ORIGINAL ARTICLE

PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells are associated with pathogenesis and progression of patients with hepatitis B virus-related hepatocellular carcinoma

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Abstract

Hepatitis B virus-associated hepatocellular carcinoma (HBV-HCC) is usually considered an inflammation-related cancer associated with chronic inflammation triggered by exposure to HBV and tumor antigens. T-cell exhaustion is implicated in immunosuppression of chronic infections and tumors. Although immunotherapies that enhance immune responses by targeting programmed cell death-1(PD-1)/PD-L1 are being applied to malignancies, these treatments have shown limited response rates, suggesting that additional inhibitory receptors are also involved in T-cell exhaustion and tumor outcome. Here, we analyzed peripheral blood samples and found that coexpression of PD-1 and T-cell immunoglobulin and immuno-receptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT) was significantly upregulated on CD4$^+$ and CD8$^+$ T cells from patients with HBV-HCC compared with those from patients with chronic HBV or HBV-liver cirrhosis. Additionally, PD-1$^+$ TIGIT$^+$ CD8$^+$ T-cell populations were elevated in patients with advanced stage and progressed HBV-HCC. Importantly, PD-1$^+$ TIGIT$^+$ CD8$^+$ T-cell populations were negatively correlated with overall survival rate and progression-free survival rates. Moreover, we showed that PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells exhibit features of exhausted T cells, as manifested by excessive activation, high expression of other inhibitory receptors, high susceptibility to apoptosis, decreased capacity for cytokine secretion, and patterns of transcription factor expression consistent with exhaustion. In conclusion, PD-1$^+$ TIGIT$^+$ CD8$^+$ T-cell populations are associated with accelerated disease progression and poor outcomes in HBV-HCC, which might not only have important clinical implications for prognosis but also provide a rationale for new targets in immunotherapy.

Keywords TIGIT · Programmed cell death-1 · Prognosis · HCC · Coexpression

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Abbreviations

AFP	α-Fetoprotein
ALB	Albumin
ALT	Alanine aminotransferase
AUROC	Area under the receiver-operating characteristic
BCLC	Barcelona clinic liver cancer
BTLA	B- and T-lymphocyte attenuator
CHB	Chronic hepatitis B virus infection
CTLA-4	Cytotoxic T-lymphocyte antigen 4
Eomes	Eomesodermin
FITC	Fluorescein isothiocyanate
HBeAg	Hepatitis B e antigen
HBV-HCC	Hepatitis B virus-associated hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HR	Hazard ratio
IFN-γ	Interferon gamma

LAG-3	Lymphocyte-activation gene 3
LC	Liver cirrhosis
MELD	Model for end-stage liver disease
NLR	Neutrophil–lymphocyte ratio
NK	Natural killer cells
OS	Overall survival
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death-1
PD-L1	Programmed death-ligand 1
PFS	Progression-free survival
TBIL	Total bilirubin
TCM	Central memory T cells
TEM	Effector memory T cells
TEMRA	Terminally differentiated effector T cells
TIGIT	T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain
TILs	Tumor-infiltrating lymphocytes
TIM-3	T-cell immunoglobulin domain and mucin domain 3
TNF-α	Tumor necrosis factor

Introduction

The incidence of HCC ranks fifth among all cancer and the mortality rate ranks second among men and sixth among women [1, 2]. Chronic HBV infection can lead to liver damage and is, therefore, major risk factors for liver cirrhosis (LC). Moreover, chronic HBV infection is associated with more than 80% of total HCC cases in China due to the high rate of HBV infection [3–5].

Inherent immune responses are usually unable to reject tumors because of the immunosuppressive tumor microenvironment, which is conducive to the proliferation and survival of tumor cells and can promote angiogenesis and metastasis and subvert the adaptive immune response [6–8]. T-cell exhaustion, which represents a state of T-cell dysfunction caused by persistent antigen stimulation during chronic infection and cancer, plays an essential role in these tumor-promoting effects [9, 10]. Inhibitory molecules that have been shown to contribute to repression of T-cell responses include T-cell immunoglobulin domain and mucin domain 3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), 2B4, B- and T-lymphocyte attenuator (BTLA), and, most recently, TIGIT [9, 11]. Recent immunotherapy strategies that can reverse T-cell exhaustion and reactivate T-cell responses by blocking T-cell inhibitory receptors such as programmed cell death 1 (PD-1)/programmed death-ligand 1 (PD-L1) or cytotoxic T-lymphocyte antigen 4 (CTLA-4) have shown limited clinical response rates of up to 35% in advanced lung cancer, renal cancer, and melanoma [12–15]. To our knowledge, these strategies have

only been evaluated in liver cancer in a single phase I/II study, in which patients with advanced liver cancer showed a complete response rate of 4% and a partial response rate of 18% when treated with nivolumab [16]. There is a substantial need to identify additional inhibitory receptors that may lead to poor prognosis by suppressing anti-tumor responses in human cancer.

TIGIT has been reported to be expressed on effector T cells, regulatory CD4+ T cells, and natural killer (NK) cells [17–20]. Recently, TIGIT expression has been observed on tumor infiltrating lymphocytes (TILs) in several mice tumor models and melanoma patients, and the protein was found to be coordinately expressed with PD-1 [19, 21, 22]. However, the expression patterns of TIGIT during the pathogenesis and progression of liver cancer are poorly characterized, and the roles of TIGIT in clinical prognosis and T-cell exhaustion in liver cancer are unknown. In the current study, we have included patients with chronic HBV infection (CHB), patients with LC, and patients with HBV-HCC to compare the frequency of PD-1+ TIGIT+ T cells during the pathogenesis of HBV-HCC. We report that PD-1+ TIGIT+ T-cell populations are increased in patients with liver cancer, especially at advanced stages. Importantly, the frequency of PD-1+ TIGIT+ CD8+ T cells is positively associated with tumor progression (including tumor invasion, recurrence, and death) and is therefore related to poor clinical outcomes in HBV-HCC. Moreover, we provide evidence that PD-1+ TIGIT+ CD8+ T cells display an exhausted phenotype, as indicated by excessive activation, high expression of other inhibitory receptors, high susceptibility to apoptosis, decreased cytokine secretion, and patterns of transcription factor expression indicative of exhaustion. Therefore, PD-1+ TIGIT+ CD8+ T cells might be highly correlated with the progression in hepatocellular carcinoma.

