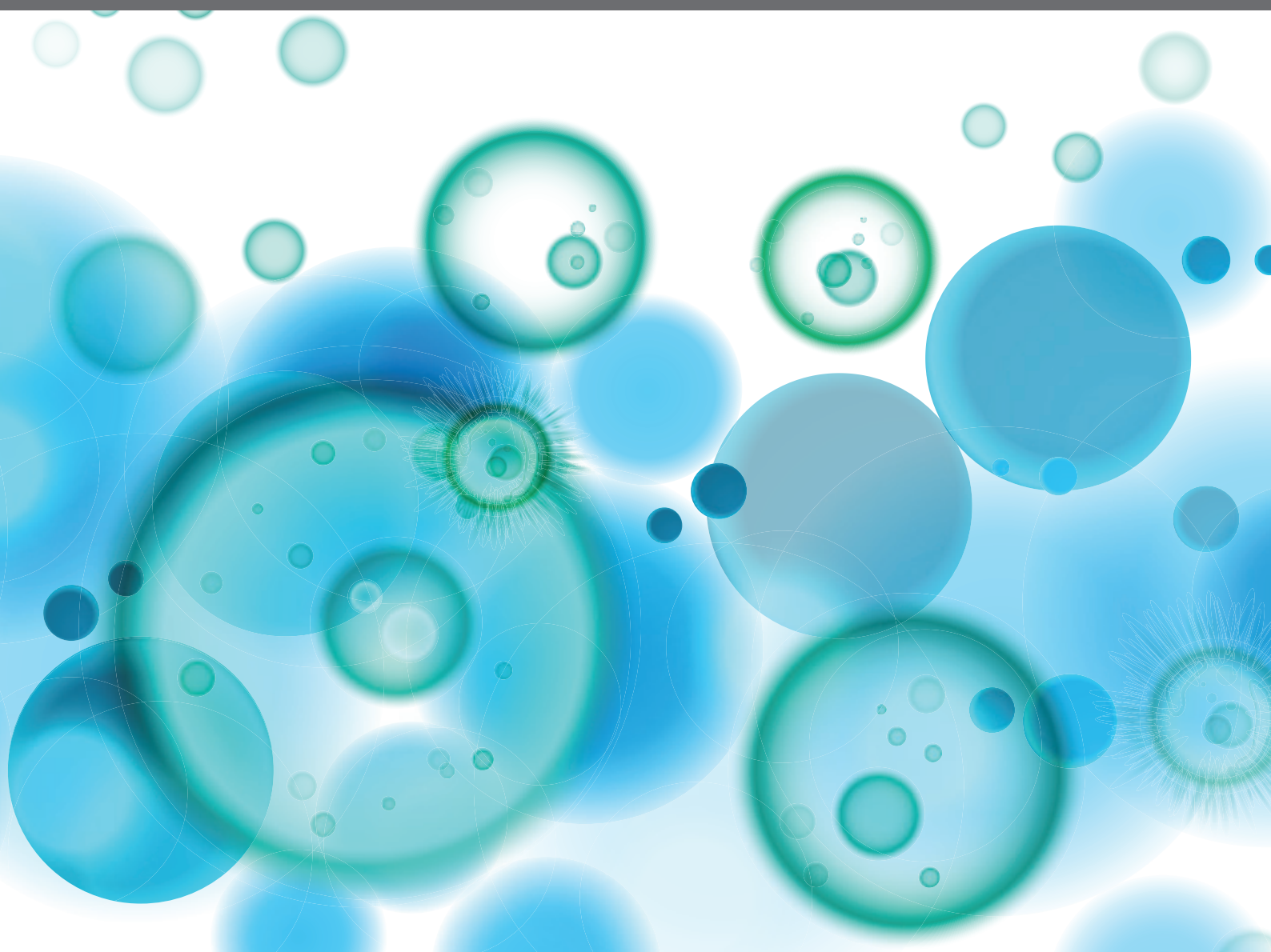


NOVEL THERAPEUTIC STRATEGIES FOR CHRONIC HBV INFECTION: AN IMMUNOLOGICAL PERSPECTIVE

EDITED BY: Seung Kew Yoon, Takanobu Kato, Yuan Quan, Fausto Baldanti
and Tomasz I. Michalak

PUBLISHED IN: Frontiers in Immunology





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ISSN 1664-8714

ISBN 978-2-88963-938-0

DOI 10.3389/978-2-88963-938-0

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NOVEL THERAPEUTIC STRATEGIES FOR CHRONIC HBV INFECTION: AN IMMUNOLOGICAL PERSPECTIVE

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Chronic hepatitis B (CHB) is a life-threatening liver disease affecting 257 million people worldwide, in particular in the Asia-Pacific regions. In endemic areas, hepatitis B virus (HBV) is usually transmitted from chronically infected mothers to neonates. Perinatal HBV infection causes chronic infection in more than 90% of exposed individuals. With perinatal infection, lifetime mortality risk due to complications of liver cirrhosis (LC) or hepatocellular carcinoma (HCC) reaches up to 40% in men and 15% in women.

For the treatment of chronic HBV infection, nucleos(t)ide analogue antivirals have been successfully used to suppress viral replication. However, HBV exists as a cccDNA, which cannot be eliminated by nucleos(t)ide analogues. Therefore, a practical goal of novel HBV therapeutics can be HBs seroconversion (loss of HBsAg and development of HBsAg-specific antibodies), which occurs during spontaneous recovery from acute HBV infection. This HBs seroconversion is referred to as “functional cure” of HBV infection. When functional cure is reached, HBsAg-specific antibodies have virus-neutralizing activity and control HBV infection even in the presence of cccDNA. Currently, peg-IFN- α is often used to induce HBs seroconversion in patients with chronic HBV infection; however, the efficacy is not satisfactory. In future, other immunological therapeutics must be considered to achieve HBs seroconversion, including therapeutic vaccines and immune checkpoint blockers.

Citation: Yoon, S. K., Kato, T., Quan, Y., Baldanti, F., Michalak, T. I., eds. (2020). Novel Therapeutic Strategies for Chronic HBV Infection: an Immunological Perspective. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-938-0

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Ex vivo Detection and Characterization of Hepatitis B Virus-Specific CD8⁺ T Cells in Patients Considered Immune Tolerant

Pil Soo Sung^{1,2†}, Dong Jun Park^{2†}, Jung-Hee Kim², Ji Won Han³, Eun Byul Lee², Gil Won Lee², Hee Chul Nam^{2,4}, Jeong Won Jang^{2,4}, Si Hyun Bae^{1,2}, Jong Young Choi^{2,4}, Eui-Cheol Shin³, Su-Hyung Park³ and Seung Kew Yoon^{2,4*}

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OPEN ACCESS

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equally to this work

Specialty section:

This article was submitted to
Viral Immunology,
a section of the journal
Frontiers in Immunology

Received: 03 February 2019

Accepted: 23 May 2019

Published: 06 June 2019

Citation:

Sung PS, Park DJ, Kim J-H, Han JW, Lee EB, Lee GW, Nam HC, Jang JW, Bae SH, Choi JY, Shin E-C, Park S-H and Yoon SK (2019) Ex vivo Detection and Characterization of Hepatitis B Virus-Specific CD8⁺ T Cells in Patients Considered Immune Tolerant. *Front. Immunol.* 10:1319. doi: 10.3389/fimmu.2019.01319

In this study, we aimed to detect and characterize ex vivo virus-specific CD8⁺ T cells in patients with immune-tolerant hepatitis B virus (HBV) infection. We investigated a Korean chronic hepatitis B cohort composed of 15 patients in the immune-tolerant phase, 17 in the immune-active phase, and 13 under antiviral treatment. We performed enzyme-linked immunospot (ELISpot) assays ex vivo and intracellular cytokine staining after *in vitro* culture. We also performed ex vivo multimer staining assays and examined the expression of programmed death-1 (PD-1) and CD127 in pentamer-positive cells. Ex vivo ELISpot revealed that HBV-specific T cell function was weaker in immune-tolerant patients than in those under antiviral treatment. *In vitro* culture of peripheral blood mononuclear cells for 10 days revealed that HBV-specific CD8⁺ T cells produced interferon- γ in some immune-tolerant patients. We detected HBV-specific CD8⁺ T cells ex vivo (using the HBV core_{18–27} pentamer) in patients from all three groups. The PD-1⁺ subset of pentamer⁺ CD8⁺ T cells was smaller ex vivo in the immune-tolerant phase than in the immune-active phase or under antiviral treatment. Interestingly, the proportion of PD-1⁺ CD8⁺ T cells in HBV-specific CD8⁺ T cells correlated with patient age when all enrolled patients were analyzed. Overall, HBV-specific CD8⁺ T cells are present in patients considered as immune-tolerant, although their ex vivo functionality is significantly weaker than that in patients under antiviral treatment ($P < 0.05$). Despite the high viral load, the proportion of PD-1 expression in HBV-specific CD8⁺ T cells is lower in the immune-tolerant phase than in other phases. Our results indicate appropriate stimulation may enhance the effector function of HBV-specific CD8⁺ T cells in patients considered as being in the immune-tolerant phase.

Keywords: hepatitis B virus, CD8⁺ T-cell response, programmed death protein-1, chronic infection, interferon- γ

INTRODUCTION

Chronic hepatitis B (CHB) is a life-threatening liver disease affecting 257 million individuals worldwide, particularly in the Asia-Pacific region (1). In endemic areas, hepatitis B virus (HBV) is typically transmitted from chronically infected mothers to neonates (2). Perinatal HBV infection causes chronic infection in more than 90% of exposed individuals (3). With perinatal infection, lifetime mortality risk due to complications of liver cirrhosis (LC) or hepatocellular carcinoma (HCC) reaches 40% in men and 15% in women (3).

Traditionally, chronic HBV infection by vertical transmission is known to have several phases (4). Initially, most children with perinatal HBV infection are asymptomatic, which is traditionally referred to as the “immune-tolerant (IT)” phase and characterized by the presence of hepatitis B surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg), and high serum HBV DNA levels with minimal liver inflammation. This phase was thought to persist for decades typically followed by the “immune-clearance” phase, which is characterized by elevated liver enzymes, declining HBV DNA, and spontaneous HBeAg seroconversion. The immune-clearance phase is followed by the “low-replicative” phase, with minimal liver inflammation. In this phase, up to 30% of patients have been reported to undergo viral reactivation with increased HBV DNA and liver enzymes. These HBeAg-negative patients with spontaneous reactivation show increased risks of LC and HCC, whereas the risk of fatal diseases for those who remain inactive is much lower (2, 3). However, this concept of immune tolerance is not generally accepted by recent guidelines from Europe (5).

Although perinatal transmission of HBV is considered to lead to chronic persistent infection, the underlying mechanism remains unclear. Until recently, HBV-infected children in the IT phase were considered to have defects in mounting effective humoral and T cell responses against the infecting virus (6, 7). Very weak type-I interferon (IFN) responses (8–10), robust immunosuppressive IL-10 induction (11), and impaired IL-21 secretion from follicular helper T cells (12) following HBV infection have been suggested to limit the induction of effective adaptive immune responses in patients in the IT phase.

Recently, however, the concept of immune tolerance in HBV-infected neonates has been challenged. Studies reported that HBV infection in younger patients was not associated with an immune profile of T-cell tolerance (13, 14). One of these studies showed that HBV-specific T cell responses in the IT phase were comparable to those in the immune-active (IA) phase (13). Another study revealed HBV DNA integration and clonal hepatocyte expansion in patients considered IT phase at a high rate (14). The authors suggested that clonal hepatocyte expansion resulted in a response to hepatocyte turnover mediated by HBV-specific T cells, which were detected in patients considered as immune-tolerant (14, 15). In agreement with these reports, a recent study showed that antiviral therapy in patients with HBeAg-positive CHB with a high viral load and alanine transaminase (ALT) level below normal reduced the risk of HCC (16). It has also been reported that substantial fibrosis and necroinflammatory activity already existed in the

liver biopsy of some patients in the IT phase (17, 18). Therefore, recent European guidelines referred to the traditional “immune tolerant” phase as HBeAg-positive chronic HBV infection (5).

In Korea and China, genotype C HBV prevails among chronic carriers of the virus, regardless of the clinical stage of liver disease (19, 20). In general, genotype C HBV infection is associated with more severe liver disease and an increased risk of HCC (20–22). Moreover, genotype C HBV is associated with lower rates of HBeAg and/or HBsAg loss than genotypes A and B (22). However, HBV-specific T cell responses in patients with genotype C HBV infection have not been explained in detail.

In a previous study by Shin et al. (23), a correlation between HCV-specific CD8 T-cell responses in the blood and molecular and functional markers of T-cell responses in the liver was demonstrated. Thus, HCV-specific CD8 T-cell responses in the blood were valid markers of intrahepatic T-cell activity. For different phases of HBV infection, another study (24) demonstrated CD8⁺ T cell dysfunction using patients' blood samples from different infection stages. A more recent report revealed an association between blood transcriptomes and liver biopsy transcriptomes at different infection stages (25). Therefore, we used peripheral blood samples to analyze *ex vivo* T cell response in patients with different phases of HBV infection.

In this study, we performed *ex vivo* functional assays and multimer staining to investigate the existence and function of HBV-specific CD8⁺ T cells in Korean patients with CHB. We also examined the expression levels of exhaustion (PD-1) and memory marker (CD127) in multimer⁺ cells in peripheral blood samples from these patients. Although their *ex vivo* function was impaired, we confirmed the presence of HBV-specific CD8⁺ T cells containing a smaller proportion of PD-1⁺ cells in IT patients. Our results indicate that HBV-specific CD8⁺ T cells in Korean IT patients may not be tolerant or exhausted, and appropriate stimulation can enhance the effector function of HBV-specific CD8⁺ T cells in patients considered as being in the IT phase.

MATERIALS AND METHODS

Patient Cohort and Sample Preparation

We recruited a cohort of 45 patients with CHB with human leukocyte antigen A2 (HLA-A2) alleles from Seoul St. Mary's hospital. **Table 1** summarizes the characteristics and laboratory findings of the cohort. Forty-four patients were categorized into three different CHB phases by serum ALT levels and serologic parameters, including HBsAg, HBeAg, anti-HBeAg, and serum copies of viral DNA (4). We adopted the traditional definitions of IT and IA phases from the American Association for the Study of Liver Diseases guidelines (4). Patients in the IT group ($n = 15$) had normal ALT levels (<40 IU/mL), HBeAg positivity, and consistently high HBV DNA levels (median HBV DNA = 9.09 log copies/mL) for at least 2 years. Patients in the IA group ($n = 17$) had elevated ALT levels. We did not divide the patients in the IA group according to HBeAg positivity. We also included patients on antiviral treatment (AT) ($n = 13$) in our cohort. Among them, 8 patients were taking entecavir (**Table 1**). The mean duration of antiviral treatment in patients on AT was 61.1 ± 43.5 weeks

TABLE 1 | Clinical parameters of study patients.

Variables	IT (n = 15)	IA (n = 17)	AT (n = 13)	P-value ^a (IT vs. AT)
Age (years), median (range)	37 (25–53)	42 (25–67)	47 (25–74)	0.005
Male sex, n (%)	8 (53)	11 (65)	8 (62)	
HBeAg/HBeAb				
+/-	15 (100)	11 (65)	1 (8)	
+/+	0 (0)	0 (0)	0 (0)	
-/+	0 (0)	6 (35)	9 (69)	
Median HBV DNA level (log copies/mL)	9.09	8.59	2.01	<0.001
ALT (U/L), median (range)	34 (14–38)	189 (52–1,599)	25 (15–43)	0.016
Antiviral therapy, n (%)				
None	15 (100)	17 (100)	0 (0)	
Adefovir	0 (0)	0 (0)	1 (8)	
Entecavir	0 (0)	0 (0)	8 (62)	
Tenofovir	0 (0)	0 (0)	4 (30)	

ALT, Alanine aminotransferase; AT, antiviral treatment; HBeAb, hepatitis B envelope antibody; HBeAg, hepatitis B envelope antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IA, immune-active; IT, immune-tolerant.

^aP-value estimated by Mann-Whitney U-test or Kruskal-Wallis test.

(mean $\pm$ standard deviation). Blood was also obtained from age-matched non-HBV-infected adult healthy controls ($n = 4$).

HBV DNA levels in serum samples were quantified using real-time PCR as previously described (7). Patients' sera were tested for HBsAg, HBeAg, and anti-HBeAg. HBV genotype was not assessed because previous reports have shown that most patients with CHB in Korea are infected with HBV genotype C (26). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient centrifugation and cryopreserved for immunologic analysis. Informed consent in writing was obtained from all patients. The present study was conducted according to the Declaration of Helsinki principles and was approved by the Institutional Review Boards (Seoul St. Mary's Hospital, KC16MIS10714).

Virus Sequencing

The genomic region covering the HBV core gene was amplified and sequenced from patients enrolled in this study. Viral DNA was extracted with the QIAamp MiniElute Virus Spin Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A 700-bp core fragment was amplified in a two-step nested polymerase chain reaction (PCR) using HBV-specific primers HBV core-forward (tgtaacgaccgaccttgagg), HBV core-reverse (tgtagctctgttcccaa), HBV core internal-forward (aggctgtaggcataaattggt), and HBV core internal-reverse (ttccacattatgagtccaag), as previously described (Supplementary Table 1) (27). PCR products were directly sequenced and aligned by Cosmogenetech (Seoul, Republic of Korea).

Flow Cytometry

The following commercially available antibodies were used for multi-color flow cytometry: BV421-conjugated anti-PD-1, BV521-conjugated anti-CD3, BV605-conjugated anti-CD4, BV786-conjugated anti-chemokine receptor 7 (CCR7), APC/Cy7-conjugated anti-mouse CD4, APC-conjugated anti-mouse PD-1 (Biolegend, San Diego, CA, USA), FITC-conjugated anti-HLA-A2, FITC-conjugated anti-CD45RA, PE-TR-conjugated anti-CD14, CD19, PE-Cy7-conjugated anti-CD127, APC-H7-conjugated anti-CD8, PE-conjugated anti-IFN- γ , PE-Cy7-conjugated anti-tumor necrosis factor α (TNF- α), PE-conjugated anti-major histocompatibility complex (MHC)-pentamer (Proimmune, Oxford, UK), Dead cells were excluded using the LIVE/DEAD red fluorescent reactive dye (Invitrogen, Carlsbad, CA, USA). Multi-color flow cytometry was performed using the LSRII instrument (BD Biosciences), and data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA). HLA-A*02 pentamers corresponding to HBV core_{18–27} FLPSPDFPSV, HBV core_{18–27} FLPSPDFPSI, and HBV polymerase_{455–463} were made by Proimmune. For detection of antigen-specific CD8⁺ T cells, PBMCs were incubated with pentamer for 30 min in the dark at 4 degrees Celsius and subsequently phenotyped. HLA class I genotyping was performed by flow cytometry using the anti-HLA-A2 monoclonal antibody.

Direct ex vivo IFN- γ Enzyme-Linked Immunospot (ELISpot) Assay

Duplicate cultures of 300,000 PBMCs/well were set up in ELISpot plates. HLA-A2 PBMCs were stimulated with a peptide mixture (ProMix HBV Peptide Pool, Proimmune, England) at a final concentration of 1 μ g/mL for 24 h (28). The sequences of HLA-A2 restricted HBV peptides are presented in Supplementary Table 2. ELISPOT assays using overlapping peptides (OLPs) of HBV core and surface proteins were carried out as previously described (7) with minor modifications. All the peptides used in our study have the sequence of HBV genotype C. After this incubation, biotinylated anti-IFN- γ detection antibody was added and streptavidin-horseradish peroxidase was used for the detection of the spots. The number of peptide-specific, IFN- γ -secreting cells was calculated by subtracting the non-stimulated control value from the stimulated sample. Positive controls were made up of cells stimulated with phytohemagglutinin (10 μ g/mL). For comparison, PBMCs were also stimulated with OLPs from cytomegalovirus (CMV) pp65 (JPT, Berlin, Germany). Wells were considered positive when the spot-forming unit (SFU) was above 7 and at least 1.5 times the mean of the unstimulated control wells.

In vitro Expansion of HBV-Specific T Cells

HBV-specific T cells were cultured as follows: 5×10^5 PBMCs were stimulated with the HBV peptide mixture in the presence of 20 IU of IL-2 in RPMI containing 10% fetal bovine serum for 10 days. The final concentration of each peptide was 1 μ g/mL. IL-2 and medium were refreshed on day 4 and 8 of culture. On day 10, intracellular cytokine staining was performed. For intracellular cytokine staining, brefeldin A (BD Biosciences) and monensin

(BD Biosciences) were added, and PBMCs were stained with surface markers after 7 h of incubation. Surface marker-stained cells were permeabilized using a Foxp3 Staining Buffer Kit (eBioscience) and further stained for intracellular cytokines or transcription factors for 30 min at 4 degrees Celsius.

