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Interleukin-7 enhances anti-tumor activity of CD8$^+$ T cells in patients with hepatocellular carcinoma

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ABSTRACT

Interleukin (IL)-7 stimulation improves virus- and tumor-specific CD8$^+$ T cell responses. However, the role of IL-7 in modulation of dysfunctional CD8$^+$ T cells in hepatocellular carcinoma (HCC) was not completely understood. In this study, a total of 37 HLA-A2 restricted patients with HCC and 16 healthy individuals were enrolled. IL-7 expression and its receptor α chain CD127 level was measured. The regulatory activity of IL-7 to peripheral and liver-resident CD8$^+$ T cells was investigated in co-culture systems which were directly or indirectly contacted with HCC cell line HepG2 *in vitro*. Serum IL-7 concentration was significantly reduced in HCC patients, while effective anti-tumor treatment up-regulated IL-7 expression. However, CD127 expression was comparable on peripheral CD8$^+$ T cells from either HCC patients and healthy individuals, and was also similar on liver-resident CD8$^+$ T cells from either normal tissues and HCC specimens. CD8$^+$ T cells purified from normal liver tissues also presented stronger cytotoxicity compared with those from HCC specimens prior to and post IL-7 treatment. Moreover, IL-7 stimulation not only augmented cytotoxicity of peripheral and liver-resident CD8$^+$ T cells, but also promoted IFN-γ and TNF-α production by CD8$^+$ T cells in direct contact co-culture system. This process was accompanied by down-regulation of programmed death-1 (PD-1) expression on CD8$^+$ T cells. Our present data indicated that IL-7 enhanced both cytolytic and noncytolytic activity of CD8$^+$ T cells to HCC probably via repression of PD-1 under direct tumor cells presentation. IL-7 might be considered as one of the therapeutic candidates for HCC treatment.

1. Introduction

Hepatocellular carcinoma (HCC) accounts for approximately 90% of primary liver malignancies, and is the second leading cause of cancer-related death all over the world [1,2]. HCC often arises in the setting of persistent viral infection and chronic inflammation. Thus, hepatocarcinogenesis is always followed by typical consequence of hepatocellular necrosis, regeneration of hepatocytes, and liver fibrosis/cirrhosis [3]. The liver plays a pivotal immunomodulatory activity through maintaining immunotolerance, which results from complex interactions between liver-resident cells and peripheral immune cells [4,5], and from complicated cytokine milieu with both proinflammatory and anti-inflammatory cytokines/chemokines expression [6]. Dysregulation of controlled immunological network leads to chronic liver diseases and HCC [3,5]. However, the reason for this dysregulation remains not fully elucidated, and identification of key factors may contribute to novel therapeutic interventions.

Evolution and progression of cancer always leads to dysfunction of CD8$^+$ T cells in both peripheral bloods and tumor infiltrating lymphocytes (TIL) [7]. Antigen-specific CD8$^+$ TIL apoptosis contributes to T cells exhaustion, and dampens anti-tumor immunity [8]. Moreover, immunosuppressive tumor microenvironment favors the inactivation of CD8$^+$ T cells, partially through enhanced expression of inhibitory receptors, including cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed death-1 (PD-1) [9]. The current therapeutic approaches are mainly focus on restoration of functional CD8$^+$ T cells by suppression negative signaling. The immune checkpoint inhibitors augments CD8$^+$ T cells responses directed against tumors [10], especially in HCC [3,11].

Interleukin (IL)-7 is vital for the development, proliferation,

Abbreviations: AFP, alpha fetoprotein; BCLC, Barcelona Clinic Liver Cancer; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; ELISA, enzyme linked immunosorbent assay; HCC, hepatocellular carcinoma; IFN, interferon; IL, interleukin; LDH, lactate dehydrogenase; PBMC, peripheral blood mononuclear cells; PD-1, programmed death-1; NC, normal controls; TIL, tumor infiltrating lymphocytes; TNF, tumor necrosis factor

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activation, and survival of $CD8^+$ T cells. IL-7 stimulation improves virus- and tumor-specific $CD8^+$ T cell responses in numerous animal models and humans [12,13]. However, few studies focus on the modulatory activity of IL-7 on HCC-specific $CD8^+$ T cells. Herein, we hypothesized that IL-7 augments anti-tumor function of $CD8^+$ T cells in HCC patients. To test this possibility, $CD8^+$ T cells isolated from HCC patients were stimulated recombinant human IL-7, and were co-cultured directly or indirectly with HCC cell line HepG2 *in vitro*. The cytolytic and noncytolytic activities of $CD8^+$ T cells were investigated prior to and post IL-7 stimulation.

2. Materials and methods

2.1. Subjects

Thirty-seven HLA-A2 restricted patients with HCC were enrolled in the current study. All patients were hospitalized or followed-up in China-Japan Union Hospital of Jilin University between July 2015 and February 2017. Blood samples, fresh HCC specimens, and patient-matched normal liver tissues were obtained from patients with HCC who underwent surgery in China-Japan Union Hospital of Jilin University, and the diagnosis of all specimens was confirmed by Department of Pathology, China-Japan Union Hospital of Jilin University. No patients received chemotherapy or radiotherapy prior to surgery. The stage of tumor was also determined according to Barcelona Clinic Liver Cancer (BCLC) staging system. For normal controls (NC), sixteen sex and age-matched HLA-A2 restricted healthy individuals were also enrolled. The baseline characteristics of enrolled subjects were shown in Table 1. The study protocol was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University, and written consent form was obtained from each enrolled subjects.

2.2. Isolation of peripheral blood mononuclear cells (PBMC) and TIL

PBMC was isolated from peripheral blood using Ficoll-Hypaque (Tiangen, Beijing, China) density gradient centrifugation. TIL was isolated from tissue specimen. Briefly, tissues were cut into small pieces, and were passage through 70 μm-pore strainers. Cells were treated with 0.002% of RNase I, 0.1% of Collagenas IV, and 0.01% of Hyaluronidase V, and then were resuspended in DMEM. TIL was isolated using Percoll (Sigma, St Louis, MO, USA) by discontinuous density gradient centrifugation.

Table 1
Baseline characteristics of enrolled subjects.

	NC	HCC
Case (n)	16	37
Gender (Male/Female)	11/5	22/15
Age (years)	38.1 ± 10.4	41.8 ± 6.9
AFP (ng/mL)	< 20 (9.12 ± 3.77)	> 400 (3145 ± 891)
BCLC staging A (n)	N.A.	10
BCLC staging B (n)	N.A.	17
BCLC staging C (n)	N.A.	5
BCLC staging D (n)	N.A.	5
Underlying liver disease (n)		
HBV	N.A.	21
HCV	N.A.	6
Alcohol	N.A.	4
Autoimmune hepatitis	N.A.	1
Budd-Chiari syndrome	N.A.	1
None	N.A.	4
Status of cirrhosis (n)		
Compensation	N.A.	5
Decompensation	N.A.	26
None	N.A.	6

2.3. Purification of $CD8^+$ cells

$CD8^+$ T cells were purified from PBMC or TIL using human $CD8^+$ T cells Isolation Kit (Miltenyi, Bergisch Gladbach, Germany) following instructions of manufacturer. The purity of enriched $CD8^+$ T cells was more than 95% according to flow cytometry analysis. Purified $CD8^+$ T cells were stimulated with recombinant human IL-7 (R&D Systems, Minneapolis, MN, USA; final concentration: 10 ng/mL) for 6 h. Cells and supernatants were harvested for further studies.

