Article

Blockade of Tim-3 Pathway Ameliorates Interferon-γ Production from Hepatic CD8$^+$ T Cells in a Mouse Model of Hepatitis B Virus Infection

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T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) has been reported to participate in the pathogenesis of inflammatory diseases. However, whether Tim-3 is involved in hepatitis B virus (HBV) infection remains unknown. Here, we studied the expression and function of Tim-3 in a hydrodynamics-based mouse model of HBV infection. A significant increase of Tim-3 expression on hepatic T lymphocytes, especially on CD8$^+$ T cells, was demonstrated in HBV model mice from day 7 to day 18. After Tim-3 knockdown by specific shRNAs, significantly increased IFN-γ production from hepatic CD8$^+$ T cells in HBV model mice was observed. Very interestingly, we found Tim-3 expression on CD8$^+$ T cells was higher in HBV model mice with higher serum anti-HBs production. Moreover, Tim-3 knockdown influenced anti-HBs production *in vivo*. Collectively, our data suggested that Tim-3 might act as a potent regulator of antiviral T-cell responses in HBV infection. *Cellular & Molecular Immunology*. 2009;6(1):35-43.

Key Words: Tim-3, HBV, CD8$^+$ T cell, hydrodynamic injection, shRNA

Introduction

Hepatitis B virus (HBV) is a noncytopathic, enveloped virus, which causes acute and chronic necroinflammatory liver diseases and affects more than 350 million people worldwide (1-3). Liver damage during HBV infection is thought to be mediated by the host cellular immune response to viral antigens (4, 5). Persistent carriers of HBV, however, show a severe HBV-specific T-cell dysfunction with low levels of antiviral cytokines and impaired cytotoxic T lymphocytes activity (6, 7). However, the mechanisms remain unknown.

T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) was originally described as a surface protein that can specifically identify Th1 cells (8). Tim-3 ligation negatively regulates Th1 responses and influences the ability to induce tolerance in both mice and humans (9-12). Recently, it was reported that Tim-3 was up-regulated on effector CD8$^+$ T cells in chronic virus infection (13). The administration of anti-Tim-3 mAbs augmented the activation of effector T cells expressing IFN-γ. So far, evidence has been provided for an important role of Tim-3 in a range of diseases, including inflammatory diseases (14-19). However, no study has been reported on the role of Tim-3 in immune repression during HBV infection.

In the present study, we studied the expression and functional role of Tim-3 in a mouse model of HBV infection based on hydrodynamic injection of HBV-DNA containing plasmid. Our results show that HBV-mediated Tim-3 up-regulation on hepatic CD8$^+$ T cells is engaged in suppressing CD8$^+$ T cell function through the inhibition of IFN-γ production, and indirectly affects neutralizing antibody production by regulating CD8$^+$ T cell-function, indicating that Tim-3 may function as a potential molecule in regulating antiviral immune response.

Materials and Methods

Plasmids

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Recombinant pcDNA3-1.1HBV, containing 110% of HBV genome (adr subtype) encoding hepatitis B core antigen (HBcAg), hepatitis B surface antigen (HBsAg), and hepatitis B e antigen (HBeAg) was constructed in our lab (20).

Tim-3 specific short hairpin RNAs (shRNAs) were designed and chemically synthesized by Allele Biotechnology and Pharmaceuticals, Inc. (San Diego, CA, USA). The shRNA sequences used are as follows: Tim-3 shRNA-1 sense: 5'-TTT GGG GAA CTG ACT CAG ACA AAC ATT GTC TGA GTC AGT TCC CCT TTT T-3'; antisense: 5'-CTA GAA AAA GGG GAA CTG ACT CAG ACA ATG TTT GTC TGA GTC AGT TCC C-3'; Tim-3 shRNA-2 sense: 5'-TTT GCA CGA TAT AAC TTG GGA ACA CAG TTC CCA AGT TAT ATC GTG CTT TTT-3'; antisense: 5'-CTA GAA AAA GCA CGA TAT AAC TTG GGA ACT GTG TTC CCA AGT TAT ATC GTG-3'; unrelated shRNA (unshRNA) sense: 5'-TTT GGT CAC GTT AAT GGT CGT TTA CAA AAC GAC CAT TAA CGT GAC CTT TTT-3'; antisense: 5'-CTA GAA AAA GGT CAC GTT AAT GGT CGT TTT GTA AAC GAC CAT TAA CGT GAC-3'. The shRNA sequences were cloned into the recombinant vector pmU6, containing murine U6 promoter. All recombinant plasmids were prepared with Qiagen EndoFree Plasmid Mega Purification Kit (Qiagen, Hilden, Germany).

Mice

Six to eight-week-old male SPF Balb/c mice were provided by the Laboratory Animal Center of Shandong University. All mice were housed under pathogen-free conditions. All procedures were pre-approved by the guidelines set up by the Institutional Animal Care and Use Committee..

Hydrodynamics-based in vivo transfection

Mice were injected with a total of 50 μg pcDNA3-1.1HBV *via* the tail vein within 5-8 s in a volume of normal saline equivalent to 10% of the mouse body weight (21-23). Mice injected with pcDNA3 served as control. Mice were sacrificed on days 1, 3, 7, 10, 14, 18 post the injection. For shRNA knockdown, mice were injected with a total of 50 μg Tim-3 shRNA or unshRNA plasmid together with the same amount of pcDNA3-1.1HBV. The inhibitory effects were detected on day 10 post the injection.

