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Regulation of HBV-specific CD8$^+$ T cell-mediated inflammation is diversified in different clinical presentations of HBV infection

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Chronic HBV infection is the leading cause of liver cirrhosis and hepatic cancer, but the individual responses toward HBV infection are highly variable, ranging from asymptomatic to chronic active hepatitis B inflammation. In this study, we hypothesized that the different individual responses to HBV infection was associated with differences in HBV-specific CD8$^+$ T cell-mediated inflammation and cytotoxicity. Blood samples were collected from subjects with asymptomatic HBV-infection, subjects undergoing active chronic HBV flares (active CHB), and subjects with HBV-infected hepatocellular carcinoma (HBV-HCC). By tetramer staining, we found that all three groups had similar frequencies of HBV-specific CD8$^+$ T cells. However, after HBV peptide stimulation, the HBV-specific CD8$^+$ T cells in asymptomatic subjects had significantly stronger interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and CD107a expression than those in active CHB and HBV-HCC patients. Examination of surface marker expression revealed that the PD-1$^-$Tim-3$^-$ double-negative cell population was the main contributor to HBV-specific inflammation. In active CHB patients and HBV-HCC patients, however, the frequencies of activated PD-1$^-$Tim-3$^-$ cells were significantly reduced. Moreover, the serum HBV DNA titer was not correlated with the frequencies of HBV-specific CD8$^+$ T cells but was inversely correlated with the frequencies of IFN-g-expressing and CD107a-express cells in response to HBV stimulation. Together, our data demonstrated that the status of HBV-specific CD8$^+$ T cell exhaustion was associated with different clinical outcomes of chronic HBV infection.

Keywords: Tim-3, PD-1, HBV, HCC

Introduction

Chronic active hepatitis B virus (HBV) infection is a major risk factor for the induction of hepatocellular carcinoma (HCC) (Kremsdorf *et al.*, 2006). Over 2 billion people worldwide were exposed to HBV at some point of their lives, and about 350 million people are currently living with chronic HBV infection (Kao and Chen, 2002). While no specific treatment is available for acute infection of HBV, some people can spontaneously clear the virus while others become chronically infected (Publicover *et al.*, 2011). Within the chronically infected group, the virus may remain quiescent with undetectable serum HBV DNA titer, or becomes detectable in the serum and initiates persistent immune responses. The reasons for the variety of clinical presentations in acute and chronic HBV infection are not completely understood, but several lines of evidence suggest that the participation of the immune system is an important driving force in modulating HBV activity (Rehermann *et al.*, 1996; Rehermann and Nascimbeni, 2005). People who spontaneously recover from acute HBV infections typically mounted more robust, multi-epitope-specific CD4$^+$ and CD8$^+$ T cell responses than patients who progress into active chronic hepatitis B diseases (Ferrari *et al.*, 1993; Rehermann *et al.*, 1995). Restoration of HBV-specific CD4$^+$ and CD8$^+$ T cell responses in the blood of chronic hepatitis B patients resulted in clinically effective reduction of HBV DNA titer (Boni *et al.*, 2003). HBV-specific CD8$^+$ T cells also mediated virus clearance during acute infection (Thimme *et al.*, 2003). Therefore, we hypothesized that the different clinical presentations of chronic HBV infection, ranging from asymptomatic to severe cirrhosis and liver cancer, might be associated with different HBV-specific T cell responses in different individuals.

CD8$^+$ T cells are widely recognized as the main virus clearance mechanism in acute HBV infections, through the production of proinflammatory cytokines interferon-gamma