2.4. Cell culture

HepG2 cells, which were also HLA-A2 restricted, were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2 condition. HepG2 cells were stimulated with recombinant human IL-7 (R&D Systems; final concentration: 10 ng/mL) for 6 h. $CD8^+$ T cells, which were purified from HLA-A2 restricted patients, were pre-treated with recombinant human IL-7 (final concentration: 10 ng/mL) for 6 h, and washed twice to remove exogenous IL-7. 10^5 of stimulated $CD8^+$ T cells were then co-cultured in direct contact or in indirect contact system with 5×10^5 of HepG2 cells in the presence of HLA-A2 restricted peptide derived from human alpha fetoprotein (AFP) [14] (sequence: FMNKFIYEI; final concentration: 10 μg/mL) for 48 h. In direct contact co-culture system, effector and target cells were cultured in common cell culture plates (Corning, Corning, NY, USA). In contrast, effector and target cells were separated by 0.4 μm-pore membrane in a Transwell chamber (Corning) in indirect contact co-culture system [15]. This allowed the passage of soluble factors only. Supernatants were harvested for further experiments.

2.5. Cellular proliferation assay

10^4 of HepG2 cells were seeded into 96-wel plates. Cellular proliferation assay was performed using Cell Counting Kit-8 (CCK-8; Beyotime, Wuhan, Hubei Province, China) following instructions of manufacturer. $OD_{450\ nm}$ reflected the proliferation of tested cells. The experiment was performed independently five times.

2.6. Transwell invasion assay

The invasion ability of HepG2 cells were assessed using 24-well Transwell chambers (Corning). The Transwell chambers contained 8 μm-pore size polycarbonate membranes and covered with 50 μL of Matrigel (1: 6 dilution; BD Biosciences, San Jose, CA). HepG2 cells were collected and suspended in 200 μL of serum-free DMEM and added into upper chamber. 200 μL of DMEM containing 10% of fetal bovine serum was added into lower chamber. Cells which passed through the membrane were stained with 0.1% crystal violet for 20 min at room temperature. The numbers of cells were calculated under Olympus BX51 microscope (Olympus, Tokyo, Japan) in five random fields. The experiment was performed independently five times.

2.7. Flow cytometry

Anti-CD3 PE-CF594 (BD Biosciences), anti-CD8 APC (BD Pharmingen, San Jose, CA, USA), anti-CD127 PerCP Cy5.5 (BD Pharmingen), anti-CD45RA PE (BD Pharmingen), anti-CD45RO FITC (BD Pharmingen), anti-PD-1 (CD279) FITC (eBioscience), were used for surface staining. Anti-CTLA-4 (CD152) PE was used for intracellular staining. Data were acquired using a FACS Aria II flow cytometer (BD Biosciences), and analyses were performed using FlowJo Software version 7.6.2 (Tree Star, Ashland, OR, USA).

2.8. Cytotoxicity assay

The cytotoxicity of HepG2 cells were assessed by measuring lactate

dehydrogenase (LDH) expression in the supernatants using LDH Cytotoxicity Assay Kit (Beyotime) following instructions of manufacturer. A low level LDH control was represented by HepG2 cells cultured alone, while a high level LDH control was represented by HepG2 cells which was treated with Triton X-100 to lyse the cells and induce maximum LDH release. The percentage of cytotoxicity was calculated in the following equation: (experimental value-low level control)/(high level control-low level control) × 100% [15].

2.9. Enzyme linked immunosorbent assay (ELISA)

Expressions of IL-7, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α were measured by commercial ELISA kits (R&D systems, Minneapolis, MN, USA) following instructions of manufacturer.

2.10. Statistical analyses

All data were analyzed using SPSS version 19.0 for Windows (Chicago, IL, USA). Shapiro-Wilk test was used for normal distribution assay. Variables following normal distribution were presented as mean ± standard deviation, and statistical significance was determined by Student *t* test, Paired *t* test, One-way ANOVA, or SNK-*q* test. Variables following skewed distribution were presented as median [Q1, Q3], and statistical significance was determined by Mann-Whitney test, Wilcoxon matched pairs test, or Kruskal-Wallis test. *P* values of less than 0.05 were considered to indicate significant differences.

3. Results

3.1. Reduced expression of IL-7 in the serum of patients with HCC

We firstly screened the expression profile of IL-7 in the serum in all enrolled subjects, including 16 of healthy individuals and 37 of HCC patients. Serum concentration of IL-7 was significantly down-regulated in HCC patients (22.08 [14.66, 36.96] pg/mL) in comparison with that in NC (36.16 [28.14, 44.36] pg/mL, Mann-Whitney test, $P = 0.0038$; Fig. 1A). However, there were no remarkable differences of serum IL-7 expression among HCC patients with different BCLC stages (Kruskal-Wallis test, $P = 0.554$; Fig. 1B). A total of seventeen patients (10 in stage A and 7 in stage B) underwent liver resection for HCC, and blood samples were harvested one month post-operation. There was a slightly elevation in serum IL-7 post-operation (31.31 [25.00, 41.31] pg/mL *vs.* 17.96 [13.10, 27.02] pg/mL, Wilcoxon matched pairs test, $P = 0.023$; Fig. 1C). Another ten HCC patients in stage B underwent radiofrequency ablation, and IL-7 concentration in the serum was also up-regulated one month post-therapy (42.00 [28.37, 52.67]pg/mL *vs.* 28.12 [15.91, 39.33]pg/mL, Wilcoxon matched pairs test, $P = 0.048$; Fig. 1D).

3.2. Comparable CD127 expression on $CD8^+$ T cells between healthy individuals and HCC patients

We then investigated expression of IL-7 receptor α chain (CD127) on $CD8^+$ T cells in all enrolled subjects. PBMC isolated from 16 of healthy individuals and 37 of HCC patients was stained with anti-CD3, anti-CD8, anti-CD45RA, anti-CD45RO, and anti-CD127. $CD45RA^+CD8^+$ T cells indicated naïve $CD8^+$ T cells, while $CD45RO^+CD8^+$ T cells indicated memorial $CD8^+$ T cells. CD127 expression on both naïve and memorial $CD8^+$ T cells was analyzed. The representative flow dots of peripheral bloods were shown in Fig. 2A, and histograms of CD127 expression in $CD45RA^+CD8^+$ and $CD45RO^+CD8^+$ cells were shown in Fig. 2B. There was no significant difference of peripheral $CD3^+CD8^+$ T cells percentage between NC and HCC patients (28.92 ± 9.14% *vs.* 30.25 ± 6.92%, Student *t* test, $P = 0.563$; Fig. 2C). The percentage of $CD45RA^+CD8^+$ T cells within $CD8^+$ T cells was significantly reduced in HCC patients (31.14 ± 7.31%) compared with that in NC (49.11 ± 8.31%, Student *t* test, $P < 0.0001$; Fig. 1D). In contrast, the frequency of $CD45RO^+CD8^+$ T cells within $CD8^+$ T cells was remarkably elevated in HCC patients in comparison with that in NC (53.30 ± 6.94% *vs.* 33.41 ± 8.04%, Student *t* test, $P < 0.0001$; Fig. 2E). Moreover, MFI value corresponding to CD127 in either $CD45RA^+CD8^+$ T cells (126.9 [111.9, 153.3] *vs.* 118.7 [90.88, 196.0], Mann-Whitney test, $P = 0.581$; Fig. 2F) or $CD45RO^+CD8^+$ T cells (229.2 [177.8, 313.2] *vs.* 224.0 [162.7, 313.6], Mann-Whitney test, $P = 0.946$; Fig. 2G) was

