

Expression Characteristics of Surface Markers of Memory T cells, CD45RO, CCR7 and CD62L, in Tumor-infiltrating Lymphocytes in Liver Cancer Tissues of Patients with Hepatocellular Carcinomas

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Abstract

Purposes: To investigate expression of surface markers on memory T cells and distribution of T cell subsets among tumor-infiltrating lymphocytes (TILs) in liver of hepatocellular-carcinoma (HCC) patients.

Methods: TILs from liver tumor and paracancerous tissue (PT) of 30 HCC patients and 30 subjects who were performed hepatectomy due to benign pathogenic lesion (control) were stained with anti-CD3, CD4, CD8, CD45RO, CD62L and CCR7 antibodies. The distribution of four T cell subsets, CD45RO⁺CD62L⁺CCR7⁺T (central memory T cells, T_{CM}), CD45RO⁺CD62L⁻CCR7⁺T (effector memory T cells, T_{EM}), CD45RO⁺CD62L⁺CCR7⁻T (CCR7⁻T) and CD45RO⁺CD62L⁻CCR7⁻T (CCR7⁺T), among TILs was measured with polychromatic flow cytometry and analyzed statistically.

Results: (1) CD4⁺T_{CM} subset in three groups accounted for <0.4% of total CD4⁺CD45RO⁺Tm. CD4⁺T_{EM} subset% in HCC, PT and control was 89.73%, 98.68% and 95.45% of CD4⁺CD45RO⁺Tm, which was significantly lower in HCC than in PT and control. CCR7⁻T subset% in HCC, PT and control was 0.04, 0.46 and 2.44% of CD4⁺CD45RO⁺Tm, which was significantly lower in HCC and PT than in control. CCR7⁺T subset% in HCC, PT and control group was 9.97%, 0.57% and 1.74% of CD4⁺CD45RO⁺Tm, which was significantly higher in HCC than in PT and control. **(2)** CD8⁺T_{CM} subset% in HCC, PT and control was 0.41%, 0.55% and 0.26% of CD8⁺CD45RO⁺Tm, which was significantly higher in HCC and PT than in control. CD8⁺T_{EM} subset% in HCC, PT and control was 92.39%, 98.14% and 98.16% of CD8⁺CD45RO⁺Tm, which was significantly lower in HCC than in PT and control. CCR7⁻T subset% in HCC, PT and control was 6.44%, 1.03% and 0.75% of CD8⁺CD45RO⁺Tm which was significantly higher in HCC than in PT and control.

Conclusion: T_{EM} was the major subset among CD45RO⁺Tm of HCC patients. CD4⁺T_{EM}, CD8⁺T_{EM} and CCR7⁻T subsets were significantly lower whereas CCR7⁺T and CCR7⁺T subsets were significantly higher in tumor than in non-tumor tissues.

Keywords: Memory T cells (Tm); CD45RO; CD62L; CCR7; Tumor-infiltration lymphocytes (TILs); Tumor microenvironment; Primary hepatocellular carcinoma (HCC)

Introduction

After contacting with antigens for the first time, the naïve T cells (T_n) within body recognize antigen presenting cells (APC). After presenting antigens, they are activated and differentiate into effector T cells (T_{EC}) and under the action of chemokines, they migrate to the antigen presenting sites where they play a role in mediating immune responses. After differentiation from T_n cells to T_{EC}, great changes take place in the expanding capability, chemotactic properties and functions. When the antigens are removed, a large number of T_{EC} undergo apoptosis via Fas-FasL and TNF- α -TNF-R and a part of T_{EC} differentiate into memory T cells (T_{mem}) [1]. T_{mem} cells are different from T_n in term of phenotype characteristics, tissue distribution, secretion of cytokines, and effector functions. Traditionally, CD45RA⁺T cells, also known as T_n cells, i.e. the naïve T cells, are thought to have the immunosuppressive functions whereas CD45RO⁺T cells known as memory T cells (T_{mem}) have the accessory inductive functions [2]. Based on the differences in homing characteristics and effector functions, CD45RO⁺Tm can be divided into four subsets: CD45RO⁺CD62L⁺CCR7⁺T cells, CD45RO⁺CD62L⁻CCR7⁺T cells, CD45RO⁺CD62L⁺CCR7⁻T cells and CD45RO⁺CD62L⁻CCR7⁻T cells. The first two subsets are the currently more studied, so called central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) [3], which are also the focal points of the concern and discussion in this

study. However, other two subsets are poorly known and their functions are practically unknown. When T_{mem} cells face the same antigen again, they are more easily activated to generate antigen-specific T helper cells and cytotoxic T lymphocytes (CTL) and induce rapid, strong, specific and effective immune responses. Thus, they play an important role in protective immunity within the body [4]. At the present, little is known about the roles of different subsets of T cells in anti-cancer immunity. Thus, in the present study, based on different surface markers of different subsets of T cells, we applied polychromatic flow cytometry (PFC) to analyze the expression characteristics of various subsets of tumor-infiltrating memory T cells of patients with HCC, aiming to gain deeper insights into immune state within the tumor microenvironment of liver cancer.

