

PD-1 and PD-L1 upregulation promotes CD8⁺ T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients

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Programmed death 1 (PD-1) and its ligand (PD-L1) play pivotal roles in regulating host immune responses. However, the inhibitory effects of this pathway on the function of cytotoxic CD8⁺ T lymphocytes, the main effector cells in hepatocellular carcinoma (HCC) patients, are not well defined. In this study, we characterized circulating and intratumor PD-1/PD-L1 expression and analyzed their association with disease progression in a cohort of hepatitis B virus-infected patients, including 56 with HCC, 20 with liver cirrhosis (LC) and 20 healthy controls (HC). The frequency of circulating PD-1⁺CD8⁺ T cells increased with disease progression from LC to HCC patients versus HC. Furthermore, tumor-infiltrating effector CD8⁺ T cells showed a drastic increase in PD-1 expression. These increases in circulating and intratumor PD-1⁺CD8⁺ T cells could predict poorer disease progression and postoperative recurrence. Immunohistochemical staining showed that PD-L1 expressing hepatoma cells and apoptotic infiltrating CD8⁺ T cells were both enriched in tumor sections. *In vitro*, CD8⁺ T cells induced PD-L1 expression on hepatoma cells in an IFN- γ -dependent manner, which in turn promoted CD8⁺ T cells apoptosis, and blocking PD-L1 reversed this effect. Therefore, this study extends our knowledge of the role of the PD-1/PD-L1 pathway in tumor evasion and provides evidence for a new therapeutic target in HCC patients.

Hepatocellular carcinoma (HCC) ranks among the top 10 cancers worldwide in terms of prevalence and mortality. The prognosis of HCC patients is generally poor with a 5-year survival rate of <5% in symptomatic HCC patients. Furthermore, these tumors have been shown to be quite resistant to radiotherapy and chemotherapy.¹ HCC patients are often found to have functional deficiencies in immune

surveillance of tumor growth.²⁻⁴ Many mechanisms have been proposed to explain their attenuated immune response to tumors, including partial antigen masking, failure of antigen processing, suppression of effector cells and inadequate costimulation.

Programmed death 1 (PD-1), a coinhibitory receptor molecule, is induced on activated T and B cells⁵ and plays a crucial role in regulating peripheral tolerance.⁶⁻⁹ The ligand for PD-1, PD-L1 (also known as B7-H1), is expressed on dendritic cells, macrophages and parenchymal cells. Substantial evidence has demonstrated that PD-L1 can deliver an inhibitory signal to PD-1 expressing T cells, leading to suppression of the immune response by inducing apoptosis, anergy, unresponsiveness and functional exhaustion of T cells.^{10,11} Several studies have shown that the PD-1/PD-L1 pathway plays critical roles in compromised tumor immunity, and blockade of this pathway by employing anti-PD-L1 antibodies could enhance antitumor immunity and inhibit tumor growth in several human cancers.¹²⁻¹⁴ In addition, patients with PD-L1-positive cancer cells were reported to have a significantly poorer prognosis than those with PD-L1-negative cancer cells in pancreatic cancer, urothelial cancer and breast and ovarian cancer patients.¹⁴⁻¹⁷ There were two recent reports focusing on the relationship between PD-L1 expression and prognosis in HCC.^{18,19} However, the changes in CD8⁺ T cell functions underlying the immunosuppressive effects through the PD-1/PD-L1 pathway remain to be clarified. Thus, in this study, we explored the PD-1/PD-L1 interactions between hepatoma

Key words: PD-1, PD-L1, hepatocellular carcinoma, apoptosis

Abbreviations: 7AAD: 7-aminoactinomycin D; DFS: disease-free survival; HC: healthy control; HCC: hepatocellular carcinoma; LC: liver cirrhosis; NIL: nontumor infiltrating lymphocytes; PD-1: programmed death 1; PD-L1: programmed death 1 ligand; TIL: tumor infiltrating lymphocytes

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Table 1. The clinical characteristics of enrolled subjects

Groups	HC	LC	HCC
Cases	20	20	56
Age (range)	45 (30–55)	53 (35–65)	55 (20–77)
Gender (male/female)	12/8	13/7	48/8
ALT (U/L)	21 (17–45)	45 (33–107)	43 (18–213)
HBeAg (+/–)	0/20	9/11	20/36
HBV viral load (copies/ml)	0	9.22×10^6 (10^2 – 10^7)	8.97×10^5 (10^1 – 10^7)
α -FP (ng/ml)	ND	ND	88 (1–1154)
Tumor size (cm)	ND	ND	4.3 (1.1–13.0)
Tumor morphology (unifocal/multifocal)	ND	ND	42/14
Vascular invasion (absence/presence)	ND	ND	29/27
Liver cirrhosis (no/yes)	ND	0/20	5/51
Child-Pugh classification (A/B)	ND	18/2	50/6
Stage, ¹ I/II/III	ND	ND	16/14/26
Therapy (resection/LT)	ND	ND	54/2

¹HCC patient disease stage was evaluated according to the Chinese criterion for diagnosis and staging primary liver cancer constituted by the Chinese Anti-Cancer Association in 2001. Abbreviations: ALT: alanine aminotransferase; α -FP, α -fetoprotein; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HC, healthy control; HCC, hepatocellular carcinoma; LC, liver cirrhosis; LT, liver transplantation; ND: no data.

cells and T cells and determined the association of this pathway with clinical prognosis and immune suppression of anti-tumor CD8⁺ T cells. Our work may provide a potential immune regulatory target for HCC therapy.

Material and Methods

Subjects

Fifty-six hepatitis B virus (HBV)-infected HCC patients, 20 liver cirrhosis (LC) patients and 20 healthy controls (HC) were enrolled in this study. All HCC patients had a history of more than 20 years of chronic HBV infection and were hospitalized or followed up in Beijing 302 Hospital. The diagnoses of HCC and LC were made on the basis of either standard imaging or biopsy examination. The clinical stage was evaluated according to the criteria for diagnosis and staging primary liver cancer constituted by the Chinese Anti-cancer Association in 2001 as reported previously.²⁰ The numbers of patients at stage I, II and III were 16, 14 and 26, respectively. Fifty-four HCC patients received surgical resections, and two received liver transplantations. None of the patients received any anticancer therapy before sampling. The clinical characteristics of enrolled subjects are listed in Table 1. The study protocol was approved by the ethical committee of our unit, and written informed consent was obtained from each subject before blood and tumor sampling.

Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation from heparinized blood. Tumor infiltrating lymphocytes (TIL) and nontumor infiltrating lymphocytes (NIL) were isolated according to our previous report.²¹ CD8⁺

T cells were isolated from PBMCs by using CD8 Microbeads (Miltenyi Biotec, Auburn, CA). All procedures were performed following the manufacturers' instructions.

Flow cytometry analysis

Antibodies conjugated with different fluoresceins, including fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC), were employed for flow cytometry analysis (FACS Calibur, BD Biosciences, San Jose, CA). All antibodies were purchased from BD Biosciences, except for PE-conjugated PD-1 and PD-L1 (eBiosciences, San Diego, CA) and FITC-conjugated CCR7 (R&D Systems, Minneapolis, MN). PD-1 expression on CD8⁺ T cells and their memory subsets and intracellular IFN- γ staining assay were analyzed by flow cytometry as previously described.²²

Proliferation assay

Proliferation of CD8⁺ T cells was analyzed by carboxyfluorescein succinimidyl ester (CFSE) labeling as described in our previous work.²² In brief, PBMCs were cultured in complete RPMI 1640 medium with 10% fetal bovine serum (Gibco, Carlsbad, CA) and stimulated with anti-CD3 and CD28 monoclonal antibodies (mAbs). The cells (5×10^5) were seeded in a 96-well plate (Costar, Lowell, MA) in the presence or absence of anti-human PD-L1 (α -PD-L1). Cells were harvested at 72, 120 or 168 hr and stained with anti-CD3-APC and anti-CD8-PerCP and then analyzed by flow cytometry. As the CFSE signal is diluted with each cell division, cells with low fluorescence intensity of CFSE (CFSE^{low}) are considered to have proliferated.

PD-L1 upregulation by IFN- γ stimulation

HepG2.2.15, a human hepatoma cell line stably transfected with the HBV genome, was supplemented with IFN- γ (25 ng/ml, Peprotech) or cocultured with CD8⁺ T cells from HCC patients at a ratio of 1:1. Cells were seeded in the presence or absence of anti-IFN- γ mAbs (5 μ g/ml, R&D Systems). After 3, 6, 9, 12 or 24 hr, the cells were harvested to analyze PD-L1 expressions on HepG2.2.15 cells by flow cytometry.

Cytotoxicity assay

For the cytotoxicity assay, HepG2.2.15 cells were used as the target cells. One of the major histocompatibility complexes of HepG2.2.15 was identified as HLA-A2; thus, the effector CD8⁺ T cells for this assay were collected from HLA-A2 positive HCC individuals. Before the assay, HepG2.2.15 cells were treated in the presence (treated) or absence (untreated) of IFN- γ (25 ng/ml) for 24 hr. Isolated CD8⁺ T cells were cocultured with treated or untreated HepG2.2.15 cells at ratios of 3:1, 6:1, 12:1 or 25:1 in the presence or absence of α -PD-L1 (5 μ g/ml). After 6 hr of incubation, the cells were harvested and stained with 7-AAD (Invitrogen, Carlsbad, CA) before flow cytometric analysis.

Apoptosis assay

IFN- γ -treated or untreated HepG2.2.15 cells were cocultured with CD8⁺ T cells isolated from HCC patients at ratios of 1:1, 5:1 or 10:1 in the presence or absence of α -PD-L1. After 24 hr of incubation, the cells were harvested and stained with Annexin-V-FITC or 7-AAD (eBiosciences) to analyze the apoptosis or necrosis of CD8⁺ T cells, respectively.

Enzyme-linked immunosorbent assay

Tumor or nontumor tissues were ground in PBS using the BD Medimachine (BD Biosciences) according to the manufacturer's instructions. Supernatants from the homogenates were subjected to enzyme-linked immunosorbent assay (ELISA) for IFN- γ detection (eBiosciences). The final results were normalized to picograms per gram of tissue (pg/g).

Immunohistochemistry

Paraffin-embedded liver sections from HCC-resected tumor and nontumor tissues (5–10 cm distal from the tumor site), biopsies of LC patients and HC tissues were used for immunohistochemical staining. All sections were obtained from clinical units of our hospital and were independent from the enrolled subjects mentioned above. Healthy liver tissue samples were obtained from the healthy donors whose livers were used for liver transplantation. The mAbs of rabbit anti-human CD8 (Abcam, Cambridge, MA), mouse anti-human PD-1 (R&D Systems), mouse anti-human PD-L1 (Biolegend, San Diego, CA) and mouse anti-human cleaved caspase-3 (Zhongshan Goldenbridge Biotech, Beijing, China) were adopted for CD8, PD-1, PD-L1 and cleaved caspase-3 staining, respectively. Double staining was performed using the

avidin-biotin-peroxidase system with two different substrates: Vector blue for blue color and Vector red for red color (Vector Laboratories, Burlingame, CA).

Statistical Analysis

The data were summarized and presented as mean \pm standard deviation and were analyzed using SPSS software (SPSS, Chicago, IL). Comparisons between HCC, LC and HC groups were performed using the Mann-Whitney *U* test. The Wilcoxon matched-pairs *t*-test was used to compare the data from the same individuals. Correlation analysis was performed using the Spearman rank correlation test. The survival curves were estimated by the Kaplan-Meier method and compared by the log-rank test. For all tests, *p* < 0.05 was considered as statistically significant.

