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ORIGINAL ARTICLE

Prognostic and therapeutic potential of imbalance between PD-1+CD8 and ICOS+Treg cells in advanced HBV-HCC

Fengna Yan[1,2,3] | Bingbing Zhu[1] | Ke Shi[1] | Yi Zhang[1] | Xuanwei Zeng[1] | Qun Zhang[1] | Zhiyun Yang[1] | Xianbo Wang[1]

[1]Center for Integrative Medicine, Beijing Ditan Hospital, Capital Medical University, Beijing, China

[2]Beijing Key Laboratory of Emerging Infectious Diseases, Institute of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing, China

[3]Beijing Institute of Infectious Diseases, Beijing, China

Correspondence
Xianbo Wang and Zhiyun Yang, Center for Integrative Medicine, Beijing Ditan Capital Medical University, No. 8, Jingshun East Street, Chaoyang District, Beijing 100015, China.
Email: wangxb@ccmu.edu.cn and 13439696988@ccmu.edu.cn

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Abstract

Over 50% of patients with hepatitis B virus-associated hepatocellular carcinoma (HBV-HCC) are diagnosed at an advanced stage, which is characterized by immune imbalance between CD8+ T cells and regulatory T (Treg) cells that accelerates disease progression. However, there is no imbalance indicator to predict clinical outcomes. Here, we show that the proportion of CD8+ T cells decreases and Treg cells increases in advanced HBV-HCC patients. During this stage, CD8+ T cells and Treg cells expressed the coinhibitory molecule PD-1 and the costimulatory molecule ICOS, respectively. Additionally, the ratio between PD-1+CD8 and ICOS+Tregs showed significant changes. Patients were further divided into high- and low-ratio groups: PD-1+CD8 and ICOS+Tregs high- (PD-1/ICOS^{hi}) and low-ratio (PD-1/ICOS^{lo}) groups according to ratio median. Compared with PD-1/ICOS^{lo} patients, the PD-1/ICOS^{hi} group had better clinical prognosis and weaker CD8+ T cells exhaustion, and the T cell-killing and proliferation functions were more conservative. Surprisingly, the small sample analysis found that PD-1/ICOS^{hi} patients exhibited a higher proportion of tissue-resident memory T (T_{RM}) cells and had more stable killing capacity and lower apoptosis capacity than PD-1/ICOS^{lo} advanced HBV-HCC patients treated with immune checkpoint inhibitors (ICIs). In conclusion, the ratio between PD-1+CD8 and ICOS+Tregs was associated with extreme immune imbalance and poor prognosis in advanced HBV-HCC. These findings provide significant clinical implications for the prognosis of advanced HBV-HCC and may serve as a theoretical basis for identifying new targets in immunotherapy.

KEYWORDS
hepatitis B virus-associated hepatocellular carcinoma, ICOS+Tregs, immune imbalance, PD-1+CD8 cells, tumor progression

Fengna Yan and Bingbing Zhu are regarded as co-first authors.

1 | INTRODUCTION

Hepatocellular carcinoma ranks as the sixth most prevalent cancer globally and the third highest cause of cancer-related mortality, making it a significant global health issue. Although nonalcoholic fatty liver disease is increasingly emerging as the primary cause of hepatocellular carcinoma, viral hepatitis remains a significant risk factor, leading to the diagnosis of most patients in intermediate to advanced stages.[1,2] Therefore, the prognosis and treatment options for patients with advanced hepatitis B virus-associated hepatocellular carcinoma (HBV-HCC) are extremely limited.

Immunotherapy with immune checkpoint inhibitors (ICIs) has recently emerged as an immunotherapy for unresectable HCC, However, over 50% of patients do not show a response to ICI treatment even when combined with other therapies.[3] Therefore, it is urgent to improve the response of ICI treatment and prolong the survival time of advanced HBV-HCC. Mechanistically, the double continuous stimulation of tumor cells and hepatitis B virus exists in the tumor microenvironment (TME) of HBV-HCC, resulting in the gradual loss of the antitumor immune effect function of CD8+ T cells.[4,5] Tumor cells obtain immune evasion by inducing and recruiting regulatory T (Treg) cells, myeloid suppressor cells, tumor-associated macrophages, and diverse immune coinhibitory and costimulatory molecules including programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), motif (ITIM) domain (TIGIT), T cell immunoglobulin and mucin domain 3 (TIM-3), inducible T cell costimulator (ICOS), and others, which disrupt the antitumor immune effect and accelerate disease progression.[6,7] Overactivation of immunosuppressive Treg cells weakens the ability of CD8+ T cells to monitor and eliminate tumor.[8] However, it is unclear what immune patterns are closely related to clinical outcomes in patients with advanced HBV-HCC. It has been previously reported that PD-1 and ICOS counter-regulate the development and differentiation of tissue-resident Treg cells during influenza,[9] thereby maintaining the immune effect during acute viral infection. It is believed that this pattern of immune balance may also be applicable in tumor microenvironments. In addition, it is indicated that the effectiveness of PD-1 blockade treatments could be predicted by the equilibrium of PD-1 expression between Treg cells and effector T cells in non-small cell lung cancer and gastric cancer.[10] The authors emphasize that the induction of dysfunctional PD-1+CD8+ T cell recovery and improving PD-1 + Treg cell-mediated immunosuppression could contribute to the poor prognosis observed in nonresponsive patients undergoing PD-1 blockade treatment. Therefore, restoring the balance between immune response and inhibition is an important means to improve the prognosis of HBV-HCC. Our study concluded that the ratio between PD-1+CD8 and ICOS+Tregs showed significant changes in advanced HBV-HCC compared with hepatitis B virus-related liver cirrhosis (HBV-LC) patients. PD-1/ICOS^{hi} patients exhibited a better prognosis than those with a low ratio. Additionally, this group of patients exhibited relatively good killing and proliferation functions. Importantly, the higher ratio of PD-1+CD8 and ICOS+Tregs in advanced HBV-HCC patients with ICI treatment exhibits faster and more powerful cytotoxicity. This reveals the significance of maintaining a proper balance between CD8+ effector T cells and immunosuppressive Treg cells within the TME and the prognosis of patients with advanced HBV-HCC.

2 | MATERIALS AND METHODS

2.1 | Patients

Peripheral blood samples were collected from 110 patients with advanced HBV-HCC, 30 patients with HBV-LC, and 30 healthy donors (HD) by the Center for Integrative Medicine, Beijing Ditan Hospital, Capital Medical University, between August 2021 and February 2022. The diagnosis of HBV-HCC and HBV-LC patients was consistent with our previous study.[11] We obtained 5-mL peripheral blood samples from all the patients. The PBMCs were obtained as previously described.[12] All participants provided written consent, and the ethics committee of Beijing Ditan Hospital, Capital Medical University approved the study. The research followed the guidelines outlined in the Declaration of Helsinki. Table 1 summarizes the clinical features of all enrolled populations.

The ICI treatment for patients was as follows: (1) Navolumab (200 mg intravenous [IV], once every 3 weeks for four cycles);

TABLE 1 Clinical characteristics of all patients.

	Healthy donors ($n=30$)	HBV-LC ($n=30$)	HBV-HCC ($n=110$)	p
Age (mean ± SD)	58.328 ± 10.066	51.333 ± 9.789	60.290 ± 9.236	<0.001
Gender (female/male)	24/6 (80/20)	5/25 (16.67/83.33)	19/91 (17.59/82.41)	0.906
HBV DNA <20	NA	24/6 (80.00/20.00)	83/27 (74.53/25.47)	0.537
Child-Pugh (A/B/C)	NA	8/17/5	61/36/11	0.015
Tumor multiplicity (solitary/multiple)	NA	NA	24/78 (23.53/76.47)	1.000
Tumor size (<5/≥5 cm)	NA	NA	61/37 (62.25/37.75)	1.000
AFP (<400/≥400; ngmL)	NA	25/5 (83.33/16.67)	40/40 (48.72/51.28)	0.428
ALT (U/L)	NA	26.00 (18.60,37.50)	25.60 (18.10,44.60)	0.702

Note: Values are represented as (mean ± SD) or median. $p < 0.05$ comparison between HBV-HCC considered statistically significant.