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(IFN-γ) and tumor-necrosis factor alpha (TNF-α) to initiate and modulate immune responses, and through the release of granzymes and perforin to initiate killing of virus-infected cells (Phillips *et al.*, 2010). CD107a, a lysosome-associated membrane glycoprotein, is expressed on the cell surface during the release of cytotoxic granule contents (Betts *et al.*, 2003; Alter *et al.*, 2004). In chronic virus infections, $CD8^+$ T cells suffer from a progressive loss of function in a mechanism termed T cell exhaustion (Wherry *et al.*, 2007; Ha *et al.*, 2008; Wirth *et al.*, 2010; Wherry, 2011). These exhausted $CD8^+$ T cells express high levels of programmed death 1 (PD-1) (Virgin *et al.*, 2009), a CD28 family costimulatory/coinhibitory molecule that promotes apoptosis in antigen-specific T cells while reduces regulatory T cell apoptosis (Francisco *et al.*, 2010; Fife and Pauken, 2011), and T cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3) (Jones *et al.*, 2008; Golden-Mason *et al.*, 2009), a negative regulator of adaptive T cell responses (Anderson, 2012; Moorman *et al.*, 2012). It was previously demonstrated that Tim-3 expression and signaling resulted in impaired HBV-specific $CD8^+$ T cell function in chronic HBV-infected patients (Nebbia *et al.*, 2012). The PD-1 and/or Tim-3 signaling pathway mediated T cell dysfunction were also directly associated with disease progression in hepatitis B (Wu *et al.*, 2011; Wang *et al.*, 2014) and poor prognosis in hepatocellular carcinoma (Li *et al.*, 2012). These previous studies suggested that $CD8^+$ T cell exhaustion in chronic active hepatitis B patients led to the impaired control of HBV, but the status of HBV-specific $CD8^+$ T cell exhaustion in asymptomatic HBV-infected patients and HBV-induced hepatocellular carcinoma patients has not been examined.

In this study, we postulated that the wide-ranging clinical presentations of HBV infection, from asymptomatic infection to chronic active hepatitis B and HBV-induced hepatocellular carcinoma, were associated with differences in HBV-specific $CD8^+$ T cell-mediated inflammation and cytotoxicity.

Materials and Methods

Patients and samples

Peripheral blood samples from 45 chronic HBV-infected patients were collected from Linyi Hospital, in which 15 subjects were HBV-infected asymptomatic patients, 15 subjects were undergoing active chronic HBV flares (active CHB), and 15 subjects were HBV-infected hepatocellular carcinoma (HBV-HCC). The diagnosis of the patients was according to the previously described criteria (Lok and McMahon, 2007). All patients were negative for HCV and HIV antibodies. Relevant clinical and demographic information are shown in Table 1. All patients gave written informed consent and all study procedures were approved by the ethics committee of Linyi Hospital. Peripheral blood mononuclear cells (PBMCs) were obtained from blood by standard Ficoll-Hypaque centrifugation. Serum HBV DNA levels were measured by the Bayer Versant HBV DNA 1.0 Assay at the date of sample collection. HLA typing were done using A*02 SSP UniTray Kit (Invitrogen).

Tetramer staining and flow cytometry

Soluble HLA-A2-peptide tetramers were synthesized as previously described (Altman *et al.*, 1996; Maini *et al.*, 2000). Briefly, recombinant HLA-A2 heavy chains and β2-microglobulin were produced in *E. coli*, and complexes were folded in vitro using HLA-A2 protein, β2-microglobulin, and synthetic peptide at 6:5:2 ratio. A previously used panel of HLA-A2 restricted peptides was purchased from Chiron Mimotopes and was used in this study (Boni *et al.*, 2007). The HLA-A2-peptide complex was then biotinylated and tetramerized with streptavidin-PE as previously described (Maini *et al.*, 2000).

A total of 5×10^5 PBMCs were incubated for 30 min at 37°C with 1 μg tetramer complex in 200 μl culture media (RPMI 1640 supplemented with 10% FBS, L-glutamine, and Pen Strep), and then washed twice with staining buffer (PBS supplemented with 2% FBS). Cells were then incubated with Fixable Violet dead cell stain (Invitrogen), and fluorescence-conjugated anti-human CD3, CD4, CD8, PD-1 (BD), and Tim-3 (R&D) antibodies for 30 min at 4°C. Samples were measured by FACSCanto II flow cytometer (BD) and analyzed by FlowJo software (Tree Star).

HBV-specific T cell stimulation

PBMCs were resuspended at 10^6 cells per 200 μl in culture media for 7 days with or without 10 μg/ml HBV peptide at 37°C and 5% CO_2. Ten μg/ml HBV peptide, CD107a antibody (BD) and brefeldin A (Sigma-Aldrich) were added to the culture 6 h prior to harvest. Cells were washed and stained with HBV tetramer and surface antibodies as described above, and then stained with IFN-γ and TNF-α antibodies using permeabilization and fixation solutions A and B (Caltag) according to the manufacturer's protocol.