Results

PD-1 expression is upregulated on effector phase CD8⁺ T cells in HCC patients, especially in TIL

We first analyzed PD-1 expression on CD8⁺ T cells. The results showed that PD-1 on circulating CD8⁺ T cells was significantly higher in HCC than those in LC (*p* = 0.008) and HC (*p* < 0.001). Furthermore, PD-1 on CD8⁺ T cells in TIL was on average 30% and 20% higher than those in the corresponding PBMC (*p* < 0.001) and NIL (*n* = 56, *p* < 0.001), respectively (Figs. 1a and 1b). We further analyzed PD-1 expressions on different CD8⁺ T cell subsets based on CCR7 and CD45RA expressions. All subjects clearly displayed four subsets (Fig. 1c). Notably, the PD-1 on CD8⁺ T-cell subsets in HCC differed from those in HC and LC subjects and predominantly increased on effector phase T cells, including T_{cm} (CCR7⁺CD45RA⁻) and T_{em} (CCR7⁻CD45RA⁻). Particularly in TIL, the PD-1 on each subset was further increased over that in NIL (*p* < 0.01). CD4⁺T cells also exhibited a similar PD-1 expression profile (data not shown). Thus, these data indicated that PD-1 is preferentially increased on effector phase CD8⁺ T cells in HCC, especially in TIL.

In situ expressions of PD-1/PD-L1 by immunohistochemical staining

We also explored the *in situ* expressions of PD-1/PD-L1. As shown in Figure 2a, PD-1 and PD-L1 were most apparently expressed in tumor sections, compared with LC and HC. In particular, PD-1 was expressed mainly on lymphocytes, compared with PD-L1 on hepatoma cells (Fig. 2a). Quantitative analysis showed that HCC patients (*n* = 20) expressed more PD-1 than LC patients (*n* = 10, *p* < 0.001) and HC (*n* = 10, *p* < 0.001, Fig. 2b). Double staining revealed that a large proportion of CD8⁺ T cells were PD-1-positive in tumor tissue but not in normal tissue of healthy liver donors (Fig. 2c; Supporting Information Fig. 1). In addition, CD8⁺ T cells were mainly distributed around the PD-L1-positive tumor nest accompanied by few infiltrated CD8⁺ T cells (Fig. 2c, right). A typical HCC case showed that tumor-associated PD-L1 expression was clearly increased in aggressive tumor regions (Fig. 2d).

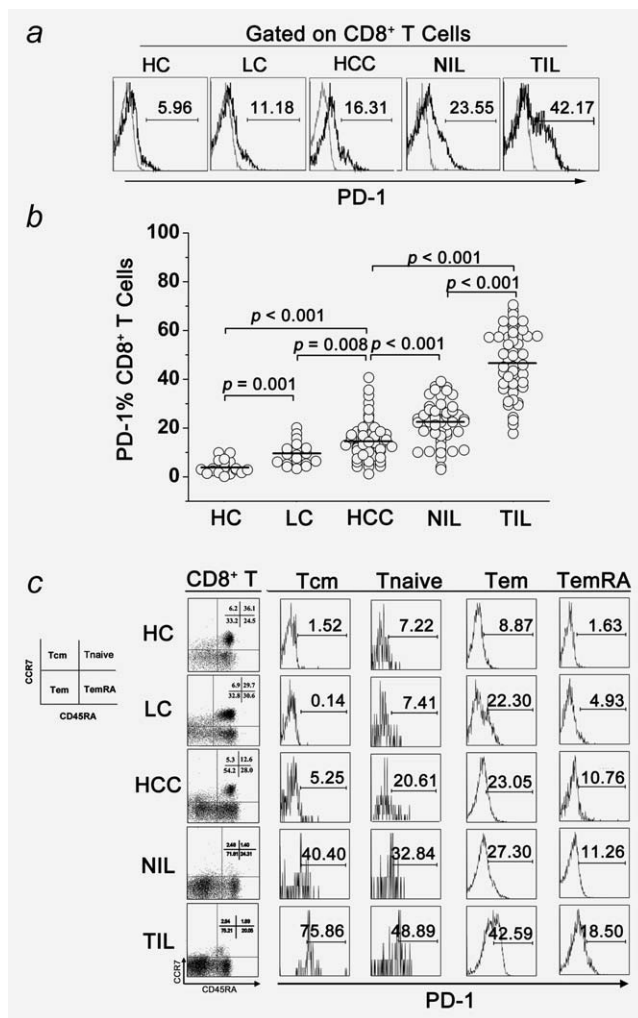


Figure 1. PD-1 upregulation on effector phase CD8⁺ T cell in peripheral blood and liver tissue in HCC patients. Representative histograms (a) and combined results with statistical analysis (b) of PD-1 expressions on CD8⁺ T cells in peripheral blood of HC ($n = 20$), LC ($n = 20$) and HCC ($n = 56$) individuals and corresponding NIL and TIL ($n = 56$). For (a), the black line represents PD-1 and the gray line is the isotype control. The number on the upper right corner is the frequency of positive cells. For (b), each dot indicates one individual. Black bars in the graph with statistical results are mean values of % PD-1 expression on CD8⁺ T cells. (c) Representative histograms of PD-1 expressions on different CD8⁺ T-cell subsets in peripheral blood and corresponding NIL and TIL. Different memory subsets are distinguished by CCR7 and CD45RA. Tcm, central memory; Tem, effector memory; Tnaive, naive T; TemRA, CD45RA⁺ effector memory. The numbers in the upper right corners are the frequencies of positive cells. The data are representative of five subjects for each group.

Blocking PD-L1 restores the proliferative abilities and IFN- γ production in CD8⁺ T cells

To investigate the impacts of PD-1/PD-L1 pathway on CD8⁺ T cells in HCC, PBMC from HC and HCC were stimulated

in the absence or presence of blocking antibody against PD-L1 (α -PD-L1). Results showed that blocking the pathway could largely enhance the proliferative capacity of CD8⁺ T cells in HCC from 72 hr (Figs. 3a and 3b). It was also found that the percentage of IFN- γ -producing CD8⁺ T cells in HCC increased in the presence of α -PD-L1 ($p < 0.001$) to the level of that in HC ($p = 0.850$) (Figs. 3c and 3d). Therefore, these data confirmed previous reports that blocking PD-L1 could restore the capacities of proliferation and IFN- γ production of CD8⁺ T cells, which were impaired in HCC.