Materials and methods

Patients

We prospectively enrolled 122 patients with HBV-HCC, 27 patients with HBV-related LC, 20 patients with CHB and 35 healthy volunteers from March 1, 2016 to May 1, 2017 at Beijing Ditan Hospital, Capital Medical University (Beijing, China). The diagnosis of HCC was the same as our previous study [23]. We excluded the patients if they meet the following criteria: (1) evidence of HCV or HIV infection; (2) other cause of chronic liver disease; (3) metastatic liver cancer or with other tumors. The diagnosis of cirrhosis was based on histopathology, radiology, or clinical complications like ascites, variceal bleeding, hepatorenal syndrome, and

Table 1 Clinical characteristics of all patients

	Healthy donors ($n=35$)	CHB ($n=20$)	HBV-LC ($n=27$)	HBV-HCC ($n=122$)	P values
Age (years)	54.6 ± 7.0	41.5 ± 9.8*	52.2 ± 11.9	56.4 ± 10.4	< 0.0001
Sex (male/female)	22/13	16/4	21/6	97/25	0.22
ALT (U/L)	NA	27.0 (18.2, 36.3)	19.8 (15.7, 38.0)	29.5 (19.3, 48.4)	0.06
HBeAg ($\pm$)	NA	11/9	19/8	94/28	0.11
HBV-DNA ($\geq 100/< 100$ IU/ml)	NA	4/16	4/23	29/93	0.58

CHB chronic hepatitis B, *HBV-LC* hepatitis B virus-related liver cirrhosis, *HBV-HCC* HBV-related hepatocellular carcinoma, *ALT* aspartate aminotransferase

*p value < 0.05 vs healthy donors, HBV-LC or HBV-HCC group

hepatic encephalopathy. The healthy donors were recruited from patients receiving a physical examination at Beijing Ditan Hospital. Clinical and demographic characteristics of all patients are summarized in Table 1.

Sample collection and separation of peripheral blood mononuclear cells (PBMCs)

We collected 5 ml peripheral blood samples from all above patients and healthy volunteers. The PBMCs were isolated as described before [24].

Flow cytometry staining and analysis

The following antibodies were used: anti-human BV786-conjugated anti-CD3, APC-H7-conjugated anti-CD4, BV510-conjugated anti-CD8, AF700-conjugated anti-CD45RA, BV421-conjugated anti-CCR7, BV711-conjugated anti-PD-1, BV650-conjugated anti-TIM-3, FITC-conjugated anti-2B4, PE-CF594-conjugated anti-CD95, PE-conjugated anti-CTLA-4, FITC-conjugated anti-CD38, PE-conjugated anti-HLA-DR (BD Biosciences), PE-conjugated anti-perforin, AF700-conjugated anti-Granzyme B (BioLegend), PE-Cy7-conjugated anti-TIGIT, APC-conjugated anti-LAG-3 (eBioscience), and the corresponding isotype controls.

For intracellular staining, PBMCs were stimulated for 5 h with anti-CD3/anti-CD28 (2 μg/mL and 5 μg/mL, respectively; eBioscience). Then, cells were stained with CD3, CD4, CD8, PD-1, and TIGIT antibodies, and intracellularly stained with AF700-conjugated anti-IFN-γ, FITC-conjugated anti-TNF-α, or PE-conjugated anti-IL-2 (BD Biosciences) antibodies. For analysis of ki67, perforin, and T-bet staining, cells were stained for surface antibodies described above, and then intracellularly stained with APC-conjugated anti-perforin, FITC-conjugated anti-ki67, AF700-conjugated anti-Granzyme B, BV421-conjugated

anti-T-bet, or PE-conjugated anti-Eomes (BD Biosciences) antibodies. We used a fixable viability dye (eFluor® 506, eBioscience) to evaluate the cell viability.

For apoptosis analysis, we used FITC Annexin V Apoptosis Detection Kit (BioLegend), according to the instruction. Data were acquired with an LSR Fortessa flow cytometer, and then analysis with FlowJo software (Tree Star).

Statistical analysis

Statistical analysis was performed by GraphPad 5.0 and SPSS version 19.0. Quantitative data accorded were expressed as the mean $\pm$ standard deviation (SD) and they were analyzed by t tests. One-way ANOVA for more than two independent samples was used by Tukey's multiple comparison test. The non-normally distributed data were expressed as median with quartile range and analyzed by Mann–Whitney U test. Pearson's and Spearman's correlation coefficients were used to evaluate correlations for normally and non-normally distributed data, respectively. We used Kaplan–Meier method for survival analyses. The prediction value of the observation indexes was estimated by areas under the receiver-operating characteristics (AUROC) curve. P values < 0.05 were considered statistically significant.

Results

Elevated levels of PD-1$^+$ TIGIT$^+$ T cells in patients with HBV-HCC

To evaluate the roles of PD-1 and TIGIT expression in the pathogenesis and progression of HBV-HCC, we performed flow cytometric analysis of PD-1 and TIGIT on CD4$^+$ and CD8$^+$ T cells from CHB, HBV-LC, and HBV-HCC patients. Age-matched healthy donors were taken as controls. The percentages of PD-1$^+$ and TIGIT$^+$ cells in the CD4$^+$ T-cell population were apparently elevated in HBV-HCC,
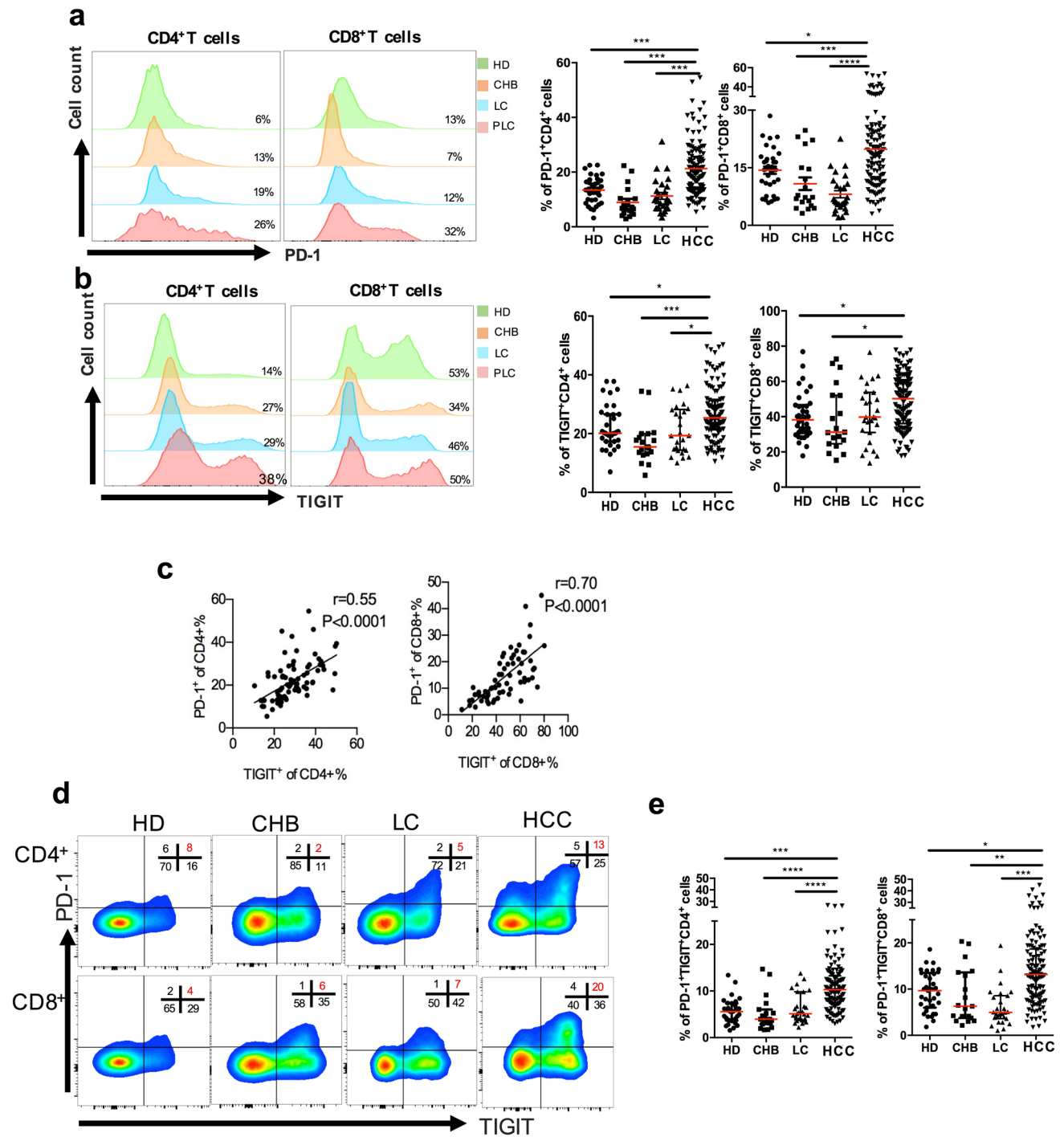
Fig. 1 Coexpression of PD-1 and TIGIT is upregulated on T cells in HBV-HCC patients. **a**, **b** The expression of PD-1 and TIGIT on CD4$^+$ and CD8$^+$ T cells from HBV-HCC ($n=122$), compared with healthy donors ($n=35$), CHB ($n=20$) LC patients ($n=27$) by flow cytometry analysis. **c** Correlation analysis of PD-1 and TIGIT on CD4$^+$ T cells (left) and CD8$^+$ T cells (right) from patients with HBV-HCC. **d** Representative flow data of PD-1 and TIGIT expression on CD4$^+$ and CD8$^+$ T cells within each group. **e** Percentages of PD-1$^+$ TIGIT$^+$ CD4$^+$ cells (left) and PD-1$^+$ TIGIT$^+$ CD8$^+$ cells (right) of all CD4 and CD8 T cells, respectively, from patients with HBV-HCC. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$

