HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Tim-3/galectin-9 Signaling Pathway Mediates T-Cell Dysfunction and Predicts Poor Prognosis in Patients with Hepatitis B Virus-Associated Hepatocellular Carcinoma

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The interaction between T cell immunoglobulin- and mucin-domain-containing molecule (Tim-3) expressed on T helper 1 (Th1) cells, and its ligand, galectin-9, negatively regulates Th1-mediated immune responses. However, it is poorly understood if and how the Tim-3/galectin-9 signaling pathway is involved in immune escape in patients with hepatocellular carcinoma (HCC). Here we studied the expression, function, and regulation of the Tim-3/galectin-9 pathway in patients with hepatitis B virus (HBV)-associated HCC. We detected different levels of galectin-9 expression on antigen-presenting cell (APC) subsets including Kupffer cells (KCs), myeloid dendritic cells (DCs), and plasmacytoid DCs in HCC. The highest galectin-9 expression was on KCs in HCC islets, not in the adjacent tissues. Furthermore, Tim-3 expression was increased on $CD4^+$ and $CD8^+$ T cells in HCC as compared to the adjacent tissues, and $Tim\text{-}3^+$ T cells were replicative senescent and expressed surface and genetic markers for senescence. Interestingly, tumor-infiltrating T-cell-derived interferon (IFN)-γ stimulated the expression of galectin-9 on APCs in the HCC microenvironment. Immunofluorescence staining revealed a colocalization of $Tim\text{-}3^+$ T cells and galectin-9^+ KCs in HCC. Functional studies demonstrated that blockade of the Tim-3/galectin-9 signaling pathway importantly increased the functionality of tumor-infiltrating $Tim\text{-}3^+$ T cells as shown by increased T-cell proliferation and effector cytokine production. Finally, we show that the numbers of $Tim\text{-}3^+$ tumor-infiltrating cells were negatively associated with patient survival. *Conclusion*: Our work demonstrates that the Tim-3/galectin-9 signaling pathway mediates T-cell senescence in HBV-associated HCC. The data suggest that this pathway could be an immunotherapeutic target in patients with HBV-associated HCC. (HEPATOLOGY 2012;56:1342-1351)

Hepatocellular carcinoma (HCC) is one of the most common cancers. More than 80% of patients are not candidates for curative treatments with the final diagnosis, and are linked to chronic infection with the hepatitis B (HBV) or hepatitis C (HCV) viruses based on different regions.[1] HCC is usually accompanied by cirrhotic liver with extensive lymphocyte infiltration due to chronic viral infection. Many studies have demonstrated that tumor-infiltrating effector $CD8^+$ T cells and T helper 17 (Th17) cells correlate with improved survival after surgical resection of tumors.[2-7] However, tumor-infiltrating effector T cells fail to control tumor growth and metastasis.[8,9] In the tumor microenvironment, suppressive antigen presenting cells (APCs),[10-12] inhibitory B7-H1 (PD-L1) and B7-H4 (B7x, B7S1)-

Abbreviations: APCs: antigen presenting cell subsets; CFSE: carboxyfluorescein succinimidyl ester; DCs: myeloid dendritic cells; GAPDH: glyceraldehydes-3-phosphate dehydrogenase; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; IDO: indoleamine-2,3-dioxygenase; IFN: interferon; IHC: immunohistochemistry; KCs: Kupffer cells; mDCs: myeloid dendritic cells; pDCs: plasmacytoid dendritic cells; Tim-3: T cell immunoglobulin- and mucin-domain-containing molecule-3.

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Received October 19, 2011; accepted April 4, 2012.

Supported by Yangtze River Scholar Award Program and the National Science Fund of P. R. China for Young Scholars (81101825).

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expressing cells,[13] and $CD4^+Foxp3^+$ regulatory T (Treg) cells[2-5,14] together form suppressive networks that can mediate tumor immune escape and temper the efficacy of vaccination and other immune therapies.[15-17] In patients with HCC, the B7-H1/PD-1 signaling pathway mediates $CD8^+$ T-cell functional exhaustion,[18,19] and Treg cells infiltrate the HCC microenvironments[3,20] and contribute to tumor immune evasion. It is thought that $CD8^+$ T cells are the main effector cells mediating antitumor immunity, whereas $CD4^+$ T cells provide the help required for effective $CD8^+$ T-cell responses against tumor. However, tumor-associated antigen (TAA)-specific $CD4^+$ T cells may elicit protective tumor immunity and directly eliminate tumors.[21-23] Although Treg cells have been extensively examined in multiple types of human tumors, including HCC,[16] the phenotype and functionality of conventional $CD4^+Foxp3^-$ T cells are not well studied in the human tumor. This work focuses on $CD4^+Foxp3^-$ T cells in the HCC environment.

Originally, Tim-3 was found to be expressed on Th1 cells and Tc1 cells, but not on Th2 cells.[24] Galectin-9 was first identified as a tumor antigen of unknown function in patients with Hodgkin's disease.[25] Galectin-9 is expressed on different types of cells and regulates cell differentiation, adhesion, aggregation, and cell death.[26,27] Recent studies have demonstrated that Tim-3 is the receptor for galectin-9, and galectin-9 induces apoptosis of $Tim\text{-}3^+$ Th1 cells.[28-30] In HIV-1 and HCV chronic infections, Tim-3 was overexpressed on $CD8^+$ T cells that correlated with $CD8^+$ T-cell exhaustion.[31-33] Blockade of Tim-3 could reverse T-cell exhaustion and restore antivirus immunity.[31-33] Tim-3 is also thought to participate in $CD8^+$ T-cell dysfunction in certain mouse tumors,[34,35] human melanoma,[36] and lymphoma.[37] Because the nature of the Tim-3/galectin-9 pathway in HCC patients is poorly defined, we studied their expression, regulation, immunological, and pathological relevance in this patient population.