CD8⁺ T-cell produced IFN- γ can induce PD-L1 expression on hepatoma cells

As some previous reports demonstrated that IFN- γ could induce PD-L1 expression, we performed a series of parallel experiments to verify whether PD-L1 upregulation could be triggered by IFN- γ released from CD8⁺ T cells. Results showed that both IFN- γ and coculturing with CD8⁺ T cells could increase PD-L1 expression on HepG2.2.15 cells after 6 hr of treatment; the PD-L1 expression gradually unregulated during the entire 24-hr period of *in vitro* incubation, whereas blocking IFN- γ inhibited upregulation by both methods (Fig. 4a). Thus, upregulation of PD-L1 is triggered by CD8⁺ T cells in an IFN- γ -dependent mechanism.

IFN- γ facilitates hepatoma cells resistance to CD8⁺ T cells

To investigate whether IFN- γ -treated hepatoma cells could weaken the cytotoxic effect of T cells, the CD8⁺ T cells isolated from HLA-A2 positive HCC subjects were cocultured with HepG2.2.15 cells at different ratios in the presence or absence of PD-L1 blocking antibody. After 24 hr of stimulation, the cells were harvested to evaluate necrosis by 7-AAD staining. The results showed that the IFN- γ -treated HepG2.2.15 cells were much more resistant to necrosis than the controls at all effector to target (E/T) ratios. Blocking PD-L1 reversed the sensitivity of HepG2.2.15 cells to CD8⁺ T cell-dependent cytotoxicity (Fig. 4b).

Hepatoma cells promotes CD8⁺ T cell apoptosis by PD-L1 upregulation

Because IFN- γ -treated hepatoma cells could upregulate the expression of PD-L1, we further investigated whether IFN- γ -treated HepG2.2.15 cells could promote the apoptosis or necrosis of CD8⁺ T cells. Our data showed that apoptosis (Annexin-V⁺, Fig. 4c left) and necrosis (7-AAD⁺, Fig. 4c right) of CD8⁺ T cells significantly increased when cocultured with IFN- γ -treated HepG2.2.15 cells, compared with the IFN- γ -untreated control. Blocking PD-L1 could largely inhibit apoptosis and necrosis of CD8⁺ T cells induced by IFN- γ -treated HepG2.2.15 cells (Fig. 4c). To detect IFN- γ *in situ*, the concentrations of IFN- γ in the supernatant of tissue homogenates were measured by ELISA. The results showed that IFN- γ increased significantly in nontumor tissue in stage III HCC, compared with stage I HCC ($p < 0.001$) and HC tissue (Fig. 4d, $p < 0.001$). However, there were no