Table 1. Characteristics of the study population

	Asymptomatic	Active CHB	HBV-HCC	P
n	15	15	15	
Age, yr	38 (22–71)	44 (25–62)	51 (38–72)	> 0.05
Gender (m/f)	8/7	9/6	10/5	> 0.05
Serum HBV DNA, IU/ml	< 300 (undetectable)	2×10^5 ($1500–3\times10^7$)	7×10^4 ($2200–2\times10^7$)	> 0.05*
HBsAg+	15	15	15	-
HBeAg+	0	12	13	-
HBeAb+	15	10	9	-
Cirrhosis (N/Y)	15/0	14/1	13/2	> 0.05

All numerical data were expressed as median (range). *Comparison between active CHB and HBV-HCC.

Statistics

Two-tailed ANOVA analysis followed by Tukey's post-test was used for single and multiple comparisons between more than two groups. Pearson correlation coefficient was calculated for correlation analyses. $P < 0.05$ was considered statistically significant. Prism 6 software (GraphPad) was used.

Results

HBV-specific T cells in study subjects were comparable across different groups

Relevant clinical and demographic information of the patients are shown in Table 1. No significant differences in age and sex were observed between all three study groups, and no significant differences in serum HBV DNA titer were observed between active CHB subjects and HBV-HCC subjects (Table 1). All subjects were HLA-A*02-positive.

We first examined the frequencies of HBV-specific $CD8^+$ T cells by HLA-A2-restricted HBV tetramer staining (Fig. 1A). $Tetramer^+$ T cells were identified as HBV-specific cells, and were observed in all patients, including the asymptomatic patients with no detectable viral load (Fig. 1B). No significant differences in the frequencies of $Tetramer^+CD8^+$ T cells between study groups were observed. To examine the state of exhaustion in HBV-specific cells, we then examined the expressions of PD-1 and Tim-3 on the $Tetramer^+CD8^+$ T cells. Active CHB patients and HBV-HCC patients had significantly higher frequencies of $PD\text{-}1^+$ single-positive cells and $PD\text{-}1^+Tim\text{-}3^+$ double-positive cells within the $Tetramer^+CD8^+$ T cell population, compared to asymptomatic subjects (Fig. 1C and E). HBV-HCC patients also had significantly higher frequencies of $Tim\text{-}3^+$ single positive cells in the $Tetramer^+CD8^+$ T cell population, than asymptomatic subjects and CHB patients (Fig. 1D). Together, these data demonstrated that although the overall frequencies of HBV-specific $CD8^+$ T cells were comparable between all groups, those in active CHB and HBV-HCC patients expressed higher levels of exhaustion markers.

Active CHB and HBV-HCC patients had significantly reduced levels of HBV-specific $CD8^+$ T cell inflammation

Next, we examined $CD8^+$ T cell responses to HBV-specific antigen stimulation. Peripheral blood mononuclear cells from study subjects were stimulated with HBV peptide pool. The expressions of antiviral cytokines IFN-γ, TNF-α, and degranulation marker CD107a were examined (Fig. 2A). We