Materials and Methods

Patients and Specimens. Tumor samples were obtained from 150 patients with pathologically confirmed HCC. None of the patients received anticancer therapy before surgical resection. Clinical stages were classified according to the International Union against Cancer. For *in vitro* experiments, 38 paired fresh tissues were used from HCC patients, including 30 HBV^+ cases and eight HBV^- alcoholic cases in different experiments. Fresh HCC tissues and surrounding nontumor adjacent liver tissues (at least 3 cm distant from the tumor site) were used for the isolation of tumor- and nontumor-infiltrating leukocytes. For survival analysis, we followed 99 HBV-associated HCC patients after surgical resection from January 2007 to April 2010 (Table 1). The research was approved by the Institutional Review Board of Tongji Medical College of Huazhong University of Science and Technology. Both written and oral consent was obtained before samples were collected.

Cell Isolation. Immune cells were obtained from peripheral blood and fresh liver tissues as described.[19] $CD14^+$ tumor-associated Kupffer cells (KCs) and $Tim\text{-}3^+CD4^+$ T cells were isolated with paramagnetic beads (StemCell Technology, Canada) and sorted. Cell purity was >90% as confirmed by flow cytometry (LSR II, Becton Dickinson).

Flow Cytometric Analysis. Immune cells were stained extracellularly with fluorochrome-conjugate-specific antibodies against human antibodies, then fixed and permeabilized with Perm/Fix solution (eBioscience), and stained for intracellular cytokines and Ki67 (eBioscience).

Functional Assays. $Tim\text{-}3^+CD4^+$ T cells (5×10^5/ml) were cocultured with $CD14^+$ KCs (10^5/mL) from the same HCC tissue from six patients for 5 days in the presence of antihuman CD3 (2.5 μg/mL, BD Biosciences) and antihuman CD28 (1.25 μg/mL) or with autologous HCC (10^5/mL). Neutralizing monoclonal antibody (mAb) against human Tim-3 (10 μg/mL, Biolegend) or isotype controls were added to the culture. The resultant cells were collected for flow cytometry analysis or for ELISPOT assay with ImmuneSpot analyzer (Cellular Technology).

T-Cell Expansion. Carboxyfluorescein succinimidyl ester (CFSE)-labeled $Tim\text{-}3^+CD4^+$ T cells were incubated with $CD14^+$ KCs from the same HCC tissue from six patients for 5 days. Cell division was determined based on CFSE dilution by flow cytometry analysis.

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DOI 10.1002/hep.25777

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Table 1. Clinical Characteristics of HBV-Associated HCC Patients

Variables	Case Numbers
No. patients	99
Age, y (median, range)	51.1, 38-72
Gender (male/female)	91/8
Cirrhosis (absent/present)	16/83
Child-Pugh score (A, B, C)*	Tim-3^{high}: 32, 11, 6 Tim-3^{low}: 24, 7, 3
ALT, U/L (median, range)	79, 42-208
AFP, ng/mL ($\leq$25 /$>$25)	22/77
Tumor size, cm ($\leq$5/$>$5)	43/56
Tumor multiplicity (solitary/multiple)	84/15
Vascular invasion (absent/present)	91/8
Intrahepatic metastasis (no/yes)	89/10
TNM stage (I, II, III, IV)*	Tim-3^{high}: 41, 6, 10, 0 Tim-3^{low}: 30, 8, 4, 0
Tumor differentiation (I, II, III, IV)*	Tim-3^{high}: 6, 38, 13, 0 Tim-3^{low}: 6, 31, 5, 0

*$P > 0.05$ in Tim3^{high} group among A, B, and C based on Child-Pugh score, among TNM stages I, II, and III, and among tumor differentiation grades. Pearson's chi-squared test.

Immunohistochemistry. Frozen tissue sections were stained with primary antibodies, rat monoclonal antihuman Tim-3 (clone: 344823, 1/200, IgG2a, R&D Systems), mouse antihuman CD4 (Clone: RPA-T4, 1/500, IgG1, eBioscience), mouse antihuman galectin (clone: 9M1-3, 1/500, IgG1, Biolegend), and CD68 (clone: Y1/82A, 1/500, IgG2b, eBioscience), and subsequently stained with secondary antibodies, Alexa Fluor 568-conjugated goat antirat IgG2a, Alexa Fluor 488-conjugated goat antimouse IgG1, and Alexa Fluor 568-conjugated goat antimouse IgG2b (all 2 μg/mL, Invitrogen). Hoechst 33342 (Invitrogen) was used for nuclear staining. Images were acquired by fluorescence microscope and positive cells were quantified by ImagePro Plus software (Media Cybernetics, Bethesda, MD) and expressed as the mean of the percentage of positive cells $\pm$ standard error of the mean (SEM) in five high-powered fields. To quantify Tim-3$^+$ cells in large numbers of patients, paraffin-embedded HCC samples were processed for conventional immunohistochemistry. HCC tissue sections were stained with rat antihuman Tim-3 (R&D), and then with HRP-conjugated goat antirat IgG (1/500, Invitrogen). Visualization was achieved with ABC-Elite Reagent (Sigma). The sections were counterstained with Mayer's hematoxylin (Sigma). The nuclei were stained with 1% ammonium hydroxide. The numbers of Tim-3$^+$ cells were counted in five fields at $\times$400 magnification.

Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR). Real-time PCR was performed as described.[14,19] Specific primers are listed in Supporting Table 1.

Transwell Experiments. Transwell chambers with a 0.4 μm pore membrane (Corning-Costar) were used. CD14$^+$ cells (5×10^5/mL) from the blood of healthy donors or normal KCs from relatively normal liver tissues with hepatic hemangiomas were plated to the lower chambers. T cells isolated from HCC tissues or adjacent tissues were added to the upper chamber and cultured with interferon (IFN)-γ (400 U/mL) for 48 hours. CD14$^+$ cells were collected and galectin-9 expression was determined by flow cytometry. Antihuman IFN-γ mAb (500 ng/mL, R&D) was added to the culture as indicated.