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; HBV-HCC, hepatitis B virus-associated hepatocellular carcinoma; HBV-LC, hepatitis B virus-related liver cirrhosis.

(2) Sintilimab (200 mg IV, once every 3 weeks for four cycles); (3) Tislelizumab (200 mg IV, once every 3 weeks for four cycles); (4) Camrelizumab (200 mg IV, once every 3 weeks for four cycles). The immunotherapy lasted for a maximum of 12 months until diagnosis of progressive disease, assessment of toxicity reactions that were deemed unsuitable for continued treatment, initiation of alternative cancer treatment, or withdrawal of informed consent.

2.2 | Flow cytometry of human PBMCs

Lymphocytes were transferred into FACS tubes and stained with fluorescence-conjugated monoclonal antibodies. The antibodies used were the following; anti-human BV785-conjugated anti-CD3 (1:100; Biolegend, # 344842), APC-Fir750-conjugated anti-CD4 (1:100; BD, # 563800), BV510-conjugated anti-CD8 (1:100; Biolegend, #344732), BV421-conjugated anti-CD25 (1:25; Biolegend, # 302630) PE-conjugated anti-CD127 (1:100; Biosciences, #557938), PE-CF594-conjugated anti-CD69 (1:100; Biolegend, #310942), AF700-conjugated anti-GranzymB (1:100; Biolegend, #372221), AF700-conjugated anti-ICOS (1:100; Biolegend, #313528), FITC-conjugated anti-ki67 (1:100; eBioscience, #350534), FITC-conjugated anti-Annexin V (1:100; eBioscience, #640906), BV605-conjugated anti-CD103 (1:100; Biosciences, #350217), BV711-conjugated anti-PD-1 (1:100; Biosciences, #564017), PE-CY7-conjugated anti-TIGIT (1:100; eBioscience, #25-9500-42), BV650-conjugated anti-Tim-3 (1:100; Biosciences, #565564), APC-conjugated anti-CTLA4 (1:100; Biosciences, #349908), APC-conjugated anti-perforin (1:100; Biosciences, #349908), and the corresponding isotype controls. Gating strategies for flow cytometry analysis are shown in the Figures S1 and S2. Data were collected using an LSR Fortessa flow cytometer and evaluated using FlowJo software (Tree Star).

2.3 | Statistical analysis

GraphPad 5.0 and SPSS version 19.0 were utilized to analyze the clinical patients' baseline statistics and flow cytometry staining results. The consistent quantitative data were presented as average ± standard deviation (SD) and examined using *t*-tests. Data that did not follow a normal distribution were represented as the median of the quartile range and examined using the Mann–Whitney *U* test ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, ns indicates no statistical significance).

3 | RESULTS

3.1 | The ratio between PD-1+CD8 and ICOS+Tregs was diminished in advanced HBV-HCC patients

A total of 170 participants were enrolled according to the inclusion and exclusion criteria: HDs ($n=30$), HBV-LC ($n=30$), HBV-HCC ($n=110$). On the day of enrollment, comprehensive clinical data of all patients were collected (Table 1). To investigate the significantly altered antitumor immune effects in the progression of advanced HBV-HCC, flow cytometry was initially employed to examine the significantly changed proportion of multiple immune cell subsets in HDs, HBV-LC patients, and HBV-HCC patients, as well as the coinhibitory molecular changes of the immune effect of CD8+ T cells (Figure S1a) and immunosuppressive Treg cells (Figure S1b). The study revealed a significant reduction in the proportion of CD8+ T cells in HBV-HCC patients ($p<0.01$) and a higher proportion of Treg cells ($CD4+CD25+CD127^{low}$; $p<0.05$; Figure 1A,B). Then, we analyzed the levels and ratios of PD-1, TIGIT, TIM-3, CTLA-4, ICOS, and GITR on CD8+ T and Treg cells in the three groups, respectively. We revealed that PD-1 and ICOS are the most significant changes of CD8+ T and Treg cells. Their proportion declined most significantly in the HBV-HCC patients (Figures 1C,D and S1e). Finally, the median ratio of the PD-1+CD8/ICOS+Tregs level (1.67) was utilized as the critical value. We separated the 110 patients with HBV-HCC into two groups: $PD\text{-}1/ICOS^{hi}$ (>1.67; $n=40$) and $PD\text{-}1/ICOS^{lo}$ (≤1.67; $n=60$) according to the ratio of PD-1+CD8/ICOS+Tregs. We divided all advanced HBV-HCC patients into progressive and nonprogressive groups and compared the CD8+/Treg+PD-1 ratio between the two groups. A significant reduction in the ratio of PD-1+CD8 and ICOS+Tregs was observed in the progressive group compared with the nonprogressive group of advanced HBV-HCC and ICI treatment patients ($p<0.01$; Figure 1E–G). It was closely related to the prognosis of the disease. In order to confirm the relationship between the ratio of PD-1+CD8/ICOS+Tregs and the prognosis of HBV-HCC patients, multicolor immunofluorescence staining was performed on HCC paracarcinoma and tumor tissues and responders versus nonresponders. It was found that the ratio of PD-1+CD8/ICOS+Tregs in HCC tumor tissues and immunotherapy responders was significantly higher than that in paracarcinoma and nonresponders ($p<0.001$; Figure 1H–J).

3.2 | The higher ratio of PD-1+CD8 and ICOS+Tregs was associated with improved progression of advanced HBV-HCC patients

We examined clinical characteristics to assess the correlation between various ratios of PD-1+CD8 and ICOS+Tregs and clinical outcomes in advanced HBV-HCC patients. Our findings indicated that there were no significant differences between the two groups regarding age, sex, hypertension, diabetes, Child-pugh score, HBV DNA levels, hepatitis B e antigen (HBeAg) status, tumor size, tumor heterogeneity, alpha-fetoprotein (AFP) levels, leukocyte counts, and neutrophil counts (Table 2). In addition, lymphocyte counts were higher in the $PD\text{-}1/ICOS^{hi}$ group. Nevertheless, no statistically significant differences were observed between the two groups.