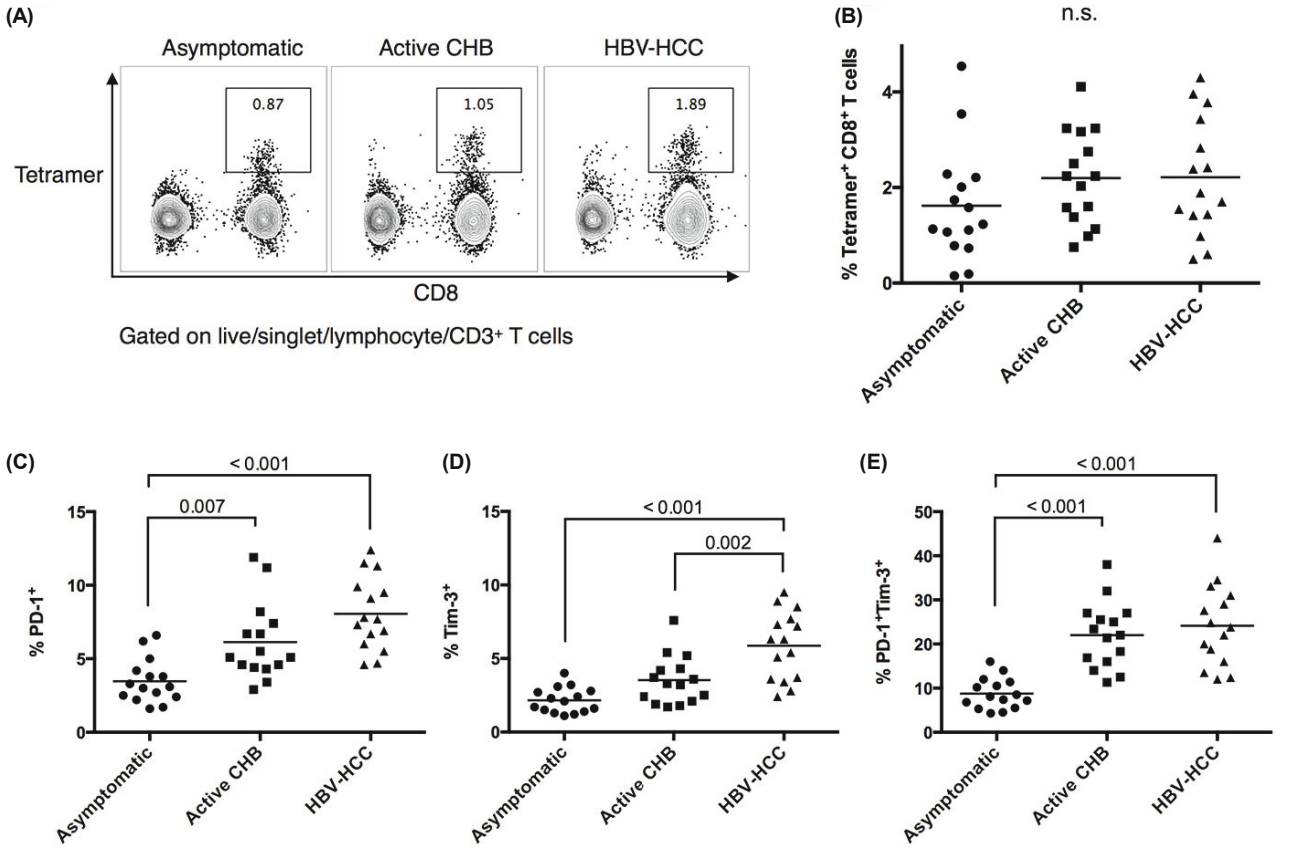

Fig. 1. HBV-specific $CD8^+$ T cells in active CHB and HBV-HCC patients exhibited elevated expression of exhaustion markers. (A) Example Tetramer-HBV peptide complex staining in one of each of asymptomatic, active CHB, and HBV-HCC subjects. Cells shown were gated on live $CD3^+$ T cells. (B) Summary of the frequencies of HBV-specific $CD8^+$ T cells, represented by the percentage of $Tetramer^+$ cells in total $CD8^+$ T cells, in all asymptomatic, active CHB, and HBV-HCC subjects. (C) Frequencies of $PD\text{-}1^+$ single-positive cells in $Tetramer^+CD8^+$ T cells in all study subjects. (D) Frequencies of $Tim\text{-}3^+$ single-positive cells in $Tetramer^+CD8^+$ T cells in all study subjects. (E) Frequencies of $PD\text{-}1^+Tim\text{-}3^+$ double-positive cells in $Tetramer^+CD8^+$ T cells in all study subjects. One-way ANOVA and Tukey's post-test. Numbers indicate *P* value. n.s.: not significant.