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Materials and Methods

Clinical data

The specimens of liver tissues were all derived from surgically operated patients who were performed hepatectomy from October 2011 to December 2012. A total of 30 patients with liver cancer were pathologically confirmed to be HCC post-operationally. Among them, 23 patients were males and 7 patients were females with the mean age of 45.4 ± 12.6 years, ranging from 35 to 58 years. They had not received any related therapies such as radiotherapy and chemotherapy. Their tumors were completely resected during the surgical operation. 30 patients with liver cancer all have the case history of hepatitis B virus (HBV) infection and were diagnosed with the diagnosis criteria for HBV set by the Ministry of Health of People's Republic of China. The mixed infection with other hepatitis virus was eliminated. Among 30 patients with HCC, the liver tumor tissues of 4 patients had fewer than 5000 infiltrating lymphocytes and thus, they were not included in the statistical analysis ($n=26$). There were 30 cases in the control group. These patients were performed hepatectomy because of the benign pathological changes but they were not infected by HBV ($n=30$). Among them, 13 patients were males and 17 patients were females with a mean age of 48.1 ± 12.4 years, ranging from 35 to 64 years. All the investigated patients had no history of autoimmune disease such as diabetes and hyperthyroidism and had no history of immunotherapy. The clinical data included sex, age, HBV infection history, serum alpha-fetoprotein (AFP), hepatitis B two half-and-half check and post-operational pathological data.

Ethic and sampling

The research protocols were approved by The Ethic Committee of Peking University Shenzhen Hospital and all the patients and the volunteer participants signed the Informed consents. Fresh liver tissues were obtained from the patients who were performed hepatectomy and liver tissues were treated within 6 hours. For the patients with HCC, specimens were obtained from the tumor tissues (inside the tumor 1 cm from the edge of tumor tissues) and the paracancerous tissue (more than 5 cm from the tumor tissues) and included in the HBV-HCC group and paracancerous tissue group, respectively. The specimens in the control group were obtained from the normal liver tissues of patients who were performed hepatectomy due to the benign pathological changes.

Detection of infiltrating lymphocytes in liver tissues with flowcytometry

Equipment and reagents: PE/Cy7 labeled anti-CD3 antibody (PE/Cy7-CD3), FITC-CD4, PE-CD8, APC/Cy7-CD62L, APC-CD197 (CCR-7), Percp/Cy5.5-CD45RO as well as the corresponding isotype controls, APC/Cy7-IgG1, APC-IgG2a, and Percp/Cy5.5-IgG2a were all obtained from Biologend (San Diego, CA, USA). Human lymphocyte isolation medium and human tumor cell isolation medium were purchased from Sigma (St Louise, MO, and USA). Fetal bovine serum (FBS), modified RPMI-1640 medium, 0.25% trypsin and phosphate buffered saline (PBS) were obtained from HyClone (Logan, UT, USA). FACSCanto flow cytometry was purchased from BD Biosciences (San Jose, CA, USA). High speed refrigerated centrifuge 5810R was obtained from Eppendorf (Hamburg, Germany). Low speed autobalancing centrifuge LDZ5-2 was obtained from Beijing Medical Centrifuge Co., Ltd (Beijing, China). BCM-1300 series biological clean bench was purchased from AirTech (Huntington Beach, CA, USA). Ultra Thermostatic water bath DKB-501A was obtained from Shanghai Jing Hong Laboratory Instrument Co., Ltd (Shanghai, China).

Hemocytometer XB-K-25 was obtained from Yuhuan County Medical Products Factory (Yuhuan, Zhejiang, China). Binocular microscope CH20 was obtained from Olympus (Tokyo, Japan). High quality micro slide glass was obtained from Shitai Industrial, Co. Ltd. (Shanmen, Zhejiang, China). Single channel Nichipet EX-Plus Pipette was purchased from NICHIRYO (Tokyo, Japan). 15 ml/50 ml centrifuge tubes and 60 mm \times 15 mm culture plates were purchased from Corning (Corning, NY, USA). HemolysinOptilyse C was obtained from Beckman Coulter (Sykesville, MD, USA). BD flow cytometry tubes were obtained from Zhejiang Gongdong Medical Technology Co., Ltd. (Huangyan, Zhejiang, China). Small animal dissecting instruments set was purchased from Shanghai JWFU Industry Co., Ltd (Shanghai, China). 100/300 mesh screening filters were obtained from Shanghai Bioleaf Biotech Co., Ltd (Shanghai, China).