To examine the impact of tumor progression on the ratio of PD-1+CD8/ICOS+Tregs, we utilized the Kaplan–Meier survival curve to evaluate the overall survival (OS) and progression-free survival (PFS) time of two groups ($PD\text{-}1/ICOS^{hi}$ and $PD\text{-}1/ICOS^{lo}$) within the clinical subgroup. In the cohort of patients with advanced HBV-HCC, we

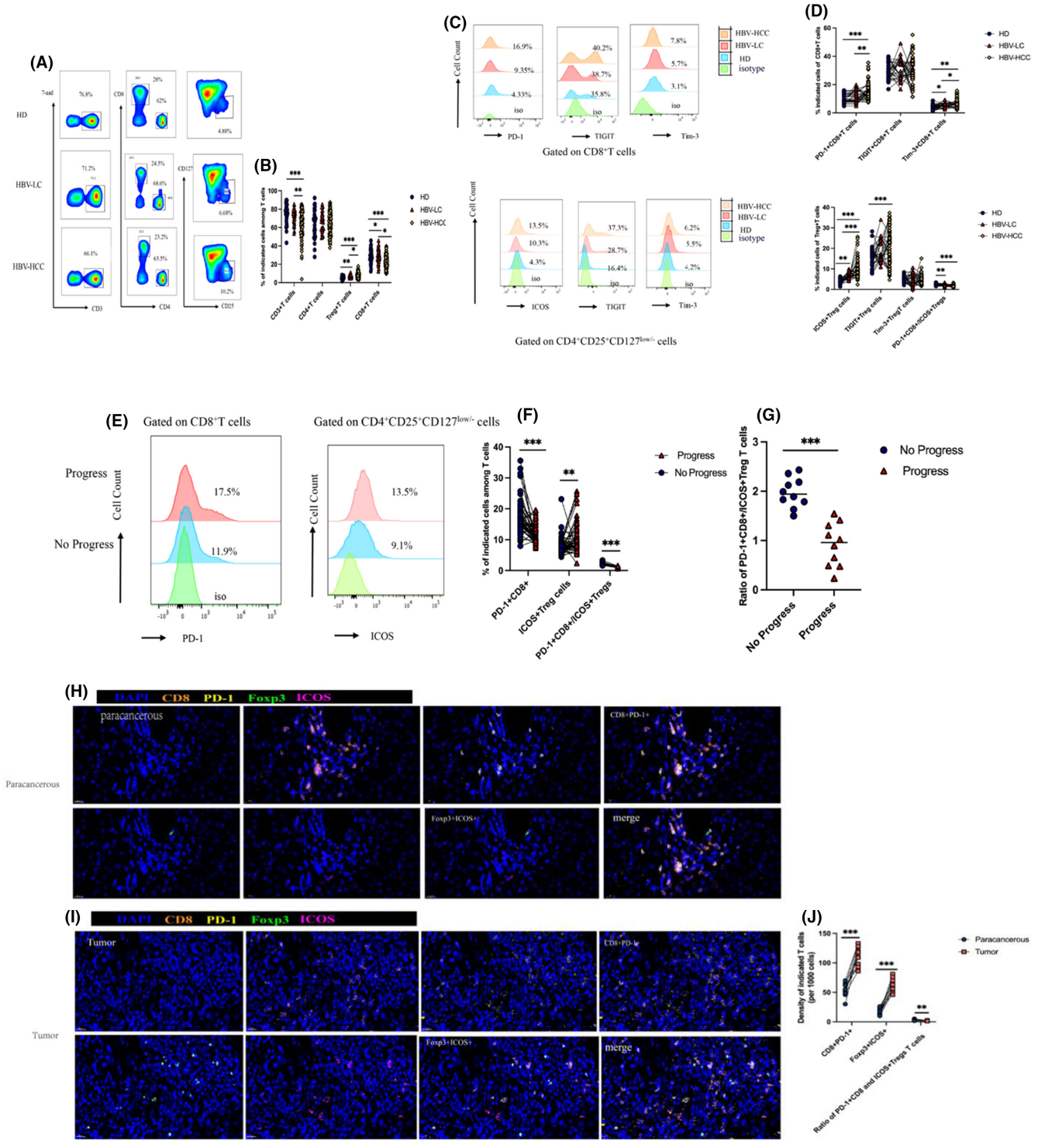

FIGURE 1 The ratio of PD-1+CD8/ICOS+Tregs is significantly reduced in advanced hepatitis B virus-associated hepatocellular carcinoma (HBV-HCC) patients. (A, B) The proportion of CD8+ T cells and Treg cell subsets (CD4+CD25+CD127^{low} cells) from HBV-HCC ($n = 110$), compared with hepatitis B virus-related liver cirrhosis (HBV-LC) patients ($n = 30$) and heathy donors ($n = 30$) by flow cytometry analysis. (C, D) The expression of PD-1, CTLA-4, Tim-3 on CD8+ T, and Treg cells. (E–G) The ratio of PD-1+CD8/ICOS+Tregs in advanced HBV-HCC patients receiving immune checkpoint inhibitor (ICI) treatment with progression ($n = 64$) and no progression ($n = 46$). (F) The ratio of PD-1+CD8/ICOS+Tregs expression of HBV-HCC patients. (G) The ratio of PD-1+CD8/ICOS+Tregs expression of HBV-HCC patients receiving ICI treatment. (H–M) Representative multiplex immunofluorescence images of paracancerous tissue (H), tumor tissue (I), responders (K), and nonresponders (L) with ratio of PD-1+CD8/ICOS+Tregs (J, M) exhibiting the discrepancies correspondingly. *p*-values were calculated using the Kruskal–Wallis nonparametric H test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

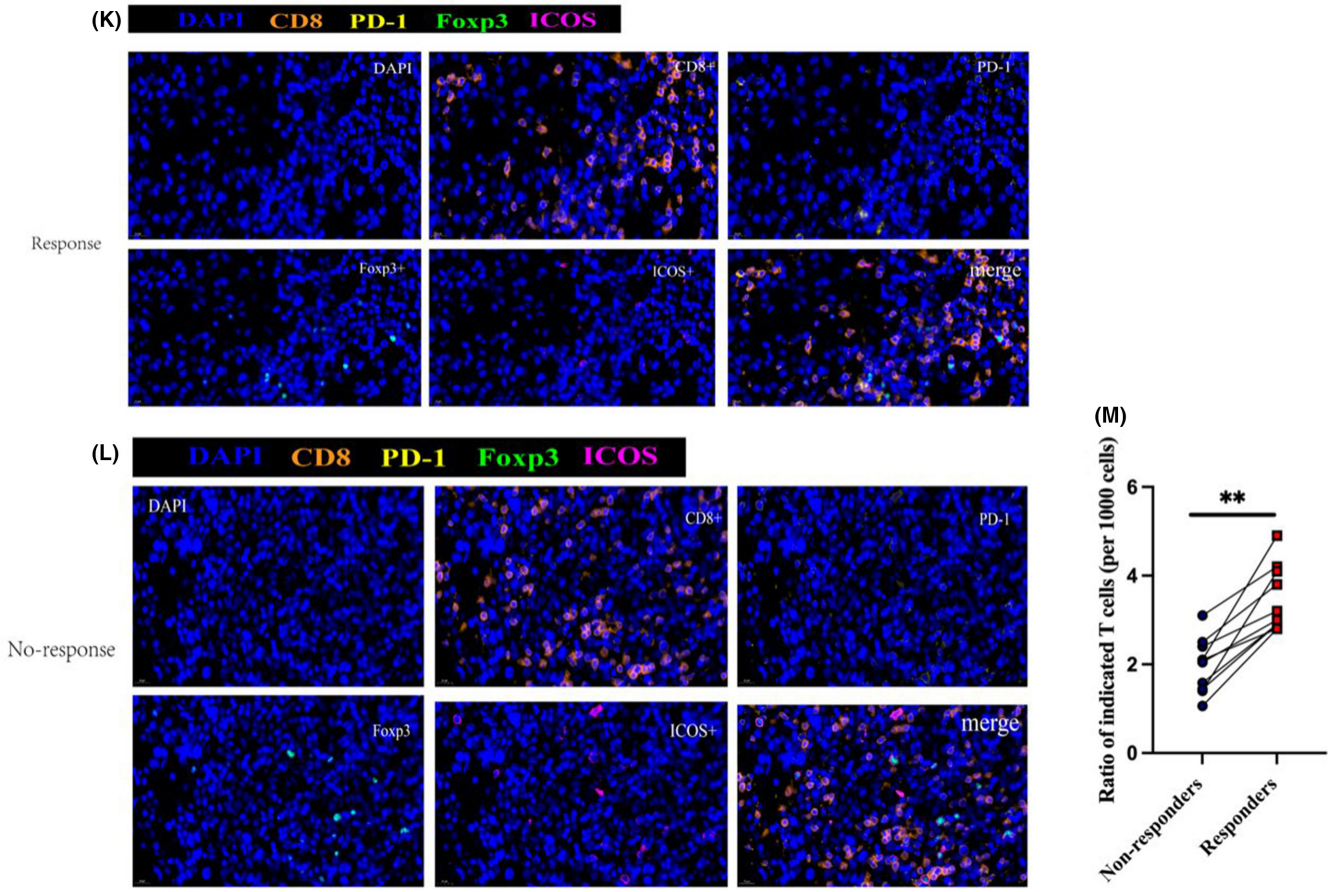

FIGURE 1 (Continued)