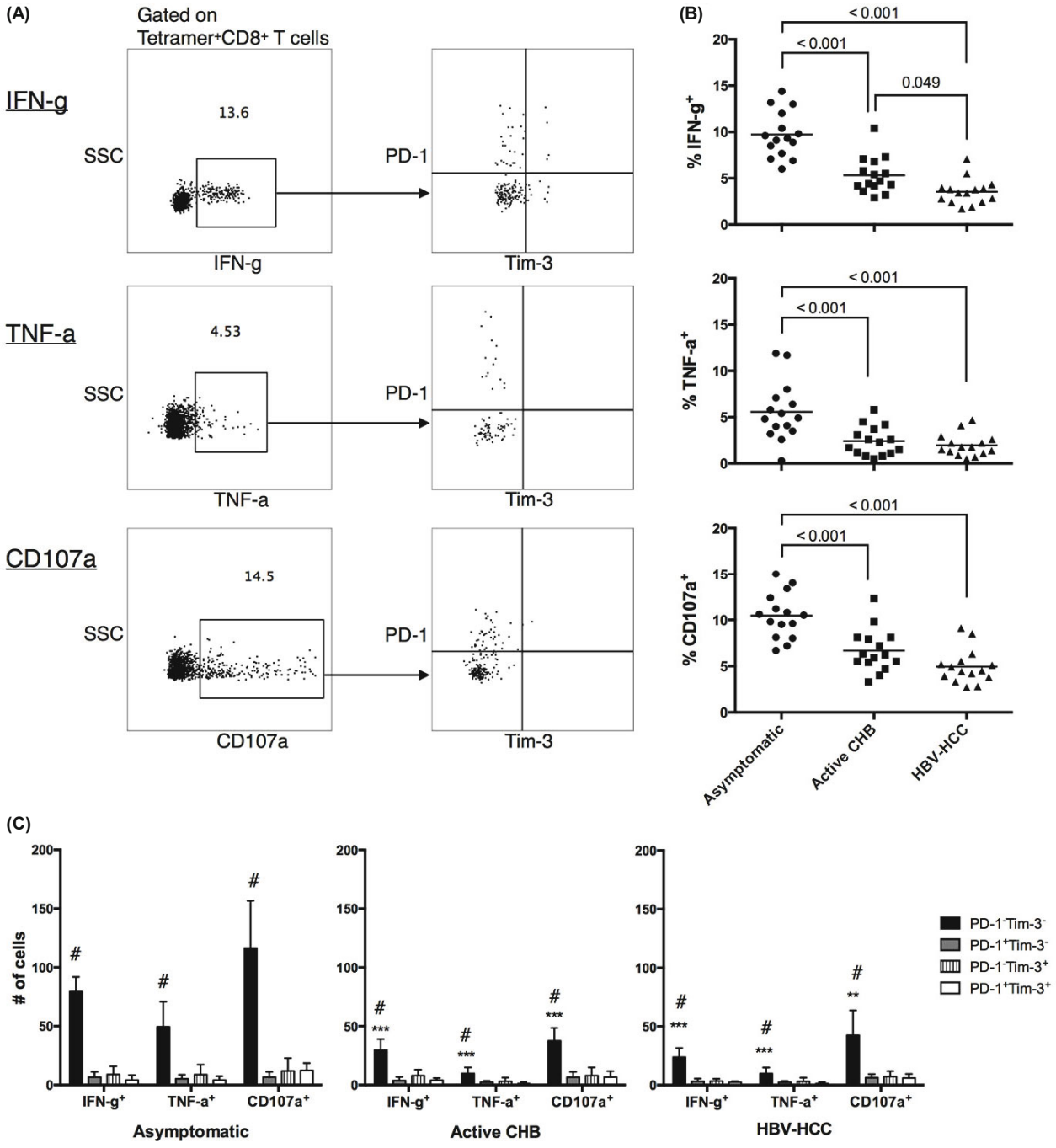

Fig. 2. HBV-specific T cells in active CHB and HBV-HCC patients had reduced IFN-γ-, TNF-α-, and CD107a-expression after HBV stimulation. (A) The expressions of IFN-γ, TNF-α and CD107a in HBV-specific $CD8^+$ T cells after HBV stimulation are shown in the left panels, and the expressions of PD-1 and Tim-3 in IFN-γ^+, TNF-α^+, and $CD107a^+$ cells are shown in the right panels. Figures shown were obtained from an asymptomatic individual. (B) Summary of the frequencies of IFN-γ^+, TNF-α^+, and $CD107a^+$ cells in $Tetramer^+CD8^+$ T cells after HBV-stimulation, in all asymptomatic, active CHB, and HBV-HCC patients. One-way ANOVA and Tukey's post-test. Numbers indicate P value. (C) The composition of IFN-γ^+, TNF-α^+, and $CD107a^+$ cells, as defined by PD-1 and Tim-3 expression. The Mean ± SD of all subjects was shown. Two-way ANOVA and Tukey's post-test. Pound sign (#) indicate that there is significant difference between the number of cells in the PD-1^-Tim-3^- fraction and that in other fractions ($P < 0.001$ for all). Asterisks (*) indicate that there is significant difference between the number of cells in the active CHB or HBV-HCC cohort and that in the asymptomatic cohort. $^{**}P < 0.01$. $^{***}P < 0.001$.

found that active CHB and HBV-HCC patients had significantly reduced frequencies of IFN-γ^+, TNF-α^+, and $CD107a^+$ cells after stimulation, compared to that in asymptomatic individuals (Fig. 2B). The expression of IFN-γ in HBV-HCC patients was further reduced from that in active CHB patients.