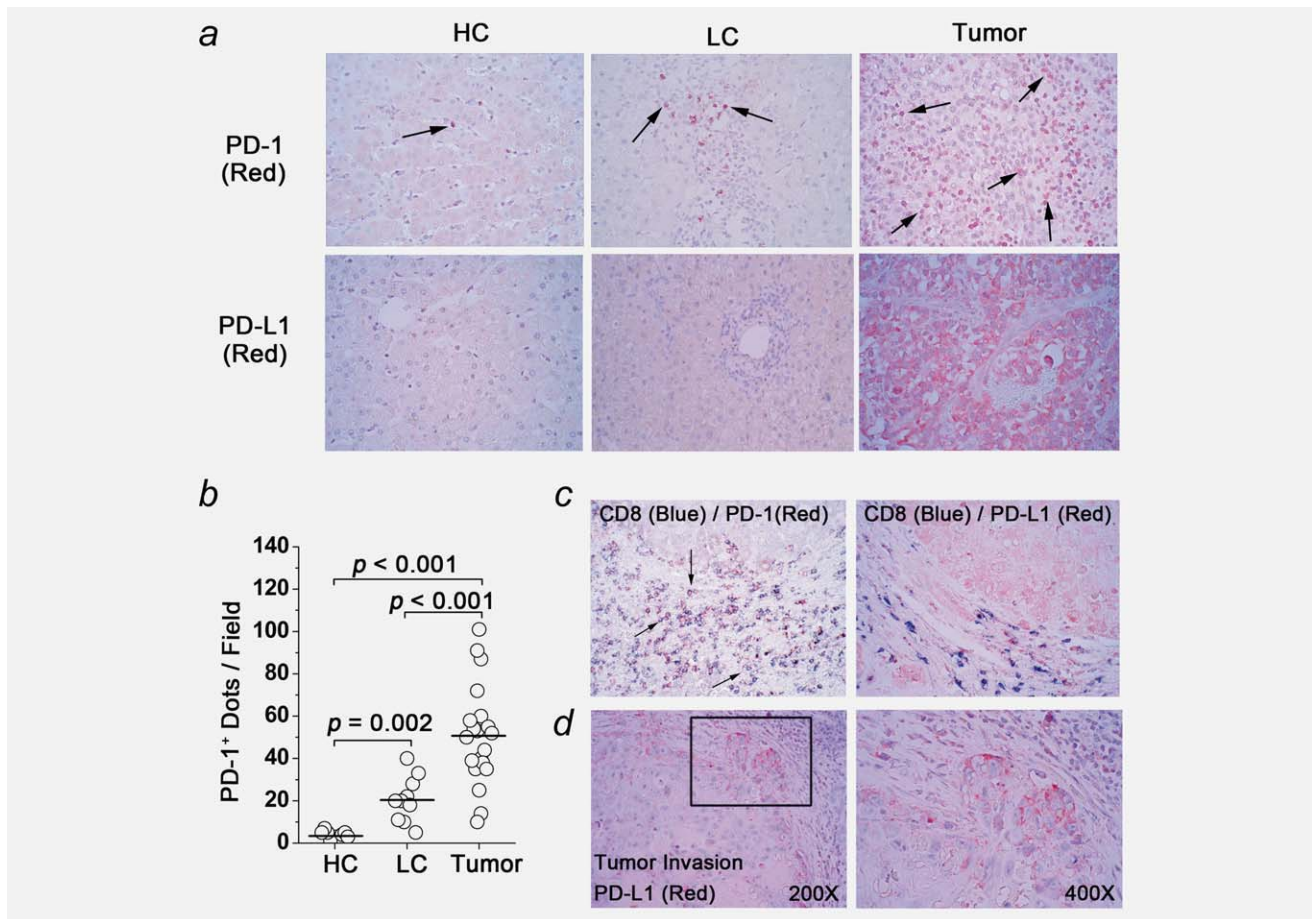


Figure 2. The *in situ* expressions of PD-1/PD-L1 by immunohistochemical staining. (a) PD-1 (upper, red) and PD-L1 (lower, red) expressions in liver sections are shown by immunohistochemical analysis. Arrows indicate PD-1 positive lymphocytes. (b) Quantitative results with statistical analysis for PD-1-positive dots in HC ($n = 10$), LC ($n = 10$) and HCC ($n = 20$). The data were collected at 200 \times power by two different investigators. One dot indicates one section from one patient. Black bars indicate the mean values. (c) Double staining for PD-1/CD8 (left: blue for CD8, red for PD-1) and PD-L1/CD8 (right: blue for CD8, red for PD-L1). Arrows show the CD8 and PD-1 double positive lymphocytes. (d) PD-L1 staining of a typical aggressive tumor section. The rectangle marks the aggressive tumor area. Left panel: 200 \times magnification; right panel: 400 \times magnification. For each assay, at least 10 sections were analyzed.

significant differences among the three stages of tumor tissues in HCC (discussed below). Furthermore, apoptosis of CD8⁺ T cells in tumor tissues was determined by double staining with anti-CD8 and anti-cleaved caspase-3 antibodies. In normal and cirrhosis liver tissues, few apoptotic cells were observed (data not shown). However, in tumor sections, most infiltrating CD8⁺ T cells around or primarily inside the tumor nest were apoptotic (Fig. 4e).

Levels of PD-1 correlate with disease progression in HCC

In this study, we found that the frequency of PD-1⁺CD8⁺ T cells in peripheral blood positively correlated with disease progression in HCC and reached the highest level in stage III patients (*vs.* stage I, $p < 0.001$; *vs.* stage II, $p = 0.04$; Fig. 5a). We further analyzed the relationship of PD-1 expressions between PBMC and corresponding liver tissues. The PD-1

expression in PBMC positively correlated with those in NIL ($r = 0.699$, $p < 0.001$) and TIL ($r = 0.742$, $p < 0.001$). Moreover, we analyzed the validity of intrahepatic and circulating PD-1 in predicting the postoperative recurrence by dividing these patients into two groups according to the median value of PD-1 frequency: high PD-1 ($n = 28$) and low PD-1 ($n = 28$) groups. For intrahepatic PD-1, log-rank analysis showed that the high PD-1 group (PD-1 frequency, 57.42% \pm 6.40%; DFS median, 13.63 months) had significantly shorter disease-free survival (DFS) intervals than the low PD-1 group (PD-1 frequency, 35.99% \pm 7.25%; DFS median, 28.70 months, $p < 0.001$). Moreover, circulating PD-1 (PD-1 frequency: high *vs.* low, 21.04% \pm 6.75% *vs.* 8.59% \pm 3.36%, respectively) could also predict similarly the poor prognosis of DFS (DFS median: high *vs.* low, 16.30 *vs.* 27.15 months, $p = 0.001$).