compared with healthy donors and CHB or HBV-LC patients ($P<0.01$) (Fig. 1a, b). The percentage of PD-1$^+$ cells in the CD8$^+$ T cell was also elevated in HBV-HCC patients

(Fig. 1a). We further analyzed the correlation between PD-1 and TIGIT on CD4$^+$ and CD8$^+$ T cells. Interestingly, the frequency of TIGIT expression on CD8$^+$ T cells from

HBV-HCC patients was strongly positively correlated with the frequency of PD-1 expression ($r = 0.70$, $P < 0.0001$), while in the case of CD4$^+$ T cells, a moderate correlation was observed ($r = 0.55$, $P < 0.0001$) (Fig. 1c). Accordingly, we observed increased coexpression of PD-1 and TIGIT on both CD4$^+$ and CD8$^+$ T cells in HBV-HCC, compared with those from healthy donors and patients with CHB or HBV-LC (Fig. 1d, e).

Elevation of PD-1$^+$ TIGIT$^+$ CD8$^+$ T-cell levels is related to HBV-HCC progression

We divided the HBV-HCC group into two subgroups based on the percentage of PD-1$^+$ TIGIT$^+$ cells in CD8$^+$ T cell: PT^{hi} ($\geq 12.8\%$) and PT^{lo} ($< 12.8\%$). We used the median percentage (12.8%) of PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells from 122 patients with HBV-HCC as the cut-off. We analyzed differences in clinical characteristics between the two subgroups (Table 2). We found that the neutrophil–lymphocyte ratio (NLR), which has been identified as a poor prognostic indicator in some tumors [25–28], was elevated in the PT^{hi} group ($P = 0.002$). The PT^{hi} group also displayed some differences in tumor features; specifically, a higher number of patients in this group showed tumors ≥ 5 cm in size and multiple tumors (approximately 50%). No differences in the vascular invasion or intrahepatic metastasis were detected. Finally, the PT^{hi} group exhibited more patients with BCLC stage C and D HCC ($P = 0.002$, Table 2).

We next used CT/MRI imaging to investigate whether PD-1$^+$ TIGIT$^+$ T-cell populations are associated with disease progression, which encompasses tumor recurrence, tumor invasion, and death. We observed a dramatically higher rate of disease progression in the PT^{hi} group compared with the PT^{lo} group [48/13 (78.7%) vs. 23/38 (37.7%), $P < 0.0001$, Table 2]. Similarly, we found that the percentage of PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells was higher in patients with BCLC stage C and D HCC and patients with progression ($P = 0.002$, Fig. S1a, b). Though the tumor size and multiplicity were associated with PD-1$^+$ TIGIT$^+$ CD4$^+$ T-cell populations, the HCC progression had a slight difference between the high and low PD-1$^+$ TIGIT$^+$ CD4$^+$ groups (Table S1). Together, our results showed a more strong correlation between PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells and prognosis in HBV-HCC than PD-1$^+$ TIGIT$^+$ CD4$^+$ T cells, suggesting that these cells may play a critical role in the pathogenesis and progression of HBV-HCC.

Increased prevalence of PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells predicts poor prognosis in HBV-HCC patients

To determine whether PD-1$^+$ TIGIT$^+$ CD8$^+$ T-cell populations are associated with prognosis in HBV-HCC, we followed up 122 patients for a median of 59 weeks (32–84 weeks). The overall survival rate at 24 weeks and 48 weeks were 78.7% and 72.1%, respectively. The progression-free survival (PFS) rates at 24 weeks and 48 weeks

Table 2 Demographic and clinical characteristics of different level of PD-1 + TIGIT + CD8 + cells in patients with HBV-HCC

	PT^{+lo} ($n = 61$)	PT^{+hi} ($n = 61$)	P values
Age (mean $\pm$ SD)	56.2 ± 9.9	56.7 ± 10.8	0.77
Sex (male/female)	50/11	49/12	0.82
Cirrhosis (present/absent)	58/3	53/8	0.114
NLR (mean $\pm$ SD)	2.86 ± 2.20	4.78 ± 4.02	0.002
ALT ($\geq 50/< 50$ U/L)	14/47	14/47	1
TBIL ($\geq 18.8/< 18.8$ μmol/L)	38/23	36/24	0.796
Albumin ($\geq 40/< 40$ g/L)	18/43	16/45	0.686
AFP ($\geq 400/< 400$ ng/ml)	13/48	18/43	0.298
HBeAg ($\pm$)	18/43	10/51	0.085
HBV-DNA ($\geq 100/< 100$ IU/ml)	15/46	14/47	0.832
Tumor size ($\leq 5/> 5$ cm)	49/12	35/26	0.007
Tumor multiplicity (solitary/multiple)	33/28	20/41	0.02
Vascular invasion (present/absent)	40/21	33/28	0.2
Intrahepatic metastasis (yes/No)	11/50	11/50	1
BCLC stage (A + B/C + D)	39/22	22/39	0.002
Progression (yes/no)	23/38	48/13	< 0.0001
Antiviral therapy (yes/no)	50/11	54/7	0.445
Treatments for HCC (resection/minimally invasive/palliative)	10/36/15	14/28/19	0.342