Statistical Analysis. Comparisons were made using the Wilcoxon test. Survival curves were compared by the Kaplan-Meier method and the log-rank test, and survival was measured in months from resection to the last review. The log-rank test was applied to compare the groups. Multivariate analysis of prognostic factors for survival data was performed using the Cox proportional hazards model. Differences in values at $P < 0.05$ were considered significant. All analyses were done using SPSS v12.0 software.

Results

Galectin-9 Expression on APC Subsets in Human HCC. To study the functional relevance of galectin-9 in patients with HCC, we examined the expression of galectin-9 on lin$^-$CD45$^-$ HCC cells and different immune cell populations including T cells, HLA-DR$^+$CD14$^+$ KCs, lin$^-$HLA$^-$DR$^+$CD4$^+$CD11c$^+$ myeloid dendritic cells (mDCs), and lin$^-$HLA$^-$DR$^+$CD4$^+$CD123$^+$ plasmacytoid dendritic cells (pDCs), in paired HBV-associated HCC tissues and surrounding nontumor adjacent tissues. Flow cytometry analysis revealed that tumor cells and T cells expressed minimal galectin-9 ($<$4%), pDCs and mDCs expressed moderate levels of galectin-9 (10%), and KCs expressed the highest levels of galectin-9 in HCC (Fig. 1A). Next we compared the expression of galectin-9 on KCs in HCC tissues and adjacent tissues from both HBV-positive and -negative patients. In HBV-positive patients the percentage of galectin-9$^+$ KCs was higher in tumor tissues than in adjacent tissues (46.8 $\pm$ 3.9% versus 10.7 $\pm$ 2.3%) (Fig. 1B). However, in HBV-negative patients (Fig. 1B) the levels of galectin-9 expression on KCs were negligible ($<$0.5%) in both HCC and adjacent tissues. Immune fluorescence staining confirmed that there were higher numbers of galectin-9$^+$CD68$^+$ KCs in HCC tumor tissues (38 $\pm$ 13%) than in adjacent nontumor tissues (11 $\pm$ 5%) (Fig. 1C). The data indicate that KCs are the