observed that the PD-1/ICOS^{lo} group had worse OS and PFS rates compared with the PD-1/ICOS^{hi} group (Figure 2A,B; HR=2.515, 95% CI 8.055–20.3, p=0.0366; HR=2.337, 95% CI 1.304–4.191, p=0.044). Next, we analyzed the impacts in both cohorts with varying liver function levels and ICI therapy. For patients with Child-Pugh A stage, the PFS in the PD-1/ICOS^{hi} group was significantly better than that in the PD-1/ICOS^{lo} group (p=0.002, Figure 2D). However, no significant difference was observed in the subgroups of patients with Child-Pugh B+C (p=0.558, Figure 2E). Additionally, advanced HBV-HCC patients with PD-1/ICOS^{hi} had better 1-year PFS after ICI treatment than patients with PD-1/ICOS^{lo} (p=0.0094, Figure 2C). Furthermore, we subdivided tumor burden, liver function, hepatitis B virus level, and inflammatory response to compare the effects of PFS on two subgroups. The PFS of the PD-1/ICOS^{hi} patients was more favorable than that of the PD-1/ICOS^{lo} patients with tumor size <5 cm (Figure 3A; HR=2.888, 95% CI 1.268–6.577, p=0.0116), tumor size ≥5 (Figure 3A; HR=2.144, 95% CI 0.8711–5279, p=0.097), tumors multiple (Figure 3B; HR=2.806, 95% CI 1.426–5.522, p=0.0028), alanine aminotransferase (ALT)>50 U/L (Figure 3C; HR=5.416, 95% CI 1.388–21.13, p=0.0115), aspartate aminotransferase (AST)>40 U/L (Figure 3D; HR=13.647, 95% CI 1.33–9.998, p=0.0119), HBV DNA level<20 IU/mL (Figure 3E; HR=2.275, 95% CI 1.16–4.463, p=0.0168;), HBeAg-positive group (Figure 3F; HR=1.22, 95% CI 1.22–4.226, p=0.0097;), lactate dehydrogenase (LDH)≥250 U/L (Figure 3G; HR=2.248, 95% CI 1.104–4.577, p=0.0255), and neutrophil-lymphocyte ratio (NLR)<3 (Figure 3H; HR=2.515, 95% CI 1.155–5.477, p=0.0118), but not in patients with tumors solitary (p=0.3293), ALT≤50 U/L (p=0.0488), AST≤40 U/L (p=0.0848), HBV-DNA level ≥20 IU/mL (p=0.1233), HBeAg-negative group (p=0.1862), LDH<250 U/L (p=0.1159), and NLR>3 (p=0.1978).

3.3 | The lower ratio of PD-1+CD8 and ICOS+Tregs in advanced HBV-HCC patients exhibits a more exhausted CD8+ T phenotype and overactivation

The persistent overexpression of CD8+PD-1 is a prominent characteristic of the immunosuppressive TME and significantly impacts the prognosis of HCC. Additionally, studies have demonstrated that the PD-1/PD-L1 pathway and Tregs are essential for maintaining immune tolerance.[12,13] The TME activation helps evade immune surveillance of transformed cells and suppress antitumor immune response.[14] Therefore, we compared the immunophenotypes of patients with the ratio of PD-1+CD8/ICOS+Tregs between the two groups. Our findings revealed that patients with a lower ratio exhibited reduced levels of peripheral CD8+ T cells, an increased proportion of Treg cells, and comparable percentages of CD3+T

TABLE 2 Demographic and clinical characteristics of different levels of ratio of PD-1+CD8/ICOS+Tregs cells in patients with advanced HBV-HCC.

	PD-1/ICOS^{lo} ($n=60$)	PD-1/ICOS^{hi} ($n=50$)	p
Age, mean (±SD)	61.69 ± 7.90	58.96 ± 10.13	0.124
Gender (female/male)	15/45	9/41	0.801
HBV DNA <20	42/18	39/11	0.505
HBeAg <1	48/12	39/11	0.201
Child-Pugh (A/B/C)	27/21/12	30/16/4	0.283
Tumor multiplicity (solitary/multiple)	23/37	11/49	0.567
Tumor size, cm (<5/≥5)	38/22	32/18	0.468
AFP (ngmL; <400/≥400)	33/27	29/21	0.361
NLR	2.37	2.19	0.995
PLT (109/L)	82 (55,146)	101 (71,123)	0.325
ALT (U/L)	25.3 (19.6,41.8)	26.1 (17.7,44.6)	0.820
AST (U/L)	31.1 (20.4,46.7)	31.5 (23.4,49.5)	0.601
TBIL (umol/L)	21.6 (12.6,34.1)	20.5 (13.6,30.7)	0.225
ALB (g/L)	35.10 ± 5.24	36.28 ± 5.14	0.242
LDH (U/L)	244.68 ± 138.63	223.61 ± 77.68	0.371
CRP (mg/L)	21.10 ± 45.56	10.96 ± 20.03	0.174
Prothrombin times (s)	13.82 ± 2.28	13.32 ± 1.76	0.215
Prothrombin activity (%)	73.43 ± 13.75	76.02 ± 14.31	0.352
Progression (yes/no)	41/19 (74.5/25.5)	28/22 (60.0/40.0)	0.044
Treatments for HCC (resection/minimally invasive/palliative)	3/15/42	2/12/26	0.478
Types of immune checkpoint inhibitors (sintilimab/tislelizumab/camrelizumab)	5/5/1	5/4/0	0.768

Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C reactive protein; HBeAg, hepatitis B e antigen; HBV-HCC, hepatitis B virus-associated hepatocellular carcinoma; ICOS, inducible T cell costimulator; LDH, lactate dehydrogenase; NLR, neutrophil-lymphocyte ratio; PD-1, programmed cell death protein 1; TBIL, total bilirubin.