Ex vivo Cytokine Secretion Assay

The cytokine secretion assay on pentamer⁺ CD8⁺ T cells was performed as described previously (29). Briefly, PBMCs were coupled with capture reagents (Cytokine Secretion Assay kit, Miltenyi Biotec, Auburn, AL, USA) for human IFN- γ and TNF- α under stimulation from the peptide mixture. The cells were incubated in a closed tube for 45 min at 37 degrees Celsius under slow continuous rotation using the MACS mix (Miltenyi Biotec). After a washing step, cells were resuspended in cold medium containing IFN- γ and TNF- α detection antibodies. Subsequently, the cells were resuspended in MACS buffer containing antibodies specific for surface markers, including pentamer, and flow cytometry was performed on a BD LSR II cytometer.

Statistical Analysis

SPSS version 20 software (IBM Corp., Armonk, NY, USA) was used for statistical analyses. The discrete variables were compared using the χ^2 test, and an independent *t*-tests were used for continuous variables. Pearson correlation tests were performed to analyze correlations between two parameters. Statistical significance was defined as a *P* < 0.05.

RESULTS

Proportion of HBV-Specific CD8⁺ T Cells in IT Phase Does Not Differ From Those in Other Clinical Phases of CHB

Initially, we examined the *ex vivo* proportion of HBV-specific CD8⁺ T cells in the peripheral blood from normal controls and patients in the IT, IA, and AT groups based on MHC class I multimer staining (Figure 1A). Because of limited sample availability, we performed multimer staining on 4 samples from normal controls, 10 samples from patients in the IT group, 8 samples from patients in the IA group, and 11 samples from patients under AT.

We used the HLA-A*02 pentamer corresponding to the HBV core_{18–27} (FLPSDFFPSV) to identify HBV-specific CD8⁺ T cells (30, 31). This pentamer can be used to detect HBV-specific CD8⁺ T cells, as it recognizes the T cell receptors of T cells induced by both the HBV core_{18–27} region/ HLA-A*02 complex: FLPSDFFPSI/ HLA-A*02 and FLPSDFFPSV/ HLA-A*02 (32) (Supplementary Figure 1). The substitution at position 27 I to V is known to affect peptide binding to HLA-A*02 molecules and does not likely affect T cell receptor recognition (31). Before multimer staining, we sequenced the corresponding epitope regions in viral DNA using sera from randomly selected patients (Supplementary Table 3). We confirmed that the sequence of the HBV core_{18–27} was FLPSDFFPSI in every sample tested (Supplementary Table 3). We compared the frequency of CD8⁺ T cells recognizing the

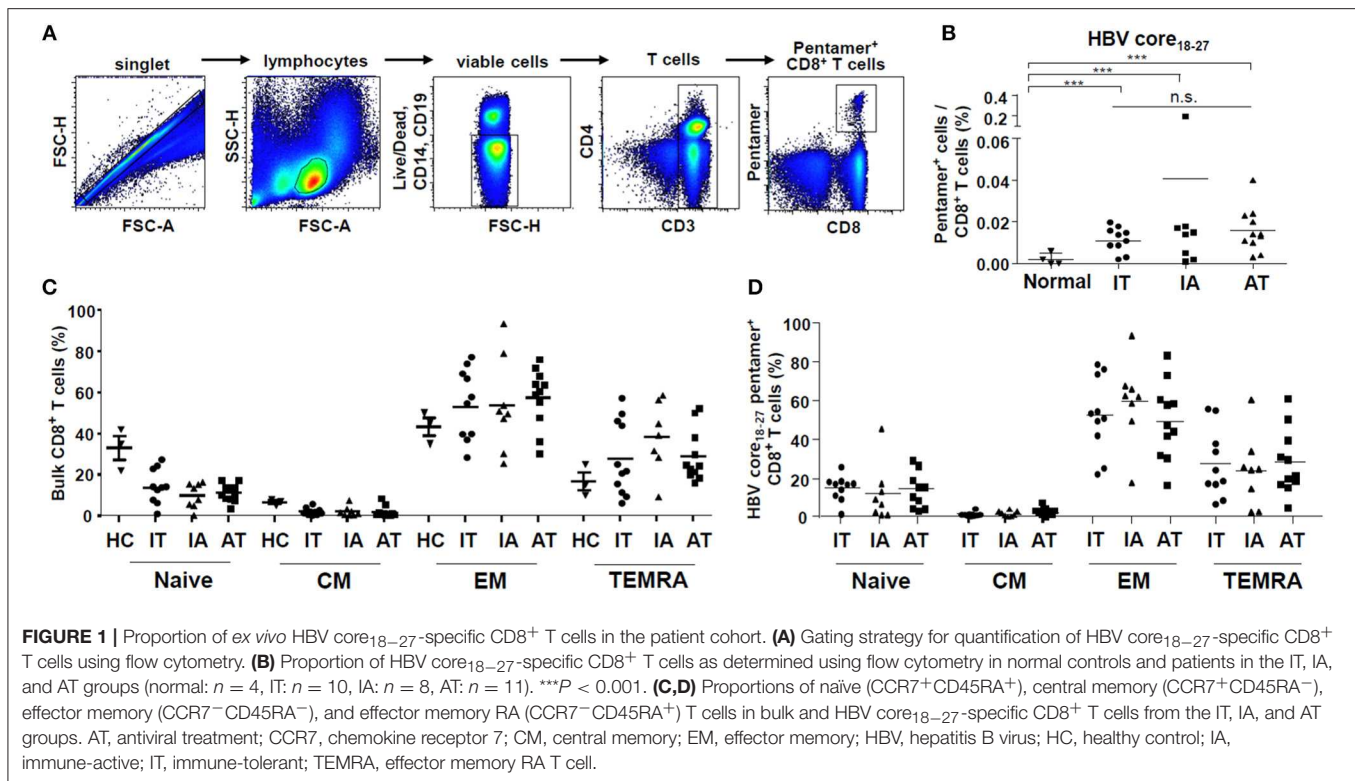
HLA-A*02 pentamer to HBV core_{18–27} FLPSDFFPSV and to HBV core_{18–27} FLPSDFFPSI (Supplementary Figure 1). There were no significant differences in the frequencies of pentamer⁺ CD8⁺ T cells in our cohort when either FLPSDFFPSV/HLA-A*02 pentamer or FLPSDFFPSI/HLA-A*02 pentamer was used.

The gating strategy used to detect CD8⁺ T cells using the HBV core_{18–27} pentamer is presented in Figure 1A. As shown in Figure 1B, the proportion of HBV core_{18–27}-specific CD8⁺ T cells in the IT phase did not differ from those in the other clinical phases of CHB, although the values were significantly higher than those in normal controls (*P* < 0.001) (Figure 1B and Supplementary Figure 2). When using a HBV polymerase_{455–463} pentamer, we certainly detected pentamer⁺CD8⁺ T cells in two patients with acute HBV infection and one patient in IA phase (Supplementary Figure 3). However, for chronic HBV infection with IT and AT phases, pentamer⁺CD8⁺ T cells were not readily detected when using polymerase_{455–463} pentamer, which agrees with the results of a recent study (33).

Next, we identified subsets of bulk and HBV core_{18–27}-specific CD8⁺ T cells based on the expression of CCR7 and CD45RA. The proportions of naïve (CCR7⁺CD45RA⁺), central memory (CCR7⁺CD45RA[−]), effector memory (CCR7[−]CD45RA[−]), and effector memory RA (CCR7[−]CD45RA⁺) T cells in bulk and HBV core_{18–27}-specific CD8⁺ T cells were calculated (Supplementary Figure 4). A recent study demonstrated that most HBV core_{18–27}-specific CD8⁺ T cells are effector memory T cells regardless of the infection stage, although the proportion significantly varies among individuals (32). We found similar results, with the effector memory T cell subset showing the highest values among IT, IA, and AT groups (Figure 1D). However, there were no significant differences in the proportions of naïve, central memory, effector memory, and effector memory RA subsets in bulk CD8⁺ T cells and HBV core_{18–27}-specific CD8⁺ T cells among the IT, IA, and AT groups (Figures 1C,D).

Ex vivo HBV-Specific CD8⁺ T Cells in the IT Phase Have Low Proportion of PD-1⁺ Cells

Next, we investigated the surface markers of *ex vivo* HBV-specific CD8⁺ T cells in PBMCs. First, we focused on the surface expression of PD-1, a well-known T cell exhaustion marker, in bulk and HBV-specific CD8⁺ T cells. In patients in the IT group, the proportion of PD-1⁺ cells was similar between HBV core_{18–27}-specific CD8⁺ T cells and bulk CD8⁺ T cells, although the proportion of PD-1⁺ cells was generally higher in HBV-specific CD8⁺ T cells than in bulk CD8⁺ T cells in the IA and AT phases (Figures 2A,B). We found that the proportion of PD-1⁺ HBV core_{18–27}-specific CD8⁺ T cells was significantly lower in patients in the IT phase than in those in the IA and AT phases (*P* < 0.05) (Figures 2A,C), although the proportions of PD-1⁺ bulk CD8⁺ T cells did not differ from those in the other phases (Figure 2D). We also examined the expression of CD127, a representative memory marker (34). CD127 is a cell surface marker that identifies CD8⁺ T cells that will become memory CD8⁺ T cells, in



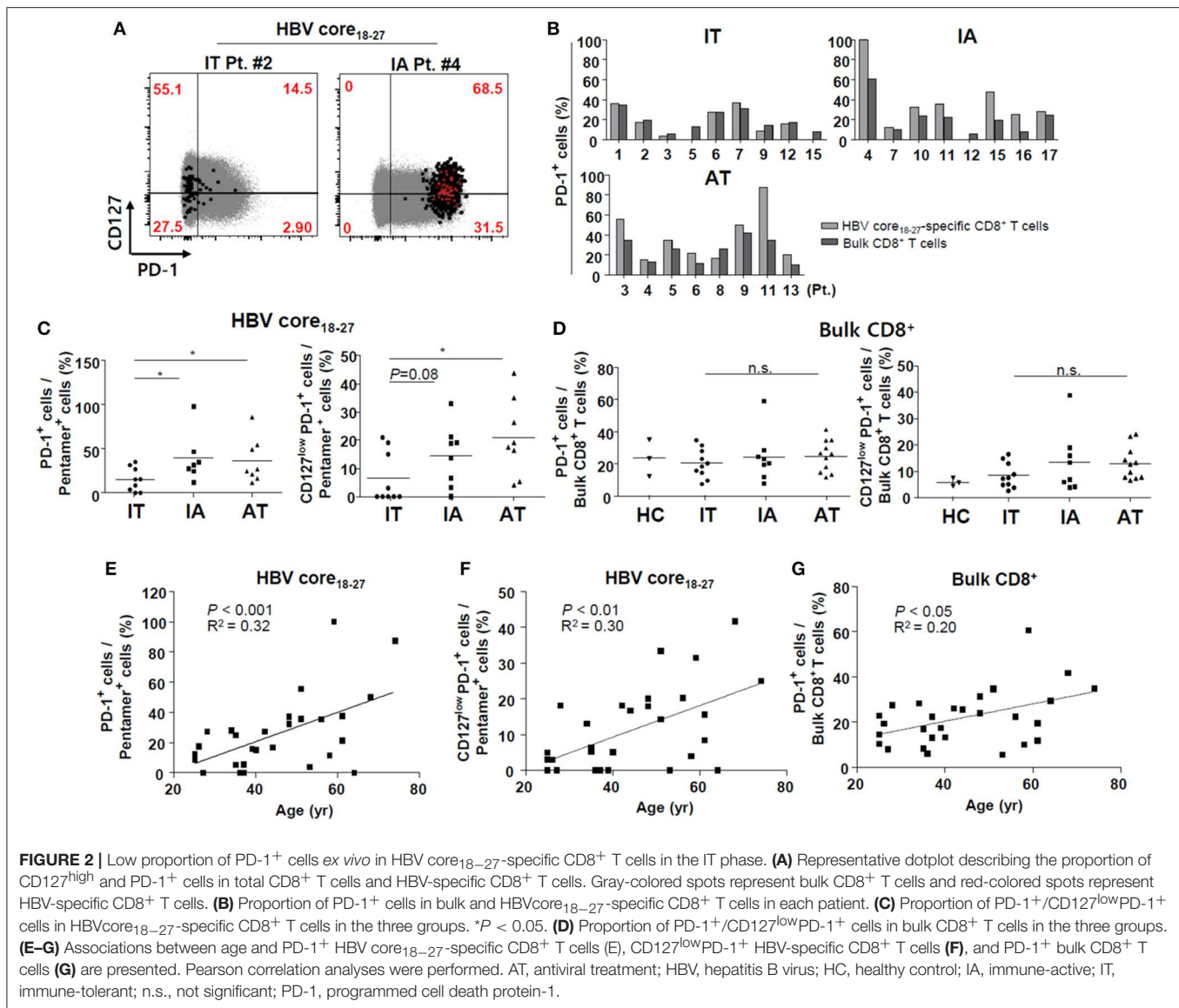
both the mouse models and in humans with acute resolving viral infection (35). In chronic HBV infection, a recent study demonstrated that the CD127⁺PD1⁺ subset is a memory-like population in chronic viral infection, and that the frequency of this subset clearly correlated with the expansion capacity of HBV core_{18–27}-specific CD8⁺ T cells in chronic HBV infection (34). In our analyses, we observed no significant changes in the proportions of CD127⁺ HBV-specific CD8⁺ T cells among different infection stages (data not shown). However, when we calculated the proportions of CD127^{low}PD-1⁺ CD8⁺ cells, the proportion of CD127^{low}PD-1⁺ HBV core_{18–27}-specific CD8⁺ T cells was significantly lower in patients in the IT phase than in those in the AT phase (Figures 2A,C).

Furthermore, we performed additional analyses with pentamer⁺ CD8⁺ T cells for Tim-3 and Lag-3 as surface markers, and T-bet and Eomes as transcription factors. The gating strategy is presented in **Supplementary Figure 5**. Unfortunately, we could not perform these analyses in all the enrolled patients because of limited sample availability. For HBV core_{18–27} specific CD8⁺ T cells, we confirmed that pentamer-specific CD8⁺ T cells showed significantly higher levels of Tim-3 expression compared to in bulk CD8⁺ T cells in most enrolled patients, and the levels were higher in IA and AT patients than in IT patients; however, the difference was not significant because of the small number of samples (**Supplementary Figure 6**). Lag-3 was not readily detected in our samples (**Supplementary Figure 6**). In general, high expression of Eomes accompanied by low levels of T-bet has been linked to T cell exhaustion (36). In our cohort, T-bet and Eomes expression was examined in selected

patients because of limited sample availability, and no significant differences were observed in T-bet and Eomes expression of HBV core_{18–27}-specific CD8⁺ T cells among IT, IA, and AT patients (**Supplementary Figure 6**).

Proportions of PD-1⁺ CD8⁺ T Cells and CD127^{low}PD-1⁺ CD8⁺ T Cells in HBV-Specific CD8⁺ T Cells Correlated With Patient age

To investigate potential contributing factors associated with the surface expression of PD-1 in HBV-specific CD8⁺ T cells, we performed correlation and regression analyses. Factors such as the level of HBV DNA in the IT and IA phases, ALT level in the IT, IA, and AT phases, and duration of AT in the AT phase, were not correlated with the PD-1 level in HBV-specific CD8⁺ T cells (data not shown). The proportions of PD-1⁺ cells and CD127^{low}PD-1⁺ CD8⁺ T cells in HBV core_{18–27}-specific CD8⁺ T cells were positively correlated with patient age (Figures 2E,F). The proportion of PD-1⁺ cells in bulk CD8⁺ T cells also correlated with patient age (Figure 2G), although the correlation coefficient was higher in HBV-specific CD8⁺ T cells. The number of SFUs in ELISpot or the proportion of pentamer⁺ cells was not significantly associated with patient age (data not shown). Overall, these data suggest that HBV-specific CD8⁺ T cells in the IT phase expressed low levels of PD-1, and that the PD-1⁺ cell population increased as the patients aged.



Virus-Specific T Cells From Patients With Chronic HBV Infection Show Defective IFN- γ Production *ex vivo*

Next, we performed *ex vivo* IFN- γ ELISpot assays using PBMCs and a mixture of HBV peptides (Supplementary Table 2). Consistent with previous reports (13, 24), *ex vivo* ELISpot did not detect robust IFN- γ responses within the IT and IA groups, although some patients on AT with very low HBV DNA levels had PBMCs showing notable *ex vivo* IFN- γ production (Figures 3A,B). The AT group had a significantly larger number of SFUs than the IT and IA groups (*P* < 0.05) (Figure 3B and Supplementary Figure 7). There were no differences in the number of SFUs among patients with different antiviral agents. We performed ELISpot using CMV_{pp65} OLPs to exclude general activation of non-HBV-specific T-cell responses and observed no difference in the CMV-specific T cell response among the three

groups (Figure 3C). *Ex vivo* ELISPOT using OLPs from HBsAg and HBcAg also revealed poor IFN- γ responses in selected patients within the IT and IA groups (Supplementary Figure 8). Together, these findings suggest that patients with chronic HBV infection are defective in *ex vivo* IFN- γ production after stimulation with HBV peptides, which can be partly restored by treatment-induced viral suppression.

Multimer-Stained CD8⁺ T Cells in the IT Phase Show Defective IFN- γ Production *ex vivo* After Peptide Stimulation

Subsequently, *ex vivo* cytokine secretion assays using a capture antibody for IFN- γ were conducted to confirm the defective secretion of IFN- γ in HBV core_{18–27} pentamer-stained cells from IT patients (Figures 4A,B). Initially, we attempted to combine intracellular cytokine staining and pentamer staining procedures

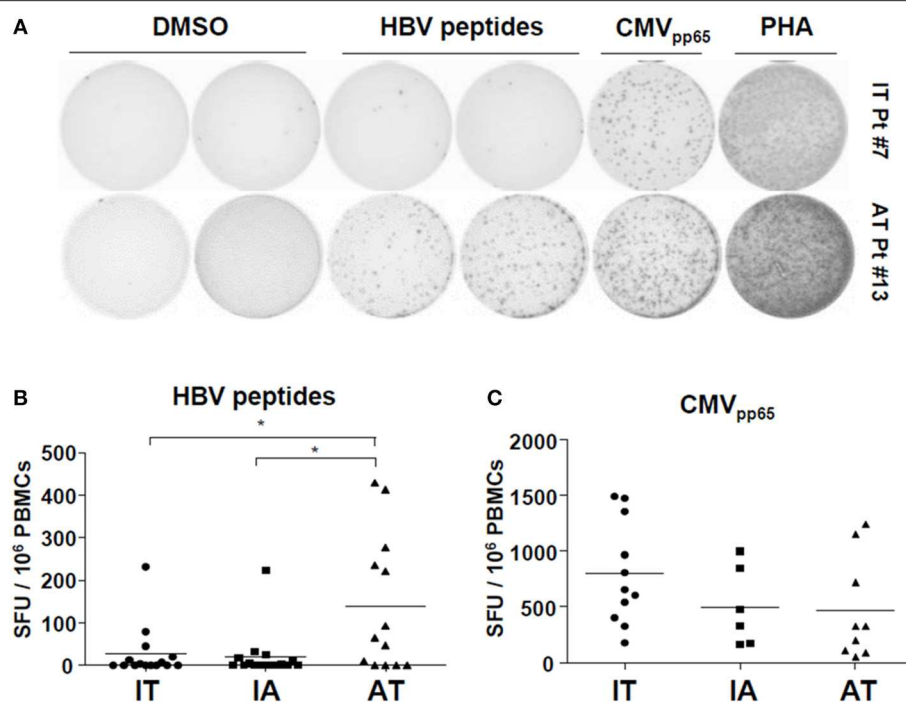


FIGURE 3 | Defective *ex vivo* IFN- γ production by HBV-specific T cells in patients with chronic HBV infection. **(A,B)** Direct *ex vivo* ELISpot assay using an HBV peptide mixture was performed on PBMCs from the patient cohort (IT: $n = 15$, IA: $n = 14$, AT: $n = 13$). PBMCs were stimulated with the peptide mixture at a final concentration of 1 $\mu\text{g/mL}$ for 24 h at 37°C. **(A)** Representative ELISpot result. **(B)** Results of *ex vivo* ELISpot using the peptide mixture in each group in the cohort. * $P < 0.05$. **(C)** Results of *ex vivo* ELISpot using CMV_{pp65} OLPs in each group in the cohort. AT, antiviral treatment; CMV, cytomegalovirus; ELISpot, enzyme-linked immunospot; HBV, hepatitis B virus; IA, immune-active; IFN, interferon; IT, immune-tolerant; OLPs, overlapping peptides; PBMC, peripheral blood mononuclear cell; SFU, spot-forming unit.

to determine whether pentamer⁺ cells in the IT phase have antiviral functions (although ELISpot did not show significant results). However, combining intracellular cytokine staining and direct pentamer staining shows some limitations (29). It is known that after stimulation, T cell receptors may be downregulated or there may be stimulation from the tetramer itself (29). Therefore, we instead performed an *ex vivo* secretion assay. Pentamer-stained HBV-specific CD8⁺ T cells were defective in IFN- γ secretion in patients in the IT group, although some patients in AT group showed *ex vivo* IFN- γ secretion after peptide stimulation (Figures 4A,B). CD3 stimulation of PBMCs from patients in the IT group led to robust IFN- γ secretion in both of bulk CD8⁺ T cells and pentamer⁺ cells (Figures 4A,B).