Measurement methods: Fresh specimens of liver tissues were put to the culture dish and washed with physiological saline solution to remove the surface blood residues. The bile duct and necrosis tissues were removed with scissors. The fresh specimens of liver tissues were chopped into 1-2 mm³ pieces and ground or digested with enzymes, and washed repeatedly with PBS solution to collect the cell suspension, filtered with 100/300 mesh screening filter and centrifuged at low speed to remove the cell aggregate and cell debris. The suspension solution was centrifuged at 2000 rpm for 5 min. The supernatant was discarded and the cell pellet was re-suspended with PBS solution. The lymphocytes were isolated with the method of Ficoll density gradient centrifugation. The resulting infiltrating-lymphocyte suspension of liver tissue was washed with 5 ml of PBS twice and centrifuged at 2000 rpm and 1500 rpm successively at room (26°C) for 5 min. The lymphocytes were suspended to 500 μ l with PBS. After being counted with hemocytometer, the lymphocyte number in the tissue infiltrating lymphocyte suspension was re-adjusted to more than 1×10^6 /ml. The specimen tube and the control tube were set. 200 μ l of infiltrated lymphocyte suspension solution was added to each tube. 5 μ l of each of 6 fluorescently labeled antibodies PE/Cy7-CD3, FITC-CD4, PE-CD8, Percp/Cy5.5-CD45RO, APC/Cy7-CD62L and APC-CCR7 was added to the specimen tube. 5 μ l of each of PE/Cy7-CD3 and FITC-CD4 and the corresponding isotype control antibodies, APC/Cy7-IgG1, APC-IgG2a, and Percp/Cy5.5-IgG2a, was added to the control tube. The mixtures were mixed gently and incubated at dark for 30 min, then, 1-2 ml of 10% hemolysin optilyse C was added and incubated in dark at room temperature for 5-10 min. The mixtures were washed with the same volume of PBS twice and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the antibody residues were washed. After being re-suspended with 200 μ l of PBS, the lymphocytes were measured with flowcytometry. More than 5000 lymphocytes were acquired and measured with FACSCanto flowcytometry and the results were analyzed with Flow jo software.

Statistical methods

SPSS13.0 software was used for performing statistical analysis of the experimental data and the results of each group were expressed as $\bar{x} \pm SD$ %. The differences of the mean values between groups were tested by t-test. When p value was <0.05 , the difference between groups was regarded as statistically significant.

Results

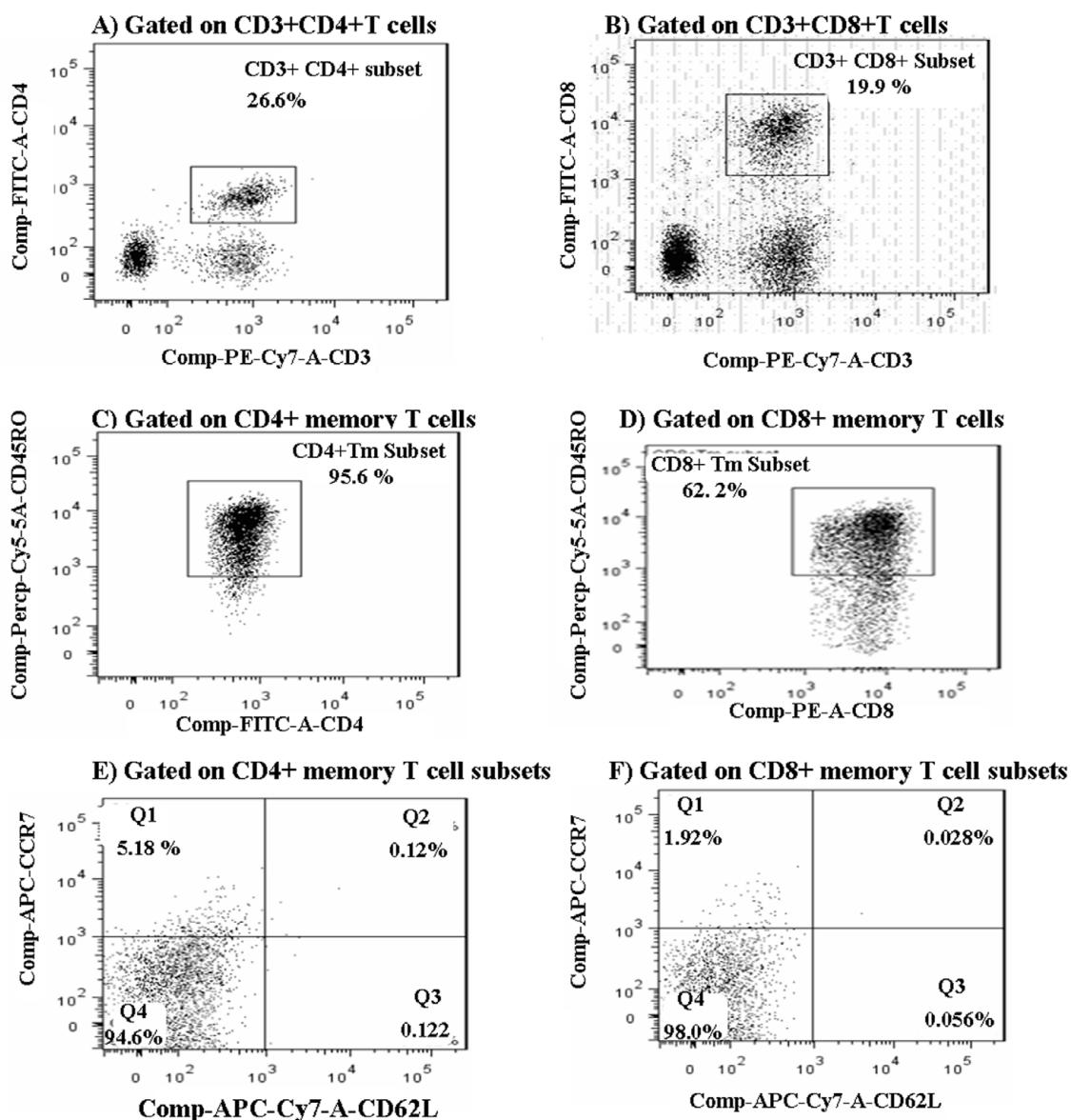
Among 30 patients with HCC, infiltrating lymphocytes isolated from their liver tumor tissues of 4 patients were fewer than 5000 and thus, they were not included in the statistical analysis. When infiltrating lymphocytes of liver tissues were analyzed, they were gated on subsets