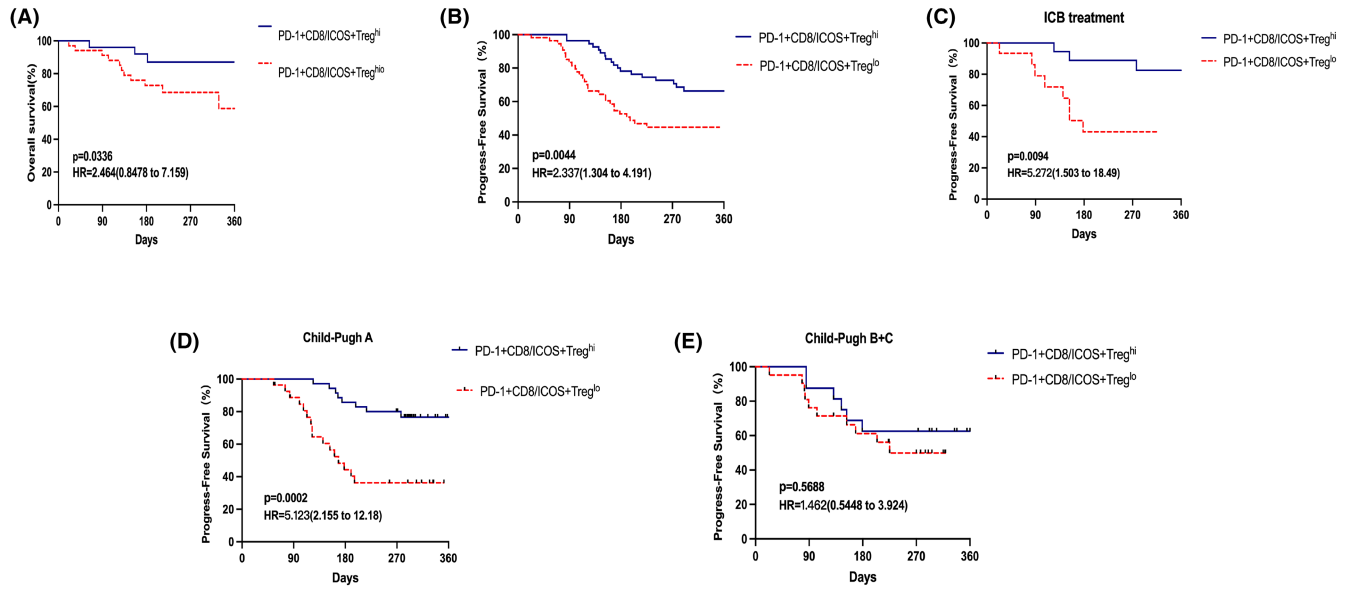

FIGURE 2 Kaplan–Meier curve analysis demonstrates the effectiveness of the ratio of PD-1+CD8/ICOS+Tregs levels as a predictor of overall survival, progression-free survival, and Immune checkpoint blockade (ICB) treatment in advanced hepatitis B virus-associated hepatocellular carcinoma (HBV-HCC) with different liver reserve functions. (A, B) Kaplan–Meier curve analysis showed the efficacy of the ratio of PD-1+CD8/ICOS+Tregs levels as a predictor of overall survival (A), progression-free survival (B), and ICB treatment (C) in advanced HBV-HCC patients. (D, E) Subgroup analysis of patients with (D) Child-Pugh and (E) Child-Pugh B+C. p-values and hazard ratios (HRs) were calculated using the log-rank test.

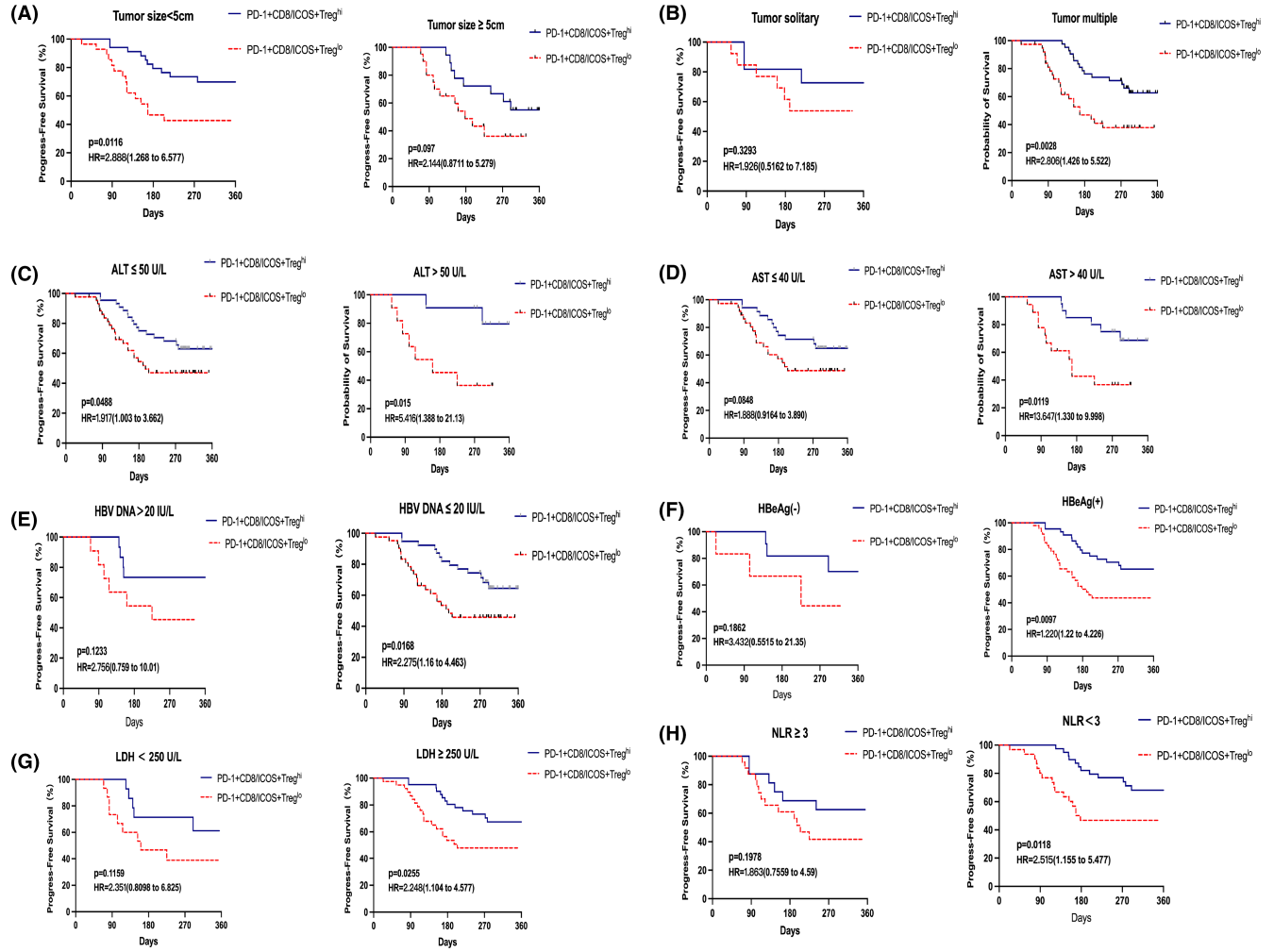

FIGURE 3 Kaplan–Meier curve analysis demonstrates the ratio of PD-1+CD8/ICOS+Tregs levels as a predictor of progression-free survival in hepatitis B virus-associated hepatocellular carcinoma (HBV-HCC) across different tumor burdens, HBV virus load, hepatitis B e antigen (HBeAg), liver function, lactate dehydrogenase (LDH), and neutrophil-lymphocyte ratio (NLR) level. (A) Patients with different tumor sizes: tumor size ≤5 cm and tumor size >5 cm; (B) patients with different tumor numbers: tumor solitary and tumor multiple; (C) patients with different alanine aminotransferase (ALT) levels: ALT ≤50 U/L and ALT >50 U/L; (D) patients with different aspartate aminotransferase (AST) levels, AST ≤40 U/L; (E) patients with different HBV-DNA levels; (F) patients with or without HBeAg; (G) patients with different LDH levels, LDH ≤ 250 U/L and LDH > 250 U/L; (H) patients with different NLR ratio levels, NLR <3 and NLR ≥3, *p*-values and HRs were calculated using the log-rank test.

and CD4+T cells when compared with patients with a higher PD-1+CD8/ICOS+Tregs ratio ($p < 0.05$, Figure 4A,B). The differentiation of CD8+ T cells plays a crucial role in the development of the immunosuppressive phenotype.[15] We observed that PD-1/ICOS^{hi} patients exhibited a significantly higher proportion of effector memory (T_{EM}) cells ($p < 0.05$, Figure 4C,D). Although the proportion of naive T (T_N) cells and central memory (T_{CM}) cells decreased, the change was not statistically significant ($p > 0.05$). Since T cell overactivation caused by sustained antigenic stimulation results in T cell exhaustion and a stepwise loss of function,[16,17] we defined the activation status of Treg cells using CD45RA and FOXP3 flushing, including native Treg cells (Foxp3^{lo}CD45RA^{+}, Fr. I), activated Treg cells (Foxp3^{hi}CD45RA^{-}, Fr. II), and non-Treg cells (CD45RA^{-}FOXP3^{lo}, Fr. III) across different ratios of PD-1+CD8/ICOS+Tregs patients. We observed a higher frequency of native Treg cells and a lower frequency of activated Treg cells among in the PD-1/ICOS^{hi} patients compared with PD-1/ICOS^{lo} patients ($p < 0.05$, Figure 4E,F). In addition, patients with a low ratio had higher expression of inhibitory receptors CTLA-4 and TIM-3 on CD8+ T cells. The tyrosine-based inhibitory motif (ITIM) domain (TIGIT) expression was not statistically significant ($p < 0.05$, Figure 4G,H).