Reduction in HBV-specific CD8$^+$ T cell inflammation is due to the decrease of activated PD-1$^-$Tim-3$^-$ double negative T cells

Previous work on HCV showed that PD-1$^+$Tim-3$^+$ T cells had reduced IFN-γ, TNF-α, and CD107a production (McMahan *et al.*, 2010). We wondered whether the decrease in HBV-specific CD8$^+$ T cell activation in active CHB and HBV-HCC patients was due to the increased frequencies of PD-1$^+$ and Tim-3$^+$ cells in the HBV-specific compartment. The numbers of IFN-γ$^+$, TNF-α$^+$, and CD107a$^+$ cells in each of the PD-1$^-$Tim-3$^-$ double-negative, PD-1$^+$Tim-3$^-$ single-positive, PD-1$^-$Tim-3$^+$ single-positive, and PD-1$^+$Tim-3$^+$ double-positive compartment were enumerated. We found that in all three study groups, the PD-1$^-$Tim-3$^-$ double-negative cells were the main contributor to HBV-specific IFN-γ, TNF-α, and CD107a expression, since the vast majority of cytokine and degranulation marker-expressing cells were concentrated in the PD-1$^-$Tim-3$^-$ double-negative compartment (Fig. 2C). In active CHB and HBV-HCC patients, the numbers of IFN-γ-, TNF-α-, and CD107a-expressing PD-1$^-$Tim-3$^-$ double negative cells were significantly reduced compared to that in asymptomatic patients. PD-1$^+$Tim-3$^-$ single positive, PD-1$^-$Tim-3$^+$ single positive, and PD-1$^+$Tim-3$^+$ double positive cells were not a major contributor of HBV-specific CD8$^+$ T cell response in any group.