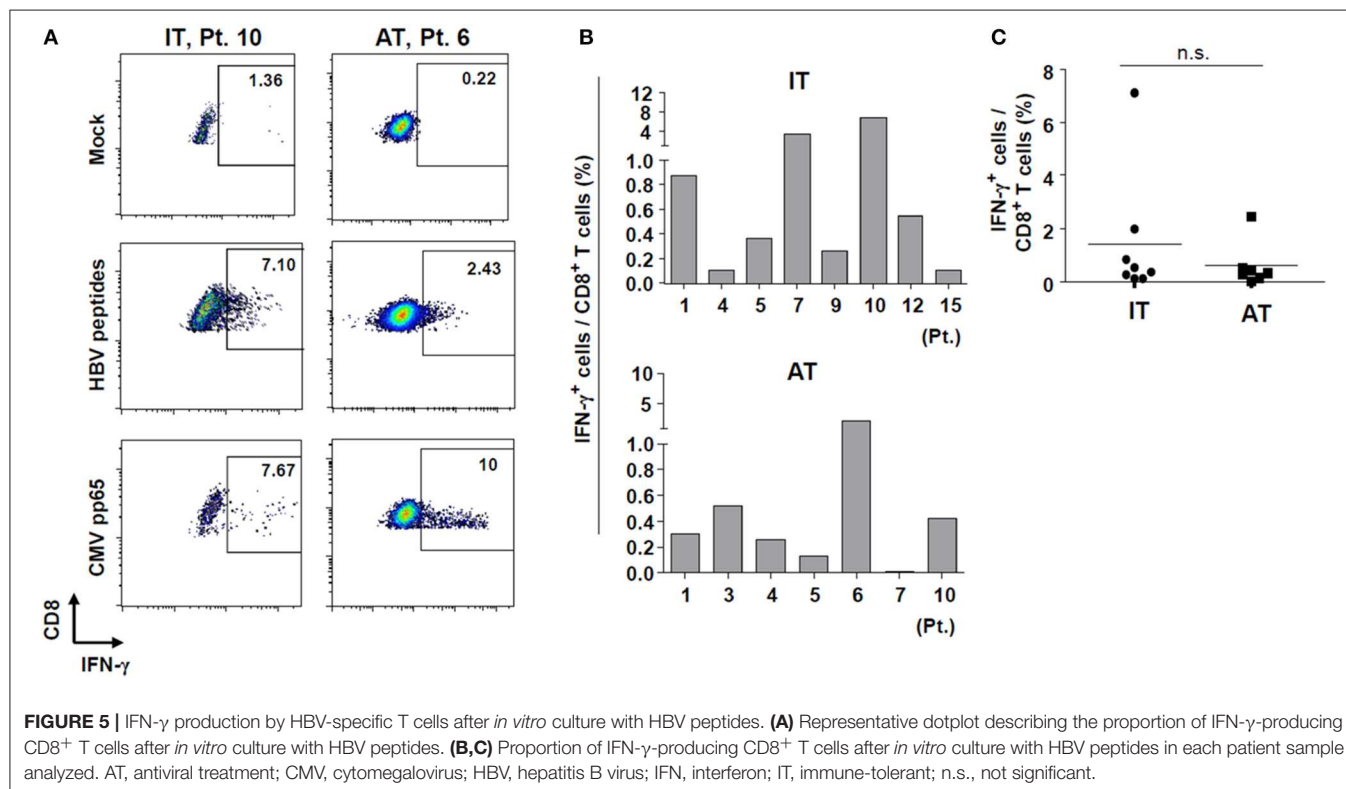
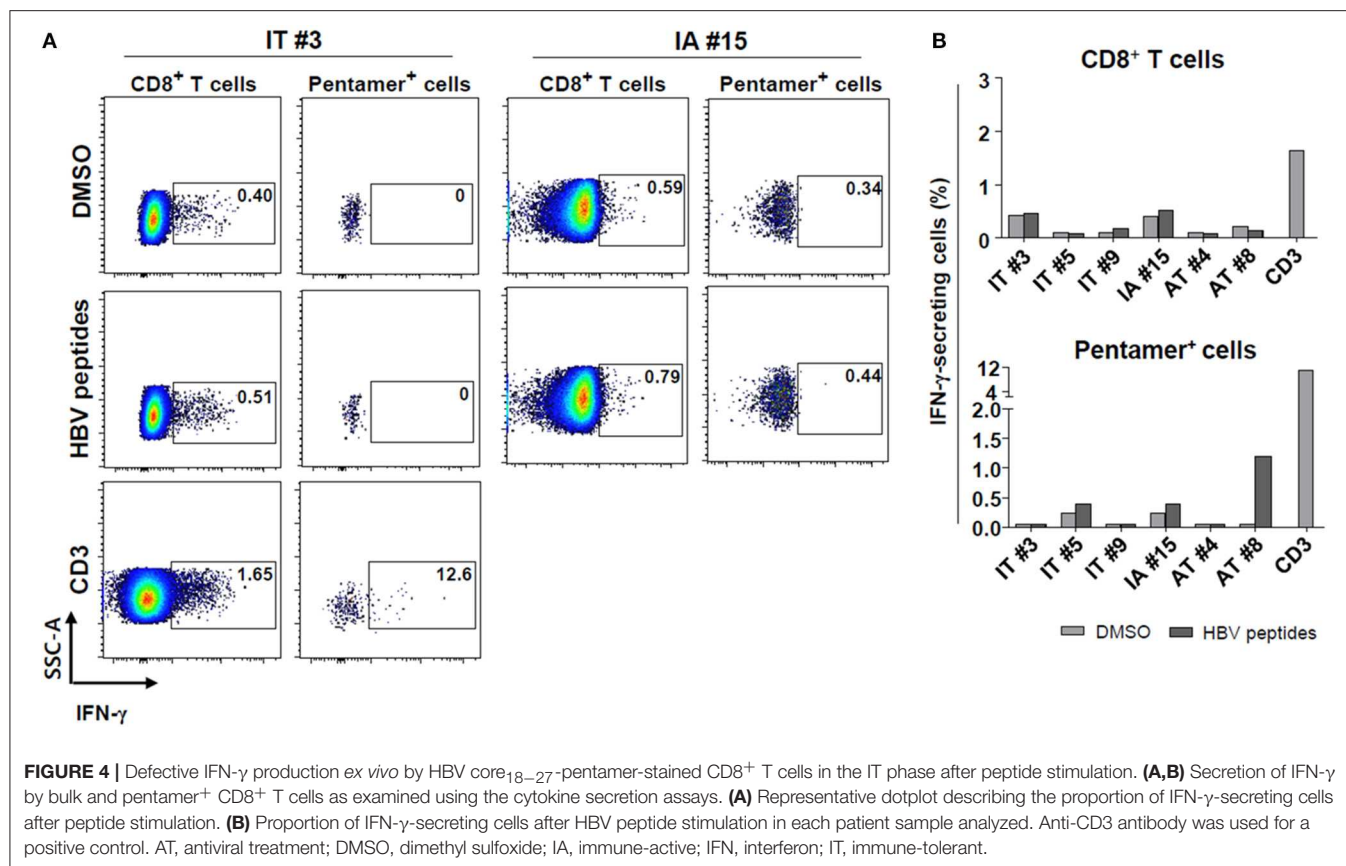
HBV-Specific CD8⁺ T Cells From Patients in the IT Phase Can Produce IFN- γ After *in vitro* Culture With HBV Peptides

Next, we performed a 10-day *in vitro* culture of PBMCs with an HBV peptide mixture (Supplementary Table 2) and IL-2. We performed *in vitro* expansion of HBV-specific CD8⁺ T cells from selected patients enrolled in the study because of limited sample availability from some patients. After 10 days of culture, the percentages of IFN- γ -producing CD8⁺ T cells from some patients in the IT group were significantly increased (Figures 5A,B) despite the defective IFN- γ production seen in

the *ex vivo* ELISpot results (Figure 3B). Cells from one patient (Patient #10) in the IT group, which showed a minimal response in the *ex vivo* ELISpot, produced IFN- γ robustly after 10-day culture (7.1% IFN- γ -producing cells among all CD8⁺ T cells). There were no significant differences in the frequency of IFN- γ -producing CD8⁺ T cells between IT and AT groups (Figure 5C). These data demonstrate that HBV-specific CD8⁺ T cells in patients considered to be in the IT phase may be activated and secrete IFN- γ when appropriately stimulated.

DISCUSSION

In this study, we confirmed the presence of HBV-specific CD8⁺ T cells and low proportion of PD-1⁺ cells in patients considered as being in the IT phase. Although HBV-specific T cells in the IT phase do not readily produce IFN- γ *ex vivo*, they can be activated and produce IFN- γ after persistent *in vitro* stimulation. Furthermore, the proportion of PD-1 expression in HBV-specific CD8⁺ T cells is lower in this phase than in subsequent phases despite the high viral load. Our data suggest that appropriate stimulation can enhance the effector function of HBV-specific CD8⁺ T cells in patients considered as IT, and future immunomodulatory approaches should target these patients.



In East Asia, chronic HBV infection is typically established in early childhood, resulting in many young patients with CHB in the IT phase. Traditionally, the IT phase has been associated with a lack of disease activity. Therefore, the international guideline did not recommend treatment of patients in the IT phase with antiviral agents (22). However, a recent study demonstrated that integration of HBV-DNA and clonal expansion of hepatocytes occurred in patients considered IT (14). Because the authors detected HBV-specific T-cell responses in PBMCs, they suggested that clonal expansion of hepatocytes may result from T-cell-mediated killing of hepatocytes (14). In agreement with the report, our results further demonstrate that HBV-specific CD8⁺ T cells are composed of a low proportion of PD-1⁺ cells *ex vivo* in these patients and the cells can be activated by appropriate stimuli as well as produce IFN- γ . Direct *ex vivo* functional analysis of HBV-specific T cells without *in vitro* expansion is performed by measuring cytokine secretion or cell proliferation upon *in vitro* stimulation with HBV antigens for a few hours. This better represents the physiological nature of immune responses.

Immune tolerance to specific pathogens encompasses both deletional and functional tolerance. Deletional T-cell tolerance mainly affects T cells with high affinity to their cognate antigen (37). Functional tolerance is caused by silencing of T cell activation in an antigen-dependent manner by cell intrinsic mechanisms, interacting with inhibitory molecules on target cells, or inhibitory molecules or regulatory cells around the T cell (37). These mechanisms appear to be maximally exploited by HBV. Previous reports demonstrated that the number of effector T cells detected by *ex vivo* ELISpot in chronic HBV infection was exceptionally low (13, 14, 24, 37–39). This may have been caused by deletion of HBV-specific T cells or functional unresponsiveness. Therefore, detecting the HBV-specific T cell response in the IT phase of HBV infection *ex vivo* is difficult using ELISpot. As an alternative, *in vitro* culture of PBMCs for 10 days was performed before the various analyses to detect T cell responses. In this study, we did not detect *ex vivo* ELISpot responses in patients considered as immune tolerant. However, although the numbers were small in selected samples, we detected HBV-specific CD8⁺ T cells *ex vivo* using multimer staining, suggesting that not all HBV-specific CD8⁺ T cells had been eliminated. Consistent with our data, Chinese groups described the *ex vivo* multimer detection (HBV core_{18–27} pentamer) of HBV-specific CD8⁺ T cells (30, 40–42). In Korea and China, most patients with HBV are infected with genotype C HBV (19), indicating that viral and host factors from different regions worldwide influence the detectability of HBV-specific CD8⁺ T cells using the HBV core pentamer.

A previous study demonstrated that the single amino acid alteration of valine to isoleucine at the position of the HBV core 27 amino acids may reduce the binding affinity to HLA-A*02 by 10-fold (31). This may result in an insufficient CD8⁺ T cell response to the FLPSPDFPSI epitope or inefficient deletion of CD8⁺ T cell precursors responsive to FLPSPDFPSI in chronic infection (31). However, a very recent study showed that sequence variations in the core_{18–27} region may not account for

the epitope-specific CD8⁺ T cell phenotypes (32). Despite the replacement of valine to isoleucine at the core 27 amino acid position, the responses to the core_{18–27} epitope displayed the most homogeneous phenotypic profiles, with strong expression of both PD-1 and CD127 in nearly all chronic patients (32). This suggests that the sequence data do not completely explain the phenotypic differences observed between HBV-specific CD8⁺ T cell responses, and that CD8⁺ T cell responses to the HBV core_{18–27} epitope in patients with FLPSPDFPSI sequence variation are a useful marker of the CD8⁺ T cell response in chronic HBV infection.

PD-1 expression is known as the classical hallmark of exhausted T cells (2). However, Rivino et al. recently demonstrated that the frequency of HBV-specific T cell responses was higher in patients without flares after stopping antiviral therapy, and these cells were most commonly found in the PD-1⁺ T cell compartment (33). They found that these PD-1⁺ T cell populations were functional, at least in terms of their proliferative capacity and ability to produce IFN- γ (33). Similarly, recent studies showed that patients with partial immune control of HBV infection display higher levels of intrahepatic PD-1⁺ CD39⁺ tissue-resident CD8⁺ T cells with the capacity to mount robust cytokine responses (43). Based on this information, *ex vivo* PD-1 expression on the surface of HBV-specific CD8⁺ T cells may not be associated with defective production of IFN- γ . Our data agree with previous reports showing that patients under AT express relatively higher levels of PD-1 in their HBV-specific CD8⁺ T cells, although their *ex vivo* IFN- γ production is higher than that of patients considered IT. The age-dependent increase in *ex vivo* PD-1 expression in HBV-specific CD8⁺ T cells suggests that PD-1 is associated with the duration of antigen exposure in patients with chronic HBV infection (44). A recent report also demonstrated that expression of T cell immunoreceptor with the Ig and ITIM domains (TIGIT), another immune checkpoint molecule, increases with age on hepatic CD8⁺ T cells in HBsAg-transgenic mice whose adaptive immunity is tolerant to HBsAg (45).

Our study had some limitations. First, most of the data presented in this study are from the multimer analysis with an HLA-A*02 multimer (HBV core_{18–27}). Recently, two independent groups demonstrated that phenotype of HBV-specific T cells may differ when the targeted epitope is changed (32, 34, 36). We also performed analyses with an HBV polymerase_{455–463} pentamer, but only in selected patients because of sample availability. Moreover, we could not perform multimer analyses to detect other epitope-specific CD8⁺ T cells. Second, we performed *ex vivo* functional analysis only by quantifying IFN- γ production. More detailed analyses were not performed because of limited sample availability. Finally, also because of sample limitations, we could not evaluate multiple exhaustion markers in all samples, although we measured Tim-3 and Lag-3 in some of the samples and observed significantly higher levels of Tim-3 expression in most enrolled patients compared to in bulk CD8⁺ T cells. These results must be validated in larger-scale studies.

In conclusion, we confirmed the presence of HBV-specific CD8⁺ T cells and low proportion of PD-1⁺ cells in patients considered as being in the IT phase. HBV-specific CD8⁺ T cells were not activated *ex vivo* but could be activated in *in vitro* culture in the IT phase. The age-dependent increase in *ex vivo* PD-1 expression in HBV-specific CD8⁺ T cells indicates that the proportion of PD-1 expression in HBV-specific CD8⁺ T cells was lower in this phase than in the following phases. Our data suggest that future immunomodulatory approaches should target IT patients because their virus-specific CD8⁺ T cells are not exhausted or tolerant. A longitudinal study is needed to confirm the changes in the phenotype and function of *ex vivo* HBV-specific T cells in patients with chronic HBV infection.

ETHICS STATEMENT

Informed consent in writing was obtained from all patients. The present study was conducted according to the Declaration of Helsinki principles and was approved by the Institutional Review Boards (Seoul St. Mary's Hospital, KC16 MISI0714).

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AUTHOR CONTRIBUTIONS

PS and SY: study design, data collection, data analysis, data interpretation, manuscript writing, and manuscript approval. DP: data collection, data analysis, data interpretation, and manuscript writing. J-HK, JH, EL, GL, and HN: data collection. JJ, SB, JC, E-CS, and S-HP: data interpretation and manuscript approval.

FUNDING

This work was supported by the Global Hightech Biomedicine Technology Development Program of the National Research Foundation (NRF) and the Korea Health Industry Development Institute (KHIDI) funded by the Korean government (MSIP & MOHW) (2015M3D6A1065146).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.01319/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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rt269I Type of Hepatitis B Virus (HBV) Leads to HBV e Antigen Negative Infections and Liver Disease Progression via Mitochondrial Stress Mediated Type I Interferon Production in Chronic Patients With Genotype C Infections

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OPEN ACCESS

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Specialty section:

This article was submitted to
Viral Immunology,
a section of the journal
Frontiers in Immunology

Received: 03 May 2019

Accepted: 09 July 2019

Published: 24 July 2019

Citation:

Lee S-Y, Choi Y-M, Oh S-J, Yang S-B,
Lee J, Choe W-H, Kook Y-H and
Kim B-J (2019) rt269I Type of
Hepatitis B Virus (HBV) Leads to HBV
e Antigen Negative Infections and
Liver Disease Progression via
Mitochondrial Stress Mediated Type I
Interferon Production in Chronic
Patients With Genotype C Infections.
Front. Immunol. 10:1735.
doi: 10.3389/fimmu.2019.01735

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Hepatitis B virus infection is a serious global health problem and causes life-threatening liver disease. In particular, genotype C shows high prevalence and severe liver disease compared with other genotypes. However, the underlying mechanisms regarding virological traits still remain unclear. This study investigated the clinical factors and capacity to modulate Type I interferon (IFN-I) between two HBV polymerase polymorphisms rt269L and rt269I in genotype C. This report compared clinical factors between rt269L and rt269I in 220 Korean chronic patients with genotype C infections. The prevalence of preC mutations between rt269L and rt269I was compared using this study's cohort and the GenBank database. For *in vitro* and *in vivo* experiments, transient transfection using HBV genome plasmid and HBV virion infection using HepG2-hNTCP-C4 and HepaRG systems and hydrodynamic injection of HBV genome into mice tails were conducted, respectively. This report's clinical data indicated that rt269I vs. rt269L was more significantly related to HBV e antigen (HBeAg) negative serostatus, lower levels of HBV DNA and HBsAg, and disease progression. Our epidemiological study showed HBeAg negative infections of rt269I infections were attributed to a higher frequency of preC mutations at 1896 (G to A). Our *in vitro* and *in vivo* studies also found that rt269I could lead to mitochondrial stress mediated STING dependent IFN-I production, resulting in decreasing HBV replication via the induction of heme-oxygenase-1. In addition, we also found that rt269I could lead to enhanced iNOS mediated NO production in an IFN-I dependent manner. These data demonstrated that rt269I can contribute to HBeAg negative infections and liver disease progression in chronic patients with genotype C infections via mitochondrial stress mediated IFN-I production.

Keywords: hepatitis B virus, HBV e antigen (HBeAg) negative infection, genotype C, mitochondrial stress, type I interferons

INTRODUCTION

Hepatitis B virus (HBV) infection is a high-risk global health issue leading to severe liver disease. In 2015, it was estimated that 350 million patients were chronically infected worldwide and 900,000 patients died (1). Although vaccines and therapeutic agents are currently available against HBV, the number of deaths caused by HBV has increased worldwide (2).