through forward scatter (FSC) and side scatter (SSC) and then were gated on the subsets of CD3⁺CD4⁺T cells (Figure 1A) and CD3⁺CD8⁺T cells (Figure 1B). The CD45RO⁺Tm cells were gated on CD3⁺CD4⁺T cells and CD3⁺CD8⁺T cells, respectively (Figures 1C and 1D). Finally, CD45RO⁺Tm cells were further sub-grouped according to the expression of CD62L and CCR7 (Figures 1E and 1F). The upper-right corner (where Q2 was located) was CD45RO⁺Tm CD62L⁺CCR7⁺ subset, i.e. T_{CM} subset; The lower-left corner (where Q4 was located) was the CD45RO⁺Tm CD62L⁻CCR7⁻ subset, i.e. T_{EM} subset; The lower-right corner (where Q3 was located) was CD45RO⁺Tm CD62L⁺CCR7⁻ subset; and the upper-left corner (where Q1 was located) was CD45RO⁺Tm CD62L⁻CCR7⁺ subset.

Analysis of T_{CM} and T_{EM} subsets of CD4⁺CD45RO⁺ Tm cells

The analyzed results of T_{CM} and T_{EM} subsets of CD4⁺CD45RO⁺

Tm cells were presented in Table 1, which indicated that CD4⁺T_{CM} subset accounted for 0.25 ± 0.17%, 0.29 ± 0.22% and 0.37 ± 0.33% of total CD4⁺CD45RO⁺Tm in HBV-HCC group, paracancerous tissue group and control group, respectively. Multiple comparisons revealed that the differences between HBV-HCC group, paracancerous tissue group, between HBV-HCC group and control group; and between paracancerous tissue group and control group were not statistically significant ($P=0.624, 0.089, 0.229; P>0.05$, respectively). CD4⁺T_{EM} subset counted for 89.73 ± 7.41%, 98.68 ± 0.55% and 95.45 ± 3.66% of CD4⁺CD45RO⁺Tm in HBV-HCC group, paracancerous tissue group and control group, respectively. The multiple comparisons among three groups ($P=0.000, 0.000, 0.013$, respectively) indicated that the ratio of CD4⁺T_{EM} relative to CD4⁺CD45RO⁺ Tm in HBV-HCC group was significantly lower than that in paracancerous tissue group and



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Figure 1: Flow cytometry plot of CD45RO⁺Tm subsets of infiltrating lymphocytes in liver tissues. (A) Gated on CD3⁺CD4⁺T cells; (B) Gated on CD3⁺CD8⁺T cells; (C) Gated on CD4⁺ memory T cells; (D) Gated on CD8⁺ memory T cells; (E) Gated on CD4⁺ memory T cell subsets; and (F) Gated on CD8⁺ memory T cell subsets.

Group	TCM Subset (% ± SD)	TEM Subset (% ± SD)
HCC	0.25 ± 0.17	89.73 ± 7.41a
Paracancerous tissue	0.25 ± 0.17	98.68 ± 0.55b
Control	0.37 ± 0.33	95.45 ± 3.66

Note: (a) compared to that of paracancerous tissue and control group $P < 0.05$; (b) compared to that of the control group, $P < 0.05$.

Table 1: The percentage of T_{CM} subset and T_{EM} subset relative to $CD4^+CD45RO^+Tm$ detected by FCM.

Group	CD62L ⁺ CCR7 ⁺ subset (% ± SD)	CD62L ⁻ CCR7 ⁺ subset (% ± SD)
HCC	0.04 ± 0.03a	9.97 ± 7.34c
Paracancerous tissue	0.46 ± 0.35b	0.57 ± 0.33
Control	2.44 ± 2.06	1.74 ± 1.93

Note: (a) compared with that of control group, $P < 0.05$; (b) compared with that of control group, $P < 0.05$; (c) compared with that of paracancerous tissue group and control groups.

Table 2: The percentage of CD62L⁺CCR7⁺subset and CD62L⁻CCR7⁺subset relative to $CD4^+CD45RO^+Tm$ detected by FCM.

Group	TCM subset (% ± SD)	TEM subset (% ± SD)
HCC	0.41 ± 0.31	92.39 ± 4.30b
Paracancerous tissue	0.55 ± 0.60a	98.14 ± 0.74
Control	0.26 ± 0.25	98.16 ± 1.05

Note: (a) compared to that of control group, $P < 0.05$; (b) compared to that of paracancerous tissue group and control group, $P < 0.05$.