Cell culture and transfection

RAW264.7 murine macrophage cell line (ATCC TIB-71) was maintained in Dulbucco's Modified Eagle's High Glucose Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Calf Serum (FCS) (Invitrogen) in 5% CO_2 atmosphere at 37ºC. For shRNA knockdown assay, cells were plated in duplicate 24-well plates and transfected with plasmid Tim-3 shRNA-1, Tim-3 shRNA-2 or unshRNA using Lipofectamine 2000 reagent (Invitrogen). Transfections were carried out according to the manufacturer's protocol. Cells treated under the same conditions without shRNA vector were set as mock control. Then above cells were harvested at 24 h, 48 h and 72 h after transfection, respectively, for the detection of Tim-3 expression by RT-PCR and western blot.

RNA preparation and RT-PCR

Total RNA was isolated from shRNA-transfected cells or mock cells using Trizol Reagent (Invitrogen). Reverse transcription of 1 μg of total RNA was performed by using M-MLV reverse transcriptase (Promega, Madison, WI). And the products of cDNA were used as a template for PCR. Primers for Tim-3 and β-actin were as follows: Tim-3, forward: 5'-CAG TTC CCT GGT CTT ATG AAT GA-3'; reverse: 5'-TCC GTG GTT AGG GTT CTT GG-3'; β-actin, forward: 5'-TGC GTG ACA TCA AAG AGA AG-3', reverse: 5'-TCC ATA CCC AAG AAG GAA GG-3'. PCR conditions were as follows: 95ºC for 60 s, followed by 30 cycles for Tim-3 or 24 cycles for β-actin at 95ºC for 60 s, 58ºC for 60 s, and 72ºC for 60 s. The PCR products were separated by electrophoresis in 2% agarose gel and ethidium bromide staining. Gels were scanned on GelDoc2000 system (Bio-Rad, Hercules, CA) and the band intensity values were transformed logarithmically. The relative Tim-3 mRNA levels were calculated as ratios of Tim-3 signals over that of the β-actin. Results presented are representative of three individual experiments.

Western blot

The transfected RAW264.7 cells were harvested at indicated time points, washed three times in PBS and incubated in the lysis buffer (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.02% sodium azide, 100 mg/ml PMSF, 1 mg/ml peptin and 1 mg/ml aprotinin) for 30 min on ice. Cell lysates were subjected to SDS-PAGE and Western blot assay with Tim-3 antibody, Tim-1 antibody or actin antibody. Briefly, cell lysates were loaded on 12% gradient polyacrylamide gel and transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was incubated with goat anti-Tim-3 IgG (R&D Systems, Minneapolis, MN), goat anti-Tim-1 IgG (R&D Systems) or goat anti-actin IgG (R&D Systems), respectively, and immunodetection was performed using rabbit-anti-goat IgG labeled with HRP (Sigma-Aldrich, St. Louis, MO). Protein bands were visualized with enhanced chemilluminescent (ECL) reagents (Sigma-Aldrich) and the band intensity values were transformed logarithmically. The relative Tim-3 (or Tim-1) protein levels were calculated as ratios of Tim-3 (or Tim-1) signals over that of the β-actin. Results presented are representative of three individual experiments.

Detection of serum HBV antigens, HBV-DNA, specific antibodies and transaminase activity

Sera from individual mouse were obtained at indicated time points. The levels of HBsAg, HBeAg and anti-HBs were determined with time-resolved immunofluorometric assay (IFMA) kit (Xinbo Biotech, Suzhou, China) according to the supplier's instructions. To eliminate residual plasmid DNA, sera were pre-treated with DNase I (Takara, Ohtsu, Japan) and then proteinase K (Takara). The copy number of serum HBV-DNA was measured with quantitative fluorescence PCR kit (Da-an Gene, Guangzhou, China) according to the manufacturer's instructions and the standard of negative used

was < 10^3 copies/ml. Serum alanine aminotransferase (ALT) activity was measured using the ALT Infinity Reagent (3 V Biotech, Weifang, China).

Immunohistochemical staining

Formalin fixed paraffin-embedded mouse liver tissue sections were stained with anti-HBc polyclonal rabbit antibody (Zsbio, Beijing, China) and HRP-labeled goat-anti-rabbit IgG (Zsbio) to detect HBcAg expression. Positive hepatocytes were observed by light microscopy and the images were recorded by digital camera.

Preparation of hepatic mononuclear cells (HMCs)

HMCs were isolated by metrizamide gradient centrifugation as reported (24). Briefly, the livers were minced, pressed through a 200-gauge stainless steel mesh. RBCs were depleted and then hepatic parenchymal cells were removed by the gradient centrifugation in 40% Percoll (Pharmacia, Uppsala, Sweden). Cells in the bottom were harvested as HMCs.

Flow cytometric analysis

Isolated HMCs were stained with anti-DX5-FITC, anti-Tim-3-PE, anti-CD8-FITC or -Cy5, anti-CD3-Cy5 and anti-CD4-Cy5 monoclonal antibodies (eBioscience, San Diego, CA). Intracellular IFN-γ staining was performed in accordance with the manufacturer's instructions (BD Biosciences, San Diego, CA). To exclude nonspecific binding, respective isotype antibodies (eBioscience) were used as controls. Stained cells were acquired by FACSCalibur (Becton Dickinson, San Jose, CA) and analyzed with WinMDI 2.8 software.