HBV is an enveloped and partially double-stranded DNA virus and preferentially replicates in hepatocytes. The HBV genome consists of four open reading frames (ORFs): surface antigens (S), core proteins (C), polymerase (Pol), and X proteins (X) (3). After infecting the hepatocytes, the genomes from HBV are converted to covalently closed circular DNA (cccDNA) by host enzymes (4). Since HBV cccDNA cannot be completely eradicated by nucleot(s)ide analog agents (5), epigenetic regulation via innate immune response modulating agents such as Type I IFN (IFN-I) is necessary for the complete viral clearance from chronic hepatitis B (CHB) patients (6).

IFN-I is a first-line defense mechanism of innate immune systems for viral infections (7) that is mediated via host recognition of viral pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) (8). However, for its survival, HBV has developed various strategies to evade host IFN-I dependent innate immunity (9). It has been reported that some HBV proteins such as HBV surface antigen (HBsAg), HBV e antigen (HBeAg), and HBV virions can lead to inhibit Toll-like receptor (TLR) mediated IFN-I production (10). In addition, HBxAg and Pol can also negatively regulate retinoic acid inducible gene I (RIG-I) mediated antiviral responses (11, 12). In RNA sensing pathways, it has also been reported that HBV Pol can block IFN-I dependent antiviral pathways via the inhibition of STING dependent cytosolic DNA sensing pathways (13).

On the basis of an 8% divergence in HBV genome sequences, HBV has been characterized into 10 genotypes as A-J (14). Various studies on HBV genotypes have reported that they have distinct pathogenic potentials as well as distinct geographic and ethnic distributions (15). Among the 10 genotypes, genotypes B and C are widespread in Asia, but two genotypes lead to distinctly different clinical outcomes (16). Compared to genotype B, genotype C showed high HBV replication capacity, with high levels of HBV DNA in the serum (17, 18). In addition, the tendency of chronicity was higher and more frequently developed into liver cirrhosis (LC) and hepatocellular carcinoma (HCC) in patients with genotype C than genotype B (19). However, the underlying mechanisms regarding distinct clinical and virological traits and distinct responses to IFN therapy between genotype C and genotype B remain unknown.

We recently introduced some mutations in the reverse transcriptase (RT) region of Pol related to HCC from genotype C infected patients [rtM80I, rtN139K/T/H, and rtM204I/V] (20). In addition to HBV mutations, it has been reported that there are several genotype dependent polymorphisms in HBV RT regions, which are generally defined as having a frequency <10% vs. wild type (Table S1). Of these, there are two polymorphisms at the Pol-269 site, rt269L (57.2%), and rt269I (42.8%), in patients

with genotype C, in which two types are present with an almost similar ratio, but only rt269I is present as a major wild type in other genotypes.

In this study, we hypothesized that the presence of two HBV Pol RT polymorphisms distinct only in HBV genotype C may play a very pivotal role in viral phenotypes, clinical outcomes, and worse responses to IFN therapy distinct in genotype C infections. Thus, we sought to investigate the clinical factors and capacity to modulate IFN-I between two HBV polymorphisms, rt269L and rt269I, in genotype C infected CHB patients.

MATERIALS AND METHODS

Patients

For this study, serum samples were collected from 410 patients chronically infected with HBV and the amino acid at 269th on RT region was identified by direct sequencing method. In 190 patients of these, their polymorphisms could not be identified by sequencing analysis due to the low sensitivity. However, 220 patients could be identified into their polymorphism, rt269L or rt269I types and selected for this study. To elucidate the correlation between the polymorphism of 269th amino acid and the characteristics of disease, their clinical factors and polymerase RT regions were assessed. This report was approved by the Institutional Review Board of Konkuk University Hospital (KUH-1010544) and Seoul National University Hospital (IRB-1808-067-965).

HBV DNA Extraction and PCR Amplification for Polymerase RT Region

For this cohort study, HBV DNA was extracted from the serum of patients using a QIAamp DNA Blood Mini kit (QIAGEN, Hilden, Germany) and dissolved in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, pH 8.0). First-round PCR was performed using primers POL-RT1 and the amplicon was used as a template for second-round PCR using primers POL-RT2 (Table S2). The PCR products were subject to direct sequencing analysis.

HBV Genotyping

The 1,032-bp polymerase RT sequences were examined by direct sequencing and were compared to the sequences of the reference strains representing each of the genotypes (A-H including the C strains) obtained from GenBank. The sequences of the RT region were compared via Bayesian method for phylogenetic/molecular evolutionary analysis using MrBayes version 3.2.7, and the phylogenetic tree was constructed using FigTree version 1.4.3. Maximum-likelihood method was also used for the phylogenetic analysis using MEGA version 10.0. Phylogenetic trees were reconstructed using 1,000 bootstrap replicates, and the mean genetic distances were estimated using Kimura two-parameter with Invariant sites and Gamma model.

Plasmid and Site-Directed Mutagenesis

The pHBV-1.2x (GenBank accession No. AY641558) containing genotype C HBV full-length genome was used for site-directed mutagenesis to generate polymerase RT mutant DNA constructs

using an *i*-pfu kit (iNtRON, Seongnam, Korea). Mutagenesis was performed using the primers rt269I based on rt269L construct. To exclude CMV promoter, HBV full genome constructs were cut by restriction enzyme *Sma*I and prepared for linear formed genome.

Cell Culture and Transfection

Human hepatocellular carcinoma HepG2 cells and mouse hepatoma Hepa-1c1c7 purchased from the Korean Cell Line Bank (KCLB, Seoul, South Korea) were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml of penicillin-streptomycin, and 25 mM HEPES at 37°C in a humidified environment containing 5% CO₂. pHBV-1.2x containing genotype C HBV full-length genome (2.5 µg) was transiently transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). To normalize the transfection efficacy, pSV-β-Galactosidase (0.25 µg) was co-transfected, and the enzyme assay was accompanied using β-Galactosidase Enzyme Assay System with Reporter Lysis buffer (Promega, Madison, WI, USA), following the manufacturers' protocol.

In vivo Assay and Hydrodynamic Injection

Female C57BL/6 mice (7 weeks old) were hydrodynamically tail-vein injected with HBV DNA (10 µg) per 8% of mouse body weight within 30 s. All of the animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University College of Medicine (SNU-170308). Mice were sacrificed on 4, 7, and 16 days after HBV-encoding DNA injection, and liver and serum were collected to analyze gene expression of ISGs and HBV viral factor, respectively.

Enzyme-Linked Immunosorbent Assay (ELISA)

To measure the HBV antigens in the blood serum or culture supernatant, ELISA was performed for HBsAg (Biokit, Barcelona, Spain) and HBeAg (AccuDiag, Oceanside, CA, USA) according to the manufacturers' instructions. The concentration of mouse and human IFN-β was measured with ELISA kit purchased from Biolegend (San Diego, CA, USA) and PBL assay Science (Piscataway, NJ, USA), respectively, according to the manufacturer's procedure.

Covalently Closed Circular DNA (cccDNA) Extraction and Real-Time Polymerase Chain Reaction

The pHBV-1.2x with rt269L or rt269I were digested with restriction enzyme *Sma*I to remove CMV promoter, and the linear DNA (2.5 µg) were transfected using lipofectamine 3000 following manufacture's instruction. The transfected cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 1% NP-40), and the nuclei were collected via centrifugation and incubated with nucleus lysis buffer (10 mM Tris-10 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, 0.5% SDS, and 0.5 mg/ml protein K). The nucleic acids were purified via ethanol precipitation and treated with 10 U Plasmid-Safe ATD dependent DNase I (PSAD, Epicentre, Madison, WI, USA). The cccDNA

was purified by PCI and ethanol precipitation and quantified via real-time PCR using SYBR and primers cccF and cccR (Table S2).

Total RNA Extraction and Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the transfected cells or mouse liver tissue using TRIzol, and the target genes were amplified using SensiFAST SYBR Lo-ROX One-Step kits (BioLine, London, UK). The transcription level was analyzed using qRT-PCR with sets of primers (Table S2) and housekeeping gene 18S ribosomal RNA was used as an internal control.

IFN-I Luciferase Reporter Assay

Cell culture supernatants from transfected cells were overlaid on top of HEK293 IFN reporter cells containing ISRE-luciferase construct (21) and incubated for 4 h. The reporter cells were lysed in passive lysis buffer (Promega, Madison, WI, USA) for 30 min at room temperature, mixed with firefly luciferin substrate (Promega, Madison, WI, USA), and measured using an illuminometer (Beckman Coulter Inc., Fullerton, CA, USA).

IFN-I Signal Block Assay

HepG2 cells were pre-treated with 10 µg of anti-IFNAR2 antibody (PBL assay Science, Piscataway, NJ, USA) and AZD1480 (Sigma, St. Louis, MO, USA) in MEM supplemented with 2% of FBS and transfected with pHBV-1.2x DNA constructs. STING-siRNA (Santa Cruz Biotechnology, Dallas, TX, USA) were co-transfected with pHBV-1.2x DNA constructs, following the manufacturers' procedures. Scrambled siRNA (Thermo-Fisher Scientific, Waltham, MA, USA) was used as control.

Preparation of HBV From Transiently Transfected Cells and Infection Assay

For an infection study, culture supernatant of HepG2 cells, transiently transfected with 1.2x-rt269L or rt269I type of HBV plasmid, was collected. The supernatant was cleared through a sterile 0.45-µm pore size filter and precipitated with 6% polyethylene glycol (PEG) 8000 overnight. The media were ultracentrifuged and the collected pellet was resuspended in PBS containing 15–25% FCS. After quantification by qPCR, 3×10^9 HBV genome equivalent per milliliter was aliquoted and stored at –80°C. HepaRG and HepG2-hNTCP-C4 cells were seeded in 12-well plates. Infection assay was performed with the concentrated virus in the presence of 4% PEG8000 at 37°C for 20 h. HepaRG was purchased from BIOPREDIC International (HPR116) and HepG2-hNTCP-C4 was kindly provided by Dr. Koichi Watashi (National Institute of Infectious Disease, Tokyo, Japan).

Flow Cytometry and Confocal Analysis

The mtROS were stained with MitoSOX (5 µM), and analyzed by flow cytometry (LSRII, Becton Dickinson, San Jose, CA, USA) and confocal microscope (Confocal-A1, Nikon, Tokyo, Japan). DAPI was used to stain nuclei and the cells were mounted in mounting medium (VECTASHIELD Antifade Mounting Medium, H-1000). The images were captured using a 100× oil immersion objective lens.

8-OHdG ELISA Assay

Genomic DNA was extracted from the transfected cells using QIAamp Blood DNA extraction kit (QIAGEN, Hilden, Germany). For the detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG) activity, a competitive ELISA for 8-OHdG analysis kit (OxiSelect Oxidative DNA Damage ELISA kit, Cell Biolabs, San Diego, CA, USA) was used according to the manufacturer's protocol.

Measurement of NO₂⁻ and NO₃⁻ Levels

The produced NO was measured in culture supernatants obtained from the transfected cells using a colorimetric Nitrite/Nitrate Assay Kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions, and measured the absorbance at 540 nm in microplate reader (Tecan Infinite M200 Pro, Tecan Group Ltd., Männedorf, Switzerland).

Statistical Analyses

For the cohort study, statistical analysis was performed using SPSS Software (IBM SPSS version 23.0.0.0 Inc., Chicago, USA). Categorical variables were analyzed using Multivariate Analysis of Variance (MANOVA). Independent *t*-test was used to compare continuous variables. Tests were two-sided.

Experimental data were analyzed using Graphpad Prims 5 (GraphPad Software, La Jolla, CA, USA). All experiments were independently repeated three times and the statistical analyses were indicated in the figure legends. The *p*-value of statistical significance was set at either; **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

RESULTS

rt269I Was Related to Enhanced Disease Progression in a Korean Cohort With Genotype C Infections

In the web page "HBV RT: Mutation prevalence according to genotype and treatment" (<https://hivdb.stanford.edu/HBV/DB/cgi-bin/MutPrevByGenotypeRxHBV.cgi>), the mutation prevalence or polymorphisms of the entire HBV RT region were analyzed according to the genotypes and antiviral drug treatments (Table S1). A comparison of all of the 344 codons of RT found one unique genotype C site at the 269th codon of RT, in which both types, one for rt269L encoding leucine and the other for rt269I encoding isoleucine, were present in patients infected with genotype C at frequencies of 57.2 and 42.8%, respectively. Although rt269 codon is located at the outer region of overlapped HBsAg, polymorphism of rt269 cannot lead to HBsAg amino acid change, not affecting the performance of the HBsAg kit or immunoblotting data. There were no other polymorphisms in patients infected with genotype C exceeding 10% of the total frequency. Therefore, we postulated that the polymorphism at the 269th codon of RT may play an important role in the clinical outcomes and pathogenesis of genotype C infections. To address this issue, we analyzed the 269th codon polymorphisms of 220 patients in our Korean cohort with genotype C via direct sequencing of the RT region (Figure S1) and compared their clinical factors between rt269L and rt269I. Overall, 138 patients (62.7%) and 82 patients (37.3%) were infected with rt269L or rt269I, respectively (Table 1). We also

TABLE 1 | Comparison of the clinical features between patients infected with the two types of genotype C, rt269L, and rt269I.

Clinical factor	rt269L (n = 138)	rt269I (n = 82)	p-value
CH:HCC	92:46	46:36	ns
Sex, men	63.04%	64.63%	ns
HBeAg negative	28.99%	52.44%	**
AST/ALT	1.16 ± 0.78	1.42 ± 1.43	ns
HBV-DNA (log10 copies/ml)	6.47 ± 1.71	5.75 ± 1.62	**
HBsAg (log10 IU/ml)	3.74 ± 0.61	3.41 ± 0.71	***
Age (years)	42.04 ± 12.45	45.89 ± 12.61	*
Bilirubin (mg/dL)	0.99 ± 0.91	1.37 ± 1.71	**
Albumin (g/dL)	4.01 ± 0.58	3.95 ± 0.63	ns
Prothrombin time (INR)	1.11 ± 0.18	1.14 ± 0.18	ns
Platelet (10 ⁹ /L)	169.93 ± 63.74	168.43 ± 81.93	ns
Fibrosis-4 score	4.05 ± 7.96	4.45 ± 5.42	ns
Disease phase†			***
1	18.12%	6.10%	
2	52.90%	41.46%	
3	24.64%	43.90%	
4	4.35%	8.54%	

CH, chronic hepatitis; HCC, hepatocellular carcinoma; AST, aspartate aminotransferase; ALT, alanine transaminase.

† 1. Immune-tolerant. 2. Immune-clearance. 3. HBeAg negative CHB. 4. Inactive carrier. Multi variate test were used.

Continuous variables were tested using the independent *t*-test and categorical variables were analyzed using MANOVA. ns, non-significant; **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

found there were several patients mixed with both rt269L and rt269I in sequencing data, maybe due to quasi species generation. In these cases, we determined their polymorphisms types into one reflecting dominance in sequencing data.

Interestingly, there were distinct disparities in some clinical factors between the patients with rt269L and rt269I (Table 1). The patients with rt269I had significantly lower levels of HBV DNA and HBsAg, HBeAg negative infection, and liver disease progression based on immune response against HBV (22). Our data suggest that both rt269L and rt269I on the RT region may be key factors determining clinical outcomes regarding disease progression and HBeAg negative infection in genotype C infected patients.

The Higher Frequency of preC Mutation (G1896A) in Patients With rt269I Infections Is Responsible for the Higher Frequency of HBeAg Negative Infections

Of the various HBV mutations, G1896A mutations on the pre-core region (preC mutation) and A1762T/G1764A double mutations on the basal core promoter (BCP) lead to HBeAg negative infection that are significantly related to liver disease progression (23). To verify whether these mutations are associated with distinct clinical factors of genotype C infections between rt269L and rt269I, their mutation rates were compared not only in chronic infectious patients in our cohort study, but also in the reference strains in GenBank (Table 2). In double mutations in BCP, only the G1764A mutation, but not the A1762T, was significantly prevalent in patients with rt269I of

TABLE 2 | The frequencies of BCP and preC mutations between rt269L and rt269I in a Korean cohort and from reference strains.

Mutation	Nucleotide	Clinical isolates			Reference strains		
		rt269L (n = 115)	rt269I (n = 69)	p-value	rt269L (n = 111)	rt269I (n = 129)	p-value
BCP mutation	A1762T	A	47 (40.87%)	ns	31(27.93%)	24 (18.60%)	ns
		T	68 (59.13%)		80(72.07%)	105 (81.40%)	
	G1764A	G	44 (38.26%)	*	29(26.13%)	17 (13.18%)	*
		A	71 (61.73%)		82(73.87%)	112 (86.82%)	
preC mutation	G1896A	G	83 (72.17%)	***	79(71.17%)	65 (50.39%)	***
		A	32 (27.83%)		32(28.83%)	64 (49.61%)	

BCP, basal core promoter; preC, pre-core.

Continuous variables were tested using the chi-squared test. ns, non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

both our cohort and GenBank. In addition, the rate of G1986A preC mutation was significant higher in patients with rt269I (49.61%) than those with rt269L (28.83%, $p < 0.01$). These results suggest that the high frequency of G1986A preC mutation is responsible for HBeAg negative infections in patients with genotype C rt269I infections.

rt269I Led to Lower Levels of HBV Replication in *in vitro* and *in vivo* Experiments

Our clinical and epidemiologic data suggest there may be differences in the HBV replication capacity between rt269I and rt269L infections. To verify this hypothesis, we performed *in vivo* and *in vitro* study with genotype C HBV full genome constructs with leucine or isoleucine at the 269th amino acid on the RT region. HBV replications between rt269I and rt269L infections were analyzed via *in vivo* and *in vitro* studies. The secreted HBsAg and HBV DNA were greatly increased in the serum from the mice infected with rt269L. However, rt269I showed relatively lower HBV replication levels (Figure 1A). These replicative capacities were also examined in HepG2 cells via transient transfection of the HBV constructs and the linear DNA constructs (Figure S2A), in which CMV promoter was deleted (24). As shown in the *in vivo* study, the secreted HBsAg and HBeAg levels also decreased in HepG2 cells, Huh-7, or Huh-7.5 cells transfected with rt269I (Figure 1B and Figures S2B,C). Intracellular intermediates of HBV amplification were also analyzed via Southern blotting, which indicated that double-stranded (DS) and single-stranded (SS) DNA were significantly reduced in rt269I at 48 h post-transfection (Figure 1C). In addition, the HBV capsid form that is an intermediate of HBV amplification was significantly reduced in rt269I at 24 h after transfection (Figure 1D). Similarly, rt269I also showed decreased pgRNA levels and cccDNA levels (Figures 1E,F).

rt269I Led to Enhanced IFN-I Signaling

Since IFN-I mediated antiviral effects by up-regulating APOBEC3G that hypermutate the HBV genome (25), we postulated that rt269I could enhance IFN-I mediated APOBEC3G signaling in infected hepatocytes and finally lead to the higher frequency of G to A mutations on preC and BCP in patients with rt269I. To address this issue, we compared the gene expressions of two representative IFN-I genes, INF- α and

INF- β , and interferon stimulated genes, ISG-15, RIG-I, and APOBEC3G that could be induced by an IFN-I signal (26). The mRNA levels of APOBEC3G, ISG-15, RIG-I, IFN- α , and IFN- β were significantly enhanced in the mice infected with rt269I on 4 days post-infection (Figure 2A). In the same manner as shown in *in vivo* assays, the transcription levels of APOBEC3G and IFN-I-related genes, such as ISG-15, RIG-I, IFN- α , and IFN- β , were also induced in HepG2 and Huh7 cells transfected with rt269I at 12 h after transfection (Figure 2B and Figure S3). To evaluate the upstream genes of the IFN-I signaling pathway, the activation or expression of STAT-1, IRF-3 and the STING genes (27, 28) were analyzed using Western blotting at 48 h post-transfection (Figure 2C). rt269I activated STAT-1, IRF-3 and up-regulated STING in protein level. The secreted IFN- β was also assessed by ELISA and treating culture supernatant from the transfected cell onto HEK293 with luciferase construct under an interferon stimulated response element (ISRE) promoter (21). As the results of activated IFN-I signal, the secreted IFN-I was significantly induced in cells transfected with rt269I at 24 h post-transfection (Figure 2D and Figure S4A). These results were consequently shown in the Huh-7 or Huh-7.5 cells defective in RIG-I signaling at 24 h after transfection (Figures S4B,C), suggesting that the enhancing effect of IFN-I found in rt269I may be due to pathways other than the RIG-I dependent pathway. Notably, rt269I showed enhanced IFN-I, even compared to genotype A wild type with the same isoleucine at the 269th amino acid of RT at 24 h after transfection (Figure S4D), suggesting that the trait capable of IFN-I induction as found in rt269I may be specific to genotype C. In addition, the HBV encoding DNA was also transfected into mouse hepatoma cell Hepa1c1c-7 and IFN- β was measured assessed by ELISA (Figure S4E). Consistent with human hepatocellular cells, mouse hepatoma transfected with rt269I vs. rt269L produced enhanced IFN- β productions. Together, our data indicated that rt269I led to enhanced IFN-I production in hepatocytes.