3.4 | The lower ratio of PD-1+CD8 and ICOS+Tregs in advanced HBV-HCC patients showed worse functional exhaustion

Previous research has reported that the exhaustion of effector function in CD8+ T cells accelerates the progression of tumor immunosuppressive microenvironment.[15,16,18] To investigate the changes of effector function in patients with different

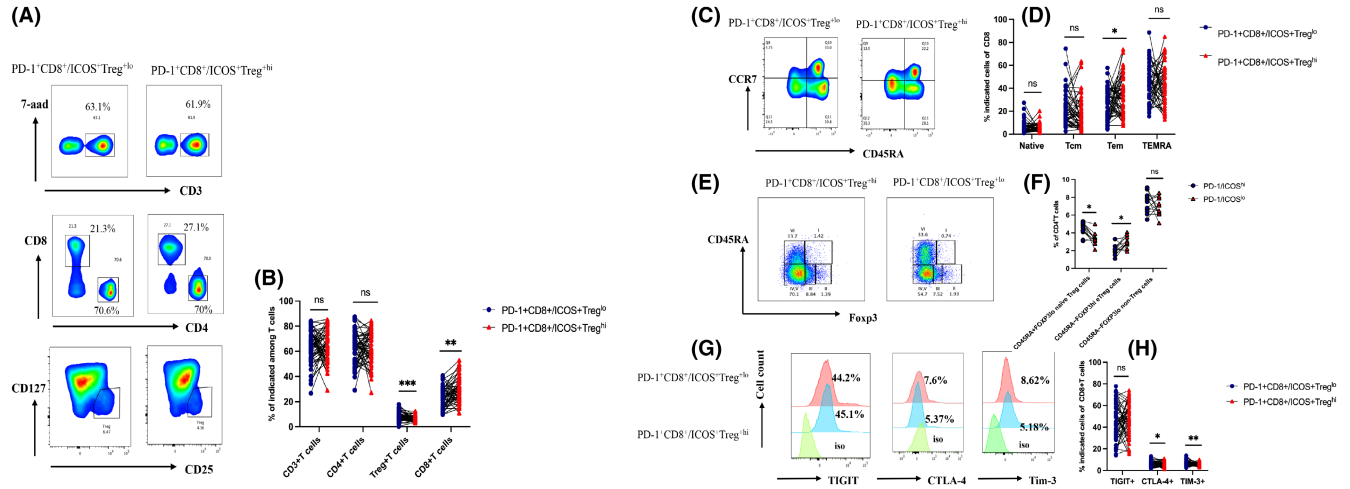

FIGURE 4 The lower ratio of PD-1+CD8/ICOS+Tregs in advanced hepatitis B virus-associated hepatocellular carcinoma (HBV-HCC) patients indicates functional exhaustion. (A, B) The proportion of CD3+, CD4+, CD8+, and Treg+ cells in peripheral blood of HBV-HCC patients with different PD-1+CD8/ICOS+Tregs ratios ($n=110$). (C, D) Distribution of naive T cells (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector (T_{EMRA}) cells in CD8+ T cell populations derived from HBV-HCC patients with different PD-1+CD8/ICOS+Tregs ratios. Representative flow cytometry data gated on CD8. (E, F) The proportions of HLA-DR+, CD38+, and CD38+HLA-DR+ subpopulations within different PD-1+CD8/ICOS+Tregs ratios from patients with HBV-HCC ($n=110$). (G, H) Frequency of TIGIT, CTLA-4, and TIM-3 on different PD-1+CD8/ICOS+Tregs ratios from HBV-HCC patients ($n=110$). *$p<0.05$, **$p<0.01$, ***$p<0.001$; ns indicates no statistical significance.

PD-1+CD8/ICOS+Tregs ratios, we detected the levels of targeted killing, proliferation, and apoptosis in the two groups of patients. Our findings demonstrated that PD-1/ICOS^{lo} patients exhibited a reduced ability to produce granzyme B and Ki67 compared with PD-1/ICOS^{hi} patients ($p<0.01$, Figure 5A,B). Further analysis showed a reduced proportion of tissue-resident memory T (T_{RM}) (CD69+CD103+) cells with sustained and rapid targeted killing ability in these patients. Moreover, their capacity to produce perforin and granzyme B was not as robust as that of PD-1/ICOS^{hi} patients ($p<0.01$, Figure 5C,D).

Since tumor CD8+ T_{RM} cells have been associated with enhanced response to immunotherapy and are generally linked to positive clinical outcomes for cancer patients,[17,19] we further examined the phenotype and functional changes of CD8+ T_{RM} cells in advanced HBV-HCC patients after ICI treatment ($n=20$). The results revealed that the proportion of peripheral CD8+ T and T_{RM} subsets in PD-1/ICOS^{hi} patients was significantly higher than that in low-proportion patients among advanced HBV-HCC patients treated with ICI. The PD-1+CD8/ICOS+Tregs ratio could effectively determine any alteration in the effect function following treatment ($p<0.05$, Figure 6A,B). However, the efficient CD3 and CD4T cells showed no statistical significance. In addition, the expression levels of coinhibitory molecules PD-1, CTLA-4, and Tim-3 on the surface of CD8+ TRM cells showed no statistical significance, suggesting that the current study results do not support the prediction of immune response in HCC patients by the immunophenotype based on the ratio of CD8+ TRM cells to Treg cells ($p>0.05$, Figure S2a–c). Specifically, PD-1/ICOS^{hi} patients exhibited a higher proportion of T_{RM} cells and granzyme B production capacity, while the apoptosis level was significantly decreased (Figure 6C,D). The proliferation of responder cells was examined with carboxyfluorescein diacetate succinimidyl ester-labeled CD8+ T cells cultured with/without ICOS CD25^{high}CD4+ T cells (eTreg cells). eTreg cells became more suppressive with ICI treatment (Figure 6E,F).

4 | DISCUSSION

More than 70% of liver cancer patients in China are initially diagnosed with advanced-stage liver cancer and miss the chance for radical surgical treatment. ICI treatment enhances the activity of CD8+ T cells by targeting the PD-1/PD-L1 pathway and restoring the tumor-killing effect, thus greatly improving the poor prognosis of patients with middle- and advanced-stage HCC.[20–22] However, the curative effect is not satisfactory. Numerous studies have shown that the level of PD-1 and ICOS significantly impact the tumor immune microenvironment and clinical prognosis.[23–25] Moreover, the proportional expression of PD-1 in CD8+ T cells and Treg cells can serve as an additional indicator for predicting the clinical prognosis of tumor patients and determining the suitability of ICI treatment.[11,26] Our findings showed significant differences in the phenotype and function of the antitumor immune effect in advanced HBV-HCC patients with different PD-1+CD8/ICOS+Tregs ratios. It can be considered a predictive biomarker for improving the clinical prognosis and immunotherapy effect of advanced HBV-HCC patients.