NLR neutrophil–lymphocyte ratio, *ALT* alanine aminotransferase, *TBIL* total bilirubin, *AFP* alpha-fetoprotein
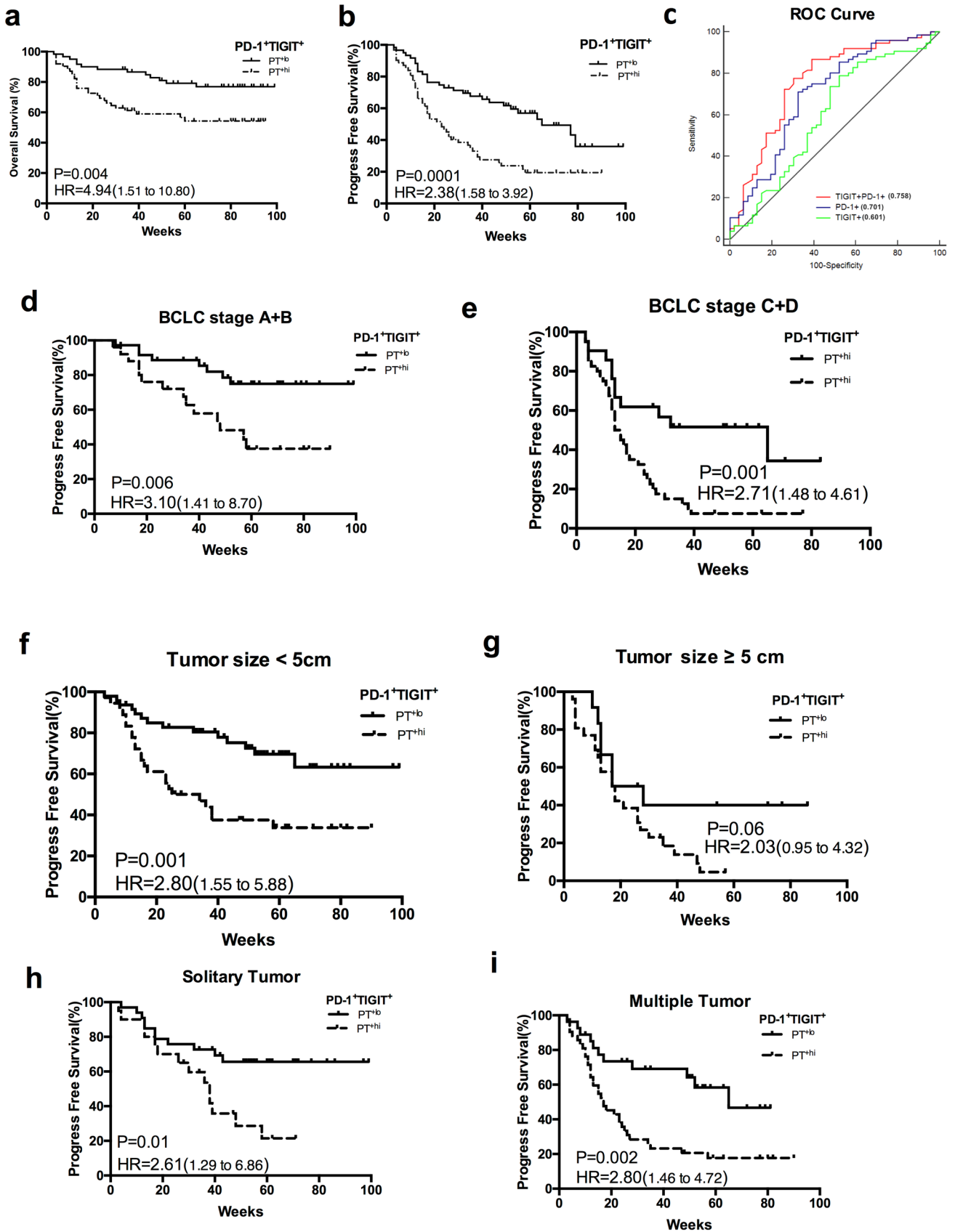
◄**Fig. 2** Kaplan–Meier curve analysis showing the efficacy of PD-1+ TIGIT+ CD8+ cell levels as a predictor of progression-free survival in HBV-HCC across different tumor burdens. **a, b** Kaplan–Meier curve analysis showing the efficacy of PD-1+ TIGIT+ CD8+ cell levels as a predictor of overall survival (**a**) and progression-free survival (**b**) in HBV-HCC patients. (**c**) ROC curve for PD-1+ TIGIT+ CD8+ T cell, PD-1+ CD8+ T cell and TIGIT+ CD8+ T-cell frequency as a predictor of progression-free survival for HBV-HCC patients. **d–i** Subgroup analysis of patients with (**d**) BCLC stage A and B HCC, (**e**) BCLC stage C and D HCC, (**f**) patients with tumor size < 5 cm, (**g**) patients with tumor size > 5 cm, (**h**) patients with single tumors, and (**i**) patients with multiple tumors. *P* values and HRs were obtained using the log-rank test

were 59.0% and 45.1%, respectively. All patients were divided into two groups (PT^{hi} and PT^{lo}) according to the percentage of PD-1+ TIGIT+ CD8+ T cell at the time of joining the study, as described above. By Kaplan–Meier survival analysis, we found that PT^{hi} group had a lower survival rate than the PT^{lo} group (Fig. 2a; HR = 4.94, 95% CI 1.51–10.80, $P = 0.004$). However, the PD-1^{+hi} group and TIGIT^{+hi} group had no difference in the overall survival rate than PD-1^{+lo} and TIGIT^{+lo} group, respectively (Fig. S2a, b). Furthermore, we showed that the PT^{hi} group had a significantly higher proportion of tumor recurrence and progression compared with the PT^{lo} group (Fig. 2b; HR = 2.38, 95% CI 1.58–3.92, $P = 0.0001$). The area under the receiver-operating characteristic (AUROC) curve for PD-1+ TIGIT+ cell population was 0.758 (95% CI 0.672–0.831), which was higher than that of PD-1+ and TIGIT+ cell populations alone (Fig. 2c; *P* values were 0.038 and 0.0006, respectively, when compared to PD-1+ TIGIT+ cell population).

To precisely determine the prognostic value of PD-1+ TIGIT+ cells in HBV-HCC, we compared the PFS of the PT^{hi} and PT^{lo} groups at different BCLC stages (Fig. 2d, e). For both patients in stages A or B and patients in stages C or D, the PFS rates of patients with high PD-1+ TIGIT+ cell populations were significantly lower than those with low PD-1+ TIGIT+ cell populations throughout the follow-up period (stages A and B, $P = 0.006$; stages C and D, $P = 0.001$). We also performed Kaplan–Meier survival analysis to assess the effect of tumor burden on PFS. The PFS rate of the PT^{hi} group was obviously lower than the PT^{lo} group in patients with tumors < 5 cm in size (Fig. 2f; HR = 2.80, 95% CI 1.55–5.88, $P = 0.001$) and solitary and multiple tumors (Fig. 2h–i), but not in the subgroups of patients with tumors ≥ 5 cm in size ($P = 0.06$; Fig. 2g).

In this study, most of the patients with HBV-HCC (111, 91.0%) had associated chronic liver disease and cirrhosis, which are usually associated with a decline in liver function. Since it is widely perceived that clinical outcomes in HBV-HCC depend not only on tumor stage but also on liver function, we assessed the efficacy of PD-1+ TIGIT+ cell populations as a predictor of PFS rates in patients with

different levels of liver function/dysfunction, as measured by the model for end-stage liver disease (MELD) scores. PT^{hi} patients had worse outcomes than PT^{lo} patients in both the subgroup of patients with below-median MELD scores (MELD < 6.16, $P = 0.003$) and the subgroup of patients with above-median MELD scores (MELD ≥ 6.16, $P = 0.001$) (Fig. 3a, b).