The Reduced Replication Capacity and Enhanced IFN-I Expression of rt269I vs. rt269L Were Also Proved in Two HBV Infection Models, HepaRG and HepG2-hNTCP-C4 Cells

To determine whether the different replication capacity between rt269L and rt269I could be reproduced in HBV

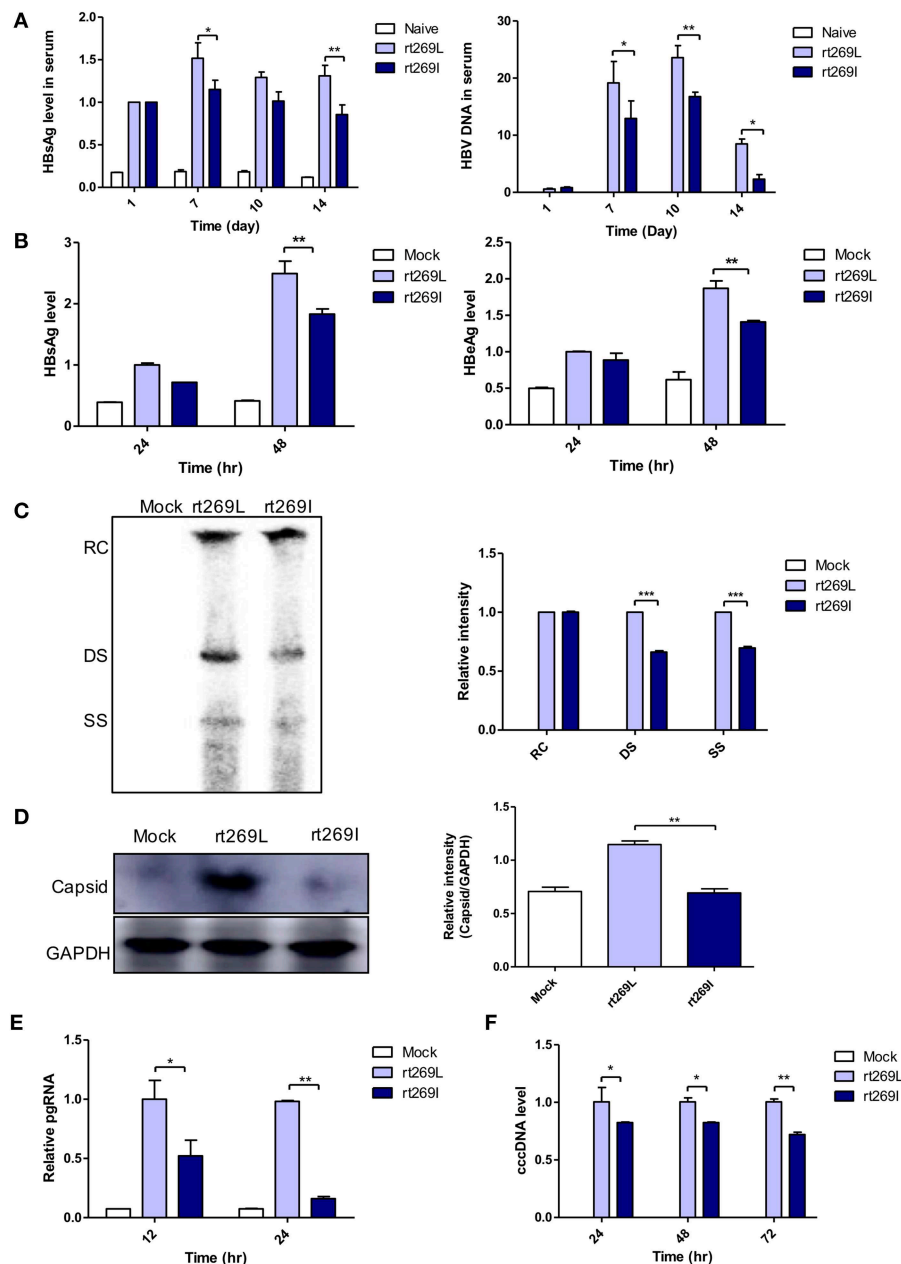


FIGURE 1 | rt269I vs. rt269L led to reduced viral replication in *in vivo* and *in vitro* analyses. **(A)** Both HBV-encoding DNA and pSV- β -Galactosidase was injected by hydrodynamic tail vein injection into C57BL/6 mice. HBsAg and HBV DNA in serum were determined by HBsAg ELISA and normalized with β -Galactosidase ($n = 5$). **(B–E)** Plasmid DNA was transfected into HepG2 cells and the replicative capacities were analyzed. **(B)** The level of secreted HBsAg and HBeAg were detected using ELISA. **(C)** HBV DNA was compared via Southern blotting at 48 h post-transfection. **(D)** Western blot analyses of intracellular capsid forms were performed by using anti-HBcAg antibody on naive gel at 24 h after transfection. **(E)** The translation mediates pgRNAs were detected by qRT-PCR. **(F)** Linear DNA removed from CMV promoter was transiently transfected into HepG2 cells. The intermediates of transcripts cccDNA were determined using qPCR. Data were normalized to β -Galactosidase enzyme assay. One- and two-way ANOVA were used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

virion infection assays, we used two cell lines, HepaRG and HepG2-hNTCP-C4 which have been widely used for the HBV infection models (29, 30). In HepG2-hNTCP-C4 cells, intracellular HBV DNA level was also significantly increased in rt269L HBV infected cells compared with that of rt269I HBV infected cells on day 3 (Figure 3A). In addition,

rt269I-HBV-infected HepG2-hNTCP-C4 cells showed lower cccDNA level compared with rt269L-HBV-infected cells on day 5 (Figure 3B). Furthermore, the secreted HBsAg was significantly elevated in rt269L vs. rt269I HBV infected group and this pattern was maintained until day 5 after infection (Figure 3C). Consistently, extracellular HBV DNA level was also

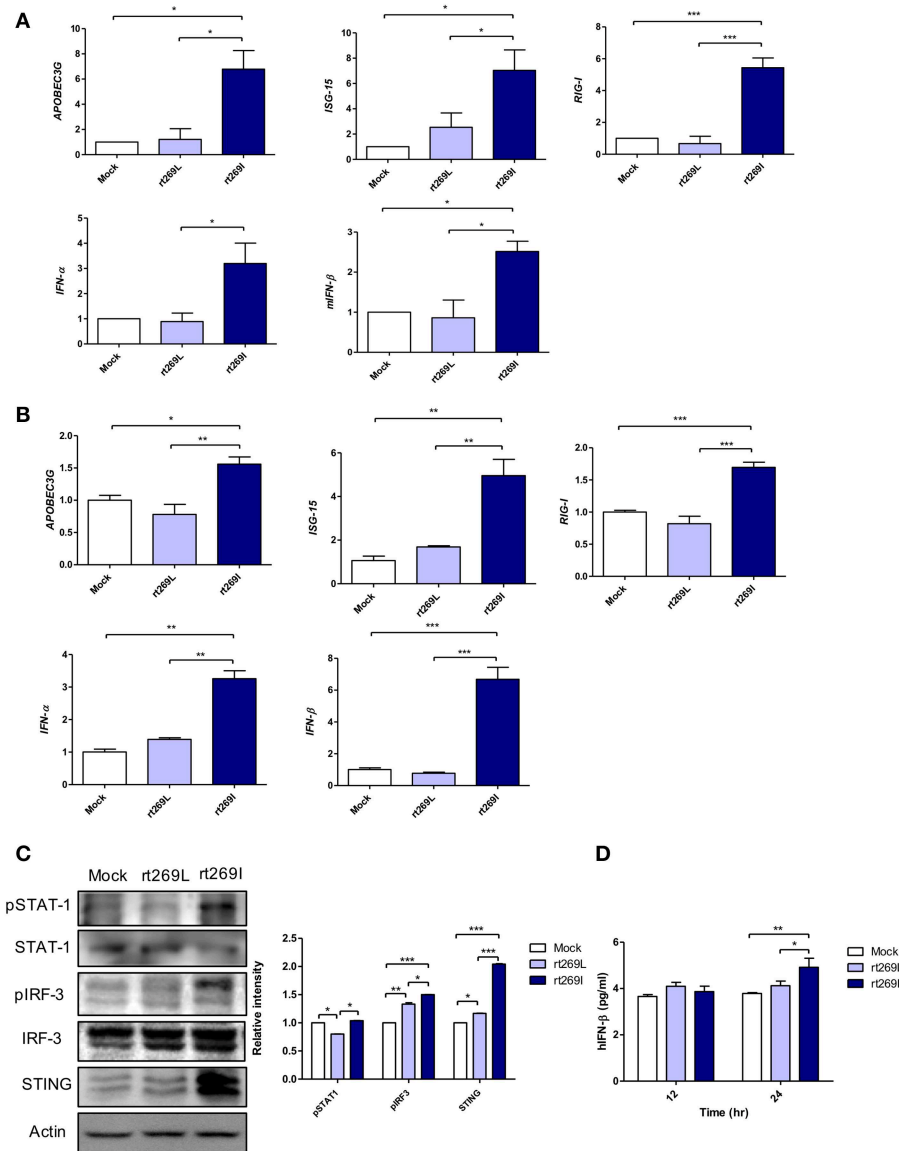


FIGURE 2 | rt269I enhanced IFN-I production in HepG2 cells. **(A)** Both HBV-encoding DNA and pSV-β-Galactosidase were injected by hydrodynamic tail vein injection into C57BL/6 mice ($n = 5$). The transcription level of APOBEC3G, ISG-15, RIG-I, IFN-α, and IFN-β were determined by qRT-PCR from mice hepatocytes on 4 days post-infection, and normalized with 18S rRNA and β-Galactosidase. **(B–D)** HepG2 cells were transfected with HBV DNA and the expression levels of IFN-I-related genes were assessed. **(B)** The mRNA level of IFN-I-related genes was determined using qRT-PCR at 12 h after transfection, and data were normalized to 18S rRNA. **(C)** The upstream proteins of the IFN-I signaling pathway were detected via Western blotting at 48 h post-transfection. **(D)** The secreted IFN-β was measured with ELISA at 24 h post-transfection. Data were and normalized to β-Galactosidase assay. One- and two-way ANOVA were used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

increased in rt269I HBV infected cells compared with that of rt269L HBV infected cells on day 3 (**Figure 3D**). The different replication capacities from infection between rt269L and rt269I HBV were further verified in HepaRG cells. Intracellular HBV DNA level was greatly increased in rt269L vs. rt269I HBV infected group, and the most distinct difference between two groups was shown on day 2 (**Figure 3E**). Reduced cccDNA level, shown in rt269I-HBV infected HepG2-hNTCP-C4 cells, was also reproduced in HepaRG cells on day 3 (**Figure 3F**). Furthermore, IFN-I secretion levels were measured by an ISRE promoter-luciferase assay. As shown in transient transfection

system, rt269I-infected-HepG2-hNTCP-C4 cells and -HepaRG cells secreted larger amount of IFN-I compared with rt269L-infected cells on Day 5 and day 0, respectively (**Figures 3G,H**). Together, we also proved that rt269I vs. rt269L could lead to reduced HBV replication and enhanced IFN-I production in HBV virion infection model system.

The Replication of HBV rt269I Was Inhibited via STING- IFN-I Axis

It has been reported that the secretion of IFN-I exerts antiviral effects against HBV (31, 32), and our above finding also showed

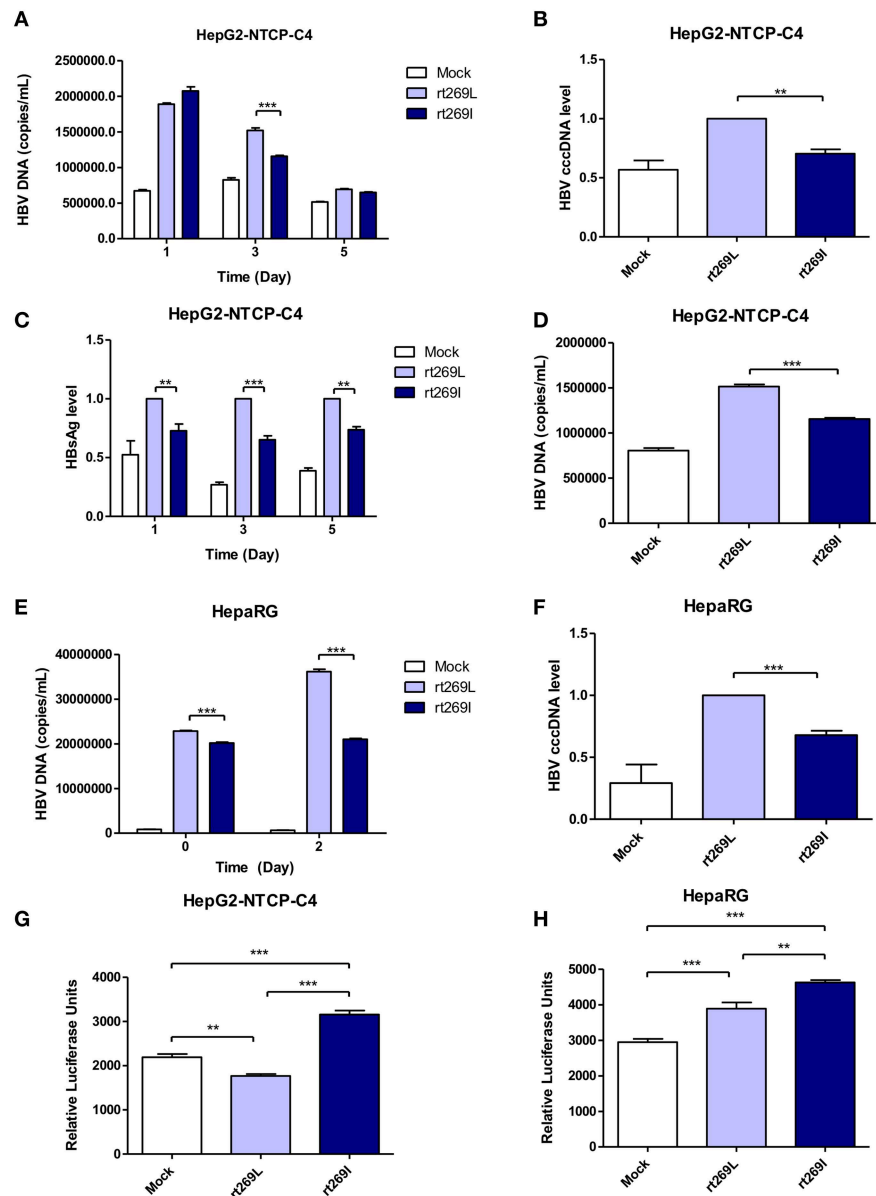


FIGURE 3 | The enhanced replication capacity of rt269L vs. rt269I shown in HBV infection models (A–D) rt269L- or rt269I- HBV variant infection of HepG2-hNTCP-C4 cells. (A) Intracellular HBV DNA level were evaluated using qPCR on day 1, 3, and 5. (B) HBV cccDNA level was measured in HepG2-hNTCP-C4 cells infected by each HBV variant on day 5. (C) HBsAg secretion in the supernatant of infected cells were measured using ELISA on day 1, 3, and 5. (D) Extracellular HBV DNA level were evaluated using qPCR on day 3. (E,F) rt269L- or rt269I- HBV variant infection of HepaRG cells. (E) Intracellular HBV DNA level was measured using qPCR on day 0 and 2. (F) HBV cccDNA level was evaluated in HepaRG cells infected by each HBV variant on day 3. (G,H) The secreted IFN-I in HepG2-hNTCP-C4 (G) and HepaRG cells (H) infected by each construct was measured using luciferase reporter assay on Day 5 and day 0, respectively. Data represents mean $\pm$ S.D. of three independent experiments. One- and two-way ANOVA were used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

that IFN-I was produced in the cells transfected with rt269I. Therefore, we verified whether replication inhibition found in rt269I is dependent on IFN-I signaling. We found that the decreased HBsAg and HBeAg levels in rt269I were neutralized or even reversed when the IFN-I signal was blocked via IFNAR2 neutralization at 48 h post-transfection (Figure 4A). To further check IFN-I signal dependence of rt269I replication, JAK-STAT pathway was inhibited using AZD1480. As shown in IFNAR2

neutralization, the inhibition of HBsAg and HBeAg level in rt269I was reversed by inhibition of JAK-STAT pathway at 48 h after transfection (Figure 4B). Furthermore, we found that the siRNA mediated knockdown of STING, but not scramble could lead to reversion of inhibition on HBV replication or HBsAg secretion as found in the rt269I type at 48 h post-transfection (Figure 4C). Together, these results indicated that rt269I led to lower levels of HBV replication via STING- IFN-I-axis in hepatocytes.

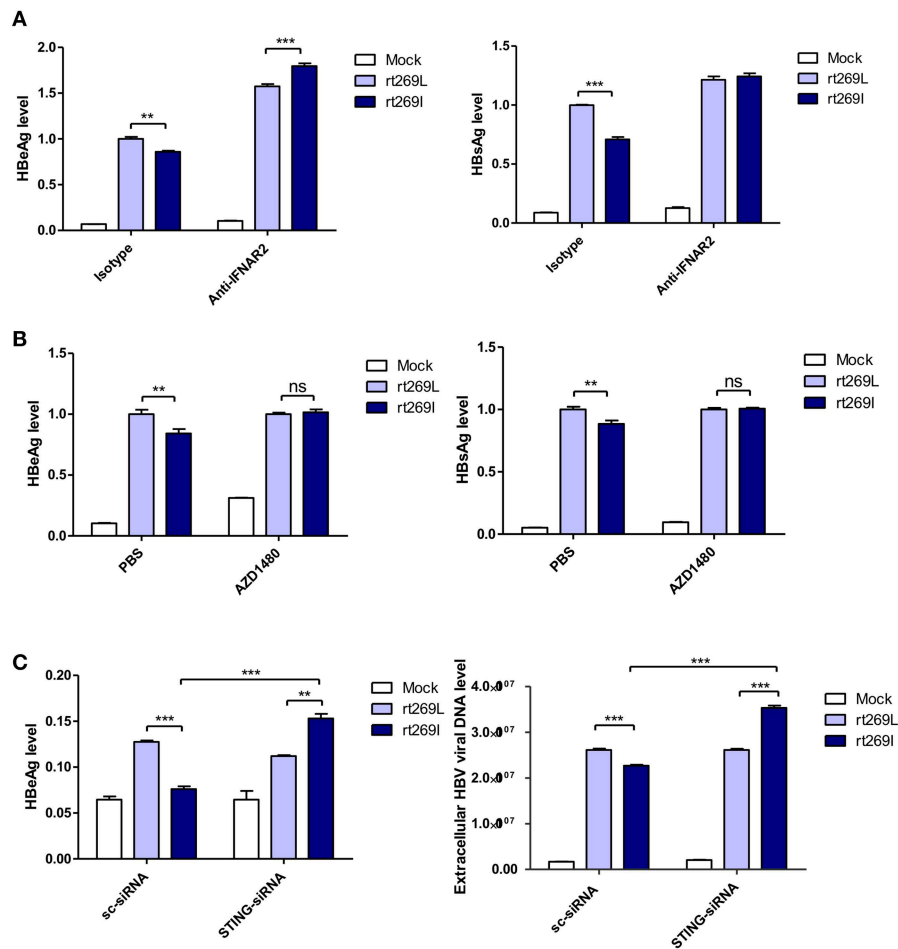


FIGURE 4 | The replication inhibition found in rt269I was mediated via STING-IFN-I axis. **(A)** IFNAR2 was blocked using anti-IFNAR2 antibody (10 μ g) and HBV-encoding DNA and pSV- β -Galactosidase were transiently transfected into HepG2 cells. HBsAg and HBeAg in the presence of isotype or anti-IFNAR2 antibody were measured using ELISA at 48 h post-transfection. **(B)** HepG2 cells pre-treated with AZD1480 (5 μ M) for 2 h were transfected with HBV DNA pSV- β -Galactosidase and constantly treated with AZD1480. HBsAg and HBeAg were measured using ELISA at 48 h post-transfection. **(C)** HBV DNA constructs and pSV- β -Galactosidase were co-transfected with either STING or scramble siRNA into HepG2 cells. The HBeAg levels and HBV virions were analyzed using ELISA and qPCR, respectively, at 48 h post-transfection. Data were normalized with β -Galactosidase enzyme assay, and represents mean $\pm$ S.D. of three independent experiments. One- and two-way ANOVA were used. * p < 0.05, ** p < 0.01, *** p < 0.001.

rt269I Led to Mitochondrial Stress Mediated Enhanced IFN-I Production and Heme Oxygenase (HO)-1

As bilirubin is one of the final byproducts of inducible HO-1 (33), our clinical data showing higher levels of bilirubin in serum of patients with rt269I (Table 1) also suggest that rt269I could enhance HO-1 expression. As expected, the expressed HO-1 significantly increased in the transcription and translation levels in cells with rt269I (Figures 5A,B). Previously, HO-1 has been reported to be induced by mitochondrial stress or ROS (34). Thus, we assessed whether mitochondrial ROS (mtROS) could be enhanced in HepG2 cells infected with rt269I. Although both groups showed similar cytosolic ROS levels (data not shown), enhanced mtROS levels were found in cells with rt269I at 24 h post-transfection (Figures 5C,D), suggesting that rt269I vs. rt269L led to enhanced mitochondrial

stress. To assess whether or not this effect is specific to genotype C, we compared mtROS production in HepG2 cells between two types of genotype C, rt269L and rt269I, and the wild type of genotype A with Pol of rt269I type via confocal analysis using MitoSOX. Of these, genotype C-rt269I produced the strongest mtROS, suggesting that the enhancing trait of mtROS found in rt269I may also be specific to genotype C at 24 h after transfection (Figure S5). It has been reported that mtROS can cause oxidative mitochondrial DNA damage, resulting in increased mitochondrial 8-OHdG that is indicative of DNA damage (35). We consistently observed that the quantification of 8-OHdG significantly increased in cells infected with rt269I at 48 h post-transfection (Figure 5E). Furthermore, we assessed if mtROS could act as upstream signaling of enhanced IFN-I and HO-1 induction induced by rt269I via treatment of MitoTEMPO, a mitochondria-targeted antioxidant.