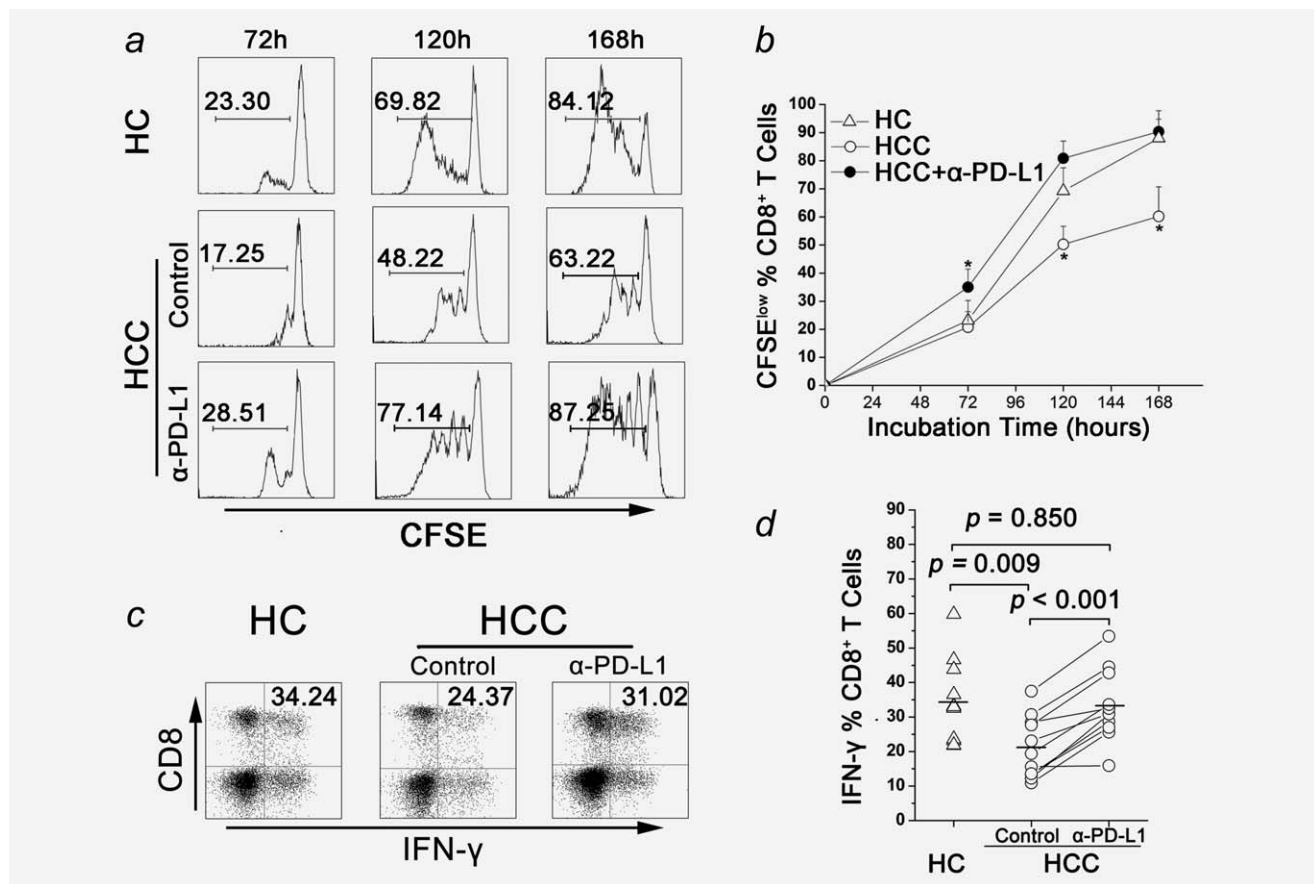


Figure 3. Proliferation and IFN- γ production by CD8⁺ T cells increased with inhibition of PD-L1. PBMC from HC ($n = 5$) and HCC ($n = 5$) were stimulated with α -CD3 (1 μ g/ml)/ α -CD28 (1 μ g/ml) in the presence or absence of blocking antibody against PD-L1, and the frequencies of CFSE^{low}CD8⁺ T cells were assessed by flow cytometry at different time points. The representative histograms (a) and summarized results with statistical analysis (b) for CD8⁺ T cell proliferation. * $p < 0.05$, compared with HC group. PBMC from HC ($n = 11$) and HCC ($n = 11$) were stimulated with PMA (50 ng/ml) and ionomycin (1 μ g/ml) for 6 hr and monensin (2 μ M) was supplemented after the first hour. The cells were then harvested for intracellular IFN- γ staining as shown by representative data in (c). Numbers on the upper right corners are the frequencies of IFN- γ ⁺CD8⁺ T cells. (d) The combined results with statistical analysis. The black bars indicate the mean values.

Discussion

The interaction between PD-1 and PD-L1 has been demonstrated to negatively regulate T-cell activation and functions, leading to inhibition of the immune response in cancer patients. Two recent reports showed that PD-L1 expressed on tumor cells or activated monocytes contributed to tumor aggressiveness and postoperative recurrence in HCC patients. However, little is known about how PD-1/PD-L1 pathway operates between CD8⁺ T cells and tumor cells in HCC patients. In this study, we characterized the profile of PD-1 expression in HCC patients and documented a significant increase in peripheral and intratumor PD-1 expression, which would promote apoptosis of CD8⁺ T cells that come into contact with hepatoma cells. Moreover, these increased circulating and intratumor PD-1⁺CD8⁺ T cells could predict poorer disease progression and postoperative recurrence. These data extends our understanding of the PD-1/PD-L1

interaction between hepatoma cells and CD8⁺ T cells during the antitumor response in HCC patients.

In this study, we first found that PD-1 was drastically up-regulated in TIL, especially on effector phase CD8⁺ T cells. As some previous reports demonstrated that PD-1 inhibits antiviral and antitumor immunity at the effector phase,^{23–25} this increased PD-1 expression might account for the poor prognosis of HCC patients. Furthermore, central memory CD8⁺ T cells have been reported to confer superior antitumor immunity compared with effector memory T cells,²⁶ and our study found that Tcm are decreased obviously in TIL. Two possible mechanisms can account for the decreased Tcm in TIL: the Tcm may have converted into Tem before being recruiting into the intratumor region or the Tcm were much more prone to apoptosis than Tem with upregulated PD-1 expression. Moreover, immunohistochemical staining showed that PD-1 and PD-L1 were largely upregulated on CD8⁺ T