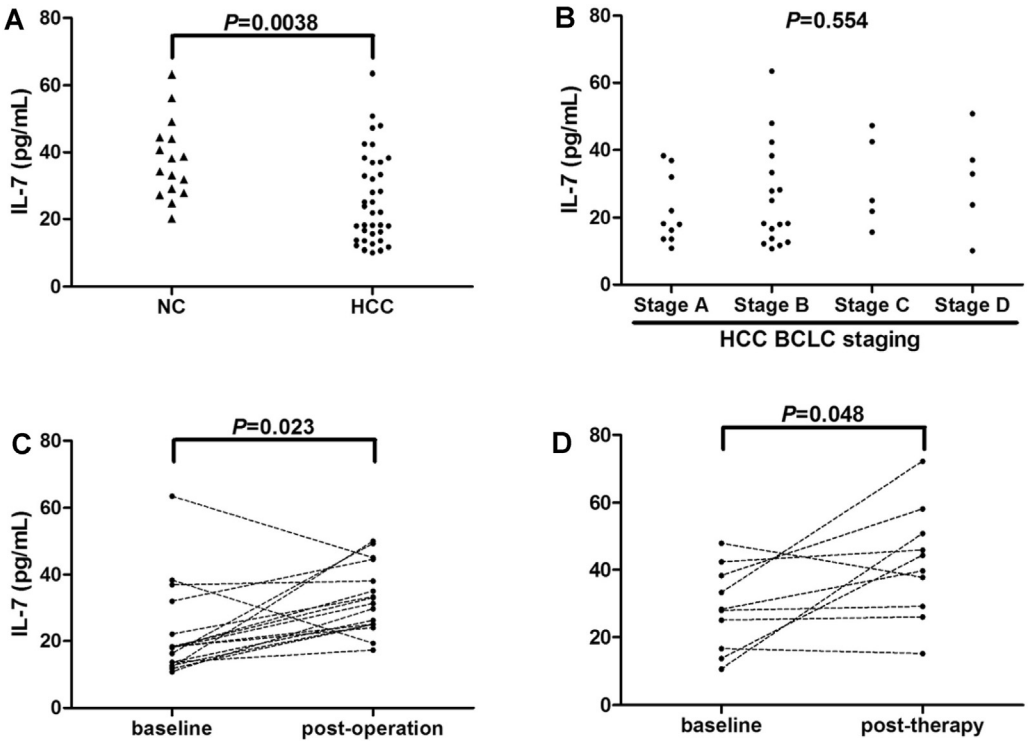

Fig. 1. IL-7 expression in HCC patients and NC. Serum samples were isolated from 16 of NC and 37 of HCC patients, and IL-7 expression was measured by ELISA. (A) Serum IL-7 expression was significantly reduced in patients with HCC in comparison with that in NC. (B) There were no remarkable differences of IL-7 concentration among HCC patients with different BCLC stages. (C) Serum IL-7 concentration was slightly increased in patients who underwent liver resection for HCC one month post-operation. (D) Serum IL-7 concentration was also increased in HCC patients who underwent radiofrequency ablation one month post-therapy. Individual level for each subject was shown.