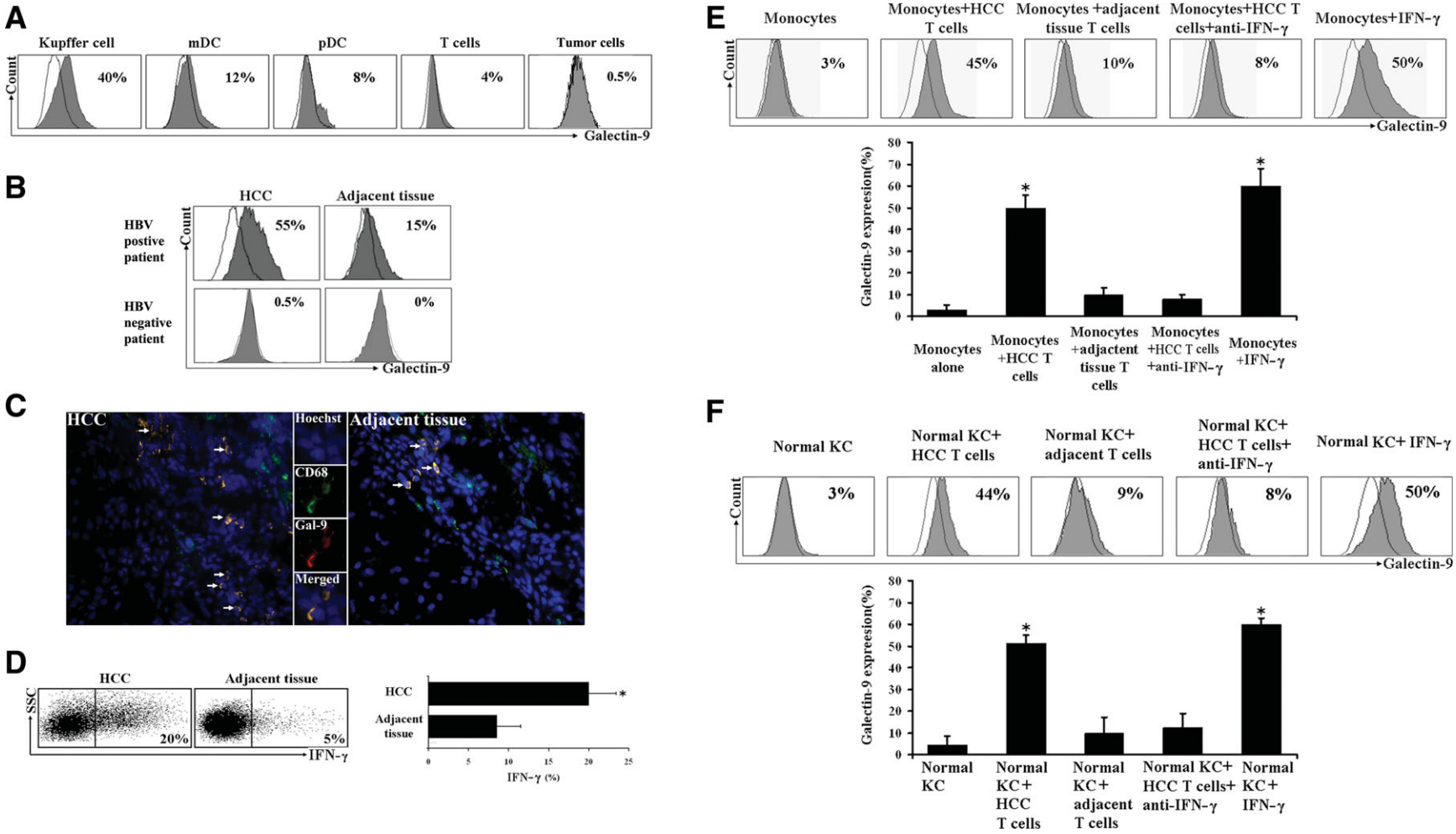

Fig. 1. HCC infiltrating T cells stimulated galectin-9 expression on KC through IFN-γ. (A) Galectin-9 expression on APC subsets in HCC tissues. Galectin-9 expression was analyzed by flow cytometry in fresh HCC tissues. The results are expressed as the percentage of galectin-9^+ cells in different APC subsets. Filled histogram, galectin-9 expression; open histogram, isotype. n = 30, $P < 0.05$. (B) Galectin-9 expression on KCs in HCC tissues. Galectin-9 expression was analyzed by flow cytometry in paired HCC and adjacent tissues from both HBV-positive (n = 9) and -negative (n = 8) patients. The results are expressed as the percentage of galectin-9^+ cells in KCs. Filled histogram, galectin-9 expression; open histogram, isotype. $P < 0.05$. (C) Galectin-9^+CD68$^+$ cells in HCC and adjacent tissues. Immunofluorescence staining was performed in fresh HCC and adjacent tissues with antibodies against human CD68 and galectin-9. CD68$^+$ KCs (red); galectin-9^+ cells (green), nuclear staining (blue). n = 9 patients. ×400. (D) High levels of IFN-γ expression by HCC infiltrating T cells. T cells were subjected to intracellular staining for IFN-γ. IFN-γ expression was analyzed by flow cytometry. The results are expressed as the percent of IFN-γ^+ cells in total T cells ± SEM. n = 15 patients. *$P < 0.05$. (E,F) Stimulation of galectin-9 expression on monocytes and KCs by HCC infiltrating T cell-derived IFN-γ. Blood CD14$^+$ monocytes were cultured for 48 hours in transwells under different conditions. Galectin-9 expression on monocytes (E) and normal KCs (F) was determined by flow cytometry. The results are expressed as the percent of galectin-9^+ cells in total monocytes ± SEM. n = 6 patients. *$P < 0.05$.