Since chronic virus infection affects expression of the coinhibitory molecules PD-1 and TIGIT, we assessed the prognostic value of PD-1+ TIGIT+ CD8+ T cells in HBV-HCC patients with different virus levels. The PFS of PT^{hi} patients was significantly lower than that of PT^{lo} patients regardless of whether the HBV-DNA level ≥ 100 IU/mL or < 100 IU/mL (Fig. 3c, d). We also examined the predictive value of PD-1+ TIGIT+ cell populations for HBV-HCC prognosis in three groups of patients with different serum AFP levels: AFP < 8.8 ng/mL, 8.8 ng/mL ≤ AFP < 400 ng/mL, and AFP ≥ 400 ng/mL (where the normal reference value is 0.9–8.8 ng/mL). At all three AFP levels, PT^{hi} patients had a higher rate of progression ($P < 0.05$) (Fig. 3f–h). For antiviral and anti-tumor treatments, PT^{hi} patients had a poorer survival in the minimally invasive treatment group and with or without antiviral therapy (Fig. 3e, i).

However, we found a minor difference in PFS between low PD-1+ TIGIT+ CD4+ T-cell groups and high groups (Table S1, Fig. S3a–c). Thus, irrespective of tumor stage, liver function, HBV virus, load and AFP, the given cut-off value for the percentage of PD-1+ TIGIT+ cells in the CD8+ T-cell population has a principal predictive value for the prognosis of HBV-HCC, with particular clinical significance for predicting the outcomes of BCLC stage A and B, HBV-DNA < 100 IU/mL, and AFP-negative patients.

PD-1+ TIGIT+ CD8+ cells from HBV-HCC patients exhibit an exhausted phenotype and overactivation

Previous studies have reported that PD-1 and TIGIT are highly expressed on antigen-experienced T cells in tumor patients [29]; therefore, we examined the expression of PD-1 and TIGIT on naïve T cells (T_N) and mature, antigen-experienced T-cell subsets. The proportion of T_N cells was decreased in patients with HBV-HCC compared with patients with CHB or HBV-LC, although this difference was not statistically significant, and the portion of terminally differentiated effector (T_{EMRA}) cells was significantly increased in the HBV-HCC group compared with the CHB group ($P < 0.01$; Fig. 4a, b). Consistent with upregulation of PD-1 and TIGIT in antigen-experienced CD8+ T cells, PD-1 and TIGIT were highly expressed on central memory (T_{CM}) and effector memory (T_{EM}) cells in all three groups of patients (Fig. 4c). The frequency of PD-1+ TIGIT+ cells in each subset of CD8+ cells was more than twofold higher in
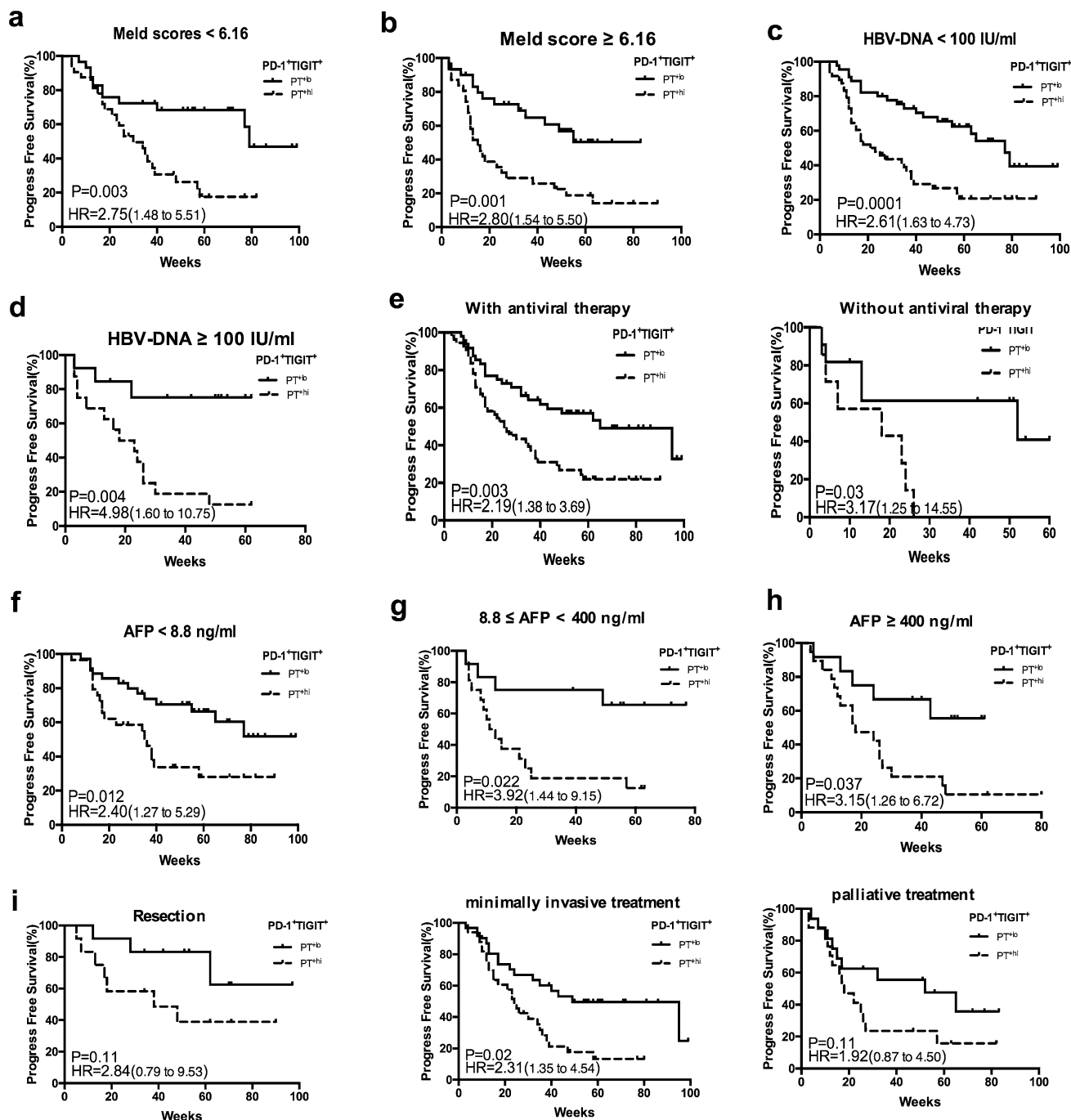
Fig. 3 Kaplan–Meier curve analysis showing the efficacy of PD-1+ TIGIT+ CD8+ cell levels as a predictor of progression-free survival in HBV-HCC across different liver function scores, HBV virus load, and AFP levels and treatments. **a, b** Patients with different MELD scores, **c, d** patients with different levels of serum HBV-DNA, (**e**) patients with or without antiviral therapy, (**f–h**) patients with different levels of serum AFP, (**i**) patients with resection, minimally invasive treatment or palliative treatment. *P* values and HRs were obtained using the log-rank test

the HBV-HCC group compared with the CHB and HBV-LC groups, with especially high frequencies observed in T_{CM} and T_{EM} cells. These results provided further evidence that the frequency of PD-1+ TIGIT+ cells is elevated in patients with HBV-HCC.