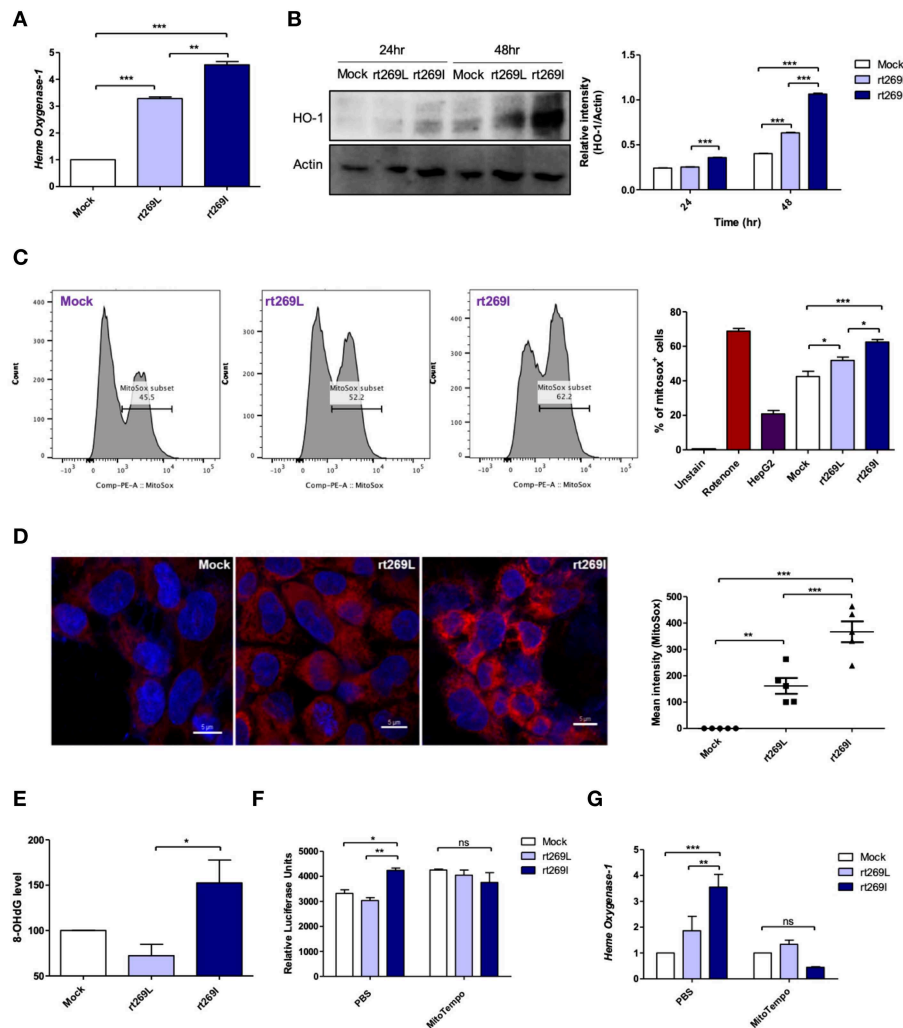


FIGURE 5 | rt269I led to enhanced HO-1 production and mitochondrial stress in infected hepatocytes. Mitochondrial stress was assessed from HepG2 cells transfected with HBV DNA. pSV- β -Galactosidase was co-transfected and used to normalize β -Galactosidase assay (A) Transcription level of HO-1 was determined using qRT-PCR at 24 h after transfection. (B) HO-1 protein was detected via Western blotting and actin was used as a control. (C,D) Mitochondrial ROS was stained with MitoSOX (5 μ M) and assessed by flow cytometry (C) and confocal microscope (D), followed by transfection for 24 h. (E) 8-OHdG ELISA was performed with genomic DNA at 48 h post-transfection. (F,G) MitoTEMPO (100 μ M) was administrated to the cells. (F) IFN-I levels were assessed using an IRSE-luciferase assay at 24 h after transfection. (G) qRT-PCR analysis of HO-1 mRNA levels were evaluated using qRT-PCR at 24 h after transfection. Data represent mean $\pm$ S.D. of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.

The treatment of MitoTEMPO abrogated the increased IFN-I induced by rt269I at 24 h post-transfection (Figure 5F). In addition, the elevated HO-1 mRNA levels induced by rt269I were also completely abrogated by MitoTEMPO treatment at 24 h post-transfection (Figure 5G). Together, these results indicated that rt269I infections could lead to mitochondrial stress and the subsequent cytosol release of oxidized mtDNA, resulting in enhanced production of IFN-I and HO-1 in hepatocytes.

rt269I Led to Enhanced iNOS Dependent NO Production

It was previously reported that HBV infections could lead to nitric oxide (NO) mediated by inducible nitric oxide synthase (iNOS), which causes the inhibition of viral replication (36).

Thus, we assessed iNOS dependent NO production between rt269I and rt269L. In an *in vivo* mouse model, the transcription of iNOS was significantly enhanced in rt269I compared to rt269L on 3 days post-injection (Figure 6A). In *in vitro* systems of transfected HepG2 cells, rt269I led to enhanced iNOS expression via Western blotting at 48 h post-transfection (Figure 6B). The secreted NO measured using ELISA was also significantly increased in rt269I at 24 h post-transfection (Figure 6C). In addition, to better understand the antiviral effects of NO, the viral replication of both types was also assessed after treatment with iNOS inhibitor, N^o-Nitro-L-arginine methyl ester hydrochloride (L-NAME). Treatment with L-NAME restored decreased HBsAg and HBeAg levels in both rt269I and rt269L types, but its effect was more pronounced in rt269I, suggesting that the lowered

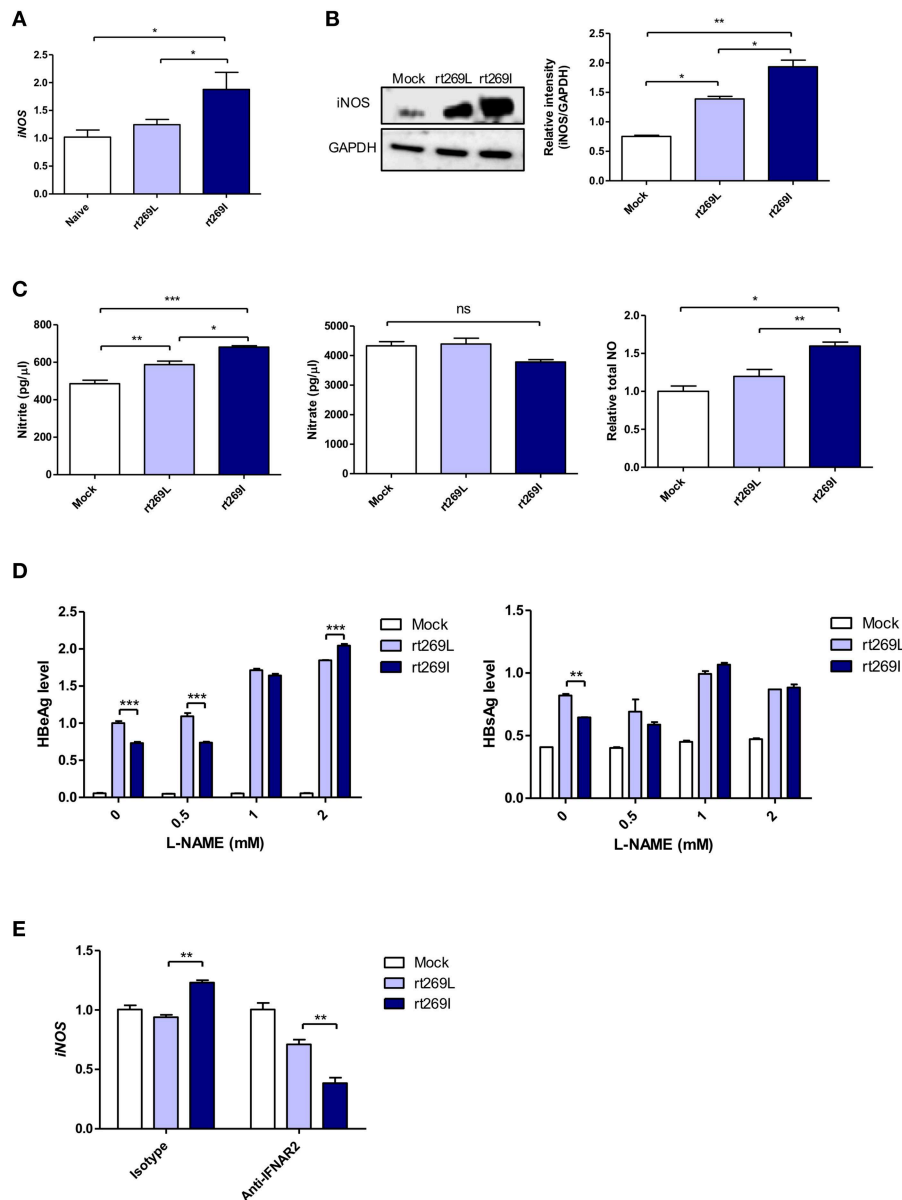


FIGURE 6 | rt269I enhanced iNOS dependent NO production in an IFN-I dependent manner. **(A)** The HBV constructs were injected with hydrodynamic tail vein into C57BL/6 mice ($n = 5$). Transcription levels of iNOS in hepatocytes were determined using qPCR and were normalized with 18S rRNA on 3 days post-injection. **(B–E)** HepG2 cells were transfected with HBV construct and pSV- β -Galactosidase was co-transfected to normalization. **(B)** iNOS synthesis was detected via Western blot analysis at 48 h after transfection. **(C)** Poly(I:C) was co-transfected with HBV DNA and the secreted NO_2^- and NO_3^- were measured using ELISA at 24 h post-transfection. **(D)** HepG2 cells pre-treated with L-NAME for 2 h were transfected with HBV DNA and constantly treated with L-NAME. The secreted HBsAg and HBeAg were detected by ELISA at 24 h. **(E)** IFN-I signaling was blocked by anti-IFNAR2 antibody (5 μg) for 12 h and iNOS gene expression was determined using qRT-PCR at 24 h after transfection. Data are shown as mean $\pm$ SD. One- and two-way ANOVA were used. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

replication shown in rt269I may be in part due to its enhanced NO production at 24 h post-transfection (Figure 6D). It has been reported that IFN-I can regulate iNOS dependent NO production as its upstream signal (37). To further verify whether iNOS induction found in rt269I is dependent on IFN-I signaling, the iNOS expression levels of both types were analyzed from infected HepG2 cells after blocking IFNAR2. The increased transcription levels of iNOS found in rt269I were abrogated by blocking IFNAR2 at 24 h post-transfection (Figure 6E). Together, our data

indicated that rt269I could lead to enhanced iNOS dependent NO production via IFN-I signaling, contributing to disease progression in chronic patients with genotype C infections.

DISCUSSION

Of 10 different HBV genotypes, genotype C and genotype B are responsible for the majority of HBV infections in endemic Asian

countries (17). In particular, it has been reported that most HBV infections in South Korea are due to genotype C (38), which may be a major reason for the lower response of IFN therapy (39) or NA therapy (40), the higher level of disease progression (41), and the higher prevalence of occult infection via vertical transmission (42, 43), observed in Korean CHB patients compared to patients in other areas. The current study investigated the virological or clinical factors and modulating capacity of antiviral IFN-I innate immunity between rt269L and rt269I in HBV genotype C infections. There are some noteworthy findings.

First, our clinical data using a Korean cohort proved that rt269L and rt269I showed distinct clinical or virological traits in HBV replication, HBsAg production, HBeAg serostatus, and bilirubin production (Table 1). This strongly suggests that there may be differences in modulating antiviral IFN-I production between rt269L and rt269I. Given a previous report indicating that the better response of genotype B than that of genotype C infections in IFN- α therapy was attributed to a higher level of HBeAg seroconversion via IFN- α mediated preC mutations (44), rt269I vs. rt269L, more related to lower HBV replication or the HBsAg level and more related to HBeAg negative serostatus was expected to lead to enhanced IFN-I production. Furthermore, a higher level of bilirubin, which is one of the final products of IFN-I mediated HO-1 metabolism, was also found in patients with rt269I type, further supporting the aforementioned findings.

Second, our further epidemiologic data showed that HBeAg seronegative status observed in patients with rt269I was due to a higher frequency of preC mutations (G to A at 1896) induced via IFN-I mediated APOBEC3G (Table 2 and Figure 2). This suggests that longer durations of HBeAg positive stages and lower levels of preC mutation frequency observed in patients with genotype C than that of genotype B infections (17) may be due to the presence of rt269L only in patients with genotype C. Actually, our further mechanism study showed that rt269L could produce lower levels of IFN-I production than rt269I, leading to a lower expression of ISGs including APOBEC3G in *in vitro* and *in vivo* systems, further supporting our epidemiological findings. Our epidemiologic data also showed that rt269L (63%) was more prevalent than rt269I (37%) in our Korean cohort, unlike other areas showing more prevalence of rt269I vs. rt269L (rt269I vs. rt269L: 57 vs. 43%). This suggests that rt269L showed not only high replication factors such as HBeAg, HBsAg, and HBV DNA level but also low preC mutation frequencies. These features of rt269L can be indicated with characteristics of genotype C, which was not shown in other genotypes. In addition, we suggest it may have a merit in infections into hosts via perinatal or vertical routes, contributing to chronic infections as a major type in South Korea. It is tempting to speculate that the higher prevalence of rt269L may contribute to some unique clinical traits found in South Korean CHB patients, including the frequent failure of IFN- α therapy or NA treatments (39, 40) and the higher

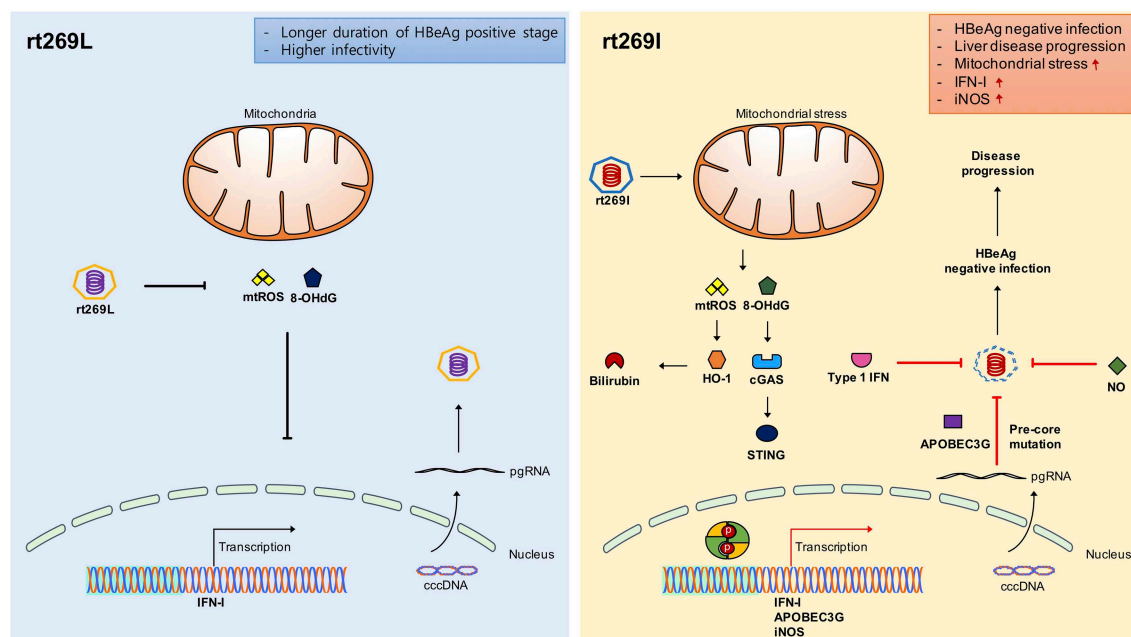


FIGURE 7 | Schematic presentation indicating distinct mitochondrial stress mediated IFN-I production and its distinct contribution to disease progression in chronic patients with genotype C infections between rt269L and rt269I. In contrast to rt269L, rt269I infection in genotype C induced mitochondrial ROS production, which led to an increased release of oxidized DNA into the cytosols of the infected hepatocytes. Sensing the oxidized DNA exposed to cytosol via the cGAS-STING pathway could lead to IFN-I production. Enhanced IFN-I production in rt269I could exert several biological activities. First, it can lead to the increased inhibition of HBV replication via the inhibition of capsid formation by HO-1 production. Second, IFN-I mediated enhanced expression of APOBEC3G and iNOS can lead to HBeAg negative infection and liver disease progression via frequent generation of preC mutations at 1896 (G to A). Thus, rt269L can contribute to HBeAg negative infection and disease progression in chronic patients infected with genotype C via mitochondrial stress mediated enhanced IFN-I production.

prevalence of occult HBV infections via vertical routes (42, 43). However, this issue demands further investigation in the future.

Third, our mechanism study indicated that distinct IFN-I production between rt269L and rt269I was attributed to different induction capacity of mitochondrial stress (**Figure 5**). Thus, rt269I vs. rt269L can lead to enhanced production of mtROS and oxidized mtDNA, resulting in induced IFN-I production via cytosolic DNA sensing through the STING-IRF3 dependent pathway. To date, distinct capacity of mitochondrial stress induced by HBV genotypes, polymorphisms, or mutations has not been elucidated. HBV Pol was recently reported to interfere with antiviral IFN-I production through DNA sensing via the inhibition of STING polyubiquitination (13). Thus, the functional difference in Pol due to SNP could contribute to differences in the two types of mitochondrial stress mediated IFN-I production. However, the molecular details regarding this issue remain to be elucidated in the future.

Fourth, we found enhanced HO-1 expression and iNOS dependent NO production as a downstream pathway of IFN-I in rt269I. The enhanced HO-1 expression in rt269I exerted anti-HBV effects via the inhibition of virion capsid formation, in line with previous reports showing the anti-HBV effects of HO-1 (45). It has also been reported that iNOS dependent NO production as another downstream pathway of IFN-I could play a pivotal role in inflammatory responses and disease progression in the liver, including fibrosis and cirrhosis (37). We also found enhanced production of iNOS and NO in HepG2 cells transfected with rt269I (**Figure 6**), in line with our clinical data showing that patients with rt269I type infections were more related to disease progression. Enhanced iNOS and NO could also increase the frequency of preC mutations shown in rt269I, leading to HBeAg negative infections in chronic patients. The iNOS dependent NO production and mtROS production can synergistically exert apoptotic cell death in the liver, also facilitating the progression of liver diseases (46), further supporting our hypothesis regarding the contribution of rt269I to disease progression (**Figure 7**).