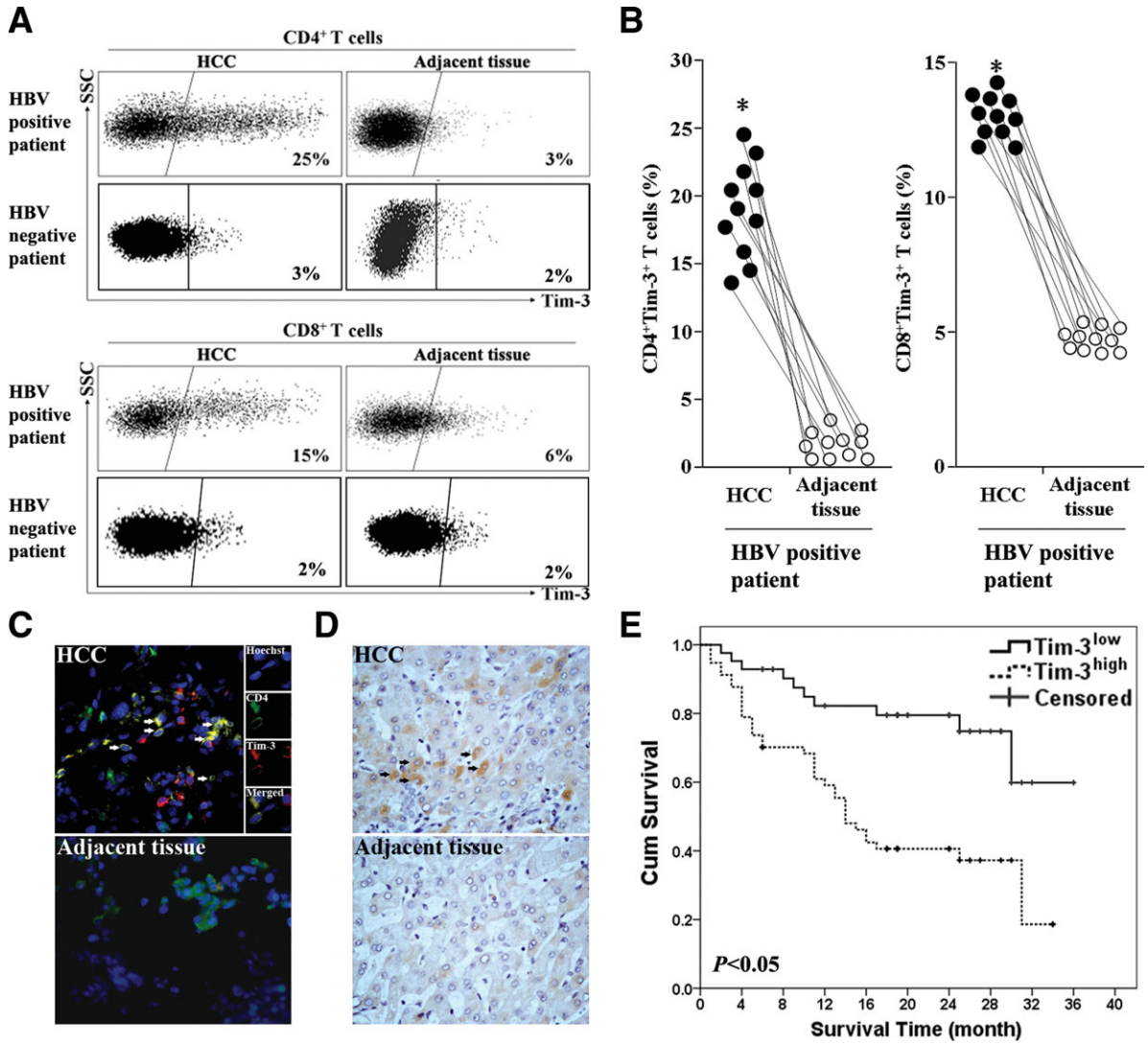

Fig. 2. T cell Tim-3 expression was correlated with poor outcome of HCC patients. (A,B) Tim-3 expression on T cells in paired HCC and adjacent tissues. T cells were isolated from HCC or adjacent tissues and were subjected to Tim-3 staining. TIM-3 expression was analyzed by flow cytometry. The results are expressed as the percent of Tim-3$^+$ cells in CD4$^+$ and CD8$^+$ T cell subsets ± SEM (B). HBV-positive patients n = 11, HBV-negative patients n = 8. *$P < 0.05$. (C) Tim-3$^+$ cells in snap-frozen HCC. Immunofluorescence staining was performed in snap-frozen HCC tissue sections for CD4 (green), Tim-3 (red), and Hoechst (blue). Arrows indicate Tim-3$^+$CD4$^+$ T cells. n = 25. ×400. (D) Tim-3$^+$ cells in paraffin-fixed HBV-associated HCC. Conventional immunochemistry was performed in paraffin-fixed HCC tissue sections for Tim-3. Arrows indicate Tim-3$^+$ cells. n = 99. ×400. (E) Tim-3$^+$ cells and HBV-associated HCC patient survival. Tim-3 expression was determined as described (D). The Kaplan-Meier survival curve was determined on the basis of Tim-3 expression. Dashed line, low expression (n = 42); solid line, high expression (n = 57). $P < 0.05$.

primary galectin-9-expressing APC subset in HBV-associated HCC.

Tumor-infiltrating T-Cell-Derived IFN-γ Induces Galectin-9 Expression on KCs. Next we investigated why KCs express high levels of galectin-9 in HCC. We hypothesized that tumor-infiltrating T-cell-derived IFN-γ induces galectin-9 expression on KCs in HCC. To test this hypothesis, we first showed that CD3$^-$ infiltrating cells (non-T cells) expressed negligible levels of IFN-γ (not shown), and tumor-infiltrating T cells expressed high levels of IFN-γ (Fig. 1D). The levels of IFN-γ$^+$ T cells were higher in HCC tissues compared to adjacent tissues (Fig. 1D). Thus, tumor-infiltrating T cells are the major source of IFN-γ in HCC. Then we examined the potential effect of tumor-infiltrating T-cell-derived IFN-γ on KC galectin-9 expression. We cocultured normal blood CD14$^+$ monocytes with T cells from HCC tissue or adjacent tissue. Tumor-infiltrating T cells were superior at inducing galectin-9 expression on monocytes as compared to adjacent T cells (Fig. 1E). The induction was blocked by neutralizing antibody against IFN-γ (Fig. 1E). To further support the stimulatory role of IFN-γ, we showed that recombinant IFN-γ induced galectin-9 expression on

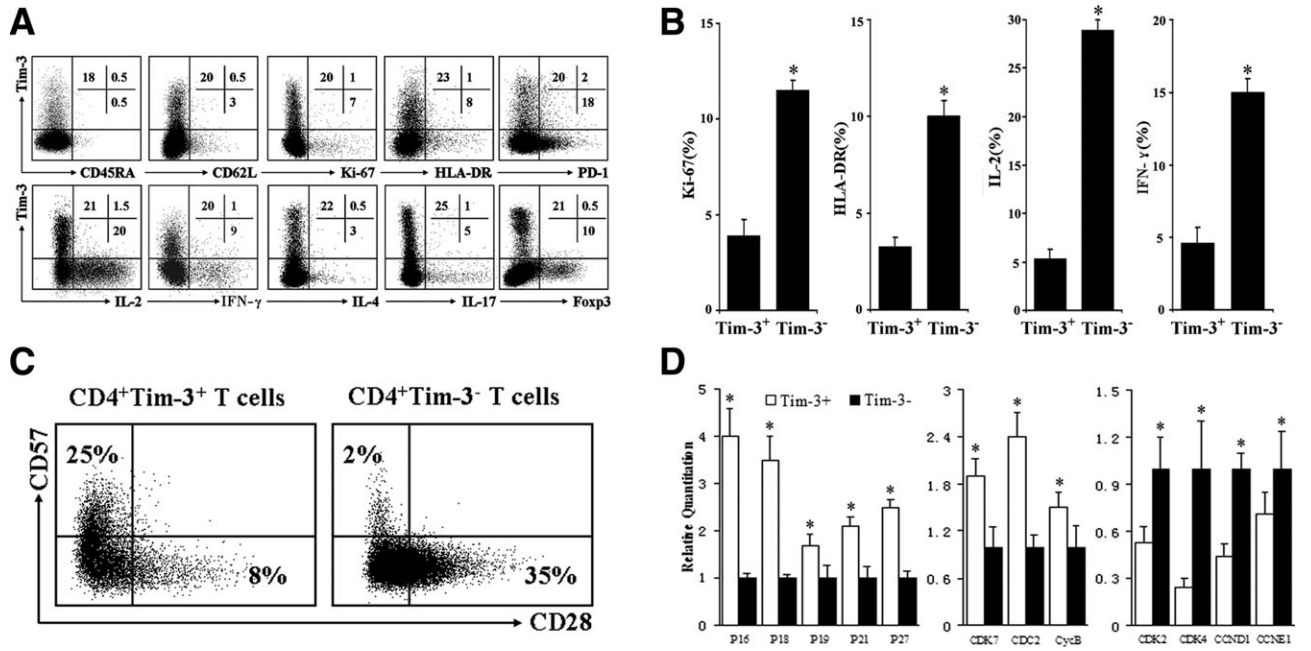

Fig. 3. Characteristics of tumor-associated Tim-3$^+$CD4$^+$ T cells in HCC. (A-C) Phenotype and cytokine profile of HCC infiltrating Tim-3$^+$CD4$^+$ T cells. HCC infiltrating T cells from 15 cirrhosis patients were stained as described. The expression levels were analyzed with flow cytometry. The results are expressed as the mean percentage of specific molecule expression in Tim-3$^+$CD4$^+$ T cells and Tim-3$^-$CD4$^+$ T cells ± SEM. (A,B, n = 15. C, n = 10, *$P < 0.05$). (D) Cell cycle associated gene transcripts in in HCC infiltrating T cells from 10 cirrhosis patients. Real-time PCR was performed in HCC Tim-3$^+$ and Tim-3$^-$ T cells. The results are expressed as the relative values ± SEM. n = 10, *$P < 0.05$.

monocytes (Fig. 1E). Additionally, we isolated KCs from relatively normal liver tissues in patients with hepatic hemangiomas, performed similar experiments, and confirmed the stimulatory effects of IFN-γ derived from HCC-associated T cells on the expression of KC galectin-9 (Fig. 1F). The results demonstrate that tumor-infiltrating T-cell-derived IFN-γ contributes to the increased galectin-9 expression on KCs in the HCC microenvironment.