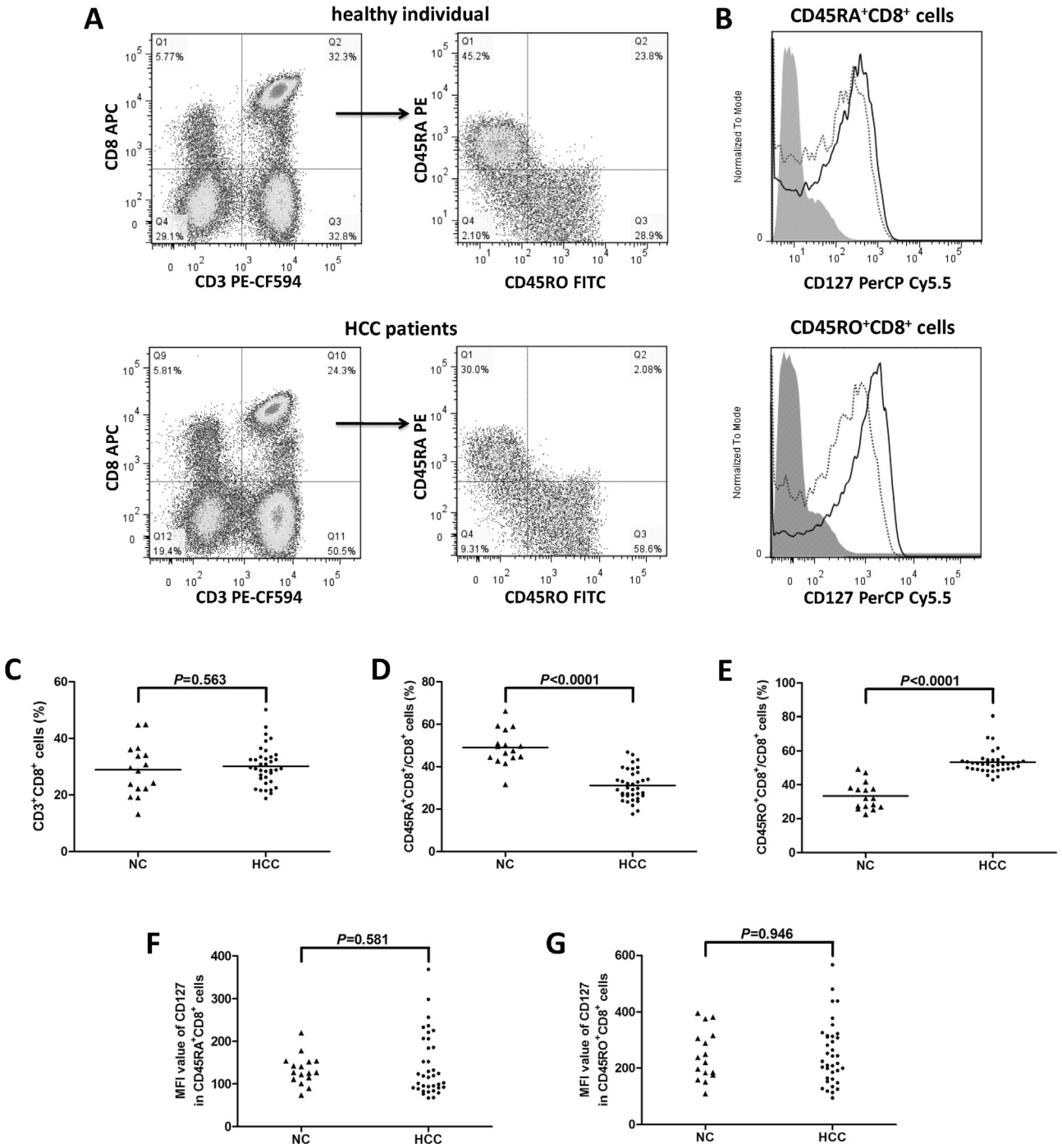

Fig. 2. CD127 expression in peripheral $CD8^+$ T cells in HCC patients and NC. PBMC was isolated from 16 of NC and 37 of HCC patients, and was stained with anti-CD3 PE-CF594, anti-CD8 APC, anti-CD127 PerCP Cy5.5, anti-CD45RA PE, and anti-CD45RO FITC. Data were acquired using FACS Calibur analyzer, and analyses were performed using FlowJo Software. (A) The representative flow dots of $CD45RA^+CD8^+$ and $CD45RO^+CD8^+$ cells in healthy individual and HCC patients were shown. (B) Histograms of CD127 expression in $CD45RA^+CD8^+$ and $CD45RO^+CD8^+$ cells were shown. The dark shadow represented expression of the isotype control, the dark gray dotted line the NC, and the black line the HCC patients. (C) There was no significant difference of $CD3^+CD8^+$ T cells percentage between NC and HCC patients. (D) The percentage of $CD45RA^+CD8^+$ T cells within $CD8^+$ T cells was significantly reduced in HCC patients compared with that in NC. (E) The percentage of $CD45RO^+CD8^+$ T cells within $CD8^+$ T cells was remarkably elevated in HCC patients in comparison with that in NC. (F) MFI value corresponding to CD127 in $CD45RA^+CD8^+$ T cells was comparable between NC and HCC patients. (G) MFI value corresponding to CD127 in $CD45RO^+CD8^+$ T cells was comparable between NC and HCC patients. Individual level for each subject was shown, and horizontal bar indicated mean level.

comparable between NC and HCC patients.

Furthermore, TIL isolated from ten HCC specimens and patient-matched normal liver tissues was also stained for CD127 expression analysis on $CD45RA^+$ naïve and $CD45RO^+$ memorial $CD8^+$ T cells. In HCC patients, the percentage of $CD3^+CD8^+$ T cells from HCC tissue was also comparable with that from normal liver tissue ($18.96 \pm 9.96\%$ *vs.* $20.03 \pm 8.24\%$, Student *t* test, $P = 0.796$; Fig. 3A). The frequency of $CD45RA^+CD8^+$ T cells within $CD8^+$ T cells was robustly decreased in TIL isolated from HCC tissues in comparison with normal liver specimens ($28.19 \pm 6.64\%$ *vs.* $41.10 \pm 7.23\%$, Student *t* test, $P = 0.0006$; Fig. 3B), while $CD45RO^+CD8^+$ T cells percentage in TIL from HCC tissues ($47.46 \pm 4.48\%$) was notably

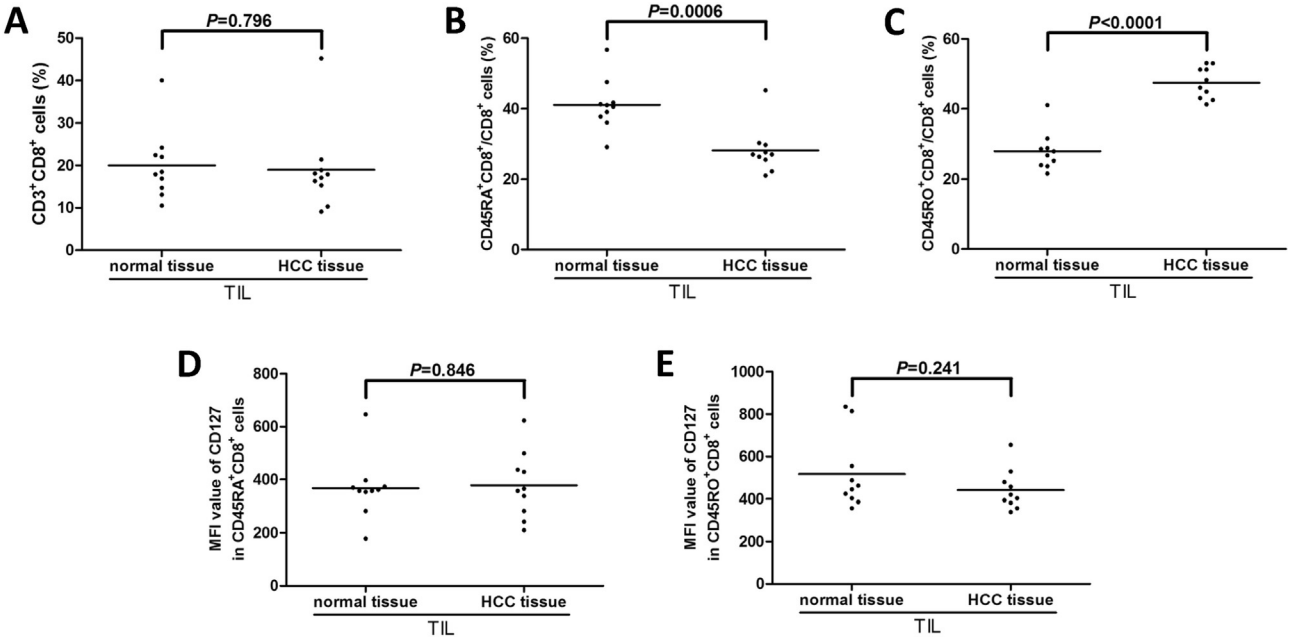

Fig. 3. CD127 expression in liver-resident $CD8^+$ T cells in HCC tissues and normal liver tissues. TIL, isolated from ten HCC specimens and patient-matched normal liver tissues, was stained for CD127 expression analysis on $CD45RA^+CD8^+$ T and $CD45RO^+CD8^+$ T cells. (A) There was no significant difference of $CD3^+CD8^+$ T cells percentage between normal liver and HCC tissues. (B) The percentage of $CD45RA^+CD8^+$ T cells within liver-resident $CD8^+$ T cells was significantly decreased in HCC tissues compared with that in normal tissues. (C) The percentage of $CD45RO^+CD8^+$ T cells within liver-resident $CD8^+$ T cells was remarkably increased in HCC tissues compared with that in normal tissues. (D) MFI value corresponding to CD127 in liver-resident $CD45RA^+CD8^+$ T cells was comparable between normal liver and HCC tissues. (E) MFI value corresponding to CD127 in liver-resident $CD45RO^+CD8^+$ T cells was also comparable between normal liver and HCC tissues. Individual level for each subject was shown, and horizontal bar indicated mean level.

increased than that from normal liver tissue (27.90 ± 5.47%, Student *t* test, $P < 0.0001$; Fig. 3C). However, there were also no significant differences of CD127 expression in either $CD45RA^+CD8^+$ T cells (368.0 ± 116.7 *vs.* 378.6 ± 124.3, Student *t* test, $P = 0.846$; Fig. 3D) or $CD45RO^+CD8^+$ T cells (517.2 ± 171.3 *vs.* 442.1 ± 94.59, Student *t* test, $P = 0.241$; Fig. 3E) between normal and HCC tissue.