Table 3: The percentage of T_{CM} subset and T_{EM} subset relative to $CD8^+CD45RO^+Tm$ detected by FCM.

Group	CD62L ⁺ CCR7 ⁻ subset (% ± SD)	CD62L ⁻ CCR7 ⁻ subset (% ± SD)
HCC	0.76 ± 0.47a	6.44 ± 4.20c
Paracancerous tissue	0.28 ± 0.24b	1.03 ± 0.45
Control	0.83 ± 0.66	0.75 ± 0.72

Note: (a) compared to that of paracancerous tissue group, $P < 0.05$; (b) compared to that of control group, $P < 0.05$; (c) compared to that of paracancerous tissue group and control group, $P < 0.05$.

Table 4: The percentage of CD62L⁺CCR7⁻ subset and CD62L⁻CCR7⁻ subset relative to $CD8^+CD45RO^+Tm$ detected by FCM.

control group ($P < 0.05$). The $CD4^+T_{EM}$ subset is the predominant subset among $CD4^+CD45RO^+Tm$ in HBV-HCC, paracancerous tissue group and control groups whereas the ratio of $CD4^+T_{CM}$ relative to $CD4^+CD45RO^+Tm$ was very small in all three groups.

Analysis of CD62L⁺CCR7 and CD62L⁻CCR7⁺T subsets of CD4⁺CD45RO⁺Tm cells

The analyzed results of CD62L⁺CCR7 and CD62L⁻CCR7⁺T subsets of $CD4^+CD45RO^+Tm$ cells were presented in Table 2, which showed that the ratio of $CD4^+CD45RO^+CD62L^+CCR7^+T$ subset relative to $CD4^+CD45RO^+Tm$ in HBV-HCC, paracancerous tissue group and control groups was $0.04 \pm 0.03\%$, $0.46 \pm 0.35\%$ and $2.44 \pm 2.06\%$, respectively. Multiple comparisons between HBV-HCC group and paracancerous tissue group, between HBV-HCC group and control group; and between paracancerous tissue group and control group ($P = 0.240, 0.000, 0.000$) revealed that this ratio of $CD4^+CD45RO^+CD62L^+CCR7^+T$ subset relative to $CD4^+CD45RO^+Tm$ in HBV-HCC and paracancerous tissue group was significantly lower than that of control group and the differences were statistically significant ($P < 0.05$). The ratios of $CD4^+CD45RO^+CD62L^+CCR7^+T$ relative to $CD4^+CD45RO^+Tm$ in HBV-FCC, paracancerous tissue

group and control group were $9.97 \pm 7.34\%$, $0.57 \pm 0.33\%$ and $1.74 \pm 1.93\%$, respectively. The multiple comparisons among three groups ($P = 0.000, 0.000, 0.313$) revealed that this ratio in HBV-HCC group was significantly higher than that in paracancerous tissue group ($P < 0.05$).

Analysis of T_{CM} and T_{EM} subsets among CD8⁺CD45RO⁺Tm

The analyzed results of T_{CM} and T_{EM} subsets among $CD8^+CD45RO^+Tm$ were presented in Table 3, which indicated that the ratio of $CD8^+T_{CM}$ subset relative to $CD8^+CD45RO^+Tm$ in HBV-HCC group, paracancerous tissue group and control group was $0.41 \pm 0.31\%$, $0.55 \pm 0.60\%$ and $0.26 \pm 0.25\%$, respectively. The multiple comparisons between HBV-HCC group and paracancerous tissue group, between HBV-HCC group and control group and between paracancerous tissue group and control group ($P = 0.221, 0.176, 0.010$) revealed that the ratio of $CD8^+T_{CM}/CD8^+CD45RO^+Tm$ in HBV-HCC group was significantly higher than that in control group ($P = 0.010, P < 0.05$). The ratio of $CD8^+T_{EM}$ relative to $CD8^+CD45RO^+Tm$ in HBV-HCC group, paracancerous tissue group and control group was $92.39 \pm 4.30\%$, $98.14 \pm 0.74\%$ and $98.16 \pm 1.05\%$, respectively. The multiple comparisons between HBV-HCC group and paracancerous tissue group, between HBV-HCC group and control group and between paracancerous tissue group and control group ($P = 0.000, 0.000, 0.974$) indicated that the ratio of $CD8^+T_{EM}/CD8^+CD45RO^+Tm$ in HBV-HCC group was significantly lower than that in paracancerous tissue group and control group ($P = 0.000, 0.000; P < 0.05$). The $CD8^+T_{EM}$ subset was the predominant subset among $CD8^+CD45RO^+Tm$ in HBV-HCC group, paracancerous tissue group and control group whereas the ratio of $CD8^+T_{CM}$ among $CD8^+CD45RO^+Tm$ was very small in all three groups. These results were similar to the distribution of T_{EM} and T_{CM} subsets among $CD4^+CD45RO^+Tm$ described above.