Since T-cell overactivation caused by sustained antigenic stimulation results in T-cell exhaustion and a stepwise loss of function [9, 11, 30, 31], we next investigated whether the activation status is different in PD-1+ TIGIT+ CD8+ T cells. We found that the frequencies of CD38+ cells, HLA-DR+

cells, and CD38$^+$ HLA-DR$^+$ cells were significantly higher in the PD-1$^+$ TIGIT$^+$ CD8$^+$ T-cell population than in the PD-1$^-$ TIGIT$^-$ CD8$^+$ T-cell population ($P < 0.0001$, Fig. 4d, e). We also found that 2B4, LAG-3, and CTLA-4 were expressed at higher frequencies in the PD-1$^+$ TIGIT$^+$ population than in the PD-1$^-$ TIGIT$^-$ population in patients with HBV-HCC ($P < 0.0001$, Fig. 4f). Finally, we compared the expression of 2B4, LAG-3, CTLA-4, and TIM-3 on PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells from patients in the PT^{hi} and PT^{lo} groups; the PT^{hi} group exhibited increased levels of each of these inhibitory receptors ($P < 0.05$, Fig. S4).

PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells from patients with HBV-HCC showed functional and transcriptional characteristics of exhaustion

To better understand the characteristics of PD-1$^+$ TIGIT$^+$ CD8$^+$ exhausted T cells, we applied functional assays and evaluated the cytokine production. PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells exhibited reduced capacity to produce IFN-γ and TNF-α compared with PD-1$^-$ TIGIT$^-$ CD8$^+$ and PD-1$^+$ TIGIT$^-$ CD8$^+$ T cells ($P < 0.05$, Fig. 5a, b). There was no change in the production of perforin and granzyme B in PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells (data not shown). We next assessed the susceptibility of PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells to apoptosis. Annexin V and CD95 expression were greatly increased on PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells, suggesting that these cells were highly susceptible to apoptosis (Fig. 5c, d). Since excessive apoptosis could lead to a reduction of T cells in HBV-HCC, we analyzed the correlation between the percentage of PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells and the absolute number of CD8$^+$ T cells. The percentage of PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells was negatively correlated with the absolute number of CD8$^+$ T cells, especially in patients with progressed HBV-HCC ($r = -0.74$, $P < 0.0001$; Fig. S5). We also found that the expression levels of PD-1 and TIGIT were higher on intratumor than in peritumor in HBV-HCC patients by immunohistochemical analysis (Fig. S6).

We further tested the intrinsic regulation of PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells by evaluating two key transcription factors, T-bet and Eomesodermin (Eomes), which involved in CD8$^+$ T-cell exhaustion. It was reported that T-bet^{dim} Eomes^{hi} CD8$^+$ T cells were linked to a terminal exhausted phenotype, whereas T-bet^{hi} Eomes^{dim} cells retain some residual T-cell functions [11, 31–33]. Consistent with previous reports, PD-1$^+$ TIGIT$^+$ cell populations contained a significantly higher percentage of T-bet^{dim} Eomes^{hi} cells than PD-1$^-$ TIGIT$^-$ and PD-1$^+$ TIGIT$^-$ cell populations ($P < 0.01$) (Fig. 5e, f). However, no differences in the percentages of T-bet^{hi} Eomes^{dim} cells were observed among the four subpopulations.

Discussion

T-cell exhaustion contributes to cancer immune escape by impeding tumor clearance [9, 10]. Increased expression of multiple inhibitory molecules is a key characteristic of this phenomenon. Recently, TIGIT expressed on TILs in various mouse tumor models and melanoma patients and to be coordinately expressed with PD-1 [21, 22]. However, the relationship between PD-1$^+$ TIGIT$^+$ T cells and the prognosis of cancer patients was not clear. In our study, we have demonstrated that HBV-HCC pathogenesis is associated with the increase of PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells. Importantly, we revealed that high levels of PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells are closely associated with a poor HBV-HCC prognosis. To our knowledge, this study provides the first evidence that PD-1$^+$ TIGIT$^+$ T cells are related to disease progression in HBV-HCC. Our results provide a potential rationale for liver cancer therapeutics that target both PD-1 and TIGIT to prevent disease progression and promote survival.

Several reports have shown that increases in circulating and intratumor PD-1$^+$ CD8$^+$ T-cell populations can predict poor disease progression and high recurrence in HCC after operation and cryoablation [34–36]. Recently, a clinical trial testing the safety and anti-tumor activity of an anti-PD-1 antibody in patients with HBV-related advanced HCC revealed that treatment with this antibody yielded a complete response rate of only 2% and a partial response rate of 12% [16]. Given the low response rate, there is an urgent need to explore the possibility of treatment with additional immune checkpoint inhibitors in combination with anti-PD-1 therapy. Recently, a small sample study of 49 patients with HCC showed that expression of both PD-1 and BTLA was upregulated on CD4$^+$ TILs and that these PD-1$^+$ BTLA$^+$ cells were highly dysfunctional [37]. However, few studies have examined the coexpression of PD-1 and other inhibitory molecules in liver cancer.