3.3. IL-7 stimulation did not affect proliferation and invasion of HepG2 cells

10^4 of HepG2 cells were seeded into 96-wel plates, and were stimulated with IL-7 for 6 h. Cells were washed twice to remove exogenous IL-7, and were cultured for another 42 h. CCK-8 results showed that there were no significant difference in HepG2 cellular proliferation with or without IL-7 stimulation (Student *t* test, $P = 0.836$; Fig. 4A). Moreover, transwell assay showed that invasion HepG2 cell counts were also comparable with or without IL-7 treatment (115.8 ± 12.66 *vs.* 110.4 ± 15.46, Student *t* test, $P = 0.561$; Fig. 4C).

3.4. IL-7 stimulation dampened PD-1 expression in tumor-infiltrating $CD8^+$ T cells

Peripheral $CD8^+$ T cells were purified from 15 healthy individuals and 17 HCC patients, while liver-resident $CD8^+$ T cells were isolated from 17 HCC specimens and patient-matched normal liver tissues, which were isolated from HCC patients underwent liver resection. Purified $CD8^+$ T cells were stimulated with recombinant human IL-7 for 6 h, and were stained for analysis of PD-1 and CTLA-4 expression. The representative flow dots of PD-1 expression on liver-resident $CD8^+$ T cells (Fig. 5A) and peripheral $CD8^+$ T cells (Fig. S1) with or without IL-7 stimulation were shown. The percentage of $PD\text{-}1^+$ on $CD8^+$ T cells were significantly higher in HCC tissues than that in patient-matched normal liver tissues (4.35 ± 1.44% *vs.* 2.67 ± 0.80%, Student *t* test, $P = 0.0002$; Fig. 5B). IL-7 stimulation remarkably down-regulated $PD\text{-}1^+$ cells percentage on both $CD8^+$ T cells from normal tissues (1.92 ± 0.85%, Student *t* test, $P = 0.012$; Fig. 5B) and from HCC tissues (2.65 ± 0.69%, Student *t* test, $P = 0.010$; Fig. 5B). Peripheral $PD\text{-}1^+CD8^+$ T cells frequency was also elevated in HCC patients (6.85 ± 1.01%) compared with that in NC (4.93 ± 0.98%, Student *t* test, $P < 0.0001$; Fig. 5C). IL-7 treatment reduced PD-1 expression on $CD8^+$ T cells from HCC patients (4.98 ± 1.26%, Student *t* test, $P < 0.0001$; Fig. 5C), but not from NC (4.41 ± 0.83%, Student *t* test, $P = 0.128$; Fig. 5B). The representative flow dots of CTLA-4 expression on liver-resident $CD8^+$ T cells (Fig. 5D) and peripheral $CD8^+$ T cells (Fig. S1) with or without IL-7 stimulation were shown. There was no remarkable difference in $CTLA\text{-}4^+$ cells frequency in liver-resident $CD8^+$ T cells between normal and HCC tissue (Student *t* test, $P = 0.681$; Fig. 5E), however, IL-7 stimulation decreased CTLA-4 expression on $CD8^+$ T cells from HCC specimens (0.95 ± 0.39% *vs.* 1.24 ± 0.31%, Student *t* test, $P = 0.024$; Fig. 5E). Although percentage of peripheral $CTLA\text{-}4^+CD8^+$ cells was higher in HCC patients compared with NC (3.03 ± 0.84% *vs.* 2.31 ± 0.83%, Student *t* test, $P = 0.021$; Fig. 5F), IL-7 treatment did not affect CTLA-4 expression on $CD8^+$ T cells either from NC ($P = 0.305$; Fig. 5F) or from HCC patients ($P = 0.461$; Fig. 5F).

3.5. IL-7 stimulation elevated the cytotoxicity and cytokines production of $CD8^+$ T cells purified from HCC patients in direct contact co-culture system

$CD8^+$ T cells were stimulated with recombinant human IL-7 for 6 h, and washed twice. 10^5 of stimulated $CD8^+$ T cells were then co-cultured in direct or indirect contact system with 5×10^5 of HepG2 cells with AFP peptide stimulation for 48 h. LDH expression in the supernatants was measured, and cytotoxicity of $CD8^+$ T cells were calculated. There were no significant differences in the percentage of cell death in HepG2 cells in indirect contact co-culture system with either liver-resident or peripheral $CD8^+$ T cells in response to IL-7 stimulation (Student *t* tests, $P > 0.05$; Fig. 6A and B). In direct contact co-culture system, $CD8^+$ T cells from normal liver tissues presented stronger