Analysis of CD62L⁺CCR7 and CD62L⁻CCR7⁺T subsets of CD8⁺CD45RO⁺Tm

The analyzed results of the ratios of CD62L⁺CCR7 and CD62L⁻CCR7⁺T subsets relative to $CD8^+CD45RO^+Tm$ were presented in Table 4, which showed that the ratio of $CD8^+CD45RO^+CD62L^+CCR7^+T$ cell subset relative to $CD8^+CD45RO^+Tm$ in HBV-HCC group, paracancerous tissue group, and control group was $0.76 \pm 0.47\%$, $0.28 \pm 0.24\%$ and $0.83 \pm 0.66\%$, respectively. The multiple comparisons between HBV-HCC group and paracancerous tissue group, between HBV-HCC group and control group and between paracancerous tissue group and control group ($P = 0.001, 0.578, 0.000; P > 0.05$) indicated that the ratio of $CD8^+CD45RO^+CD62L^+CCR7^+T/CD8^+CD45RO^+Tm$ in paracancerous tissue group was significantly lower than that in HBV-HCC and control groups and the differences were statistically significant. The ratio of $CD8^+CD45RO^+CD62L^+CCR7^+T$ cell subset/ $CD8^+CD45RO^+Tm$ in HBV-HCC group, paracancerous tissue group and control group was $6.44 \pm 4.20\%$, $1.03 \pm 0.45\%$ and $0.75 \pm 0.72\%$, respectively. The multiple comparisons between HBV-HCC group and paracancerous tissue group, between HBV-HCC and control group and between paracancerous tissue group and control group ($P = 0.000, 0.000, 0.658; P > 0.05$) revealed that the ratio of $CD8^+CD45RO^+CD62L^+CCR7^+T/CD8^+CD45RO^+Tm$ in HBV-HCC group was significantly higher than that in paracancerous tissue group and control group.

Discussion

T cells are consisted of various subsets with different phenotypes and functions. Based on the functions and the differences of major histocompatibility complex (MHC) restriction, T cells can be divided into $CD4^+T$ cells and $CD8^+T$ cells. Based on whether or not T cells have

been stimulated by antigens, T cells can be divided into naïve T cells, effector T cells and memory T cells. The naïve T cells are those T cells that have just left thymus but have not been stimulated by antigens. These T cells play a role in immunologic surveillance through re-circulation between blood and the secondary lymphatic organs. It is generally thought that CD45RA is the surface marker of naïve T cells, which is mainly circulated within the lymphatic systems whereas CD45RO is the surface marker of memory T cells, which is evenly distributed among lymphatic and non-lymphatic tissues [5]. Co-stimulatory signals are necessary for the activation of naïve T cells whereas the dependence of memory T cells on the co-stimulatory signals such as CD28 and CD154 is significantly reduced and the activation threshold for memory T cells is very low. The second stimulation can occur in the outside of the secondary lymphatic organs (or tissues) such as lymphatic nodes but they do not need the assistant of specific MHC. After *in vitro* stimulation, the naïve T cells do not generate IFN- γ , IL-4 and IL-5 but only generate lower level of IL-2, indicating that these cells are different from effector T cells and memory T cells not only in phenotypes but also in tissue distribution and functions.

CD45 molecule, also known as leukocyte common antigen (LCA), is encoded by a single gene located in chromosome 1q32. Alternative splicing of the first three consecutive exons (A, B and C) nearby the amino acid terminal of its full length mRNA leads to generation of a CD45 isoform called CD45RO that lacks these three exons. Another CD45 isoform called CD45RA only expresses exon A. Both CD45RA and CD45RO are two isoforms of CD45, which are widely present on the surface of leukocytes and belong to type I trans-membrane proteins. In cytoplasm, they also possess tyrosine phosphatase activity. They play important roles in the development and maturation of lymphocytes, signal transduction and functional regulation. Based on the expression of CD45RA and CD45RO, CD4⁺ and CD8⁺T cells can be divided into naïve T cells and memory T cells. Based on the homing of the receptor of CD62L and the chemokine receptor 7 (CCR7), the memory T cells can be further divided into T_{CM} and T_{EM} subsets. The surface marker of T_{CM} is CD45RO⁺CD62L⁺CCR7⁺ whereas the surface marker of T_{EM} is CD45RO⁺CD62L⁻CCR7⁻. CCR-7 is a member of the chemokine receptor family. CD62L, i.e. L-selectin, is a member of selectin family. Both CCR-7 and CD62L are involved in the homing of lymphocytes to peripheral lymphatic nodes. T_{EM} is present in the non-lymphatic tissues of the infected sites and plays a role in immunologic surveillance. When they contact an antigen for the first time, under the stimulation of TCR, T_{EM} can rapidly generate IFN- γ , IL-4 and IL-5 and fulfills its responding function, whereas T_{CM} is mainly located in the secondary lymphatic organs and can rapidly proliferate and chemotaxi to the effector sites where they supplement the effector T cells of the surrounding organs to play a role in removing antigens. Even under the stimulation of TCR, T_{CM} does not immediately generate prototypic cytokines such as IFN- γ etc. of the effector cells

