cells were labeled with CFSE. The cells were suspended to 1×10^6 cells/ml with RPMI-1640 and stained with 1 µl of staining solution. The solution was incubated at 37°C with 5% CO_2 for 20 min. Centrifugation was performed at 1200 rpm for 5 min, and the supernatant was discarded. The

proliferation of CD4+T cells in blood of each group was detected by flow cytometry.

PDCD1 protein expression detection by immunohistochemistry

Paraffin samples were prepared from the liver tissues of each group. Each paraffin tissue was cut into 2 pieces with a thickness of about 5 μm and used for PDCD1 protein staining. The paraffin sections were washed repeatedly with ionized water, and the blocking antibody was added into the milk liquid for 5 min. PDCD1 antibody was added and incubated at 37°C for 2 h. The samples were washed with PBS three times. Fluorescent staining labeled secondary antibody was added and incubated at 4°C for 30 min. Diaminobenzidine (DAB) chromogenic agent was added after PBS washing. The slices were dehydrated by gradient ethanol, sealed with neutral gum and observed under a microscope.

Detection of PDCD1, ECM1, E-cadherin protein expression by western blot

Tissue (20 mg) was centrifuged and lysate added (RIPA: PMSF = 99 : 1, 200 μl). Three magnetic beads were added to lysis tissue (4°C, 45 Hz, 60 s). Then a BCA kit was used to measure the protein concentration, and 5 × SDS-PAGE protein plus sample buffer was added. After being dried in a hot bath at 100°C for 15 min, samples were stored at –80°C for subsequent electrophoresis. A total of 50 μg of protein from each sample was loaded and transferred to the PVDF membrane. The primary antibodies used for immunoblotting were as follows: PDCD1, ECM1, E-cadherin1 (1 : 1000), GAPDH (1 : 1000). After the second antibody was incubated, the cells were slowly shaken and incubated at room temperature for 1 h. Image J software was used to analyze the gray levels of protein bands in each group.

Serum levels of inflammatory cytokines IL-10 and IFN-γ

After anesthesia, blood was taken from the abdominal aorta of mice, centrifuged (3600 $r \cdot min^{-1}$ × 10 min), and the serum was frozen at –80°C. After balancing the reagents and serum samples to room temperature, the procedure was performed according to the ELISA kit instructions. The absorbance was detected at 450 nm and 630 nm, and the sample OD value was calculated according to the standard curve.

Statistical analysis

All experiments were replicated independently at least three times. The data were analyzed using one-way analysis of variance (ANOVA) and are presented as the mean ± standard deviation (SD). Statistical significance was defined as $p < 0.05$.

Results

Tumor recurrence and metastasis rates in mice

The tumor recurrence rate was 80% and the metastasis rate was 70% in the model group. In the auto+HLA-DQB1 group, the recurrence rate was 70% and the metastasis rate was 60%. The recurrence and metastasis rates of the autotransfusion group were the lowest (30% and 40%, respectively), as shown in Table I.

Number of HLA-DQB1 positive cells

Flow cytometry results showed that the number of HLA-DQB1 positive cells was the largest in the autotransfusion group and autotransfusion could activate HLA-DQB1 expression. HLA-DQB1 is a tumor cell specific antigen. The fewest HLA-DQB1 positive cells were in the auto+HLA-DQB1 group, which showed no difference from the control group, as shown in Figure 2.

Content of CD4+CD25+T cells

The maximum content of CD4+CD25+T cells was in the autotransfusion group (98.4%), indicating that autotransfusion could activate immune system expression. The second group was the model group (96.6%), and the CD4+CD25+T content was the lowest in the auto+HLA-DQB1 group (92.5%), which showed no difference from the control group, as shown in Figure 3.

Proliferation of CD4+T cells

The proliferation of CD4+T cells in blood of mice was the highest in the autotransfusion group, indicating that autotransfusion can activate the im-

Table I. Recurrence and metastasis of liver cancer in mice after operation

Group	*n*	Number of cases	Recurrence rate (%)	Number of cases	Metastasis rate (%)
Control	10	10	_	10	–
Model	10	8	80	7	70
Autotransfusion	10	3	30	4	40
Auto+HLA-DQB1	10	7	70	6	60

mune system. The second group was the model group, and CD4+T proliferation was the lowest in the auto+HLA-DQB1 group, which showed no difference from the control group, as shown in Figure 4.