Accumulating evidence has demonstrated that the interaction between PD-1 and PD-L1 hampers the activation of effector T cells to inhibit antitumor T cell immunity, while blocking PD-1–PD-L1 reactivates antitumor T cell immunity by inhibiting tumor growth.[27,28] The clinical prognosis of PD-1-blocking therapy is significantly improved when tumors exhibit high expression of PD-L1. However, the therapeutic effect of this treatment is not widely applicable across a

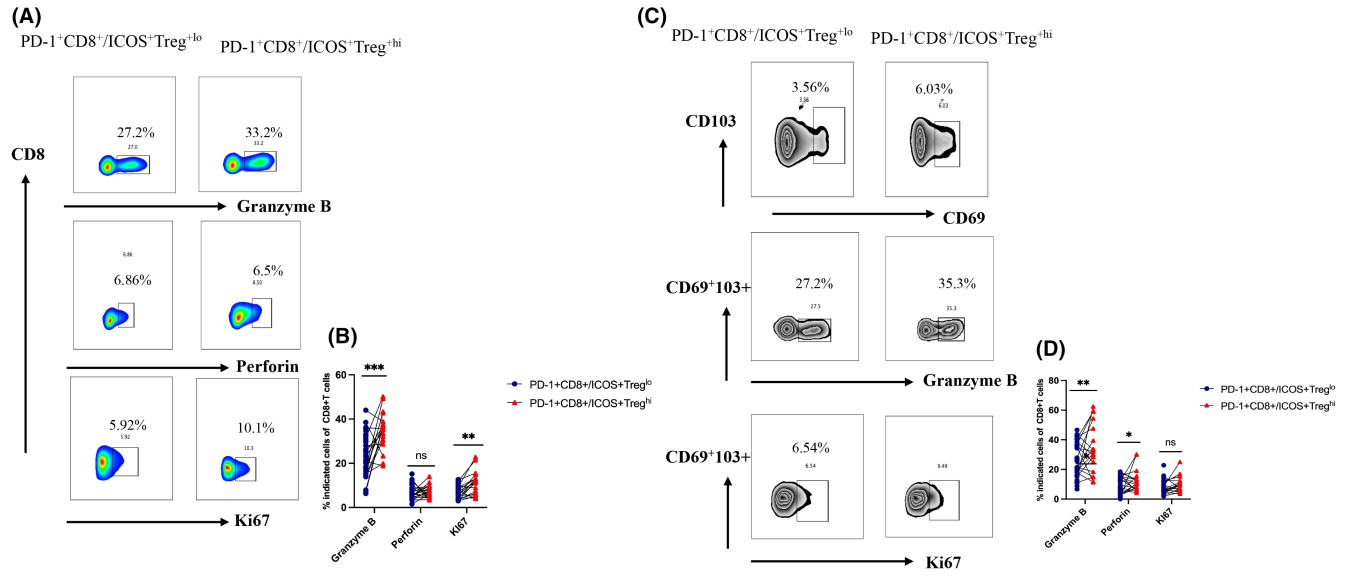

FIGURE 5 Lower ratio of PD-1+CD8/ICOS+Tregs in advanced hepatitis B virus-associated hepatocellular carcinoma (HBV-HCC) patients indicates functional exhaustion. (A, B) Intracellular staining for perforin, granzyme B, and Ki67 in different PD-1+CD8/ICOS+Tregs ratios from advanced HBV-HCC patients ($n = 60$). (C, D) Percentage of expression of tissue-resident memory T cells (T_{RM}), granzyme B, and Ki67 of TRM in different PD-1+CD8/ICOS+Tregs ratios from advanced HBV-HCC patients ($n = 60$). *p*-values were obtained by the Kruskal–Wallis ANOVA test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

broad spectrum. The strong immune response induced by tumor mutational burden, antigen presentation mechanisms, and mutations in interferon-gamma mutation burden, antigen presentation mechanism, interferon γ (IFN-γ) signaling molecule mutation, infiltration of immunosuppressive cells such as Treg cells, myeloid suppressor cells, tumor-associated macrophages can induce a stronger immune response.[29,30] An increased population of intratumoral PD-1+CD8+ T cells is associated with a higher risk of disease progression and recurrence following HCC surgery.[31,32] However, collecting sufficient tumor tissue for flow cytometry analysis is difficult, which hampers the clinical application of tumor-infiltrating lymphocytes. These findings suggest that solely CD8+PD-1+ levels cannot accurately predict the prognosis and response to PD-1 blockade therapy in patients with advanced HBV-HCC. Our study revealed that the balance of CD8+ T cells and Treg cells in peripheral blood could predict the clinical outcome of advanced HBV-HCC patients, which combines the complex immunosuppressive microenvironment with clinical application. Further, we confirmed the phenomenon that the higher PD-1+CD8/ICOS+Tregs ratio in peripheral have a better prognosis and response with immunotherapy than lower patients by multiplex immunofluorescence staining on tumor tissue of HBV-HCC patients. This ratio is more comprehensive and effective in predicting HBV-HCC prognosis and immunotherapy response than a single PD-1+CD8+T cell indicator. This is consistent with previous reports that PD-1 expression balance between effector and Treg cells predicts the clinical efficacy of PD-1 blockade therapies.[10]

Treg cells suppress effector T cells, including CD8+ T cells, which are crucial in eliminating cancer cells within the host. The level of PD-1 in CD8+ T and Treg cells represents the balance of antitumor immunity. Once this balance is broken, HBV-HCC disease progression and ICI treatment failure occur. Previous research has indicated that intestinal microbiota, transforming growth factor-β, and transarterial chemoembolization can affect the imbalance between effector CD4+ and CD8+ T cells and Tregs cells, leading to immune tolerance and promoting HCC progression.[33,34] Interestingly, studies have reported that lung tissue-resident Treg cells (TR-Treg) accumulated and expressed high levels of coinhibitory and costimulatory receptors post primary and secondary infections. Blockade of PD-1 or ICOS signaling reveals that PD-1 and ICOS signaling pathways counter-regulate TR-Treg cell expansion and IL-10 production during secondary influenza infection.[9] In addition, several studies reported that ICI therapy can be interfered with by inhibitory Treg cells, reducing therapeutic effectivenes.[35] It is concluded that immune balance is not only concerned with effector cells themselves but also with immunosuppressive mediators and their support and restriction to effector cells. In our study, we also found that the fluorescence intensity of CTLA-4 and GITR expression in peripheral Treg cells of HBV-HCC patients was significantly higher than that of healthy controls, but the expression of CTLA-4 was not more obvious than that of ICOS level. Meanwhile, we also compared the expression ratio of GITR in Treg cells with PD-1 in CD8T cells. The two could not significantly change in the course of the disease, but the ratio of PD-1+CD8 to ICOS+Tregs was more significant clinically. Modifying the exhaustion level in effector and Treg cells could be a potential therapeutic approach for improving outcomes in advanced HBV-HCC patients. Our study found that patients with higher ratio of PD-1+CD8/ICOS+Tregs showed better antitumor immune function of targeted killing, proliferation, and apoptosis than low-ratio patients. Our study demonstrates that an imbalance of CD8+ T and Treg cells could cause immune suppression. Moreover, the imbalance of exhaustion function also contributes to the poor prognosis of HBV-HCC and results in the ineffectiveness of immunotherapy.