Fifth, our finding showing rt269L vs. rt269I type led to enhanced HBV replication via inhibiting IFN-I production via STING-IFN-I axis is contrast to that reported by Ahn et al (47), previously. The difference between the two studies may be due to the use of HBV genome plasmid from different patients with distinct genome sequences or difference of used cell lines. The pHBV-1.2x (GenBank accession No. AY641558) plasmid construct used in this study has no special mutations affecting HBV virology and virulence and have been widely used for genotype C mutation analysis and virulence studies (24, 48–50). Furthermore, together with our HBV genome transient study into diverse human hepatocytes and mouse hepatoma cell lines and *in vitro* HBV virion infection study, our *in vivo* hydrodynamic injection study, and even our clinical data consistently supported enhanced HBV replication of rt269L vs. rt269I type.

In conclusion, our data showed that rt269I could contribute to HBeAg negative infections and liver disease progression in CHB patients with genotype C infections via mitochondrial stress mediated IFN-I production. In addition, enhanced iNOS dependent NO production induced by rt269I could also provide an additive role in disease progression. Furthermore, our findings could also provide a likely explanation into characteristic features of genotype C with higher frequency of rt269L type, including longer durations of HBeAg positive stages and higher infectivity (**Figure 7**).

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This report was approved by the Institutional Review Board of Konkuk University Hospital (KUH-1010544) and Seoul National University Hospital (IRB-1808-067-965). All of the animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University College of Medicine (SNU-170308).

AUTHOR CONTRIBUTIONS

S-YL and B-JK designed the study. S-YL performed statistical analysis. S-YL, Y-MC, and S-JO performed all experiments and analyzed data. S-YL, Y-MC, S-JO, S-BY, and JL edited the manuscript. W-HC provided clinical expertise and samples. Y-HK and B-JK interpreted the experiments. S-YL, Y-MC, and B-JK wrote manuscript.

FUNDING

This work was supported by the Ministry of Science and ICT (Grant No. 800-20180159).

ACKNOWLEDGMENTS

We appreciate statistical consultation from the Medical Research Collaborating Center at the Seoul National University Hospital and the Seoul National University College of Medicine. Y-MC, S-JO, and S-BY received a scholarship from the BK21-plus education program provided by the National Research Foundation of Korea.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.01735/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer KW and handling editor declared their shared affiliation.

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Hyperactive Follicular Helper T Cells Contribute to Dysregulated Humoral Immunity in Patients With Liver Cirrhosis

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OPEN ACCESS

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Specialty section:

This article was submitted to
Viral Immunology,
a section of the journal
Frontiers in Immunology

Received: 06 June 2019

Accepted: 29 July 2019

Published: 13 August 2019

Citation:

Zhao J, Shi J, Qu M, Zhao X, Wang H,
Huang M, Liu Z, Li Z, He Q, Zhang S
and Zhang Z (2019) Hyperactive
Follicular Helper T Cells Contribute to
Dysregulated Humoral Immunity in
Patients With Liver Cirrhosis.
Front. Immunol. 10:1915.
doi: 10.3389/fimmu.2019.01915

Objectives: Liver cirrhosis (LC) is usually accompanied by cirrhosis associated immune dysfunction (CAID), including reduced naïve T cells and memory B cells. However, little is known regarding on follicular helper T (Tfh) cell compartments in cirrhotic patients, especially in the secondary lymphoid organs such as spleen. This study characterizes splenic Tfh cells and explores its association with humoral immunity and disease progression in cirrhotic patients.

Methods: Using flow cytometry and histological staining, we analyzed the frequency and cytokine production of splenic Tfh cells from LC patients and healthy controls (HCs). Co-culture experiments of sorted Tfh and B cells were performed for functional analysis *in vitro*. The correlations between Tfh cells and disease progression markers as well as B cell subset perturbations were also examined.

Results: PD-1^{high}ICOS⁺CXCR5⁺ Tfh cells were preferentially enriched in the spleen of cirrhotic patients, where they expressed higher levels of CXCR3 and produced more interleukin (IL)-21. Histologically, more splenic Tfh cells occupied the B cell follicular structure in LC patients where they shaped more active germinal centers (GCs) than those in HC spleens. *In vitro*, splenic Tfh cells in cirrhotic patients robustly induce plasma cell differentiation through IL-21 dependent manner. Finally, increased Tfh cell frequency is positively correlated with the plasma cells and disease severity in LC patients.

Conclusions: We conclude that hyperactive Tfh cells contribute to dysregulated humoral immunity in patients with liver cirrhosis.

Keywords: liver cirrhosis, cirrhosis associated-immune dysfunction, Tfh cell, humoral immunity, spleen

INTRODUCTION

Liver cirrhosis (LC) is the common outcome of liver inflammation induced by various etiologies such as hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol, drug, and fat (1). While LC impairs the liver physiological function, it also causes cirrhosis associated immune dysfunction (CAID). CAID is a complicated process accompanied by the increased systemic inflammation and immune paralysis and/or immunodeficiency (2). The mechanisms leading to CAID remains unclear but possibly associates with the increased bacterial translocation due to the impaired gut barrier, reduced gut motility, and altered gut flora (3).

The occurrence of CAID is associated with systemic inflammation during LC disease progression, which is reflected by the persistent activation of peripheral immune cells and the elevated serum pro-inflammatory cytokines (2, 4). Compromised immune cell function was frequently reported in LC. For example, neutrophils from LC patients show impaired phagocytosis of opsonized bacteria (5). Peripheral non-classical CD14⁺CD16⁺ monocytes are increased in LC patients and display abnormal functions (6). LC profoundly depletes the circulating CD27⁺ memory B cells (7, 8). Accompanying this, hyper-globulinemia and elevated IgG and IgA levels were observed in advancing cirrhosis, irrespective of the underlying etiology (9). LC also causes T cell lymphopenia and disrupts T cell compartments. Particularly, naïve T cell depletion is more pronounced than the memory T compartment (10). Circulating natural killer (NK) cells are also compromised in liver cirrhosis and display poor responses to cytokine stimulation *in vitro* (11). These data partially define the characteristics of the CAID in peripheral blood, but little is known about its impact on the secondary lymph organ (SLO) such as spleen of cirrhotic patients, especially for T cells and B cell compartments, the two most important arms in the adaptive immune system.

Spleen is the largest SLO in the body. Spleen sits next to the liver and its blood was delivered to liver. It is well-known to play roles in immune defense against blood borne infections (12). However, spleen blood flow was congested due to portal hypertension during liver cirrhosis, resulting in splenomegaly and peripheral cytopenia (13). So far, it is unclear whether the cellular composition and underlying structure of spleen have been affected by cirrhosis. It is also debated whether the spleen plays a detrimental role in the liver pathophysiology during LC (14). Follicular T helper (Tfh) cells are one of main cell compartments of the spleen, and are usually defined as PD-1^{high}ICOS⁺ CD4⁺ T cells with the expression of C-X-C motif chemokine receptor 5 (CXCR5) (15). Splenic Tfh cells play key roles in T-dependent antibody responses (16). The interaction between Tfh cells and cognate B cells induces robust B cell proliferation and instructs them to undergo differentiation through CD40 engagement and IL-21 supply (17). The persistent activation of Tfh cells is required to promote broadly and affinity matured neutralizing antibodies throughout chronic viral infection, and to adapt specificity to emerging viral variants (18). However, the high levels of Tfh cells present in chronic viral infections can also render the activation of germinal

center (GC) B cells and the selection process less stringent, thus resulting in aberrant B cell activation, the generation of non-virus-specific antibodies and even autoimmune reactive antibodies, hyper-gammaglobulinemia in some cases (18). For example, in human immunodeficiency virus-1 (HIV-1) and simian immunodeficiency virus (SIV) infection, the expansion of Tfh cells present in lymph node of infected subjects correlates with hyper-gammaglobulinemia, polyclonal B cell activation, and the deletion of peripheral memory B cells (19). Similarly, the proportion of peripheral Tfh cells correlates with the emergence of autoantibodies in persistent HBV infection (20). Considering the contribution of Tfh cells to dysregulated B cell responses, the persistent interaction of Tfh cells and GC B cells is one of the key reasons for the emergence of autoreactive antibodies during autoimmune diseases (21).

Here, we hypothesized that the dysregulated Tfh cell responses might contribute to disruption of B cell compartments in cirrhosis. Our findings support the notion that the enhanced Tfh cell responses results in the persistent activation of humoral immunity, potentially depleting memory B cell pools in cirrhotic patients, and therefore is associated with LC severity.

MATERIALS AND METHODS

Study Subjects

A total of 28 HBV associated LC (HBV-LC) patients and 23 non-HBV associated LC (non-HBV-LC) patients were recruited for this study in Shenzhen 3rd People's Hospital and in Beijing 302 Hospital. According to our described criteria previously, all patients were diagnosed (22, 23) and had not received immunosuppressive drugs within 6 months before taking samples. Forty-two age- and gender-matched individuals were enrolled as healthy controls (HCs). The study protocol was approved by the ethics committee of our institutions, and written informed consent was obtained from each subject. The basic clinical information of the enrolled individuals is listed in **Table 1**. Peripheral blood mononuclear cells (PBMCs) were isolated from all enrolled individuals. Spleen samples were collected from 28 HBV-LC and 13 non-HBV-LC patients with portal hypertension who underwent splenectomy. Twenty-two healthy spleen tissues were obtained from donors whose livers were used for transplantation.

Fluorescence Antibodies and Flow Cytometry

All of the fluorescence-conjugated antibodies were purchased from BD Bioscience or Pharmingen (San Diego, CA), eBioscience (San Diego, CA), and BioLegend (San Diego, CA). For T cell subset staining, allophycocyanin-Cy7 (APC-Cy7)-conjugated anti-CD45, amCyan-conjugated anti-CD3, Pacific Blue (PB)-conjugated anti-CD4, fluorescein isothiocyanate (FITC)-conjugated anti-CXCR5 (CD185), phycoerythrin (PE)-conjugated anti-CCR6 (CD196), peridinin chlorophyll protein-Cy5.5 (PerCP-Cy5.5)-conjugated anti-CXCR3 (CD183), phycoerythrin-Cy7 (PE-Cy7)-conjugated anti-PD-1, APC-conjugated anti-ICOS were used. For intracellular

TABLE 1 | Basic information of enrolled subjects.

	HC	HBV-LC	Non-HBV-LC
Cases	42	28	23
Spleen usage*	22	28	13
Age (years)	48.5 (23-39)	40.3 (18-61)	46 (23-62)
Gender (M/F)	31/11	22/6	15/8
Platelet ($\times 10^{12}$)	ND	45.0 (15-97)	49.8 (28-83)
ALT (IU/L)	21.8 (12-60)	19.3 (9-50)	1.9 (10-31)
AST (IU/L)	44.1 (23-64)	25.4 (14-43)	29.6 (15-56)
Total bilirubin ($\mu\text{mol/L}$)	16.4 (10.6-29.1)	17.3 (7.3-37.9)	15.7 (3.7-29.8)
Albumin (g/L)	29.1 (22-34.5)	38.0 (32-48)	37.9 (35-42)
Prothrombin time (second)	13.9 (12.1-16.8)	13.0 (11.5-14.7)	12.9 (11.1-16.8)
Prothrombin activity	ND	68.2 (54.6-53.2)	70.2 (47.6-87.8)
HBeAg ($\pm$)	NA	6/22	NA
Child score (5/6/7)	NA	8/15/5	4/13/6

M, male; F, female; HBeAg, hepatitis B e antigen; ND, not determined; NA, not applicable.

*Clinical data in HC subjects were from only donors with spleen usage.

cytokine staining (ICCS), PE-conjugated anti-IL-21, APC-conjugated anti-IL-17a, and PE-Cy7-conjugated anti-IFN- γ were additionally used. For B cell subset staining, FITC-conjugated anti-CD19, PE-conjugated anti-IgD, PE-Cy7-conjugated anti-CD38, and APC-Cy7-conjugated anti-CD27 were used. The phenotypic analysis were performed at the optimal monoclonal antibody (mAb) concentrations according to the previously reported standard protocols (24). For surface marker staining, PBMCs, or spleen cells were incubated with fluorescence-conjugated surface antibodies. For intracellular staining, the cells were stained surface markers first, permeabilized and were then stained with the corresponding intracellular antibodies. Aliquots of cells were used for the analysis using FACSCanto II and FlowJo software (Tristar, San Carlos, CA). At least 50,000 events were analyzed for per sample.

Definition of T Cell Subsets and Analysis of Tfh Cells

According to previous studies (15), CD4⁺ and CD8⁺ T cell subsets were defined by the expression of chemokine receptors CXCR5, CCR6, and CXCR3. Among CD4⁺ T cells, CXCR5⁺ cells were defined Tfh-like cells. Within CXCR5⁺CD4⁺ T cells, CCR6⁺CXCR3⁻ cells, CCR6⁺CXCR3⁺ cells, CCR6⁻CXCR3⁺ cells, CCR6⁻CXCR3⁻ cells were defined as Th17 cells, Th1/Th17 cells, Th1 cells, and Th2 cells, respectively. Similarly, among CD4⁻ T cells (most are CD8⁺ T cells), CXCR5⁺ cells were defined Tfc-like cells, and CCR6⁺CXCR3⁻, CCR6⁺CXCR3⁺, CCR6⁻CXCR3⁺, CCR6⁻CXCR3⁻ CXCR5⁻ T cells were defined as Tc17 cells, Tc1/Tc17 cells, Tc1 cells, and Tc2 cells, respectively. Quantitation of peripheral blood Th and Tc cell subset percentages was performed in 24 HBV-LC patients, 23 non-HBV-LC patients, and 42 HCs. Among the available spleens, the splenic cells were further comprehensively analyzed for the expression of PD-1 and ICOS among the CXCR5⁺CD4⁺ T cells which were defined Tfh cells.

Cell Stimulation

To evaluate the IFN- γ , IL-17a, and IL-21 secreting function of CD4⁺CXCR5⁺PD-1^{high} Tfh cells, spleen cells (1×10^6) were cultured in a medium alone or with PMA (50 ng/ml) and ionomycin (1 $\mu\text{g/ml}$) in 96-well plate for 6 h. After 6 h incubation, the cells were collected and stained with surface and intracellular antibodies with anti-IFN- γ , anti-IL-17a, or anti-IL-21. Then the percentages of IFN- γ , IL-17a, or IL-21-producing cells were analyzed between HC subjects and HBV-LC patients.

Cell Sorting

Peripheral blood mononuclear cells (1×10^7) were first used for CD4⁺ T cell enrichment using microbeads (Cat# 130-096-533, Miltenyi Biotec, Germany). Then the isolated CD4⁺ T cells are stained with optimal concentrations of AmCyan-conjugated anti-CD3, PerCP-conjugated anti-CD4, FITC-conjugated anti-CXCR5, and PE-conjugated anti-PD-1 antibodies for 25 min. Alternatively, PBMCs were also stained with APC-conjugated anti-CD19. A FACS Aria II cell sorter was used to purify CD4⁺CXCR5⁺PD-1^{high} cells, CD4⁺CXCR5⁺PD-1⁺ cells, CD4⁺CXCR5⁺PD-1⁻ cells, and CD4⁺CXCR5⁻ cells as well as CD19⁺ B cells. The purity of the sorted cell subsets was more than 90%.

The Co-culture of Tfh Cells With B Cells

Similar to previous studies (17), the sorted CD4⁺CXCR5⁺PD-1^{high} cells, CD4⁺CXCR5⁺PD-1⁺ cells, CD4⁺CXCR5⁺PD-1⁻ cells, and CD4⁺CXCR5⁻ cells from 4 HBV-LC patients or 4 HC donors were co-cultured with allogeneic or autologous CD19⁺ B cells at the ratio of 1:1 in RPMI-1640 medium containing 10% fetal bovine serum (FCS) and staphylococcal enterotoxin B (SEB) (100 ng/ml). Alternatively, rIL-21R-Fc chimera (10 $\mu\text{g/ml}$; R&D Systems, Minneapolis, MN) was added when the CD4⁺CXCR5⁺PD-1^{high} cells co-cultured with B cells (24). After 7–8 day culture, the cells were collected to assess the generation of plasma cells.

Enzyme-Linked Immunosorbent Assays (ELISA)

An ELISA quantitative kit for the detection of human IgG and IgM (Bethyl Laboratories, Montgomery, TX) was used to measure the concentrations of total IgG and IgM from culture supernatants and plasma. The ELISA kits of human fatty acid binding protein 2 (FABP2, Cat#: DY3078) and CXCL13 (Cat#: DCX130) were purchased from R&D System Inc (Minneapolis, MN, USA), and were used measure their concentration in the plasma from LC and HC subjects.

Immunohistochemistry

Hematoxylin-eosin (HE) and immunohistochemistry staining were performed using paraffin-embedded 4 μm tissue sections. The sections were first incubated with optimal concentrations of primary mAbs, including anti-IL-21 (abcam, ab118510), anti-CD20 (clone L26), anti-CD4 (clone IF6), and anti-PD-1 (clone NAT105) (Zhongshan Goldenbridge Biotech, Beijing, China) for 1 h at RT. Then the sections were incubated with

biotinylated goat-rabbit, goat anti-rat, or biotinylated rabbit anti-mouse antibodies (Zhongshan Goldenbridge Biotech, Beijing, China), respectively. The GC was observed independently by two pathologists from 5 of representative high-power fields (200 × magnification) (24).

Correlation Analysis of Splenic Tfh Cells and B Cell Subsets

To analyze the correlation between splenic PD-1^{high}ICOS⁺CXCR5⁺CD4⁺ Tfh cells and relevant B-cell subsets, 28 of HBV-LC patients with available spleen were studied. The correlations between the percentage of Tfh cells and total B cells or B cell subsets or plasma IgG and IgM levels were analyzed. CD19⁺CD10[−]CD38^{lo} mature B cells were divided into IgD⁺CD27[−] naïve B cells (naïve B cells), IgD⁺CD27⁺ marginal zone B cells (MZBs), and IgD[−]CD27⁺ class-switched memory B cells (cMBCs) and IgD[−]CD27[−] atypical memory B cells (aMBCs). CD19⁺IgD[−]CD38^{high} phenotype defined the plasmablasts (24).

Correlation Analysis of Splenic Tfh Cells and Liver Functions

The correlations between the percentage of spleen Tfh cells and several clinical biochemical parameters including serum albumin (ALB), total bilirubin (TBIL), and prothrombin activity (PTA) and Child-Pugh scores were analyzed in the 28 HBV-LC patients. In addition, we also evaluated the levels splenic Tfh percentages in HBV-LC patients with various Child-Pugh scores.

Statistical Analysis

All statistics were analyzed using SPSS 16.0 software. The data are presented as mean values and standard deviations. Multiple comparisons were first performed among the different groups using the non-parametric Kruskal–Wallis *H*-test. Comparisons between various groups were made using the Mann–Whitney *U*-test, whereas comparisons between the same individual were made using the Wilcoxon's matched-pairs test. The correlations between two variables were analyzed using the Spearman rank correlation test. *P* < 0.05 at two-sides was considered to be significant for all analyses.

RESULTS

CXCR5⁺ CD4 Tfh-like Cells Are Enriched in Spleen and Peripheral Blood in LC Patients

We first analyzed the frequencies of peripheral and splenic CD4⁺ Th (Figure 1A) and CD8⁺ Tc (Supplemental Figure 1A) cell subsets using flow cytometry according to the expression of chemokine receptors CXCR5, CCR6, and CXCR3. As shown in Figure 1B, among peripheral CD4⁺ T cells, the percentages of Tfh-like cells, Th1/Th17 cells, and Th17 cells in HBV-LC and non-HBV-LC patients were all significantly higher than those in HC subjects (*P* < 0.05). By contrast, Th2 cell percentages were significantly decreased (*P* < 0.001) and Th1 cells were comparable between LC patients and HC subjects. Similar trends were also found among peripheral CD8⁺ Tc subsets (Supplemental Figure 1B). Notably, when splenic CD4⁺ and

CD8⁺ T cell subsets were analyzed, it is found that only Tfh-like cell proportions were significantly increased in LC patients than those in HC subjects; while other CD4⁺ and CD8⁺ T cell subsets were unchanged (Figure 1B and Supplemental Figure 1B). These data indicate that CXCR5⁺CD4⁺ Tfh-like cells are preferentially enriched in both peripheral blood and spleen from HBV-LC and non-HBV-LC patients.