TIGIT is an inhibitory receptor that expressed at the highest levels on effector T cells and NK cells. Inhibition of NK cell-mediated tumor killing via engagement of PVR, the receptor of TIGIT, on target cells and reduction of IFN-γ secretion is the most well-understood aspect of TIGIT signaling [38–40]. TIGIT can suppress immune responses by limiting T-cell priming and depressing effector function in chronically stimulated CD8$^+$ T cells [41]. TIGIT can also restrain anti-tumor responses, as indicated by the observation that TIGIT deficiency resulted in significantly delayed tumor growth in melanoma and colon carcinoma mice models [21, 22]. In this study, we showed that PD-1$^+$ TIGIT$^+$ T-cell populations are increased in liver cancer, especially at advanced stages. Moreover, PD-1/TIGIT double-positive T cells had a more excellent and broader prognostic value than PD-1$^+$ or TIGIT$^+$ CD8$^+$ T cells alone, as indicated by the
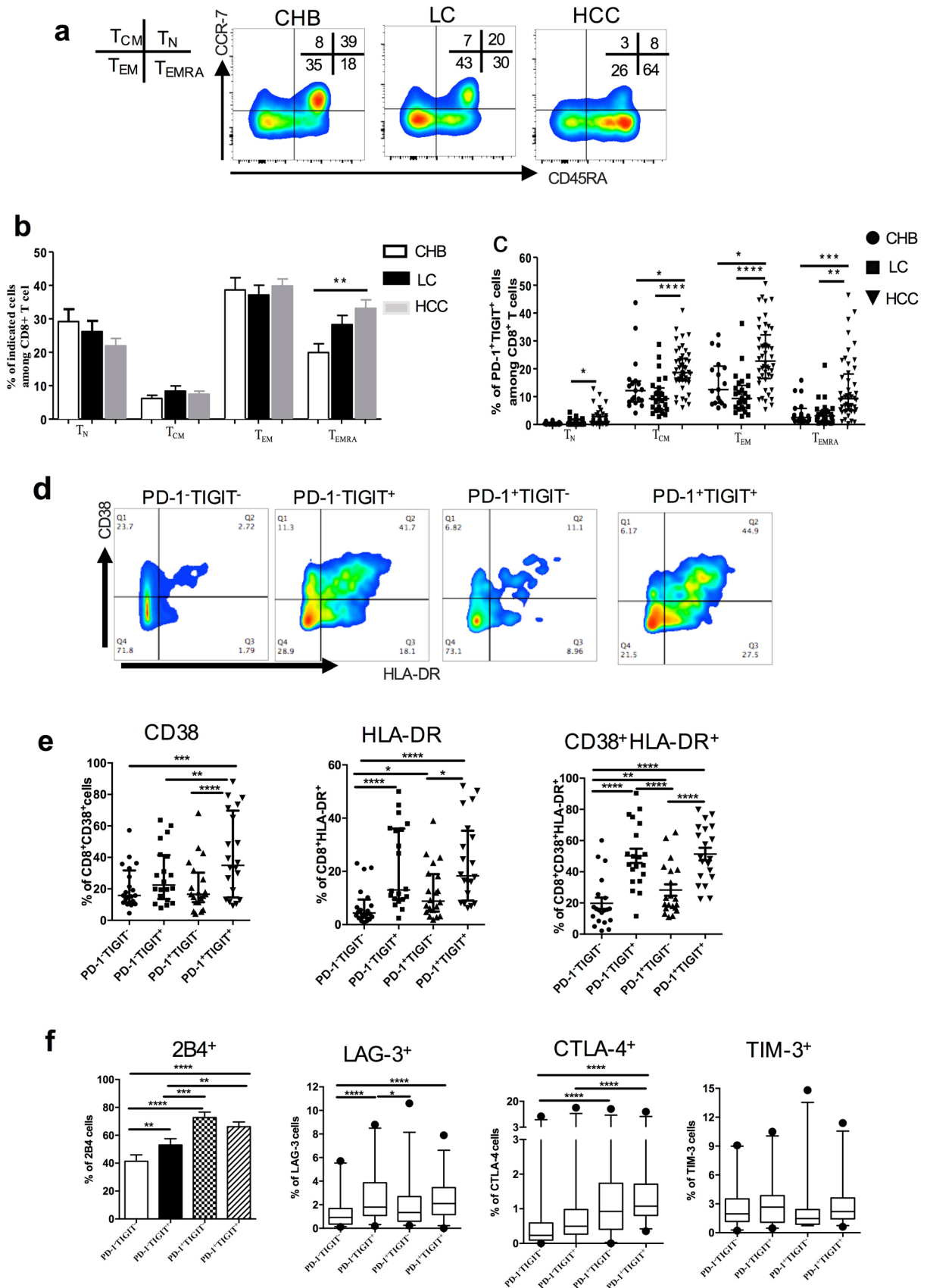
◂**Fig. 4** PD-1$^+$ TIGIT$^+$ CD8$^+$ cells in HBV-HCC patients exhibit an exhausted phenotype and overactivation. **a–b** Distribution of T_N, T_{CM}, T_{EM}, and T_{EMRA} cells in CD8$^+$ T-cell populations derived from patients with CHB, LC, and HCC. Representative flow cytometry data gated on CD8 (**a**) and histograms showing the percentage of each T-cell subset in each group (**b**) are shown ($n = 20$, 27, and 48 for the CHB, LC, and HCC groups, respectively). **c** Percentages of PD-1$^+$ TIGIT$^+$ on each subset (T_N, T_{CM}, T_{EM}, and T_{EMRA}) of CD8$^+$ T cells. **d–e** Representative flow cytometry data (d) and plots (e) showing the percentage of HLA-DR$^+$, CD38$^+$, and CD38$^+$ HLA-DR$^+$ subpopulations within PD-1$^-$ TIGIT$^-$, PD-1$^+$ TIGIT$^-$, PD-1$^-$ TIGIT$^+$, and PD-1$^+$ TIGIT$^+$ CD8$^+$ T-cell populations from patients with HBV-HCC ($n = 20$). **f** Frequency of 2B4, LAG-3, CTLA-4 and TIM-3 on PD-1$^-$ TIGIT$^-$, PD-1$^+$ TIGIT$^-$, PD-1$^-$ TIGIT$^+$, and PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells from HBV-HCC patients. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$

fact that it can predict the overall survival rate when compared with the single one. In addition, our results showed that the NLR of the PT^{hi} group was nearly twofold higher than that of the PT^{lo} group. Several reports have shown that the NLR, as a marker of the inflammatory microenvironment, predicts poor survival and recurrence in liver cancer [25–27]. In a previous study, we confirmed that the NLR is related to the pathogenesis and progression of HBV-HCC and that increases in NLR were associated with reductions in T cells [42].

Chronic infection and cancers are usually considered to cause T-cell exhaustion due to constant antigen exposure and inflammation. In this study, some patients exhibited high levels of serum HBV-DNA, which may contribute to dual antigen stimulation by viruses and tumors. Consistent with the effects of severe antigen stimulation, the HR for high PD-1$^+$ TIGIT$^+$ populations in patients with HBV-DNA levels ≥ 100 IU/mL was very high. Based on this observation, we may infer that T-cell exhaustion plays a more important role in HBV-HCC patients who have not received antiviral therapy, providing further confirmation of the importance of antiviral treatment. Antigen burden is a key determinant of the severity of T-cell exhaustion resulting from chronic infection with lymphocytic choriomeningitis [43, 44]. To determine whether the same is true for patients with HBV-HCC, we analyzed tumor characteristics in patients with different levels of PD-1$^+$ TIGIT$^+$ T cells. Indeed, PT^{hi} patients had a higher tumor burden than PT^{lo} patients, with a twofold higher frequency of tumors ≥ 5 cm in size and a nearly 1.5-fold higher frequency of tumors more than two in number. Furthermore, recent evidence has indicated that tumor burden, defined as the sum of the lengths of the long axes of tumor lesions, is related to circulating Tex cells in melanoma patients [44]. Interestingly, we found no difference in PFS between patients with high and low levels of PD-1$^+$ TIGIT$^+$ T cells in the subgroup of patients with tumor > 5 cm in size, which may be explained by the fact that large tumors have a relatively high susceptibility to progression and death;

therefore, differences between the PT^{hi} and PT^{lo} groups did not reach statistical significance. Using subgroup analysis, we discovered that patients with higher PD-1$^+$ TIGIT$^+$ T-cell populations experienced significantly higher progression rates in subgroups characterized by different levels of serum AFP. These results showed that, especially in AFP-negative patients, PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells are valuable tools for predicting the prognosis of HBV-HCC patients. Thus, our finding that high levels of PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells predict poor prognosis in HBV-associated primary liver cancer has considerable clinical significance.