Tim-3 Expression Is Inversely Associated with Clinical Outcome of Patients with HBV-Associated HCC. Galectin-9 is the ligand for Tim-3. After determining the expression and regulation of galectin-9 in the HCC microenvironment, we further studied the expression of Tim-3. Flow cytometry analysis showed that Tim-3 was expressed on tumor-infiltrating CD4$^+$ and CD8$^+$ T cells. In HBV-positive patients, the levels of Tim-3$^+$CD4$^+$ T cells were higher than that of CD8$^+$ T cells (Fig. 2A,B). Furthermore, Tim-3$^+$ T cells were largely found in HCC tissues, not in the adjacent tissues (Fig. 2A,B). In HBV-negative patients, the percentages of Tim-3$^+$ T cells were less than 3% in both HCC and adjacent tissues (Fig. 2A). In line with this, multiple-color fluorescent staining demonstrated that there were higher numbers of Tim-3$^+$CD4$^+$ cells in snap-frozen HCC tissues than adjacent tissues (15 ± 3% versus 4 ± 2%) (Fig. 2C). As Tim-3$^+$ T cells were basically detected in HBV-associated HCC, we extended our studies further to include large numbers of paraffin-fixed HBV-associated HCC tissues with conventional immunohistochemistry staining (Fig. 2D). In line with flow analysis and multiple-color fluorescent staining, there were higher numbers of Tim-3$^+$ cells in HCC tissues than adjacent tissues (12 ± 8 versus 2 ± 2) (Fig. 2D). These results indicate that Tim-3 expression is increased on T cells infiltrating the HCC microenvironment.

We further evaluated the pathological relevance of Tim-3 expression in HBV-associated HCC. Based on conventional immunohistochemistry staining in paraffin-fixed HCC tissues (Fig. 2D), we correlated the numbers of Tim-3$^+$ cells with disease stage and patient survival. There was no significant difference in Tim-3$^+$ cells among different clinical stages, Child Pugh Scores, or tumor differentiation stages (Table 1). HCC patients were divided into low (<7, n = 42) and high (>7, n = 57) groups based on the median levels of Tim-3$^+$ cells. Log-rank analysis demonstrated that the high Tim-3-expressing group experienced shorter survival when compared to the low Tim-3-expressing group (Fig. 2E). The Tim-3$^+$ cell number was positively associated with tumor size ($P < 0.05$) but no correlation to any other parameters (including age, gender, α-fetal protein level, tumor multiplicity,

vascular invasion, intrahepatic metastasis, and tumor TNM stage) (Table 1). Multivariate analysis revealed that the number of Tim-3^+ cells in HCC tissues was a negative prognostic factor of overall survival.

Characteristics of Tumor-Infiltrating Tim-3^+CD4^+ T cells in HCC. To understand the functionality of Tim-3^+CD4^+ T cells in HCC, we examined their phenotype, cytokine profile, and cell cycling genes. We observed that Tim-3^+CD4^+ T cells were basically confined to $CD45RA^-$ and $CD62L^-$ T cells (Fig. 3A), suggesting that Tim-3^+CD4^+ T cells are memory cells. Next, we compared the *in vivo* proliferation potential and activation status of Tim-3^+CD4^+ T cells versus Tim-3^-CD4^+ T cells in HCC. Ki67 and HLA-DR are markers of cell proliferating and activation, respectively. There were fewer $Ki67^+$ cells and $HLA\text{-}DR^+$ cells in Tim-3^+CD4^+ T cells than Tim-3^-CD4^+ T cells (Fig. 3A,B). This suggests that Tim-3^+CD4^+ T cells have reduced proliferation and activation potential in HCC.

PD-1 has been identified as a marker for functionally exhausted T cells in HCC.[7] We found that Tim-3^+ and PD-1^+ T cells were two different T-cell subsets with minimal overlapping in HCC. In addition, Tim-3^+CD4^+ T cells did not express interleukin (IL)-4, IL-17, or Foxp3 (Fig. 3A). Tumor-infiltrating Tim-3^+CD4^+ T cells expressed less IL-2 and IFN-γ as compared to Tim-3^-CD4^+ T cells (Fig. 3A,B). Together, the data indicate that HCC infiltrating Tim-3^+CD4^+ T cells are different from $Foxp3^+$ regulatory T cells, functionally exhausted PD-1^+ cells, and Th2 and Th17 cells. Tim-3^+CD4^+ T cell is a unique T-cell population with poor effector function and reduced proliferating potential in HCC.

Low expression of CD28 and high expression of CD57 are thought to be associated with T-cell senescence.[38] To determine if Tim-3 expression is linked to T-cell senescence in HCC, we examined the relationship between the expression of CD28, CD57, and Tim-3 on tumor-infiltrating T cells. We showed that there were more $CD57^+CD28^-$ cells and fewer $CD57^-CD28^+$ cells in tumor-infiltrating Tim-3^+CD4^+ T cells than Tim-3^-CD4^+ T cells (Fig. 3C). The results suggest that Tim-3^+CD4^+ T cells may contain senescent cells with limited proliferating potential.