Statistical analysis

The Mann-Whitney *U* test was performed to determine statistical differences between the groups. Values were considered to be significantly different when the *p* value was < 0.05.

Results

Hydrodynamic injection of pcDNA3-1.1HBV leads to viral antigen expression, HBV DNA replication and anti-HBs production in vivo

To study the role of Tim-3 in HBV infection, the plasmid pcDNA3-1.1HBV, which can express HBsAg, HBcAg and HBeAg (20), was injected into Balb/c mice by using a hydrodynamics-based procedure to establish a mouse model of HBV infection (21-23). Similar to natural HBV infection in humans, both HBV antigens (HBsAg, HBeAg) and HBV DNA could be detected in the sera of HBV model mice (Figures 1A-1C). HBV DNA appeared after day 1, peaked on day 7, and became undetectable on day 18 (< 10^3 copies/ml), indicating HBV replication in model mice (Figure 1C). More importantly, the production of neutralizing antibody against HBsAg (anti-HBs) became detectable from day 7 (Figure 1D), accompanying with the decline of HBsAg. Immuno-

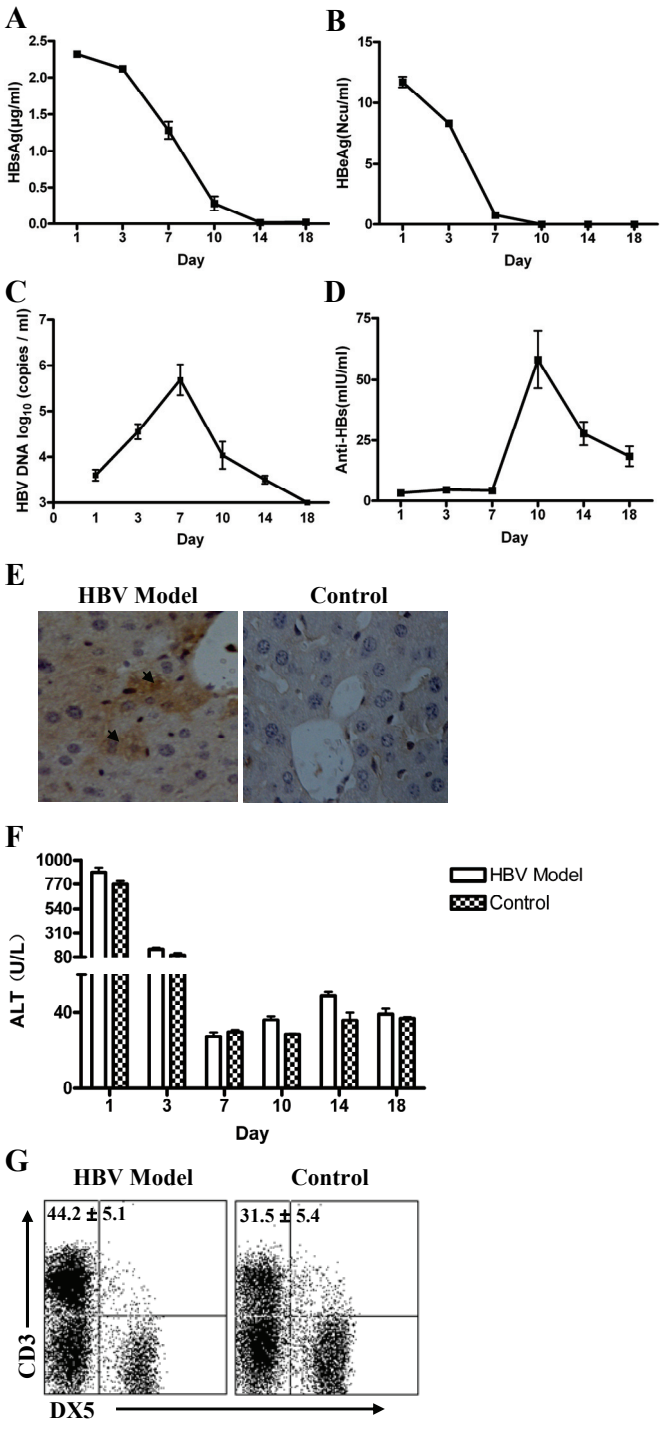

Figure 1. HBV infection in mice was induced by hydrodynamics injection of HBV expression plasmids. Mice were injected hydrodynamically with 50 μg of HBV expression palsmid. Then they were sacrificed at indicated time points to detect serum HBs antigen (A), HBe antigen (B), HBV-DNA (C) and anti-HBs antibody (D). (E) HBc expression was examined by immunohistochemical staining in sections of liver tissue from mice after plasmid injection. $HBcAg^+$ hepatocytes in the liver on day 3 were shown. Original magnification, 40×. (F) ALT activities in the sera. (G) Representative FACS profiles of T cells within hepatic lymphocytes. Data represent mean ± SD from three independent experiments with four to six mice per group.