The results from this research showed that PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells derived from HBV-HCC patients exhibit features of T-cell exhaustion. Given that previous reports have demonstrated that PD-1 and TIGIT inhibit antiviral and anti-tumor immune responses at the effector phase [45, 46], upregulation of PD-1 and TIGIT on T_{EM} cells might account for the pathogenesis and progression of liver cancer. In chronic viral infections and cancer, the coinhibitory receptors CTLA-4, 2B4, LAG-3, and TIM-3 are induced by persistent antigen stimulation. In HBV-HCC patients, PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells exhibit higher coexpression of CTLA-4, 2B4, and LAG-3, indicative of greater exhaustion. Furthermore, consistent with exhaustion, PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells is more prone to apoptosis, which may explain the reduced absolute T-cell counts and elevated NLRs of patients with HBV-HCC, as reported in our early research [42]. In contrast, PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells retained cytotoxic capacity or exhibited higher cytotoxic capacity. Two possible mechanisms can account for this discrepancy. Firstly, T cell lose the function in a progressive and hierarchical form, with exhausted T cells losing some characteristics while retaining others at early stages [9]; the ability to degranulate is often lost at later stages of dysfunction. Second, the PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells analyzed in our study are not tumor antigen-specific CD8$^+$ T cells, which could contribute to heterogeneity. Few studies have reported on the cytotoxic functions of antigen-specific T cells in HBV-HCC patients, because HBV-specific responses are rarely detected.

The main focus of this study in peripheral blood rather than the liver compartment should be acknowledged as a limitation. A recent study showed that immune-cell subsets become progressively suppressive from circulating blood to non-tumor and to tumor microenvironment in liver cancer [47]. The change of coinhibitory receptors in circulating T cells is consistent with that in tumor-infiltrating T cells. Furthermore, detection of PD-1$^+$ TIGIT$^+$ T cells in peripheral blood, which is accurate, simple, and noninvasive, is more readily applied in a clinical setting.
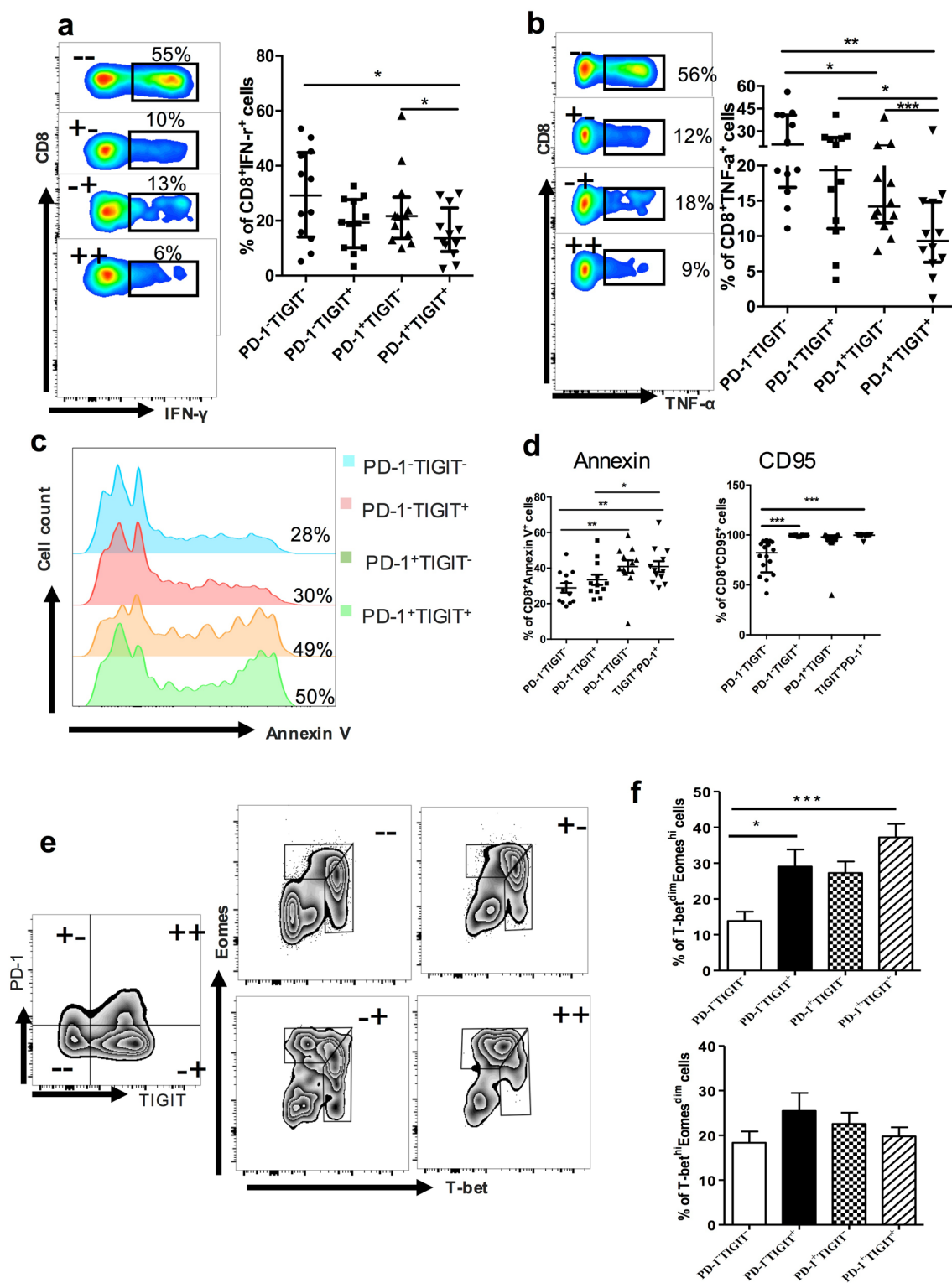
Fig. 5 PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells are defective in cytokine production, susceptible to apoptosis, and display unique patterns of T-bet/Eomes expression. **a**, **b** Intracellular staining for IFN-γ (**a**) and TNF-α (**b**) in PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells from HBV-HCC patients ($n = 19$) upon in vitro anti-CD3/anti-CD28 stimulation. **c–d** Percentage of apoptotic (Annexin V$^+$ 7AAD$^-$) cells and expression of CD95 on PD-1$^-$ TIGIT$^-$, PD-1$^+$ TIGIT$^-$, PD-1$^-$ TIGIT$^+$ and PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells from patients with HBV-HCC ($n = 13$). **e–f** Representative flow cytometry data (**e**) and histogram (**f**) showing the percentage of T-bet^{dim}/Eomes^{hi} and T-bet^{hi}/Eomes^{dim} cells in different subpopulations of PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells from patients with HBV-HCC ($n = 20$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Conclusion

In conclusion, we have profiled the phenotypes and functions of PD-1$^+$ TIGIT$^+$ T cells and their role in the pathogenesis and progression of HBV-related liver cancer. We identified that PD-1$^+$ TIGIT$^+$ CD8$^+$ T-cell populations are elevated in patients with recurrent and progressed HBV-HCC and demonstrated that this T-cell subset exhibits features indicative of T-cell exhaustion. These findings highlight the negative immune effect of PD-1$^+$ TIGIT$^+$ CD8$^+$ T cells as a predictor of poor outcome.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethics approval and consent standards The study was approved by the ethics committee of Beijing Ditan Hospital, Capital Medical University (2016-1-28).

Informed consent Each patient and healthy donors signed an informed consent. They all agreed to use their specimens and clinical information for this research.

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