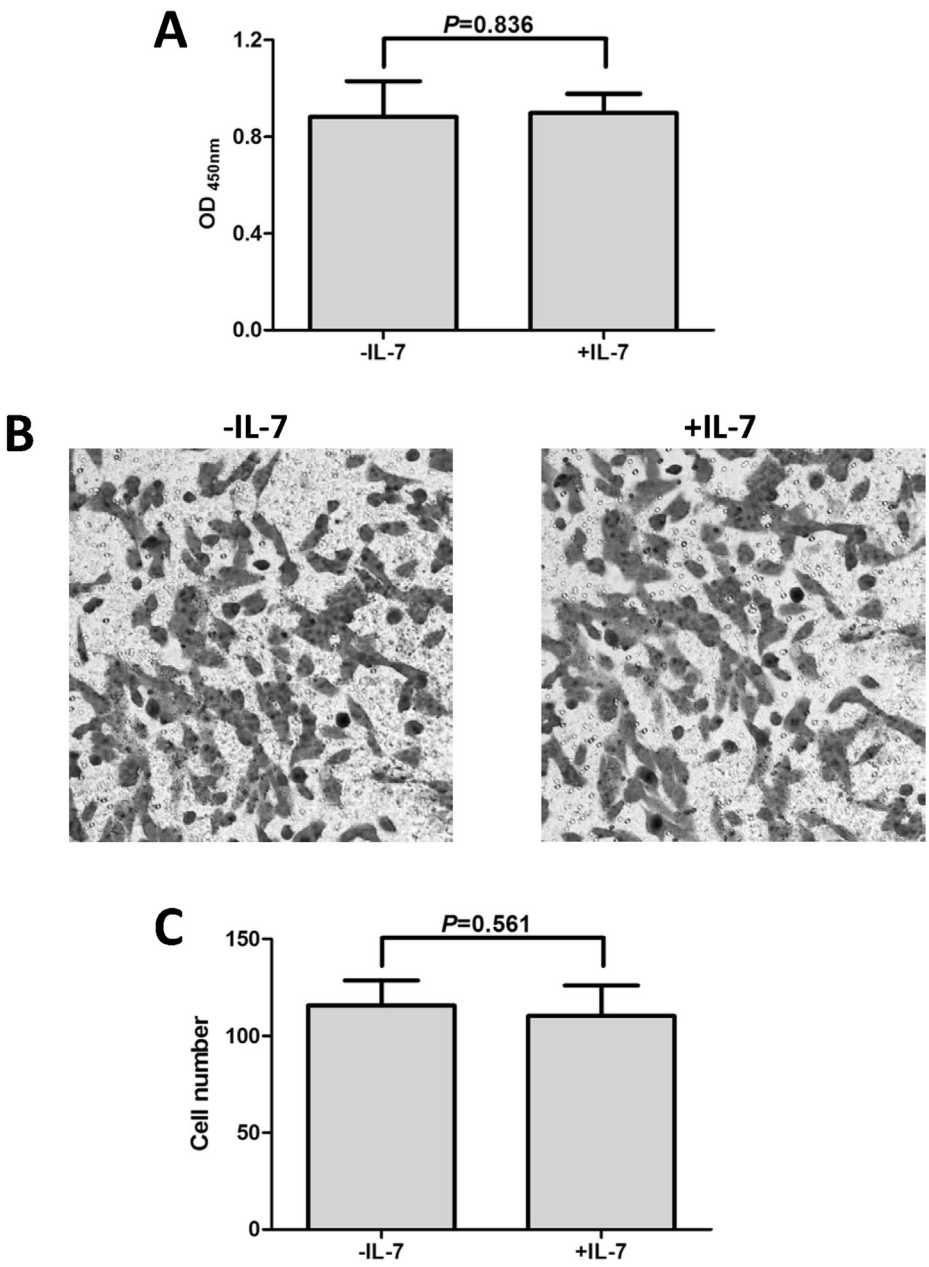

Fig. 4. Influence of IL-7 on proliferation and invasion of HepG2 cells. HepG2 cells were stimulated with recombinant IL-7 for 6 h. The proliferation was measured by CCK-8. (A) $OD_{450\ nm}$ reflected the proliferation of tested HepG2 cells. There was no significant difference in cellular proliferation with or without IL-7 stimulation. The invasion of HepG2 cells was measured by Transwell assay. (B) Cells were stained with crystal violet and imaged by microscopy without or with IL-7 stimulation. (C) Statistical analysis of invasion cell counts without or with IL-7 stimulation. There was comparable absolute invasion HepG2 cell numbers without or with IL-7 stimulation. All experiments were performed independently five times. The column indicated mean level, and the bar indicated standard error.

cytotoxicity than those from HCC tissues (Student *t* test, $P = 0.039$; Fig. 6A). Moreover, the cytotoxicity of $CD8^+$ T cells from both normal tissues (31.47 ± 6.55% *vs.* 23.94 ± 7.50%, Student *t* test, $P = 0.0038$; Fig. 6A) and HCC tissues (26.88 ± 6.80% *vs.* 19.00 ± 5.76%, Student *t* test, $P = 0.0009$; Fig. 6A) were remarkably increased by IL-7 stimulation. Although the cytotoxicity of peripheral $CD8^+$ T cells was comparable between NC and HCC patients (Student *t* test, $P = 0.363$; Fig. 6B), IL-7 stimulation elevated the percentage of HepG2 cell death in direct contact co-culture system with peripheral $CD8^+$ T cells from both NC (32.80 ± 9.68% *vs.* 21.00 ± 8.13%, Student *t* test, $P = 0.0012$; Fig. 6B) and HCC patients (30.06 ± 8.91% *vs.* 18.35 ± 8.06%, Student *t* test, $P = 0.0003$; Fig. 6B). Moreover, IFN-γ and TNF-α expression in the supernatants of co-culture systems was measured by ELISA. In direct contact co-culture system, IFN-γ production was significantly elevated by $CD8^+$ T cells purified from normal liver tissues compared with that from HCC specimens (4.90 ± 0.95% *vs.* 3.99 ± 0.99%, Student *t* test, $P = 0.011$; Fig. 6C). However, there was no remarkable difference of IFN-γ secretion by $CD8^+$ T cells between NC and HCC patients (4.67 ± 1.32% *vs.* 4.83 ± 1.24%, Student *t* test, $P = 0.695$; Fig. 6D). IL-7 stimulation promoted IFN-γ production in both liver-resident $CD8^+$ T cells (Student *t* tests, $P < 0.0001$; Fig. 6C) and peripheral $CD8^+$ T cells (Student *t* tests, $P < 0.05$; Fig. 6D). In indirect contact co-culture system, IL-7 treatment only increased IFN-γ secretion by liver-resident $CD8^+$ T cells purified from HCC tissues (Student *t* tests, $P = 0.0095$; Fig. 6C). TNF-α expression was elevated in response to IL-7 stimulation by both liver-resident and peripheral $CD8^+$ T cells (Student *t* tests, all $P < 0.05$; Fig. 6E and F).

4. Discussion

In the current study, reduced serum IL-7 level, however, comparable membrane-bound IL-7 receptor α (CD127) was observed in patients with HCC. IL-7 did not reveal direct anti-tumor activity to HCC. However, IL-7 stimulation enhanced direct cytolytic and noncytolytic functions of peripheral $CD8^+$ T cells and $CD8^+$ TIL purified from HCC patients, which presented as increased cytotoxicity and cytokines production in direct contact co-culture systems. This process was