PDCD1 protein expression detection by immunohistochemistry

Immunohistochemical analysis of PDCD1 protein showed a brownish yellow color. The expression of PDCD1 protein was the lowest in the autotransfusion group, and there was no significant difference between the normal group and the autotransfusion group. The expression of PDCD1 protein was highest in auto-HLA-DQB1, followed by the model group, as shown in Figure 5. This is because the immunosuppression of the auto-HLA-DQB1 group leads to high expression of the PDCD1 protein, which leads to immune escape of tumor cells and tumor proliferation.

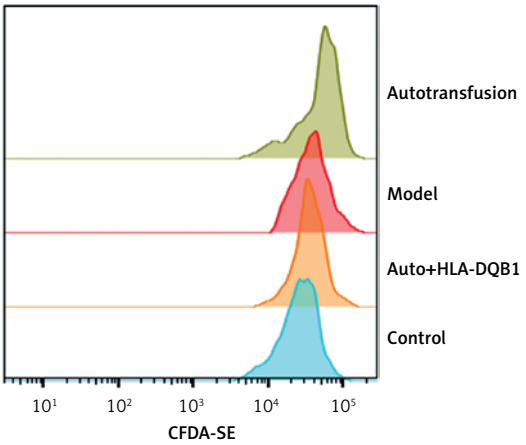

Figure 2. The number of HLA-DQB1 positive cells was detected by flow cytometry

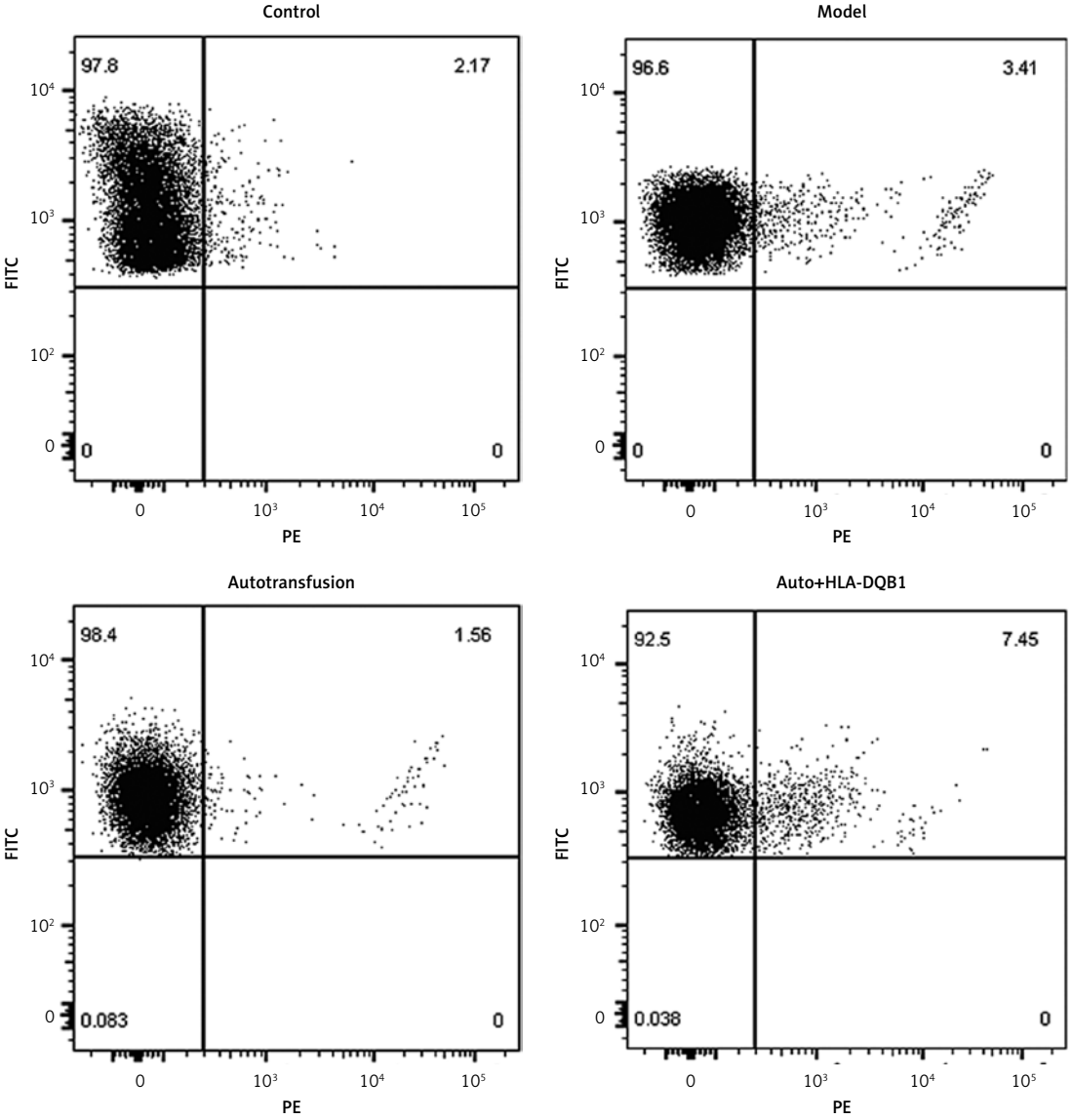

Figure 3. The content of CD4+CD25+T cells was analyzed by flow cytometry

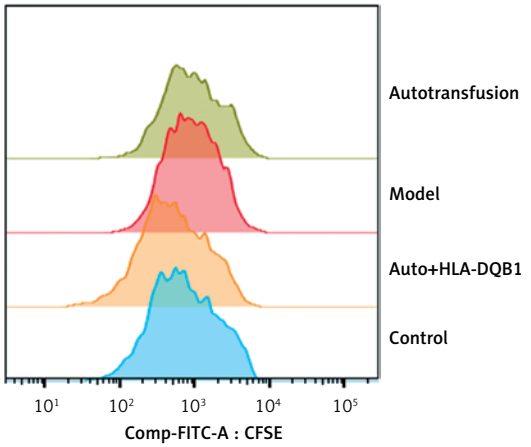

Figure 4. The proliferation of CD4+T cells was analyzed by flow cytometry

Western blot analysis of PDCD1, ECM1, E-cadherin protein expression

Expression of PDCD1, ECM1, and E-cadherin proteins in liver tissue of mice is shown in Figure 6. The expression levels of PDCD1, ECM1, and E-cadherin protein were highest in the auto+HLA-DQB1 group, and the second was the model group.

There was a significant difference, compared with the control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Compared with the model group, $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$.

Serum levels of inflammatory cytokines IL-10 and IFN-γ

The content of IL-10 was highest in the auto+HLA-DQB1 group (114.80 ±12.16 ng/l). The second was the model group (82.27 ±13.48 ng/l). The content of IL-10 in the autotransfusion group (34.01 ±11.75 ng/l) was similar to that in the control group (33.06 ±7.23 ng/l), as shown in Figure 7A. The content of IFN-γ and IL-10 showed the same trend. The control group was 1130.50 ±261.97 ng/l, the model group was 3288.45 ±615.18 ng/l, the autotransfusion group was 1075.09 ±196.79 ng/l, and the auto+HLA-DQB1 group was 2306.32 ±548.25 ng/l, as shown in Figure 7B.

Discussion

At present, the common methods of blood transfusion include allogeneic transfusion and autotransfusion. Blood tension and transmission of blood-borne diseases often exist in allogeneic transfusion. Autotransfusion has unique advantages over allogeneic blood transfusion; e.g. the storage time of autologous blood is shorter than

