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-\(\gamma\)-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 cells\(^5\) and plays a crucial role in regulating peripheral tolerance.\(^5\,\,9\) 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...
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. 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.

### 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-\(\gamma\) 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 \(\times\) 10\(^5\)) were seeded in a 96-well plate (Costar, Lowell, MA) in the presence or absence of anti-human PD-L1 (\(\alpha\)-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.

### Table 1. The clinical characteristics of enrolled subjects

<table>
<thead>
<tr>
<th>Groups</th>
<th>HC</th>
<th>LC</th>
<th>HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>20</td>
<td>20</td>
<td>56</td>
</tr>
<tr>
<td>Age (range)</td>
<td>45 (30–55)</td>
<td>53 (35–65)</td>
<td>55 (20–77)</td>
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<tr>
<td>Gender (male/female)</td>
<td>12/8</td>
<td>13/7</td>
<td>48/8</td>
</tr>
<tr>
<td>HBV viral load (copies/ml)</td>
<td>0</td>
<td>9.22 (\times) 10(^6) (10(^2)–10(^7))</td>
<td>8.97 (\times) 10(^5) (10(^1)–10(^7))</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>ND</td>
<td>ND</td>
<td>4.3 (1.1–13.0)</td>
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<tr>
<td>Tumor morphology (unifocal/multifocal)</td>
<td>ND</td>
<td>ND</td>
<td>42/14</td>
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<tr>
<td>Vascular invasion (absence/presence)</td>
<td>ND</td>
<td>ND</td>
<td>29/27</td>
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<tr>
<td>Liver cirrhosis (no/yes)</td>
<td>ND</td>
<td>0/20</td>
<td>5/51</td>
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<tr>
<td>Child-Pugh classification (A/B)</td>
<td>ND</td>
<td>18/2</td>
<td>50/6</td>
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<tr>
<td>Stage,(^1) I/II/III</td>
<td>ND</td>
<td>ND</td>
<td>16/14/26</td>
</tr>
<tr>
<td>Therapy (resection/LT)</td>
<td>ND</td>
<td>ND</td>
<td>54/2</td>
</tr>
</tbody>
</table>

\(^1\)HCC 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; \(\alpha\)-FP, \(\alpha\)-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.
**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 μg/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 μg/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 immunosorbant 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 ± 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 Tcm (CCR7+CD45RA−) and Tem (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).
Tumor Immunology

T cells in HCC, PBMC from HC and HCC were stimulated
To investigate the impacts of PD-1/PD-L1 pathway on CD8
IFN-$\gamma$
Blocking PD-L1 restores the proliferative abilities and
Tcm, central memory; Tem, effector memory; Tnaive, naïve T;
Different memory subsets are distinguished by CCR7 and CD45RA.
T-cell subsets in peripheral blood and corresponding NIL and TIL.
PD-1 upregulation on effector phase CD8
representative of five subjects for each group.
corners are the frequencies of positive cells. The data are
mean values of % PD-1 expression on CD8
each individual. Black bars in the graph with statistical results are
line is the isotype control. The number on the upper right
dots indicate
þ
T cells. (b) (c)
PD-1 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-$\gamma$ inhibited upregulation by both methods (Fig.
Thus, upregulation of PD-L1 is triggered by CD8+$^+$ T cells in an IFN-$\gamma$-dependent mechanism.

CD8+$^+$ T-cell produced IFN-$\gamma$ can induce PD-L1
expression on hepatoma cells
As some previous reports demonstrated that IFN-$\gamma$ could
induce PD-L1 expression, we performed a series of parallel experiments to verify whether PD-L1 upregulation could be
triggered by IFN-$\gamma$ released from CD8+$^+$ T cells. Results
showed that both IFN-$\gamma$ 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-$\gamma$ inhibited upregulation by both methods (Fig.
Thus, upregulation of PD-L1 is triggered by CD8+$^+$ T cells in an IFN-$\gamma$-dependent mechanism.

IFN-$\gamma$ facilitates hepatoma cells resistance to CD8+$^+$ T cells
To investigate whether IFN-$\gamma$-treated hepatoma cells could
weaken the cytotoxic effect of T cells, the CD8+$^+$ T cells iso-
lated 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-$\gamma$-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-$\gamma$-treated hepatoma cells could upregulate the expression of PD-L1, we further investigated whether IFN-$\gamma$-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-$\gamma$-treated HepG2.2.15 cells, compared with the IFN-$\gamma$-untreated control. Blocking PD-L1 could largely inhibit apoptosis and necrosis of CD8+$^+$ T cells induced by IFN-$\gamma$-treated HepG2.2.15 cells (Fig. 4c). To detect IFN-$\gamma$ in situ, the concentrations of IFN-$\gamma$ in the supernatant of tissue homogenates were measured by ELISA. The results showed that IFN-$\gamma$ 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
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\% ± 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\% ± 7.25\%; DFS median, 28.70 months, \(p < 0.001\)). Moreover, circulating PD-1 (PD-1 frequency: high vs. low, 21.04\% ± 6.75\% vs. 8.59\% ± 3.36\%, respectively) could also predict similarly the poor prognosis of DFS (DFS median: high vs. low, 16.30 vs. 27.15 months, \(p = 0.001\)).
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 extend 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 upregulated 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, 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, 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 cells during the antitumor response in HCC patients.

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and hepatoma cells, respectively. Similar upregulation of PD-1 expression was also observed in other several cancers, including melanoma, prostate cancer and renal cancer. The mechanisms for PD-1 upregulation on T cells may be due to TCR activation by tumor-associated antigens or stimulations by common \( \gamma \)-chain cytokines, 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 4. IFN-\( \gamma \)–treated hepatoma cells promotes CD8\(^+\) T-cell apoptosis. (a) CD8\(^+\) T cells upregulate PD-L1 expression on HepG2.2.15 cells in an IFN-\( \gamma \)–dependent manner. * \( p < 0.05 \), compared with the HepG2.2.15 control group. (b) IFN-\( \gamma \)–treated HepG2.2.15 cells were much more resistant to CD8\(^+\) T cell-dependent cytotoxicity. * \( p < 0.05 \), compared with the IFN-\( \gamma \)–treated group. (c) IFN-\( \gamma \)–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-\( \gamma \)–treated group. At least five trials were run in the above experiments. (d) The IFN-\( \gamma \) 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.
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 peripheral 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 HepaG2.2.15 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 hepa-
toma 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 upregula-
tion 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 eva-
sion used by tumors for attenuating T-cell responses and
promoting antitumor effector CD8⁺ T-cell apoptosis. Fur-
thermore, 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. 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 tu-
mor evasion, and blocking interaction with anti-PD-L1 anti-
body 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.
Fur-
ther understanding of the immunopathogenesis of HCC will
be critically important for the development of effective therapeu-
tic strategies against the disease.

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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 tu-
mor evasion, and blocking interaction with anti-PD-L1 anti-
body 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.
Fur-
ther understanding of the immunopathogenesis of HCC will
be critically important for the development of effective therapeu-
tic strategies against the disease.

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