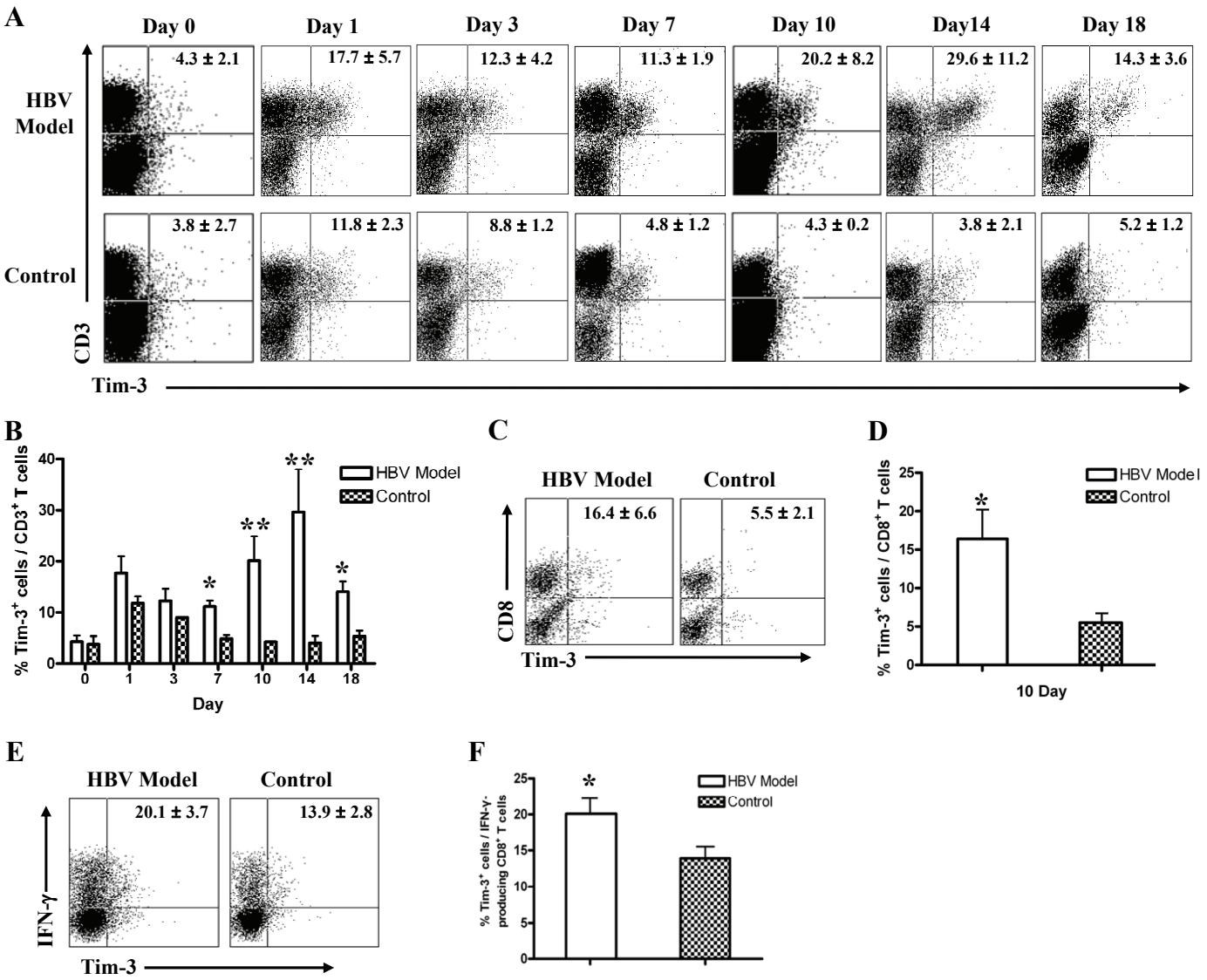

Figure 2. Tim-3 expression is increased on hepatic CD8$^+$ T cells in HBV model mice. (A) Representative FACS profiles of Tim-3 expression on hepatic T cells. HMCs from HBV model mice and control mice at indicated time points were analyzed by flow cytometry. (B) The histograms represent the percent of Tim-3$^+$ cells within hepatic T cells. (C) Representative FACS profiles of Tim-3 expression on CD8$^+$ T cells from HBV model mice and control mice on day 10 post the injection. (D) The histograms represent the percent of Tim-3$^+$ cells within hepatic CD8$^+$ T cells. (E) Representative FACS profiles of Tim-3$^+$ cells in IFN-γ-producing CD8$^+$ T cells from HBV model mice and control mice on day 10 post the injection. (F) The histograms represent the percent of Tim-3$^+$ cells in IFN-γ-producing CD8$^+$ T cells. Data represent mean ± SD from three independent experiments using six to eight mice per group. *$p < 0.05$, **$p < 0.01$, Mann-Whitney U test, compared with control mice.

histochemical staining clearly showed HBcAg$^+$ hepatocytes randomly distributed throughout the liver lobule in HBV model mice (Figure 1E), illustrating HBV expression in hepatocytes. As shown in Figure 1F, ALT activities were slightly increased in both HBV model mice and control mice on days 1 and 3. We also detected similar ALT increase in mice receiving hydrodynamic injection of normal saline (data not shown). Thus, we hypothesized that early ALT increase might result from mechanic injury by hydrodynamic injection but not from HBV infection or plasmid-mediated toxin. However, from day 10 HBV model mice seemed to have higher ALT activities than control mice, but there is no significant difference between two groups (Figure 1F). Accounting for the increased amount of infiltrating T lymphocytes in the liver (Figure 1G), this might suggested the insufficient T cell-mediated inflammation in this model.