Given that Tim-3^+CD4^+ T cells were less proliferative and contained senescent cells, we further quantified the expression of key genes controlling cell cycle and cellular senescence. Real-time PCR revealed that the expression of cyclin-dependent kinase inhibitors (p16, p18, p19, p21, and p27) was increased in Tim-3^+CD4^+ T cells over Tim-3^-CD4^+ T cells (Fig. 3D). This suggests that Tim-3^+CD4^+ T cells failed to actively enter the cell cycle. In line with this possibility, the expression of G_1/S phase-associated genes CDK2, CDK4, CCND1, CCNE1 was increased and that of G_2/M phase-associated genes CDC2, CycB, CDK7 was decreased (Fig. 3D). The results support the possibility that Tim-3^+CD4^+ T cells contain senescent cells and experience cell cycle arrest in G_1/S phase.

Tim-3 and Galectin-9 Interaction Impairs T-Cell Effector Function in HCC. To evaluate the functional relevance of the interaction between Tim-3 and galectin-9 interaction, galectin-9^+ KCs and Tim-3^+CD4^+ T cells were sorted from HCC, and T-cell function was analyzed in the *ex vivo* cocultured system. Blockade of this interaction with specific anti-Tim-3 mAb resulted in enhanced Ki67 expression on T cells (Fig. 4A). In some experiments, T cells were initially labeled with carboxyfluorescein succinimidyl ester (CFSE), and we showed that there were more T cells entering cell division in the presence of anti-Tim-3 mAb as compared to isotype control (Fig. 4B). Furthermore, this blockade increased the expression of T cell effector cytokines IL-2 and IFN-γ (Fig. 4C,D). ELISPOT assay confirmed that anti-Tim-3 mAb increased tumor-specific T cell IFN-γ-spots (Fig. 4E). When we cocultured Tim-3^+ and $Tim3^-$ T cells with the Hepa G_2 cell line, Tim-3^+ and $Tim3^-$ T cells had no effect on the proliferation of Hepa G_2 cell lines (not shown). The data indicate that disruption of the interaction between Tim-3 and galectin-9 can restore T cell effector functions in HCC.

Discussion

The interaction between Tim-3 and galectin-9 has been reported in multiple pathological scenarios.[24,28-37,39] However, the nature of the Tim-3 and galectin-9 signaling pathway remains undefined in patients with HCC. We evaluated the expression, function, and clinical relevance of the Tim-3/galectin-9 signaling pathway in HCC.

In HCC, galectin-9 expression is found on myeloid APCs including DCs and KCs; however, the main galectin-9-expressing cells are KCs. Galectin-9 is a defined ligand for Tim-3.[28] Interestingly, we found high numbers of Tim-3^+ T cells in HBV-associated HCC. Furthermore, galectin-9^+ KC and Tim-3^+ T cells are colocalized in the HCC. Tim-3^+CD4^+ T cells expressed senescent markers and exhibited decreased proliferative ability and effector function when compared to Tim-3^- T cells. Importantly, blocking the Tim-3/galectin-9 signaling pathway can recover