The mechanisms that control the conversion of effector T cells to the phenotype of memory T cells are still not clearly understood and even very little is known about how T_{CM} and T_{EM} subsets are formed. The prerequisite for the conversion of effector T cells into memory T cells is that they must escape the programmed cell death (apoptosis). Acquiring the survival advantage in time is extremely important for memory T cells. IL-7 is the key factors for the conversion of effector cells into memory T cells. It enables the CD4⁺ and CD8⁺ effector T cells to acquire long-term survival and steady proliferation [6,7]. There are data indicating that CD8⁺ memory T cells are directly originated from CD8⁺ effector T cells [8]. These CD8⁺ memory T cells express effector protein molecules such as perforin, granzyme-B and cytokines and,

at the same time, highly express IL-17 receptor and a large amount of anti-apoptotic molecules [9]. In addition to cytokines, the generation of CD8⁺ memory T cells also requires the assistant of CD4⁺T cells. If the assistant of CD4⁺T cells is lacking, CD8⁺T cells would not be converted to immune memory T cells [10]. Transcriptional factors play important roles in the formation of CD8⁺ memory T cells. Sullivan et al. [11] found that lacking of transcriptional factor FOXO3 can increase the number of CD8⁺ memory T cells but does not influence the phenotypes and functions of CD8⁺T memory T cells, implying that during the clonal expanding period, apoptosis of effector CD8⁺T cells can be controlled by regulating the expression level of transcriptional factor FOXO3 to influence the proliferation or vanish of memory T cells and finally influence of the formation of memory T cells.

The mechanisms by which the effector CD4⁺ T cells are converted into CD4⁺ memory T cells require to be further investigated. CD4⁺T cells are a complex immune regulatory network and the main members include helper T cells 1 (Th1), Th2, Th17, follicular helper T cells (Tfh) and regulatory T cells (Treg). It has been demonstrated that Th1-like effector cells can generate Th1 memory cells and these Th1 memory cells possess the “traditional characteristics” of T_{EM} [12]. Similarly, there is evidence demonstrating that after the contraction phase of immune responding reactions, Th2 effector cells can form T_{EM} [13]. While it is not clear how Th17 effector cells enter the memory cell pool, there are many lines of evidence supporting that the *in vitro* derived Th17 cells could become IFN- γ -secreting cells after being introduced into mice [14,15]. These studies have indicated that Th1 effector cells, Th2 effector cells and Th17 effector cells can be possibly converted into CD4⁺T_{EM}. Currently, there is very rare evidence for the entrance of the transcriptional factor FOXP3-expressing Treg cells to the memory T cell pool, because there is experimental evidence confirming that FOXP3⁺ cells are not present in the memory T cell populations after infection [16]. However, another study reported that Treg cells had lost their expression of FOXP3 before they became memory T cells [17]. Regarding to the origin of T_{CM}, several investigators believed that Tfh cells that have the characteristics of expressing chemokine receptor CXCR5 was one of the origins for T_{CM} [18,19] whereas other investigators believed that there was the existence of precursor cells of T_{CM} [20,21]. Additionally, B cells also play similarly important roles in the generation of memory T cells and in “determining” T_{CM}-T_{EM} [22-24].

In this study, we found that among the tumor infiltrating CD45RO⁺Tm cells, T_{EM} subset was the predominant subset in both CD4⁺-CD45RO⁺Tm cells and CD8⁺-CD45RO⁺Tm cells whereas the proportion of T_{CM} subset was very small. This may be related to the heterogeneity of the generation processes and tissue distribution of T_{EM} and T_{CM}. After leaving the thymus, naïve T cells express CCR7 and CD62L and are re-circulated in blood and the secondary lymphatic organs to perform the immune surveillance function. After being activated by the antigen-presenting dendritic cells (DC), naïve T cells, CD28 enhances the activation of T cells and the activated T cells migrate to the target organs where they play the effector functions. After the antigens have been removed, more than 90% of effector T cells are removed by the mean of apoptosis and only 5% to 10% of the effector T cells differentiate into resting memory T cells. Among which, a part of memory T cells also express CCR7 and CD62L, i.e. T_{CM} subset, and are homing to lymphatic nodes. Another part of memory T cells, i.e. T_{EM} subset, are selectively remained in the tissues [25]. T_{EM} cells can also express high levels of α 1- and α 2-integrin. Integrin plays a role in mediating the adherence of T cells to extracellular matrixes and is a cell surface receptor required for the entrance of T cells into the inflammatory tissues. The CCR7-Tm including T_{EM} cells highly

express the tissue-specific homing receptors CD103 and CLA that help T cells to enter the normal or inflammatory tissues and also express inflammatory cytokine receptors that have the activity of chemotactic attraction. These receptors stimulate T_{EM} to enter non-lymphatic tissues [26]. In this study, we observed that the ratios of $CD4^{+}$ - and $CD8^{+}T_{EM}$ subsets in $CD45RO^{+}Tm$ of liver tissues were very high, indicating that they play the major effector roles. In contrast, T_{CM} express two lymphatic node-homing receptors, CD62L and CCR7. Thus, T_{CM} are selectively homing to lymphatic nodes and thus, their distribution in liver tissues is much smaller.