Taken together, using hydrodynamics-based procedure, we mimicked the natural HBV chronic infection in mice with persistence of HBV DNA, production of neutralizing antibody and a low level of inflammation.

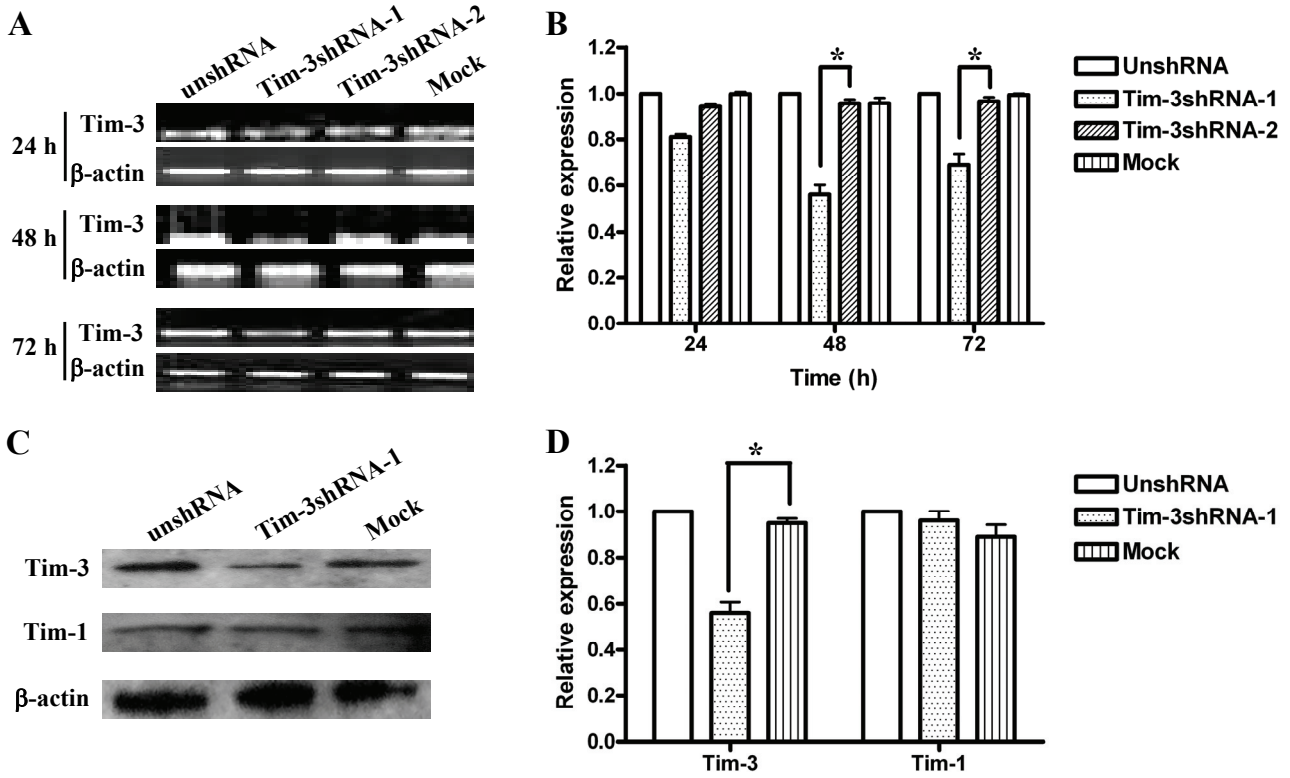

Figure 3. Tim-3 short hairpin RNAs (shRNAs) specifically suppress Tim-3 expression in murine macrophage cell line RAW264.7. The shRNA expression plasmid unshRNA, Tim-3 shRNA-1, Tim-3 shRNA-2 or mock plasmid was transfected into Tim-3- and Tim-1-coexpressing murine macrophage cell line RAW264.7, respectively. (A) Photographs of RT-PCR products obtained using primers specific for Tim-3 and β-actin at indicated time points in a representative experiment. (B) The histograms represent the ratio of Tim-3 to β-actin in mRNA level as assessed with pooled densitometric data. (C) Immunoblotting with antibodies against Tim-3, Tim-1 or β-actin in RAW264.7 cells at 48 h after transfection. (D) The histograms represent the ratio of Tim-3 or Tim-1 to β-actin in protein level as assessed with pooled densitometric data. Data represent mean ± SD from three independent experiments. *$p < 0.05$, Mann-Whitney *U* test, as compared with unshRNA treatment.

Increased Tim-3 expression on hepatic T cells, especially on CD8$^+$ T cells, in HBV model mice

To investigate the role of Tim-3 in HBV infection, Tim-3 expression on HMCs was examined at indicated time points. Flow cytometric analysis showed that Tim-3 was slightly induced on hepatic T cells from both HBV model mice and control mice on days 1 and 3 (Figure 2A and 2B). However, no difference in Tim-3 expression was found in two groups. Similar Tim-3 up-regulation on HMCs was also found in mice receiving hydrodynamic injection of normal saline (data not shown). Thus, we hypothesized that early Tim-3 up-regulation might result from mechanic injury by hydrodynamic injection but not from HBV infection. In contrast, Tim-3 expression was dramatically increased in HBV model mice from day 7 to day 18 (HBV model mice *vs* control, $p < 0.05$ on days 7 and 18, $p < 0.01$ on days 10 and 14) (Figures 2A and 2B). The increase in Tim-3 expression was prominent on CD8$^+$ T cells (Figures 2C and 2D) ($p < 0.05$ on day 10), especially on IFN-γ-producing CD8$^+$ T cells (Figures 2E and 2F) ($p < 0.05$ on day 10). However, we did not detect any change in Tim-3 expression in the spleen (data not shown). This difference may result from HBV infection that might directly trigger unknown factors in liver microenvironment to induce T cells to express high levels of Tim-3. These results suggest that Tim-3 may play a role in the development of HBV infection.