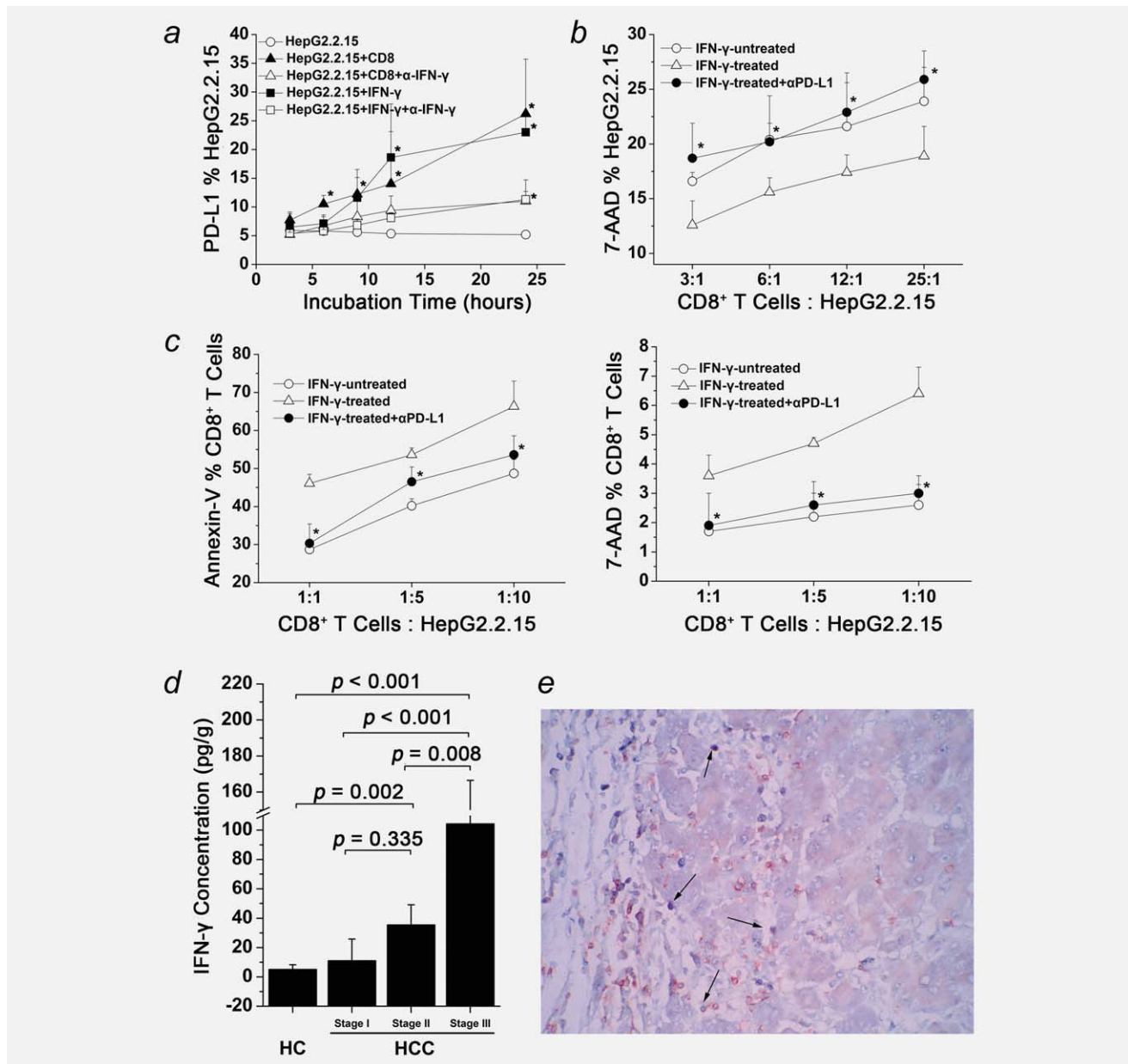


Figure 4. IFN- γ -treated hepatoma cells promotes CD8⁺ T-cell apoptosis. (a) CD8⁺ T cells upregulate PD-L1 expression on HepG2.2.15 cells in an IFN- γ -dependent manner. * $p < 0.05$, compared with the HepG2.2.15 control group. (b) IFN- γ -treated HepG2.2.15 cells were much more resistant to CD8⁺ T cell-dependent cytotoxicity. * $p < 0.05$, compared with the IFN- γ -treated group. (c) IFN- γ -treated HepG2.2.15 cells promoted CD8⁺ T cells apoptosis (Annexin-V⁺, left panel) and necrosis (7AAD⁺, right panel) by a PD-L1-dependent pathway. * $p < 0.05$, compared with IFN- γ -treated group. At least five trials were run in the above experiments. (d) The IFN- γ concentration increased significantly in nontumor tissue in stage III, compared with stage I and healthy control tissue. (HC, $n = 5$; stage I, $n = 6$; stage II, $n = 6$; stage III, $n = 5$). The data indicate mean values \pm SD. (e) CD8/cleaved caspase-3 double stainings *in situ* were measured in tumor sections (red for CD8, blue for cleaved caspase-3). Arrows show double positive cells.

cells and hepatoma cells, respectively. Similar upregulation of PD-1 expression was also observed in other several cancers, including melanoma, prostate cancer and renal cancer.^{13,27,28} The mechanisms for PD-1 upregulation on T cells may be due to TCR activation^{5,29} by tumor-associated antigens or stimulations by common γ -chain cytokines,³⁰ including IL-2,

IL-7, IL-15 and IL-21 in tumor microenvironment. From the pathological analysis, we directly observed that the PD-L1-expressing tumor nest was an apparent forbidden area for lymphocytes, especially for PD-1⁺CD8⁺ T cells. Furthermore, *in situ* results also showed that CD8⁺ T cells were prone to apoptosis in the tumor region, which was consistent with