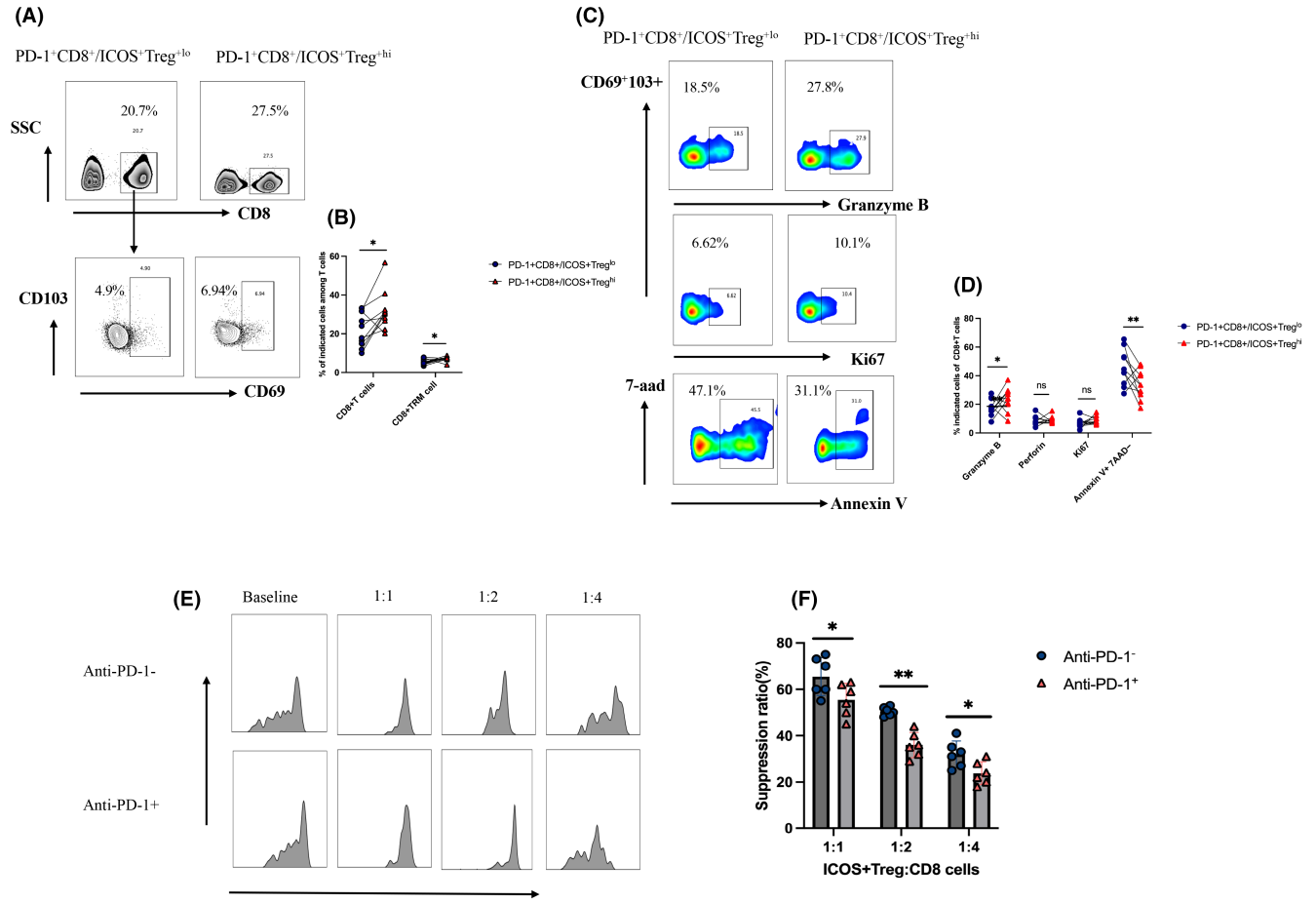

FIGURE 6 Higher ratio of PD-1+CD8/ICOS+Tregs in advanced hepatitis B virus-associated hepatocellular carcinoma (HBV-HCC) patients with immune checkpoint inhibitor (ICI) treatment demonstrates more efficient targeted killing effects. (A, B) The proportion from baseline and representative flow cytometry plots of CD8+T cells and tissue-resident memory T (T_{RM}) cells (CD8+CD69+CD103+) in different PD-1+CD8/ICOS+Tregs ratios from advanced HBV-HCC patients treated with ICI. (C, D) Intracellular staining for perforin, granzyme B, Ki67, and Annexin V of T_{RM} cells in different PD-1+CD8/ICOS+Tregs ratios from advanced HBV-HCC patients treated with ICI ($n = 20$). (E, F) Suppression assays with human ICOS+ Treg cells with ICI treatment condition were performed. Representative histograms (E) and summaries (F) of suppressive function are shown ($N = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns indicates no statistical significance.

T_{RM} is a sentinel cell with a unique phenotype and function in tumor tissues, which can be retained for extended periods and is suitable for rapid and effective first-line immune monitoring.[36,37] Although expressed peripherally, it is not as sufficient as the local tumor. Increasing evidence indicates that the number and characteristics of T_{RM} contribute to enhanced antitumor effects in HBV-HCC patients and provide new strategies for developing more effective cancer immunotherapy.[38,39] Limited clinical samples were investigated whether T_{RM} cells were responsible for different ratios of PD-1+CD8/ICOS+Tregs in patients with opposite prognoses. Indeed, we confirmed that the proportion of T_{RM} cells and effector functions were significantly different in the two groups. However, a large sample of patients is still needed to validate this result and fully investigate the underlying immunomodulatory mechanisms.

One limitation of this study is its focus on the ratios of PD-1+CD8/ICOS+Tregs in peripheral rather than local tumors. A recent study shows that immune cell subsets are gradually inhibited from circulating blood to nontumor and tumor microenvironments in HCC.[39] The alterations in coinhibitory receptors observed on circulating T cells correlate with those observed on tumor-infiltrating T cells. In clinical practice, peripheral blood testing is the standard method due to its accuracy, simplicity, noninvasiveness, and ease of use.

In conclusion, we have characterized the phenotype and function of patients with different ratios of PD-1+CD8/ICOS+Tregs and evaluated its impact on the progression and prognosis of advanced HBV-HCC. We discovered a decrease in this ratio among relapsed and advanced HBV-HCC patients, which indicated the balance of T cell immune effects and suppression was disrupted and failure was more pronounced in these patients. These findings highlight the association between the immune balance of the ratio of PD-1+CD8/ICOS+Tregs and accelerated disease progression and immunotherapy failure in advanced HBV-HCC.

AUTHOR CONTRIBUTIONS

Fengna Yan: Conceptualization; data curation; formal analysis; writing – original draft. **Bingbing Zhu:** Formal analysis; methodology; project administration. **Ke Shi:** Project administration; resources; software. **Yi Zhang:** Project administration; resources. **Xuanwei Zeng:** Project administration. **Qun Zhang:** Methodology; resources. **Zhiyun Yang:** Conceptualization; funding acquisition; supervision; writing – review and editing. **Xianbo Wang:** Conceptualization; funding acquisition; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

DATA AVAILABILITY STATEMENT

Any data within the article will be shared in anonymized format upon request from qualified investigators. If desired, please contact the corresponding author of this article.

ETHICS STATEMENTS

Approval of the research protocol by an Institutional Reviewer Board: The study was approved by the ethics committee of Beijing Ditan Hospital, Capital Medical University (2020-01-16).

Informed Consent: All patients were willing to sign an informed consent.

Registry and the Registration No. of the study/trial: N/A.

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ORCID

Xianbo Wang https://orcid.org/0000-0002-3593-5741

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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