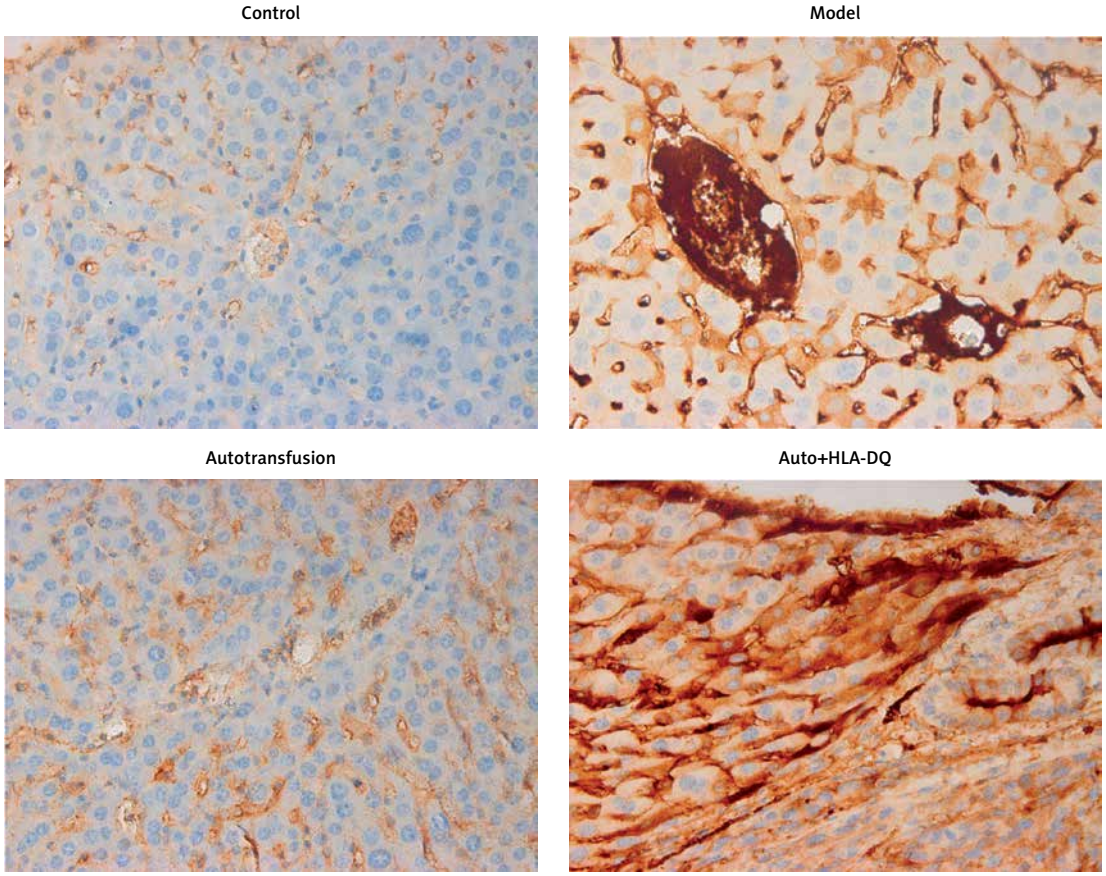

Figure 5. Immunohistochemical staining (× 200)

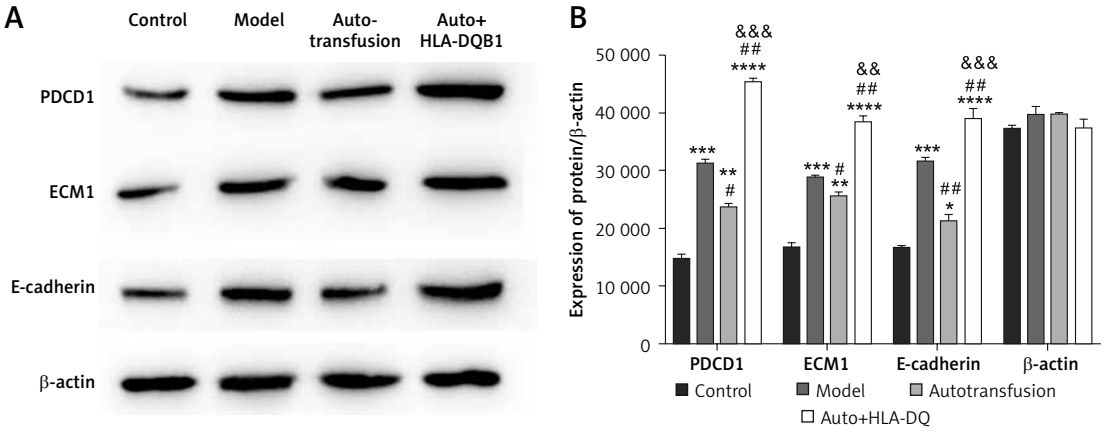

Figure 6. Protein levels of PDCD1, ECM1, E-cadherin in different groups determined by western blot (mean ± SD, $n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group; $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$ compared with model group