Our study has also demonstrated that among the tumor infiltrating $CD45RO^{+}Tm$ in HCC patients, the ratios of $CD4^{+}T_{EM}$ and $CD8^{+}T_{EM}$ relative to $CD45RO^{+}Tm$ in liver tumor tissues were significantly lower than those in non-tumor tissues whereas the ratios of $CD4^{+}T_{CM}$ and $CD8^{+}T_{CM}$ relative to $CD45RO^{+}Tm$ in liver tumor tissues were not different from those in non-tumor tissues. T_{CM} are homing to the secondary lymphatic organs. They play a major role in mediating reactive memory [27]. When they contact the antigen for the second time, they proliferate again and differentiate into effector T cells but play little effector role and some T_{CM} do not have effector function at all. Different from T_{CM} , T_{EM} cells migrate to the surrounding tissues and are mainly involved in mediating protective memory [27]. When they are stimulated by the antigen again, they rapidly generate effector factors and immediately confer effector functions. For example, $CD4^{+}T_{EM}$ and $CD8^{+}T_{EM}$ can rapidly generate IFN- γ , IL-4 and IL-5. $CD8^{+}T_{EM}$ can also immediately secrete perforin to play a killing role. In the HBV-liver cancer tissues, the ratios of $CD4^{+}T_{EM}$ and $CD8^{+}T_{EM}$ relative to $CD45RO^{+}Tm$ were significantly smaller than those in non-tumor tissues, indicating that under the influence of tumor micro-environment, the number of T_{EM} was significantly reduced and their ability to immediately confer effector function is reduced when they face secondary response and are unable to play a role in killing tumor cells. In patients with HCC, the reduced number and the decreased functions of T_{EM} cells are related with several factors. The up-regulation of FoxP3 $^{+}$ Treg cells in cancerous tissue during the progression of HCC significantly was found to inhibit the tumor infiltrating $CD8^{+}T$ cells [28]. The functions of cancer-infiltrating $CD8^{+}T$ cells are also influenced and they express lower levels of IFN- γ and perforin [29]. These effects are related to the action of the cytokine, transforming growth factor- β 1 (TGF- β 1), produced by HCC cells because TGF- β 1 up-regulates the expression of FOXP3 gene [30]. While the mechanisms involved are currently unknown, they may be related the tumor immune suppression.

Immune memory is one of the important characteristics of acquired immunity in higher animals. When the animals contact the same antigen again, they rapidly generate effective immune responses, which are accomplished by a series specific memory T cells. Thus, the memory T cells have received much concern from the researcher circles and drawn increasing attentions. Compared to the naïve T cells, the memory T cells have lower activation threshold and stronger effector functions. During the process of preventing the reinfection of pathogens, memory T cells respond rapidly, functioning strongly and play an important role. Thus, the current studies on memory T cells have mainly focused in the fields of infectious diseases such as viral hepatitis, AIDS and tuberculosis etc. [31]. Tumor infiltrating lymphocytes (TIL) isolated from tumor tissues are thought to have stronger capability of killing tumor cells. It has been found that the prognosis for the HCC patients whose tumor tissues have significant infiltrating lymphocytes is more superior over that of those HCC patients who had almost no infiltrating lymphocytes in liver tumor tissues [32]. This observation indicates that TILs play an important role in regional immune microenvironment

within tumor tissues. Analysis of the distribution characteristics of the tumor infiltrating memory T cell subsets is helpful for a better understanding of the state of tumor immune microenvironment and for judging prognosis. After being activated, adoptive infusion of the tumor-specific memory T cells isolated from TILs into the tumor-carrying patients will increase the effectiveness of the adoptive immunotherapy because these cells have already been sensitized and activated by autologous tumor cells. Vaccinations against HBV have already been one of the most effective measures for preventing the invasion of HBV. However, for patients who have already been infected with HBV, the risk of developing HCC is still very high. Perhaps, in the near future, we are able to prepare the HCC-related antigens, use them to vaccinate the susceptible populations and generate T_{EM} cells existing in the liver tissues for a long run, preventing the occurrence of liver cancer. In the cancerous tissue of patients with HCC, T_{EM} cells are in the immune suppressive state. Further study to understand the mechanisms underlying the suppressive state of T_{EM} cells will be helpful for us to change this state and to enhance the anti-cancer immune effects of T_{EM} cells. Additionally, the *in vitro* culturing the functionally competent T_{EM} cells and transplanting them to the patients with HCC may also be a promising immune therapy method for HCC in the future. For instance, Klebanoff et al. [33] performed adoptive cell transfer (ACT) with T_{CM} , T_{EM} and T_{SCM} (stem cell memory cells) to mouse B16 melanoma and obtained relatively satisfactory results, i.e. ACT of these cells caused a significant regression of tumors. Their further study [34] also has indicated that it is the $CD8^{+}T_{CM}$ rather than $CD8^{+}T_{EM}$ cells that have more potent anti-tumor effects. Of courses, only a few studies have been conducted on memory T cells in the cancer fields, many unknown mechanisms remain to be further elucidated by the cancer researcher circles.

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