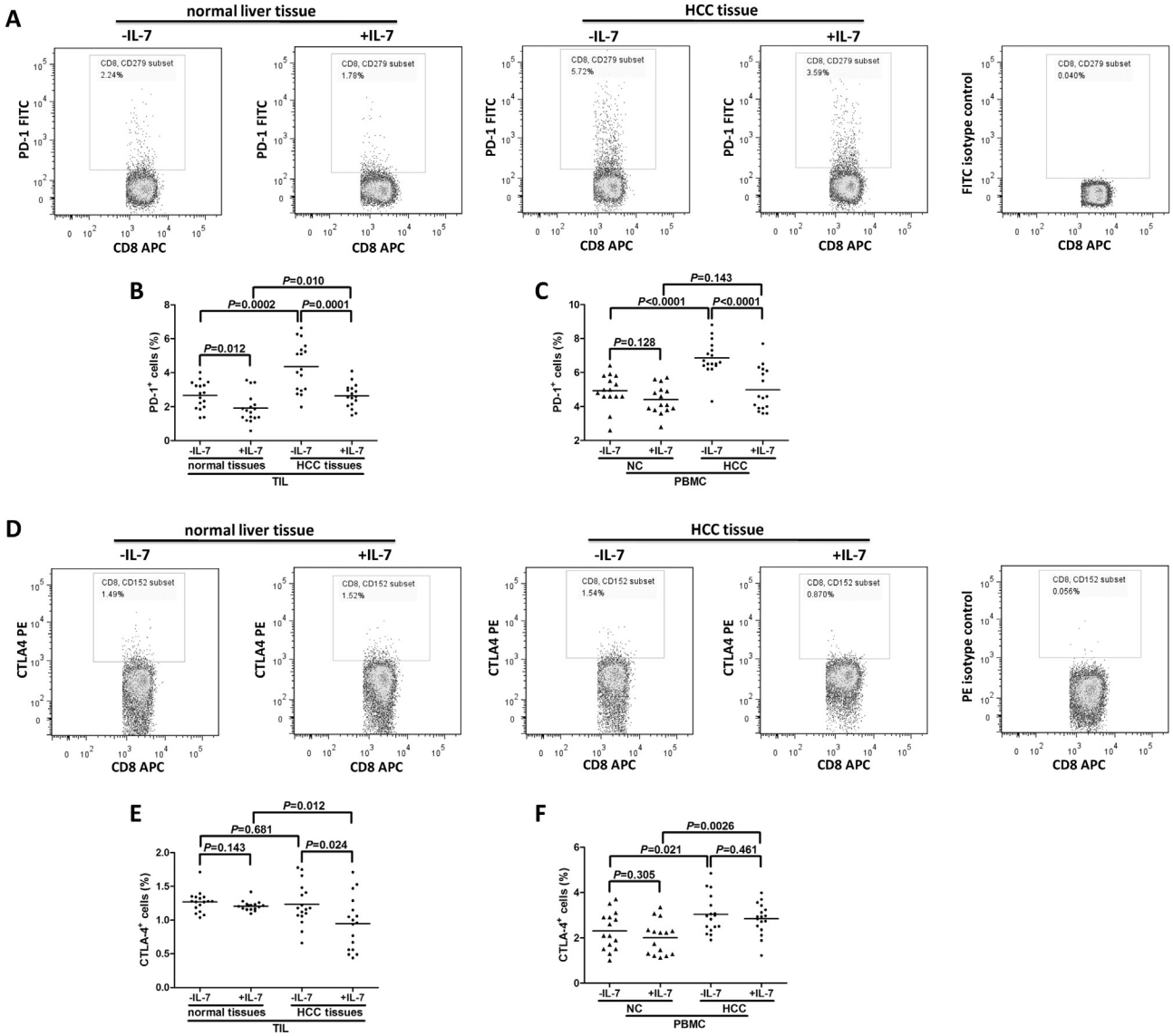

Fig. 5. Changes of PD-1 and CTLA-4 expression in peripheral and liver-resident CD8$^+$ T cells in response to IL-7 stimulation. Peripheral CD8$^+$ T cells were purified from 15 healthy individuals and 17 HCC patients. Liver-resident CD8$^+$ T cells were isolated from 17 HCC specimens and patient-matched normal liver tissues. CD8$^+$ T cells were stimulated with IL-7 for 6 h, and were stained with anti-PD-1 FITC and anti-CTLA-4 PE. Data were acquired using FACS Calibur analyzer, and analyses were performed using FlowJo Software. (A) The representative flow dots of PD-1 expression in liver-resident CD8$^+$ T cells with or without IL-7 stimulation were shown. The percentages of PD-1$^+$ cells within (B) liver-resident and (C) peripheral CD8$^+$ T cells in response to IL-7 stimulation were analyzed. (D) The representative flow dots of CTLA-4 expression in liver-resident CD8$^+$ T cells with or without IL-7 stimulation were shown. The percentages of CTLA-4$^+$ cells within (E) liver-resident and (F) peripheral CD8$^+$ T cells in response to IL-7 stimulation were analyzed. Individual level for each subject was shown, and horizontal bar indicated mean level.

accompanied by decreased PD-1 expression on CD8$^+$ T cells. The present results revealed an important immunoregulatory property and a potential therapeutic strategy of IL-7 in HCC.

IL-7 belongs to common receptor γ chain family of cytokines, which collectively control development, differentiation, growth, and survival of lymphocytes, and are related to broad aspects of diseases, including infection, autoimmune, and cancer [16]. Elevated systemic IL-7 was observed in patients with colorectal cancer, and was associated with metastatic disease and tumor location [17]. In contrast, fibroblastic reticular cells led to reduced IL-7 secretion in tumor draining lymph nodes in patients with cancer [18]. We found that serum IL-7 level was reduced in HCC patients, but there was no remarkable correlation with disease stages. Effective anti-tumor therapies enhanced systemic IL-7 expression, indicating a potential activity in the pathogenesis of neoplasia in HCC. However, controversy remained as to direct pro-tumor or anti-tumor effects of IL-7 [19]. IL-7 induced glucocorticoid resistance in early thymic precursor T-cell acute lymphoblastic leukemias [20]. It was also appeared that IL-7 prevented apoptosis and promoted tumor cell proliferation in human non-small cell lung cancer [21]. In contrast, IL-7 administration *in vivo* resulted in decreased cancer cells growth in mouse model [22]. Our current results suggested that IL-7 did not present direct anti-tumor function to HCC, without affecting proliferation and invasion of HepG2 cells *in vitro*.

High level of intratumor activated CD8$^+$ T cells predicted better prognosis of HCC with less recurrent rate and longer survival time [23,24]. CD8$^+$ TIL mediated tumor injection via antigen recognition and perforin secretion, leading to the direct destruction of tumor cells [25]. However, this cytolytic activity of CD8$^+$ TIL seemed to be deficient in different tumor mouse model [26]. Furthermore, CD8$^+$ TIL also secreted IFN-γ and TNF-α with tumor cells stimulation, inducing tumor regressions *in vitro* and *in vivo* [27]. This noncytolytic function of CD8$^+$ TIL not only contributed to the direct anti-tumor activity, but also promoted the function of antigen-specific CD8$^+$ TIL. However, few studies focused on the involvement of cytolytic and noncytolytic