Tim-3 knockdown up-regulates IFN-γ production from CD8$^+$ T cells in HBV model mice

To further study the role of Tim-3 in CD8$^+$ T cell function in HBV model mice, Tim-3 shRNA plasmids were constructed and named Tim-3 shRNA-1 and Tim-3 shRNA-2. The transfection of Tim-3 shRNA-1 suppressed 47.5% of Tim-3 mRNA expression and 44% of Tim-3 protein expression at 48 h after transfection in Tim-3 expressing murine macrophage cell line RAW264.7 compared to control shRNA (unshRNA) ($p < 0.05$) (Figure 3). Considering the fact that non-viral methods for *in vitro* gene transfection of RAW264.7 cells generally provide a low transfection efficiency (< 50%), the level of interference efficency of shRNA in the present method seems relatively high. This suppression was specific because the transfection of Tim-3 shRNA-1 had no inhibitory effect on Tim-1, another Tim family member (Figures 3C and 3D).

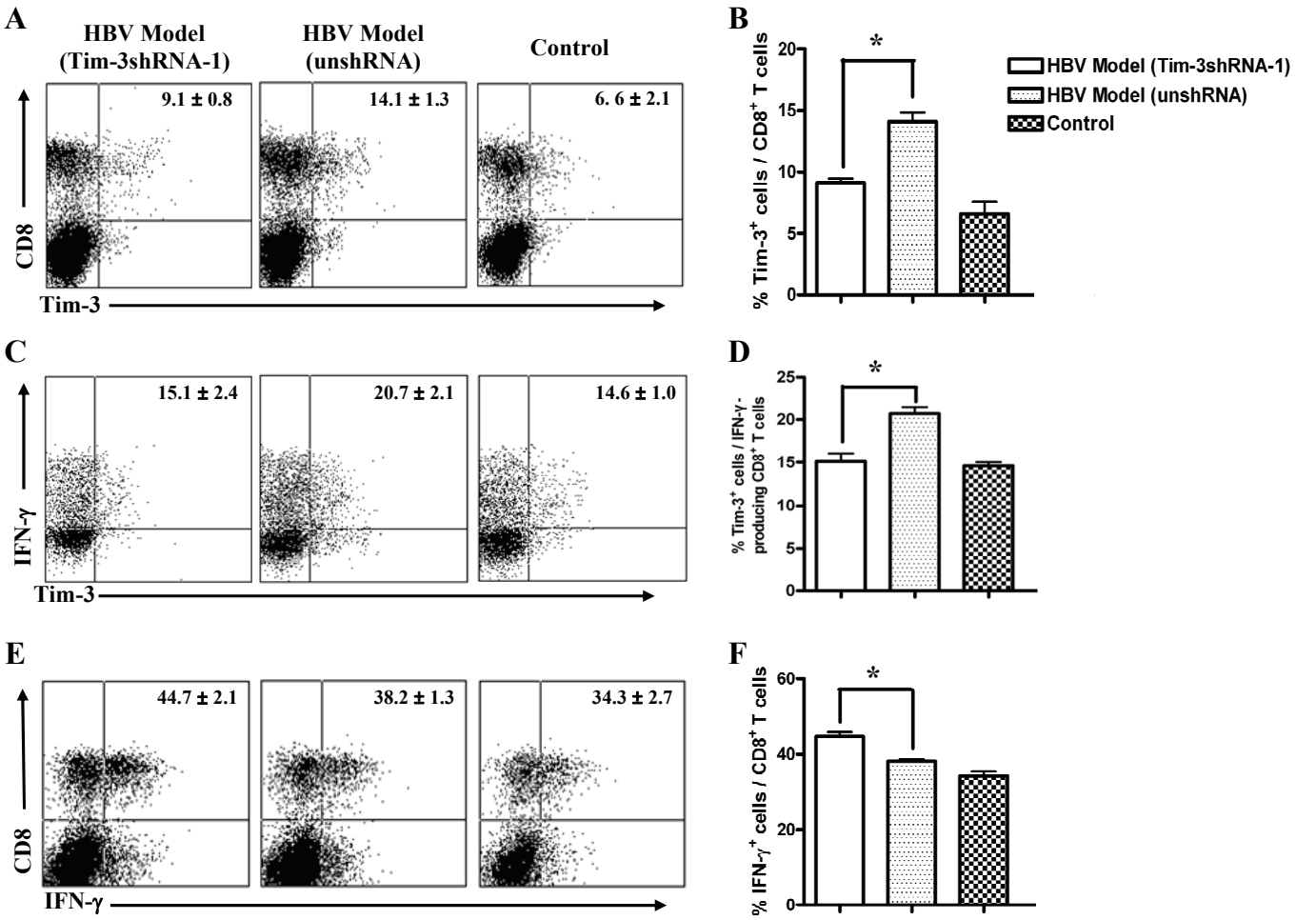

Figure 4. Tim-3 knockdown increases IFN-γ production from hepatic CD8$^+$ T cells *in vivo*. (A) Representative FACS profiles of Tim-3 expression on hepatic CD8$^+$ T cells from mice with shRNA treatment. HMCs were analyzed by flow cytometry at day 10 post the coinjection of pcDNA3-1.1HBV with Tim-3 shRNA-1 or unshRNA. (B) The histograms represent the percent of Tim-3$^+$ cells within hepatic CD8$^+$ T cells. (C) Representative FACS profiles of Tim-3$^+$ cells in IFN-γ-producing CD8$^+$ T cells. (D) The histograms represent the percent of Tim-3$^+$ cells in IFN-γ-producing CD8$^+$ T cells. (E) Representative FACS profiles of IFN-γ production from hepatic CD8$^+$ T cells with shRNA treatment. (F) The histograms represent the percent of IFN-γ$^+$ cells within hepatic CD8$^+$ T cells. Data represent mean ± SD from three independent experiments using six to eight mice per group. *$p < 0.05$, Mann-Whitney *U* test, as compared with unshRNA-treated HBV model mice.