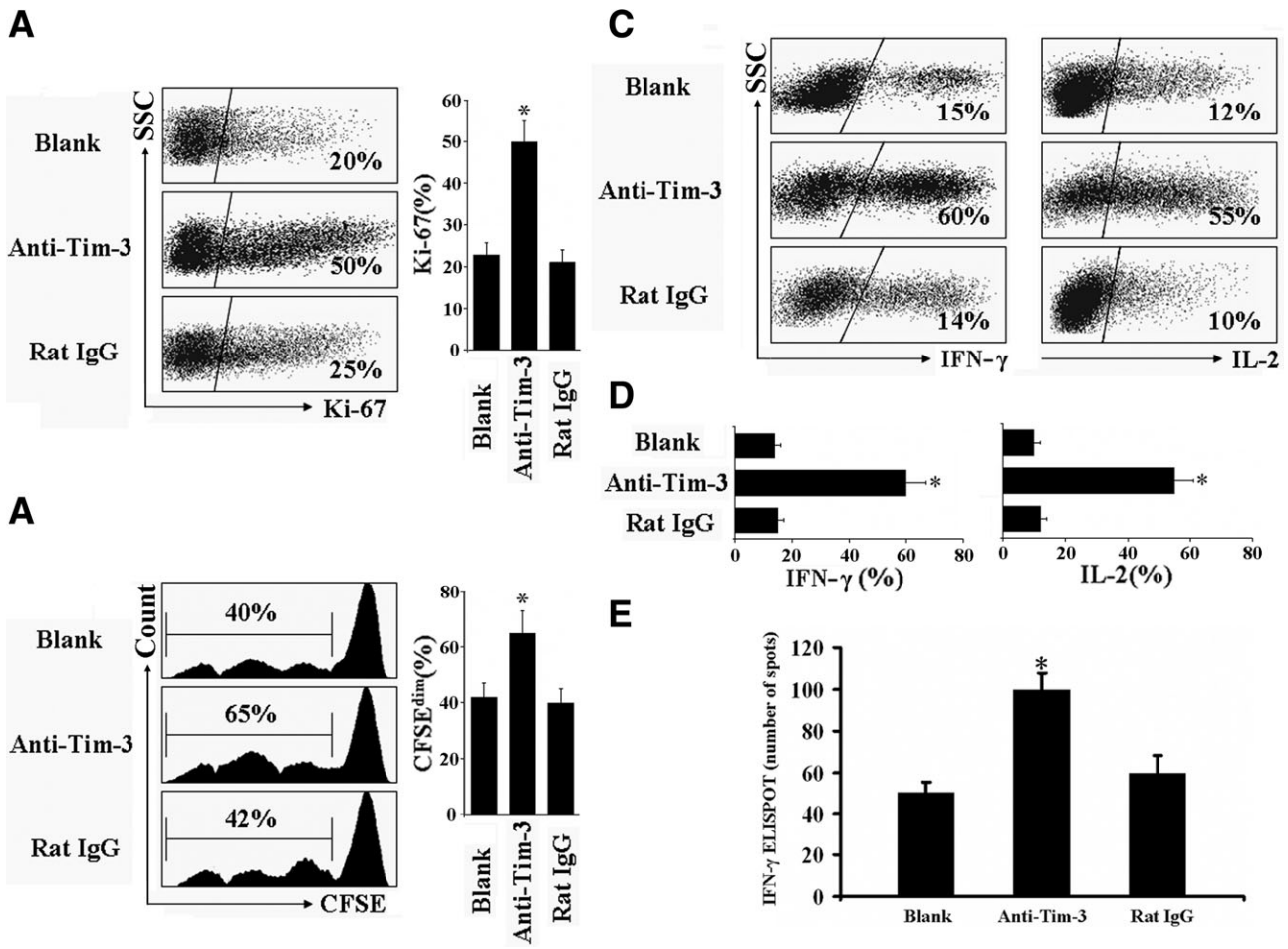

Fig. 4. Galectin-9/Tim-3 signaling pathway mediates T-cell dysfunction in HCC. $CD4^+$ T cells and KCs were sorted from HCC and cocultured for 5 days in the presence of anti-CD3 and anti-CD28 antibodies. Neutralizing anti-human Tim-3 and isotype mAbs were added into the culture. T-cell proliferation (A,B) and cytokine profile (C,D) were determined by flow cytometry analysis. (A,B) T-cell proliferation. T cells were stained for Ki67 expression after the coculture. The results are expressed as the mean percentage of $Ki67^+$ T cells ± SEM (A). Or, T cells were initially labeled with CFSE before the coculture. The results are expressed as the mean percentage of T cells experiencing three or more divisions ± SEM (B). n = 6, $*P < 0.05$. (C,D) Effector cytokines. T cells were stained for IFN-γ and IL-2 expression. (C) One of six dotplots is shown. (D) The results are expressed as the mean percentage of cytokine expressing T cells ± SEM (D). n = 6, $*P < 0.05$. (E) IFN-γ ELISPOT. $Tim\text{-}3^+CD4^+$ T cells and KCs were sorted from HCC and cocultured with autologous HCC tumor cells lysate for 5 days. Neutralizing antihuman Tim-3 and isotype mAbs were added to the culture. HCC-specific IFN-γ was determined by ELISPOT assay (n = 6 experiments, $*P < 0.05$).

effector T-cell function. The results raise two possibilities: (1) HCC-associated $Tim\text{-}3^+CD4^+$ T cells have senescent features but are not at the terminal stage of senescence. (2) Or, T-cell senescence may be reprogrammed and the functionality of senescent T cells may be partially recovered with appropriate treatment, as proposed in the human T-cell literature.[40] Nonetheless, the data indicate that Tim-3/galectin-9 interaction contributes to immune dysfunction in human HCC.

Galectin-9 is not the sole suppressive molecule expressed on HCC-associated KCs. We have previously demonstrated that KCs express high levels of B7-H1 (PD-L1) in HCC, interact with $PD\text{-}1^+$ T cells, and mediate T-cell exhaustion.[17] The pathological relevance of the B7-H1/PD-1 signaling pathway has been observed in many other types of human cancer, and the B7-H1/PD-1 signaling pathway is the justified target for treating human cancer.[17] Interestingly, $PD\text{-}1^+$ and $Tim\text{-}3^+$ T cells are two distinct T-cell populations in HCC. Our studies support the notion that KCs play a negative role in anti-HCC immunity through two distinct molecular pathways, namely, Tim-3/galectin-9 and B7-H1/PD-1. As the expression of B7-H1[18,19] and Tim-3 (this work) is negatively associated with HCC patient outcome, it is tempting to speculate that simultaneous blockade of the B7-H1/PD-1 and Tim-3/galectin-9 signaling pathways may synergistically recover T-cell immunity and improve HBV-associated HCC patient outcome.

In addition to its clinical relevance, our work raises an important issue that IFN-γ may play dual roles in tumor immunity.[41] The immune stimulatory role of

IFN-γ has been well appreciated. IFN-γ is elevated in patients with HCC. It is thought that uncontrolled immune activation, including large amounts of IFN-γ, may result in liver damage.[42,43] We have observed that tumor-infiltrating T-cell-derived IFN-γ potently stimulates galectin-3 expression on KCs in HCC. Interestingly, IFN-γ also induces B7-H1 expression on APCs.[44,45] Thus, galectin-3 and B7-H1 induced by T-cell-derived IFN-γ may be able to fine-tune and temper local immune response, and to avoid over T-cell activation-mediated liver damage. However, this mechanism may be beneficial for tumors to evade tumor immunity. In further support of this possibility, in addition to galectin-3 and B7-H1, IFN-γ can induce the expression of noncognate MHC class I,[46] indoleamine-2,3-dioxygenase (IDO),[47] and arginase,[48] which limit T-cell activation, proliferation, and effector function.[49] Furthermore, a recent study has shown that UVB irradiation induces an IFN-γ-associated gene signature in murine melanocytes and results in increased tumorigenesis.[50] These immune regulatory roles of IFN-γ are consistent with several studies in patients with cancer. Most clinical trials of IFN-γ treatment did not demonstrate clinical efficacy in patients with melanoma.[51-54] Multiple clinical trials have demonstrated that IFN-γ-treated patients fare worse than untreated patients.[55,56] Thus, although IFN-γ is a potent immune effector cytokine, it induces B7-H1, IDO, arginase, nonclassical MHC expression, and galectin-3, which may participate in a feedback mechanism to efficiently down-regulate antitumor immunity. In order to achieve clinical efficacy, novel regimens of tumor immune vaccination and therapy may have to maximize beneficial effects and minimize detrimental effects of IFN-γ.

In summary, we have demonstrated high numbers of galectin-9^+ KCs and Tim-3^+ T cells infiltrating HCC. The Tim-3/galectin-9 signaling pathway mediates T cell senescence and predicts poor survival of HBV-associated HCC patients. Thus, Tim-3/galectin-9 signaling pathway is a novel immune therapeutic target for treating patients with HBV-associated HCC.

Acknowledgment: We thank Drs. Yu Hu, Jiahong Xia, and Kai Huang for support.

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