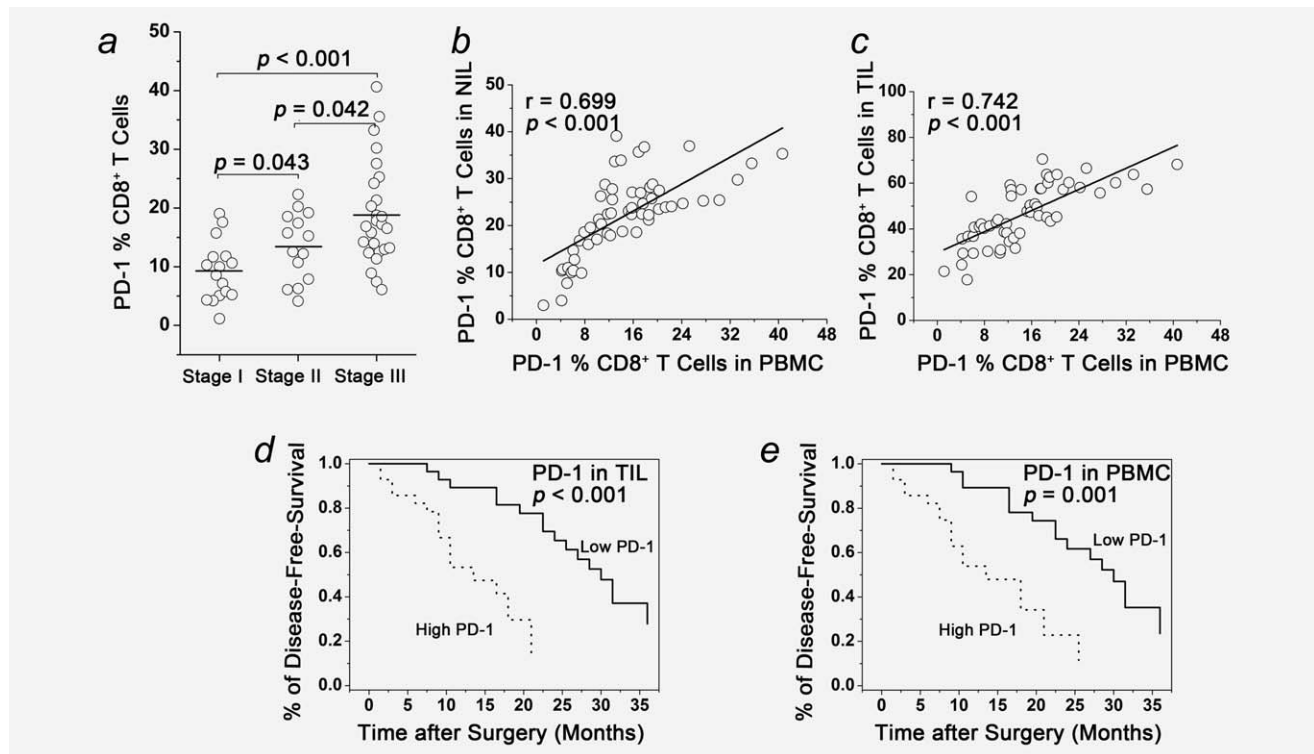


Figure 5. PD-1 expressions correlate with prognosis of HCC. (a) The frequencies of circulating PD-1 on CD8⁺ T cells according to different clinical stages of HCC (stage I, $n = 16$; stage II, $n = 14$; stage III, $n = 26$). Black bars indicate mean values. PD-1 expressions in PBMC positively correlated (Spearman rank test) with those in corresponding NIL (b) and TIL (c) ($n = 56$). Both PD-1 expressions in PBMC and TIL were divided into two groups, high and low PD-1, according to the median values. The high PD-1 ($n = 28$) expressions in both TIL (d) and PBMC (e) could predict the poor prognosis of disease-free survival (DFS). Actual DFS were analyzed by the Kaplan-Meier method, and DFS was measured in months from tumor resection or liver transplantation to relapse. The log-rank test was used to compare between the two groups.

some former reports.^{10,31,32} We provided evidence with one typical case that the aggressive tumor invasion was dependent on the high expression of PD-L1 on the boundary of the tumor nest, which seemed to form a molecular shield to protect the tumor cells from immune surveillance.

The relationship between tumor-associated PD-L1 and pathological features has been recently studied in several cancers, and it was found that patients with PD-L1-positive (high) cancer cells had a significantly poorer prognosis than those with the negative (low) cancer cells.^{16–18,33} Similar results were also found in our study. Although the detection of PD-L1 requires an invasive operation for liver sections and would be a complex test for clinical applications, it is worth investigating whether PD-1 expression can predict the prognosis of HCC patients. Therefore, we further evaluated the validity of circulating PD-1 in predicting the prognosis of HCC patients. Our data showed that the frequency of PD-1⁺CD8⁺ T cells in the peripheral blood in stage III HCC patients was two times as high as those in stage I, suggesting that CD8⁺ T cells in advanced stages of disease were prone to be functionally impaired. We further analyzed PD-1 expression in PBMC, NIL and TIL simultaneously in a total of 56 patients. We found that PD-1 expression in pe-

ripheral blood positively correlated with those in NIL and TIL. In addition, we found that PD-1 expression on CD8⁺ T cell in PBMC and TIL had an inverse correlation with the time interval of DFS. Circulating PD-1 could also predict the time interval of DFS, which may serve as a new valid marker for evaluating disease prognosis.

In vitro functional assay showed that blocking PD-L1 could enhance CD8⁺ T cell proliferation and IFN- γ secretion. We also found that blockage of PD-L1 pathway could increase the frequency of tumor-specific T cells, which is consistent with former report³⁴ (Supporting Information Fig. 2). Although the precise mechanism regulating PD-L1 expression in tumor cells is unknown, several cytokines, including IFN- γ and TNF- α , have been implicated as potent inducers of PD-L1 expression on the surface of several tumor cells.^{32,35,36} In this study, we found that CD8⁺ T cells could stimulate HepG2.2.15 cells to upregulate PD-L1 by an IFN- γ -dependent mechanism, which may facilitate HepG2.2.15 cellular resistance to CD8⁺ T cell-dependent cytotoxicity and conversely induced CD8⁺ T cells apoptosis. We also found similar results in other hepatoma cell lines such as HepG2 and Huh7. In this study, the upregulation of PD-L1 might be

one negative feedback reaction, which is fully utilized by hepatoma cells to escape from immune surveillance. We found that IFN- γ was upregulated in nontumor tissues of stage III HCC patients, which was coincident with PD-L1 upregulation in the tumor tissues. However, such differences were not found in the peripheral blood. The data also showed that IFN- γ concentrations in tumor tissues were very low, which was due to quick absorption by tumor cells within 6 hr (Fig. 4a) and narrow or disappeared interspaces within the tumor nest. In addition, incubation with α -PD-L1 could reduce HepaG2.2.15-induced apoptosis of CD8⁺ T cells and increased the cytotoxicity of CD8⁺ T cells. Collectively, induction of PD-L1 may be one mechanism for immune evasion used by tumors for attenuating T-cell responses and promoting antitumor effector CD8⁺ T-cell apoptosis. Furthermore, our data demonstrated that CD8⁺ T cells could make two distinct impacts on hepatoma cells: eradicate the hepatoma cells or induce PD-L1 expressions on hepatoma cells. One previous report illustrated that sustained low IFN- γ levels could upregulate PD-L1 on hepatoma cells, compared with high levels, which mediated significant antitumor

responses.³⁶ As a result, a crucial part of the antitumor response seemed to be whether the CD8⁺ T cells can secrete sufficient amounts of IFN- γ before the upregulation of PD-L1 on hepatoma cells. If true, more experiments should be carried out to determine this effective level of IFN- γ .

In conclusion, we described for the first time in our study the role of the PD-1/PD-L1 pathway in the interaction between hepatoma cells and CD8⁺ T cells in HCC patients. We demonstrated that PD-1/PD-L1 plays a pivotal role in tumor evasion, and blocking interaction with anti-PD-L1 antibody would revitalize CD8⁺ T cells to give them a second chance to clear the tumor. Moreover, both peripheral and intrahepatic PD-1 expressions could act as indicative markers for prognosis of HCC patients after surgical resections. Further understanding of the immunopathogenesis of HCC will be critically important for the development of effective therapeutic strategies against the disease.

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