In Tim-3 shRNA-1-treated HBV model mice, Tim-3 up-regulation was reversed on hepatic CD8$^+$ T cells comparable to control mice on day 10, while unrelated shRNA (unshRNA) had no such effects (Tim-3 shRNA-1 *vs* unshRNA, $p < 0.05$; Tim-3 shRNA-1 *vs* control, $p > 0.05$) (Figures 4A and 4B). Previous studies have shown that Tim-3-galectin-9 pathway modulates T cell secretion of IFN-γ (9, 13, 14), which is of particular importance to immunity against viral infection. Consistent with this, our results showed that Tim-3 knockdown *in vivo* significantly decreased the percent of Tim-3 expressing cells in IFN-γ-producing CD8$^+$ T cells (Tim-3 shRNA-1 *vs* unshRNA, $p < 0.05$; Tim-3 shRNA-1 *vs* control, $p > 0.05$) (Figures 4C and 4D) and increased IFN-γ expression on hepatic CD8$^+$ T cells (Tim-3 shRNA-1 *vs* unshRNA, $p < 0.05$) (Figures 4E and 4F). Therefore, our data illustrate that Tim-3 expression suppresses IFN-γ production on CD8$^+$ T cells in HBV model mice.

Relationship between Tim-3 expression and anti-HBs production in HBV model mice

A low increase of ALT activities in HBV model mice indicated a low level of inflammation in liver. However, certain amount of neutralizing antibody against HBsAg could be detected in HBV model mice although antibody titer differed in various mice. HBV model mice were divided into two groups based on the serum anti-HBs levels. A total of 50 mIU/ml was taken as the cutoff value of anti-HBs production since it closes to the average anti-HBs production (58 mIU/ml) of three independent experiments in the study. As shown in Figures 5A and 5B, mice with serum anti-HBs

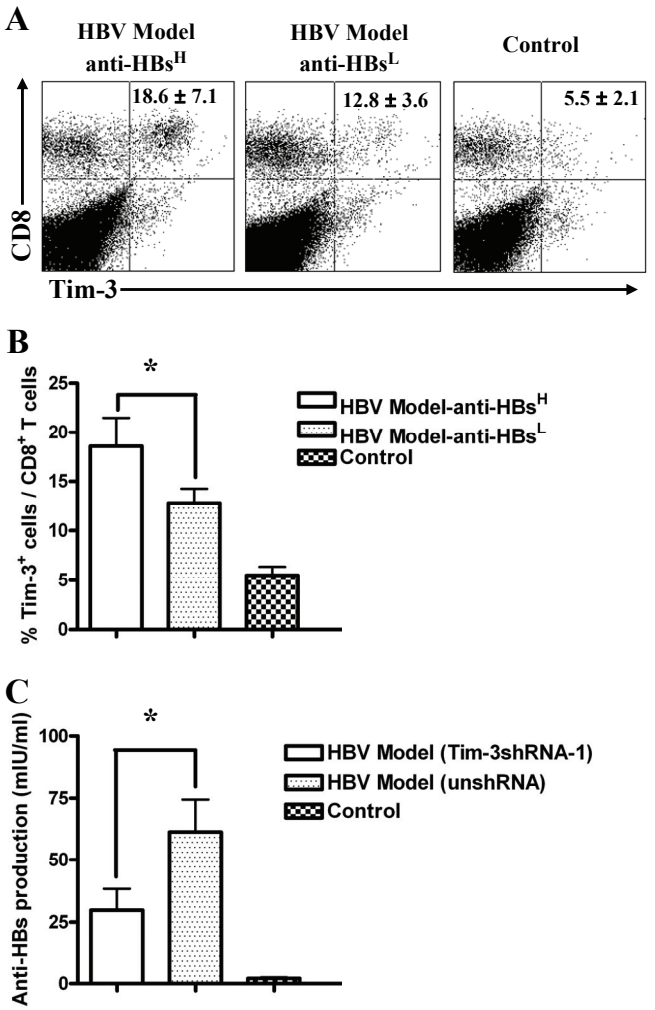

Figure 5. Relationship between Tim-3 expression and anti-HBs production in HBV model mice. (A) Representative FACS profiles of Tim-3 expression on hepatic $CD8^+$ T cells from HBV model mice with higher serum anti-HBs production (HBV model-anti-HBs^H), with low anti-HBs production (HBV model-anti-HBs^L), and control mice on day 10 post the injection. (B) The histograms represent the percent of Tim-3^+ cells within hepatic $CD8^+$ T cells in different group. *$p < 0.05$, Mann-Whitney *U* test, as compared with HBV model-anti-HBs^L. (C) Serum anti-HBs production in HBV model mice with shRNA treatment. Data represent mean ± SD from three independent experiments using six to eight mice per group. *$p < 0.05$, Mann-Whitney *U* test, as compared with unshRNA-treated HBV model mice.