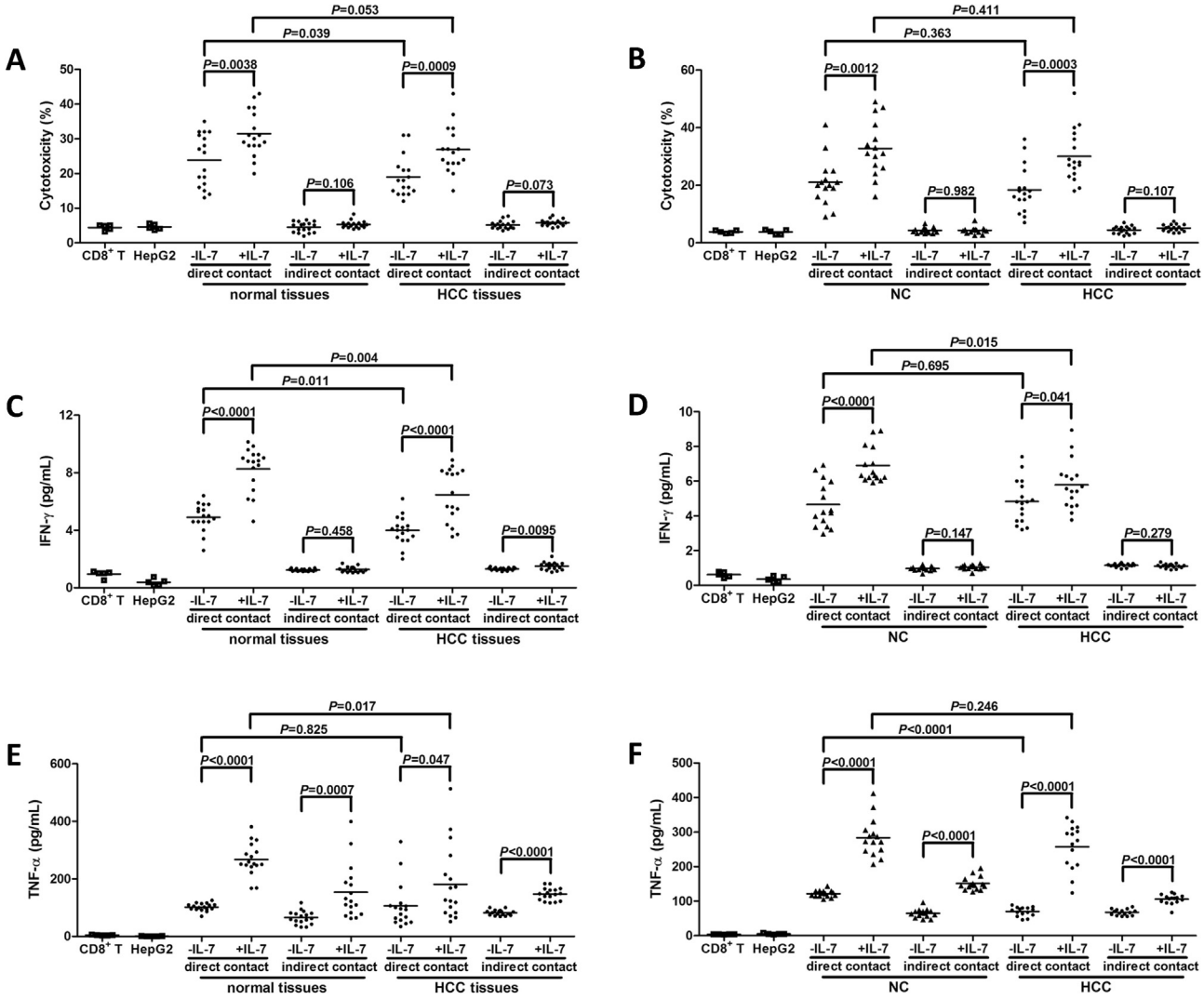

Fig. 6. Changes of cytolytic and non-cytolytic activities of liver-resident and peripheral CD8$^+$ T cells in response to IL-7 stimulation. CD8$^+$ T cells, which were purified from tissue and peripheral bloods, were stimulated with recombinant human IL-7 for 6 h. 10^5 of stimulated CD8$^+$ T cells were then co-cultured in direct or indirect contact system with 5×10^5 of HepG2 cells with AFP peptide stimulation for 48 h. Cytotoxicity of (A) liver-resident and (B) peripheral CD8$^+$ T cells was reflected by cell death percentage, which was calculated by measuring LDH expression in the supernantants. Levels of (C and D) IFN-γ and (E and F) TNF-α were measured by ELISA. Individual level for each subject was shown, and horizontal bar indicated mean level.

function of peripheral and liver-resident CD8$^+$ T cells in HCC. Yu et al. [9] used *in vitro* co-culture system to independently dissect the cytolytic (direct contact co-culture) and noncytolytic (indirect contact co-culture) activity of CD8$^+$ T cells purified from colorectal carcinoma. In the present study, we also used these co-culture systems to distinguish the two different functions of peripheral and liver-resident CD8$^+$ T cells targeting HCC cell line. In direct contact co-culture system, CD8$^+$ T cells purified from normal liver tissues revealed stronger cytotoxicity and secreted more IFN-γ than those from HCC specimens, indicating dysfunction or exhaustion of CD8$^+$ TIL in HCC. IL-7 stimulation increased cell death and IFN-γ/TNF-α production by both peripheral and liver-resident CD8$^+$ T cells, revealing both cell killing and cytokine-induced mechanism were activated by IL-7 to destroy tumor cells. In indirect contact co-culture system, CD8$^+$ T cells exerted anti-tumor effect only through secreting cytokines. Little cytotoxicity of HepG2 cells was observed in indirect contact co-culture system, suggesting that CD8$^+$ T cells-mediated IFN-γ and TNF-α production was insufficient for tumor cells regression. Moreover, these secreting cytokines also did not affected by IL-7 stimulation in indirect contact co-culture system, revealing a potential stimulatory function of tumor cells for CD8$^+$ T cells activation and for IL-7-induced regulation. However, previous study demonstrated that IL-7 adjuvant therapy augmented long-term antigen-specific CD8$^+$ T cells activity, while this enhancement depended on CD127 expression on effector CD8$^+$ T cells [28]. Although differential distribution of naïve and memorial CD8$^+$ T cells between NC and HCC, CD127 expression was comparable in both CD8$^+$ T cell subsets. This indicated that direct IL-7 regulation on CD8$^+$ T cells from HCC patients might be independent on its receptor α chain expression.

The potential mechanisms of IL-7-mediated elevation of CD8$^+$ T cells function were also investigated. Multiple mechanisms, such as elevation of effector T cells numbers and function, promotion of cytokines secretion, were involved in IL-7-induced viral clearance in chronic lymphocytic choriomeningitis virus (LCMV) infected mouse model [29]. IL-7 enhanced polyfunctional viral specific CD8$^+$ T cells responses, which was associated with Bcl-2 induction but not with altered PD-1 expression in chronic LCMV infection [30]. In contrast, Type I interferon induced hepatic IL-7 production, resulted in maintaining antiviral CD8$^+$ T cells responses via regressing PD-1 expression in acute viral hepatitis *in vivo* [31]. Herein, we demonstrated that expression of PD-1, but not CTLA-4, on peripheral CD8$^+$ T cells and CD8$^+$ TIL was elevated in HCC patients, indicating the potential immunosuppressive contribution of PD-1 to T cells dysfunction or exhaustion. Moreover, IL-

7 stimulation down-regulated PD-1$^+$CD8$^+$ T cells proportion, suggesting an inhibitory activity of IL-7 to PD-1/PD-L1 signaling pathway in HCC. However, further *in vivo* experiments are needed for elucidation of potential role of IL-7 in regulation of CD8$^+$ T cells function in HCC.

5. Conclusion

In summary, tumor-infiltrating CD8$^+$ T cells revealed dysfunctional or exhaustive activity for tumor injection in HCC patients. IL-7 stimulation promoted both cytolytic and noncytolytic activity of peripheral and liver-resident CD8$^+$ T cells probably via suppression of PD-1 expression under direct tumor cells presentation. IL-7 might be considered as one of the therapeutic candidates for HCC treatment.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.04.003.

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