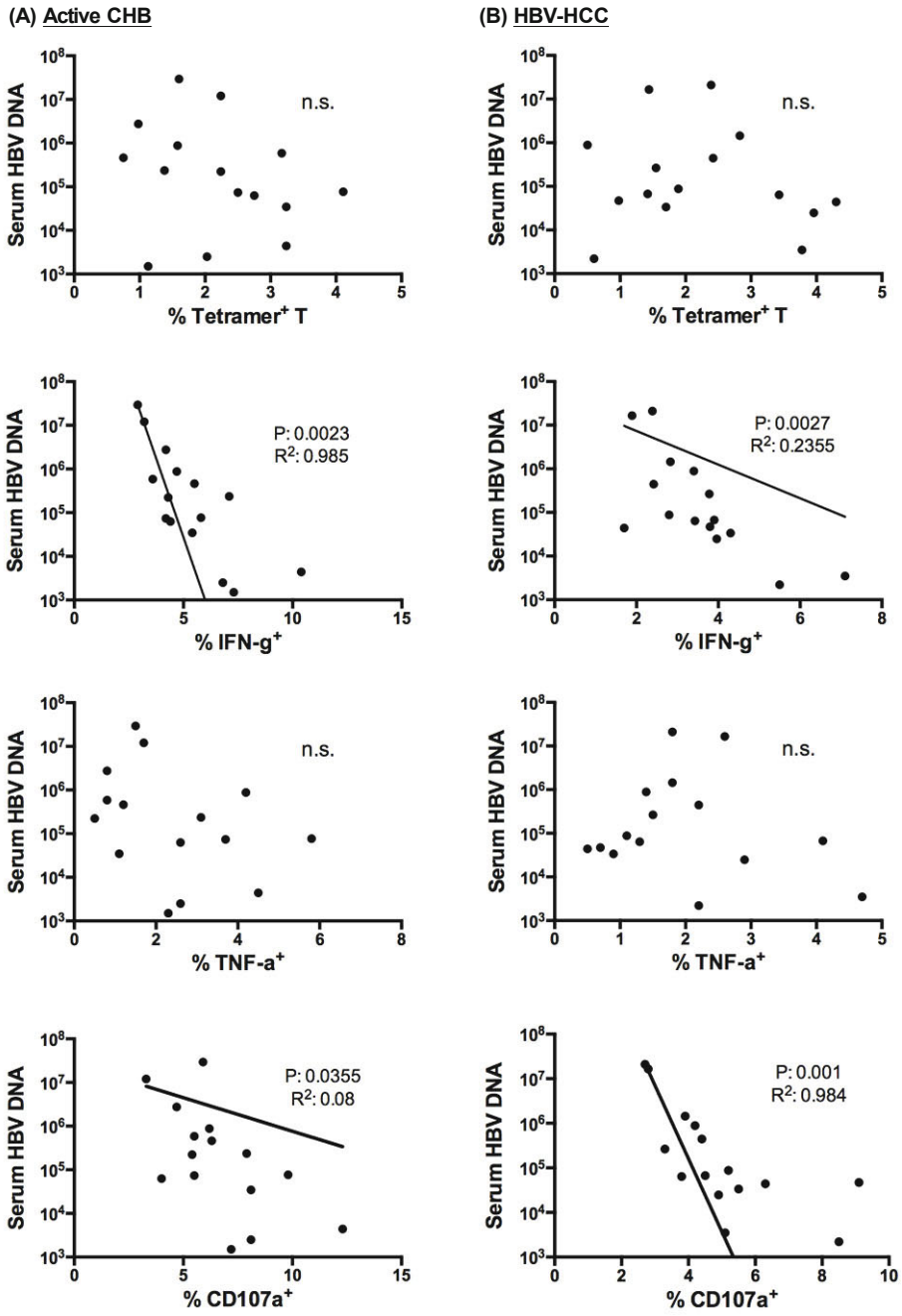

Fig. 3. Correlations between serum HBV DNA titer and HBV-specific immune response parameters. The correlations between serum HBV DNA titer and each of the four HBV-specific immune response parameters, including the percentages of total Tetramer$^+$CD8$^+$ T cells and the frequencies of IFN-γ$^+$, TNF-α$^+$, and CD107a$^+$ cells after HBV stimulation, in (A) active CHB and (B) HBV-HCC patients. Serum HBV DNA level is inversely correlated with the frequencies of IFN-γ- and CD107a-expressing cells after HBV-stimulation, but not with the frequencies of total HBV-specific CD8$^+$ T cells and TNF-α-expressing cells. *P* values indicate Pearson correlation coefficient. n.s.: not significant.

HBV-specific IFN-γ- and CD107a-expression were inversely correlated with disease progression

Serum HBV DNA level by itself is a strong indicator for the risk of hepatocellular carcinoma development, independent of alcohol consumption, cigarette smoking, HBeAg status, serum alanine aminotransferase level, and liver cirrhosis (Chen, 2006). Its modulation also overlaps with serum ALT activity, an indicator of liver damage (Rehermann and Nascimbeni, 2005). Hence, we examined the correlation between HBV-specific CD8$^+$ T cell responses and serum HBV DNA titer in active CHB and HBV-HCC subjects. We found that the serum HBV DNA titer in active CHB and HBV-HCC was not correlated with the frequencies of Tetramer$^+$CD8$^+$ T cells, but was inversely correlated with the frequencies of IFN-γ-expressing and CD107a-expressing T cells in the Tetramer$^+$CD8$^+$ T cell compartment (Fig. 3).