production > 50 mIU/ml (HBV model-anti-HBs^H) had a higher expression of Tim-3 on hepatic $CD8^+$ T cells than mice with serum anti-HBs production < 50 mIU/ml (HBV model-anti-HBs^L) ($p < 0.05$). Next, we detected serum anti-HBs in Tim-3 shRNA-treated mice. It was found that production of serum anti-HBs decreased in Tim-3 shRNA-1-treated HBV model mice compared to that in unshRNA-treated HBV model mice ($p < 0.05$) (Figure 5C). Previous reports have shown that neutralizing antibodies increased in titer when the function of $CD8^+$ T cells is impaired or absent in low-cytopathic virus infection (25-28), indicating the competitive relationship between $CD8^+$ T cell-function and serum anti-HBs. Therefore, our results suggest that Tim-3 might indirectly influence neutralizing antibody production in HBV infection by regulating $CD8^+$ T cell function.

Discussion

Tim-3, as an important immune regulatory molecule, is expressed on effector Th1 cells and Tc1 cells (8-14), both of which play important roles in the immune response to HBV. However, whether Tim-3 plays a role in the development of HBV infection still remains unclear. In the present study, we provide evidence that in HBV model mice Tim-3 expression was up-regulated on hepatic T lymphocytes, especially on $CD8^+$ T cells. As a ligand for Tim-3, galectin-9 is widely distributed in various tissues and is particularly abundant in the liver (29). Previous studies have shown that Tim-3-galectin-9 pathway modulates T cell secretion of IFN-γ (9, 13, 14), which is of particular importance to immunity against viral infection. Consistent with these reports, our data verified the simultaneous staining of Tim-3 and IFN-γ in $CD8^+$ T cells (Figures 2 and 4), which might explain why Tim-3 pathway influences IFN-γ production from $CD8^+$ T cells. Therefore, it is possible that galectin-9 in the liver directly interacts with Tim-3 expressed on $CD8^+$ T cells, and their interactions may suppress immunity of infiltrating, IFN-γ-secreting $CD8^+$ T cells. Accordingly, Tim-3 knock-down might 'rescue' infiltrating HBV-specific T cell function and augment host immunity against HBV infection. The finding is important because it shows, for the first time, the negative roles of Tim-3 in the development of HBV infection by regulating IFN-γ secretion of $CD8^+$ T cells.

With Tim-3 knockdown *in vivo*, increased IFN-γ production from hepatic $CD8^+$ T cells was observed in HBV model mice. This is particularly interesting in light of a recent report suggesting that IFN-γ acts directly on $CD8^+$ T cells to increase their abundance during viral infection (30). Consistent with this report, our data show that with IFN-γ increase mediated by Tim-3 knockdown, $CD8^+$ T cells accumulate in liver (22.87 ± 4.12% *vs* 16.38 ± 3.29%) (data from flow cytometric analysis of HMCs). Thus, it is possible that the effect of Tim-3 knockdown on IFN-γ production by $CD8^+$ T cells indirectly enhances $CD8^+$ T cells accumulation in liver, which should result in a corresponding increase in liver damage and, potentially, the control of HBV infection. However, we failed to detect any significant changes of ALT activities and HBV DNA titers in Tim-3 shRNA-treated mice, probably because of insufficient recovery of IFN-γ by the hydrodynamics-based transfection or there are other negative regulators, which we have not known yet.

Similar to a natural infection, hydrodynamics-based HBV model mice produced high levels of antigen and antibody in the sera. Interestingly, a good relationship between serum anti-HBs and Tim-3 expression on $CD8^+$ T was found in our HBV model mice (Figure 5). Studies have shown that, in low-cytopathic virus infection, neutralizing antibodies

increase in titer when $CD8^+$ T cell function is impaired or absent (25-28). This competitive coexistence may be beneficial to the host by averting a combination of strong cytotoxicity and antibody responses that may favor immunopathology (31, 32). Recent study has shown that Tim-3 upregulation in HIV infection might be the result of a physiological response to chronic immune activation necessary to hold $CD8^+$ T cell-dependent immunopathology in check (13), indicating that Tim-3 might indirectly affect neutralizing antibody production by regulating $CD8^+$ T cell function. However, the detailed mechanism needs to be further studied.

In conclusion, we have highlighted for the first time the role of Tim-3 in hepatic $CD8^+$ T cells during HBV infection. Our results suggest that in HBV infection Tim-3 may act as a potent regulator of hepatic $CD8^+$ T cells and indirectly affect neutralizing antibody production by regulating $CD8^+$ T cell function. But the deliberate suppression roles in immune response and the therapeutic potency of Tim-3 may be worth careful studying.

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