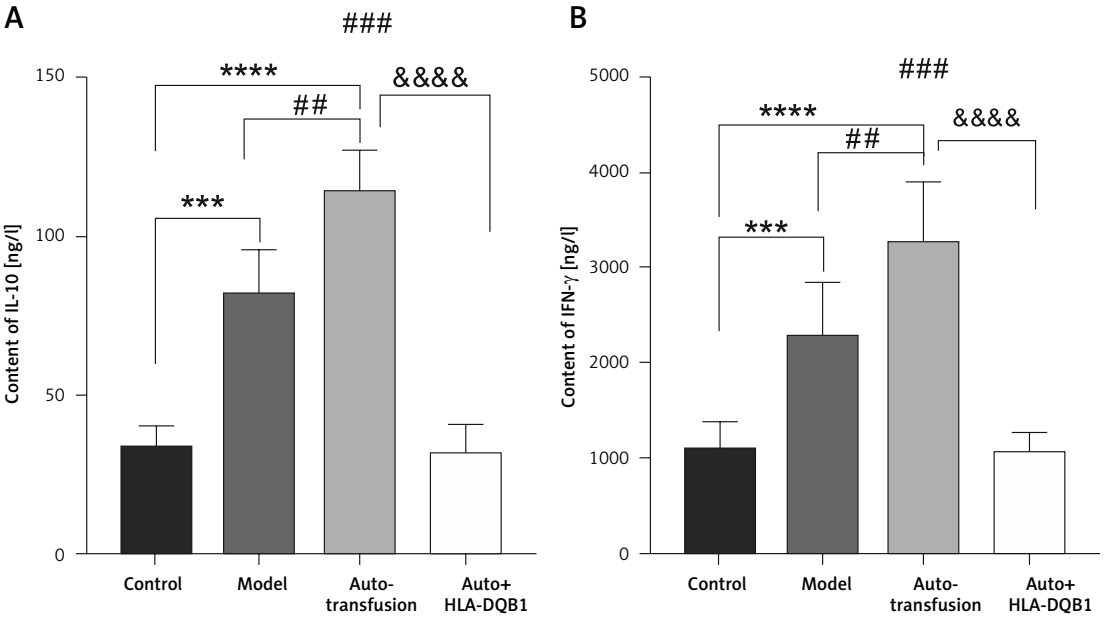

Figure 7. Serum levels of inflammatory cytokines IL-10 and IFN-γ. **A** – Content of IL-10, **B** – content of IFN-γ

that of stock blood, and the biochemical indexes are closer to those of the body. Blood transfusion is an irreplaceable treatment to solve this problem. Studies have found that reclaimed autotransfusion can reduce the expression of PDCD1 protein and reduce the tumor migration and recurrence rate.

PDCD1 is an immunoglobulin expressed primarily on the surface of activated CD4+/CD8+T cells [18]. Low PDCD1 expression and enhanced immune surveillance inhibit the migration and invasion of tumor cells. The expression level of PDCD1 in peripheral blood T cells of tumor patients was higher than that of control subjects. Recent studies have shown that abnormal expression of PDCD1 is closely related to tumors of various systems [19–21]. It was found that PDCD1 expression was elevated in stage II–III colon cancer and inhibited the T cell immune response to the tumor [22]. Immunohistochemical results confirmed that the expression of PDCD1 protein increased in the model group, the expression of PDCD1 protein was the lowest in the autotransfusion group, and the expression of PDCD1 protein was the highest in auto-HLA-DQB1.

Flow cytometry results showed that the number of HLA-DQB1 positive cells was the largest in the autotransfusion group HCC model, which proved that autotransfusion can activate HLA-DQB1 expression. The proliferation of CD4+T and the content of CD4+CD25+T in the blood of mice were the highest in the autotransfusion group, indicating that autotransfusion can activate the expression of the immune system. In conclusion,

the above two experiments demonstrated that HLA-DQB1 activated the CD4+T immune system, reduced the expression of PDCD1, and inhibited the migration and invasion of tumor cells.

In addition, contents of IFN-γ and IL-10 were increased in liver cancer models and the auto+HLA-DQB1 group. This may be because IFN-γ and IL-10 are mainly secreted by helper T lymphocytes (Th) 17, which regulates the interaction between different types of immune cells and promotes the formation of tumor microvessels, and is involved in the occurrence, invasion and metastasis of tumors [23].

In conclusion, autotransfusion activated expression of the signaling molecule HLA-DQB1, enhanced the immune surveillance of CD4+T cells, reduced the expression of PDCD1 on the surface of liver cancer cells, and reduced the metastasis and recurrence of liver cancer.

Acknowledgments

This work is supported by the National Natural Science Foundation of China (No. 82170225) and Key Discipline Group Construction Project of Pudong Health Bureau of Shanghai (No. PWZxq2022-8).

Laiwei You, Yu Bai and Ying-Hui Cui contributed equally to this study.

Conflict of interest

The authors declare no conflict of interest.

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