Discussion

Chronic HBV infection is a severe disease with highly variable clinical exhibitions. While a subset of chronic HBV-infected patients may control the infection and remain asymptomatic for a very long period of time, others with active chronic infection will develop severe liver damage and cirrhosis, with increased risk of liver cancer (Kao and Chen, 2002; Chen, 2006; Kremsdorf *et al.*, 2006; Publicover *et al.*, 2011). To obtain insight in how CD8$^+$ T cell-mediated virus control might affect the clinical outcome, we recruited three chronic HBV-infected groups of patients with distinct clinical progressions, and examined their HBV-specific responses. We observed that although asymptomatic subjects had no detectable serum HBV DNA copies, their HBV-specific CD8$^+$ T cell population was maintained in PBMCs at a comparable frequency with that in active CHB and HBV-HCC patients. When stimulated with HBV peptide, the HBV-specific CD8$^+$ T cells in asymptomatic subjects mounted strong IFN-γ, TNF-α, and CD107a responses, which mainly attributed to the PD-1$^-$Tim-3$^-$ double-negative cell population. On the other hand, in active CHB and HBV-HCC patients, the expressions of PD-1 and Tim-3 were significantly upregulated on HBV-specific CD8$^+$ T cells. When stimulated with HBV peptide, the HBV-specific CD8$^+$ T cells in active CHB and HBV-HCC patients exhibited reduced IFN-γ, TNF-α, and CD107a expression compared to those in asymptomatic patients, due to the loss of PD-1$^-$Tim-3$^-$ double-negative cells and the upregulation of PD-1 and Tim-3. Moreover, using serum HBV DNA level as an indicator of disease progression, we found that the severity of disease was not directly correlated with the frequencies of total HBV-specific CD8$^+$ T cells, but rather, it was inversely correlated with the level of HBV-specific IFN-γ and CD107a responses. Together, our data revealed that although active CHB and HBV-HCC patients had similar frequencies of HBV-specific CD8$^+$ T cells with asymptomatic subjects, their HBV-specific CD8$^+$ T cells expressed higher levels of T cell exhaustion markers and had impaired function in response to HBV stimulation. Previously, the breadth of the HBV epitopes covered by T cell receptors was associated with clinical outcomes (Ferrari *et al.*, 1993; Rehermann *et al.*, 1995). Here, we discovered an additional layer of regulation associated with the clinical outcome of chronic HBV infection.

When discussing HBV-specific CD8$^+$ T cell responses, it is important to note that diverse classes of molecules, such as food metabolites, toxic molecules, and pathogenic antigens, are passed through the liver. A strong immune response targeted toward a liver-expressing antigen is considered the main contributor to liver damage (Protzer *et al.*, 2012). As a result, the liver has evolved multiple mechanisms to prevent such a response. Antigens presented by liver sinusoidal endothelial cells (LSECs) and hepatocytes induces immune tolerance (Thomson and Knolle, 2010). Amino acid digestion enzymes are expressed highly in the liver and can deprive immune cells of essential nutrients required for proliferation and degranulation (Popov *et al.*, 2006). Local IL-10-producing cells and regulatory T cells prevent expansion of effector T cells (Knolle *et al.*, 1995). Moreover, PD-1 ligand 1 (PD-L1) is expressed by liver-resident Kupffer cells, LSECs, stellate cells, and hepatocytes (Iwai *et al.*, 2003; Diehl *et al.*, 2007). Kupffer cells also express galectin-9, a Tim-3 ligand (Mengshol *et al.*, 2010). Our discovery that HBV-specific CD8$^+$ T cells could be maintained in the absence of serum HBV load and were able to mount strong HBV-specific responses suggests that HBV infection does not directly induce T cell exhaustion. Whether there may be individual differences in liver-induced immunotolerance, and how that may impact the HBV-specific CD8$^+$ T cell function, ultimately leading to different clinical presentations in different individuals would require further studies.

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