Tfh Cells Are Significantly Expanded With High Levels of CXCR3 Expression in the Spleen of LC Patients

PD-1 and ICOS are recognized as main markers to define Tfh cells, we therefore analyzed their expression in these CD4⁺ Th cell and CD8⁺ Tc cell subsets from both peripheral blood and spleen (Supplemental Figure 2). PD-1^{high}ICOS⁺ cells formed a distinct population within the splenic Tfh-like cells; their numbers in peripheral Tfh-like cells and splenic Tfc cells were much fewer and almost absent from other peripheral and splenic CD4⁺ and CD8⁺ T cell subsets (Supplemental Figure 2). We then compared the percentages of PD-1^{high}ICOS⁺CXCR5⁺ Tfh cells in LC patients and HC subjects (Figure 2A and Supplemental Figure 3A). We found that the proportion of PD-1^{high}ICOS⁺CXCR5⁺ Tfh cells was significantly increased in both spleen (Figure 2B) and peripheral blood (Supplemental Figure 3B) from LC patients compared to HC subjects (both *P* < 0.01). PD-1^{high}ICOS⁺CXCR5⁺ Tfc cells were also significantly increased in the spleen of LC patients (Figure 2B) compared to those from HC subjects (*P* < 0.01) but not in blood (Supplemental Figure 3B). These data indicated that PD-1^{high}ICOS⁺CXCR5⁺ Tfh cells are preferentially enriched in the splenic CD4⁺ T cells, which is further increased in LC patients.

We further analyzed the proportion of CXCR3- and CCR6-expressing cells in splenic Tfh cells from LC and HC subjects (Figure 2C). It is found that CXCR3⁺ cells and CCR6⁺ cells account for nearly 80 and 10% of splenic PD-1^{high}ICOS⁺ Tfh cells, respectively, regardless of disease status (Figure 2C). The proportion of CXCR3[−]CCR6[−] Tfh2 cells was higher among CXCR5⁺ Tfh-like cells in both spleen and blood from HC donors vs. LC patients. The CXCR3⁺CCR6[−] Tfh1 cells was significantly expanded in the spleen of LC patients. While in peripheral blood, the proportion of CCR6⁺ cells (including both CXCR3⁺CCR6⁺Tfh1/Tfh17 cells and CXCR3[−]CCR6⁺ Tfh17 cells) were significantly increased in LC patients (Supplemental Figures 4A,B). These data indicated that splenic Tfh-like cells expressed higher levels of CXCR3 in LC patients.

Splenic Tfh Cells From LC Patients Produced Higher Levels of IL-21

Next, we analyzed cytokine production by splenic Tfh cells including IL-21, IFN-γ, and IL-17a in responses to PMA/ionomycin *in vitro* (Figure 3A). As shown in Figure 3B, the levels of IL-21 produced by splenic Tfh cells in HBV-LC patients were significantly higher than those in HCs (*P* < 0.05). IFN-γ production by splenic Tfh cells was significantly reduced in HBV-LC patients as compared to HC subjects. There is no

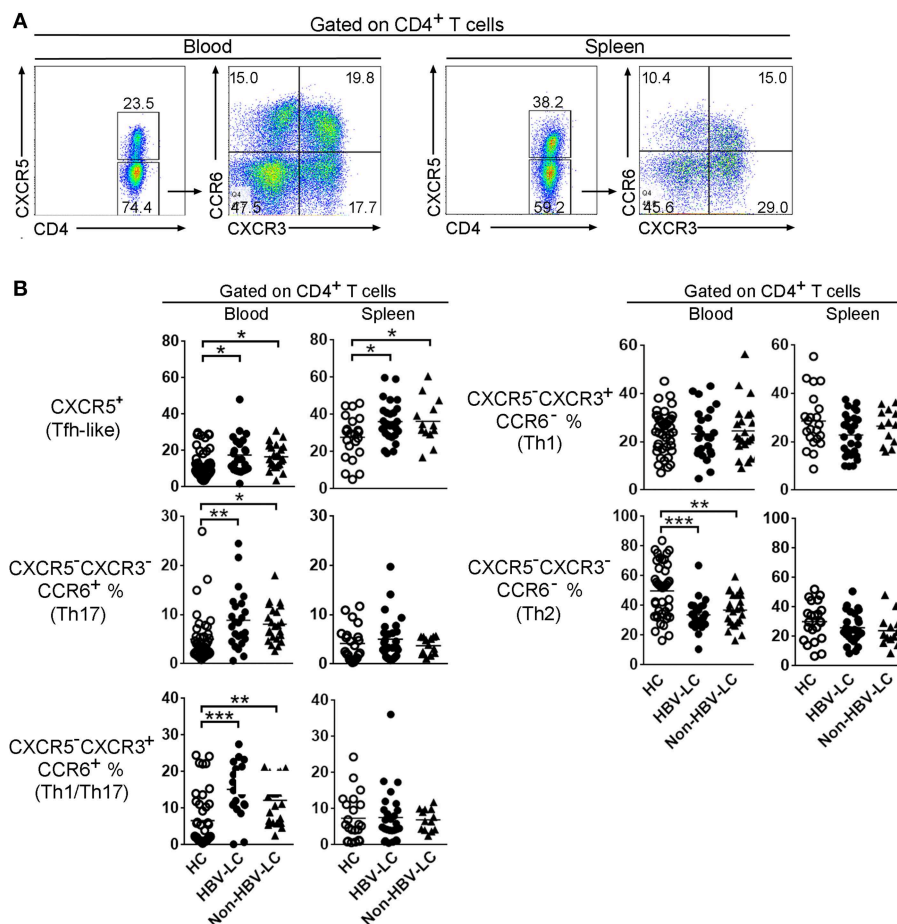


FIGURE 1 | Disruption of Th cell subset distribution in patients with liver cirrhosis. **(A)** Representative dot plots show the gating strategy of different CD4⁺ subsets based on CXCR5, CCR6, and CXCR3 expression in spleen and peripheral blood. Values in the quadrants represent the percentages of each subset. Among CD4⁺ T cells, CXCR5⁺ marks Tfh-like cells. For CXCR5⁻CD4⁺ T cells, CCR6⁺CXCR3⁻ Th17 cells, CCR6⁺CXCR3⁺ Th1/Th17 cells, CCR6⁻CXCR3⁺ Th1 cells, CCR6⁻CXCR3⁻ Th2 cells were defined, respectively. **(B)** Summarized data show the percentages of CD4⁺ T cell subsets in the peripheral blood from HC ($n = 42$), HBV-LC ($n = 24$), and Non-HBV-LC groups ($n = 23$) and in the spleen from HC ($n = 22$), HBV-LC ($n = 27$), and Non-HBV-LC groups ($n = 13$). Each dot represents one subject, and line represents mean value. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Mann-Whitney U-test.

significant difference for IL-17a production by splenic Tfh cells in LC patients as compared to those in HC subjects. These data indicated the splenic Tfh cells produced more IL-21 in LC patients, which may shape the specific microenvironment promoting humoral immunity when liver disease progresses into cirrhotic phases.

More Splenic Tfh Cells Moved Into B Cell Zone and Shaped Active Germinal Centers (GCs) in LC Patients

We investigated the distribution of splenic Tfh cells using immunohistochemical staining. More follicle-like structure showing larger active GCs was present in the spleen of LC patients than that in HC spleen where the GCs were mostly degenerative. Outside of the GCs, a ring-like layer densely populated by smaller-sized cells (likely the reported mantle zone formed mostly by naive B cells) was present in LC spleen and

absent from HC spleen (**Figure 4A**). We further stained several classical markers for Tfh cells and B cells in spleen including CD4, PD-1, and IL-21 as well as CD20 using continuous section so as to observe the localization of Tfh cells and B cells *in-situ* from LC patients vs. HC subjects. CD20 staining indicated that the follicles were indeed splenic B cell zones. It is found that less CD4⁺, PD-1⁺, and IL-21⁺ Tfh cells were present in healthy spleen. By contrast, more PD-1⁺ and IL-21⁺ cells were accumulated within the GC area in LC patients (**Figure 4B**). Consistent with the role of Tfh cells in promoting GC responses, we observed significantly increased follicular Ki67⁺ B cells in LC spleens as compared to HCs (**Figure 4C**). Plasma CXCL13, a B cell attracting cytokine, has been considered as a marker indicating GC activity (25). We measured the CXCL13 levels in the plasma of HBV-LC and HC subjects, and found that plasma CXCL13 levels was significantly higher in HBV-LC patients than that in HC subjects (**Figure 4D**). Simultaneously, the levels of plasma FABP2, a marker indicating active bacterial translocation, were also increased in HBV-LC

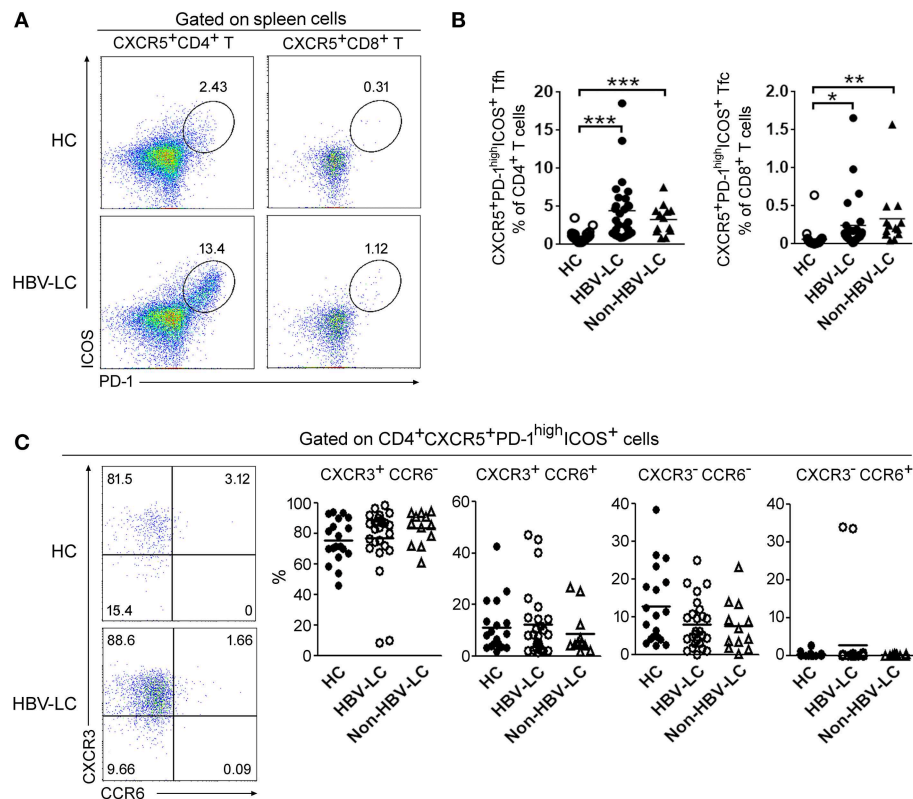


FIGURE 2 | Splenic Tfh cells were preferentially expanded in LC patients. **(A)** Representative dot plots show the proportion of PD-1^{high}ICOS⁺ cells among CXCR5⁺CD4⁺ Tfh-like cells and CXCR5⁺CD8⁺ Tfc-like cells in spleen from a LC patient. Values in the quadrants represent the percentages of splenic Tfh and Tfc cell subset. **(B)** Summarized data show the percentages of PD-1^{high}ICOS⁺ CXCR5⁺CD4⁺ T cells and PD-1^{high}ICOS⁺ CXCR5⁺CD8⁺ T cells in the spleen from HC ($n = 22$), HBV-LC ($n = 27$), and Non-HBV-LC groups ($n = 13$). **(C)** CCR6 and CXCR3 expression within splenic PD-1^{high}ICOS⁺CD185⁺CD4⁺ Tfh cells. Representative dot plots show the expression of CCR6 and CXCR3 when gated on splenic PD-1^{high}ICOS⁺CXCR5⁺CD4⁺ cells in spleen from a LC patient (Left). Values in the quadrants represent the percentages of each subset. Summarized data (Right) show the percentages of Tfh cell subsets in the spleen from HC ($n = 22$), HBV-LC ($n = 27$), and Non-HBV-LC groups ($n = 13$). **(B, C)** Each dot represents one subject, and line represents mean value. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Mann-Whitney U -test.

patients than that in HC subjects (Figure 4D). These data clearly indicated that more splenic Tfh cells moved into B cell zone in the spleen and shaped more active GCs in LC patients.

The Levels of Tfh Cells Were Positively Correlated With Increased Plasma Cells in the Spleen of HBV-LC Patients

To further explore the relationships between the increased Tfh cells and perturbed B cells, we analyzed the proportion of splenic B cell subsets in LC patients and HC subjects. CD19⁺ B cells include IgD[−]CD38^{high} plasma cells, IgD[−]CD27⁺ cMBCs, IgD⁺CD27⁺ MZBs, IgD⁺CD27[−] naïve B cells, and IgD[−]CD27[−] aMBCs (Figure 5A). Pooled data indicated that the percentages of plasma cells and naïve B cells are significantly increased in the spleen of LC patients as compared to those in HC subjects. In contrast, the percentages of MZBs and cMBCs were significantly decreased in the LC patients (Figure 5B), indicating that splenic B cell subsets were perturbed in LC patients.

We then investigated whether the disturbed B cell proportion was associated with the increased Tfh cells in these HBV-LC patients. It is found that the levels of splenic Tfh

cells were positively associated with plasma cells (defined as IgD[−]CD38^{high}CD19⁺ B cells) in LC patients ($r = 0.7053$, $P = 0.0005$, $n = 20$; Figure 5C). Although, splenic cMBC and MZB cell percentages were decreased in HBV-LC patients, there was no significant correlation between the percentages of splenic Tfh cells and the percentages of most of B cell subsets including MZBs, aMBCs, cMBCs, naïve B cells, and CXCR5⁺CD4⁺ T cells. In addition, we also found that splenic Tfh cell proportion was correlated with plasma total IgG ($r = 0.4158$, $P = 0.0346$, $n = 20$) but not IgM levels (Figure 5C). These data suggested that splenic B cell subsets were disturbed in LC patients, particularly the increased plasma cells were found to be positively associated with increased Tfh cells in the spleen of HBV-LC patients.

Splenic Tfh Cells Promote Plasma Cell Differentiation Dependent on IL-21 in LC Patients

The immunological outcome of functionally increased splenic Tfh cells remains unclear in LC patients. Tfh cells moved to the B-cell follicles in LC patients where Tfh cells may interact with GC-B cells. A significantly positive correlation between

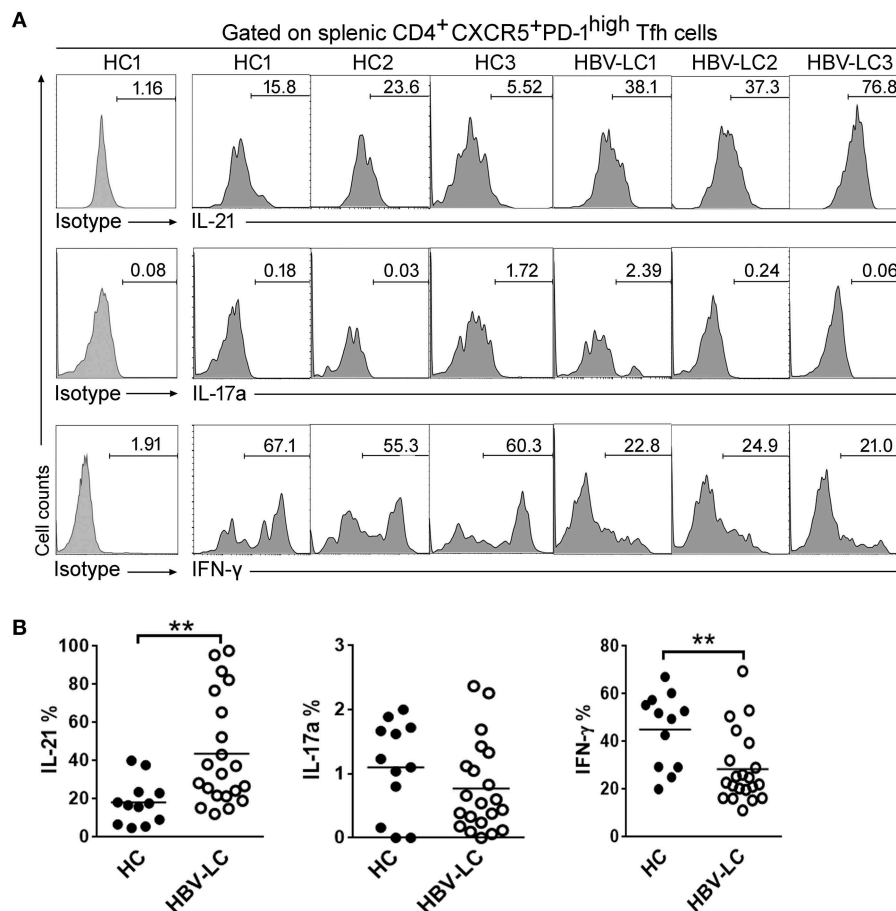


FIGURE 3 | Increased IL-21 production by splenic PD-1^{high}ICOS⁺CXCR5⁺CD4⁺ Tfh cells in LC patients. **(A)** Representative histograms from 3 HBV-LC and 3 HC subjects reflect IL-21, IFN-γ, and IL-17a production by splenic PD-1^{high}ICOS⁺CXCR5⁺CD4⁺ Tfh cells in responses to PMA/ionomycin. **(B)** Pool data indicated that splenic PD-1^{high}ICOS⁺CXCR5⁺CD4⁺ Tfh cells release more IL-21, less IFN-γ, and similar levels of IL-17a in HBV-LC ($n = 18$) than in HCs ($n = 10$) when stimulated with PMA/ionomycin. Each dot represents one subject, and line represents mean value. ** $P < 0.01$, Mann-Whitney U -test.

splenic Tfh cells with plasma cells and serum IgG levels also indicated that splenic Tfh cells have potentials to induce naive B cells to differentiate into plasmablasts in HBV-LC patients. We therefore investigated the influence of expanded splenic Tfh cells on B cells in LC patients. Splenic CXCR5⁺PD-1⁺ CD4⁺ T cells, CXCR5⁺PD-1⁺ CD4⁺ T cells, CXCR5⁺PD-1⁺ CD4⁺ T cells, and CXCR5⁺PD-1^{high} Tfh cells were co-cultured with allogeneic or autologous CD19⁺ B cells from LC or HC donors for 7 days, respectively (**Figure 6A**). rIL-21R-Fc fusion protein was added in CXCR5⁺PD-1^{high} Tfh cells co-culturing wells to block IL-21 signal. As shown in **Figure 6B**, plasma cell proportion was significantly increased in both CXCR5⁺PD-1⁺ CD4⁺ T cell and Tfh cell co-culture systems compared to CXCR5⁺PD-1⁺ CD4⁺ T cell and CXCR5⁺PD-1⁺ CD4⁺ T cell with autologous (**Figure 6B**) and allogeneic (**Figure 6C**) B cells. Blocking IL-21 signaling using rIL-21R-Fc led to the decreased proportion of plasma cells in both autologous and allogeneic B cell co-cultures *in vitro* (**Figures 6B,C**). Compared to HC subjects, we noted that splenic Tfh cells from LC patients induced more plasma cells

differentiation in both autologous and allogeneic co-culture systems than that from healthy subjects ($P < 0.05$, **Figure 6D**). Similar data were also found when splenic CXCR5⁺ CD4⁺ Tfh-like cells from LC or HC subjects were co-cultured with autologous or allogeneic B cells (**Figure 6E**). These data suggest that splenic Tfh cells from LC patients could promote more plasma cell differentiation dependent on IL-21.

The Increased Splenic Tfh Cells Are Correlated With Disease Severity in HBV-LC Patients

We finally analyzed the correlations between splenic Tfh cell proportion and several clinical markers reflecting disease severity such as Child-Pugh score, ALB, PTA, TBIL, et al. We found that HBV-LC patients with higher Child-Pugh scores displayed more splenic Tfh cell proportion than those with lower scores (**Figure 7A**), although there were no significant correlations between splenic Tfh cell proportion and the levels

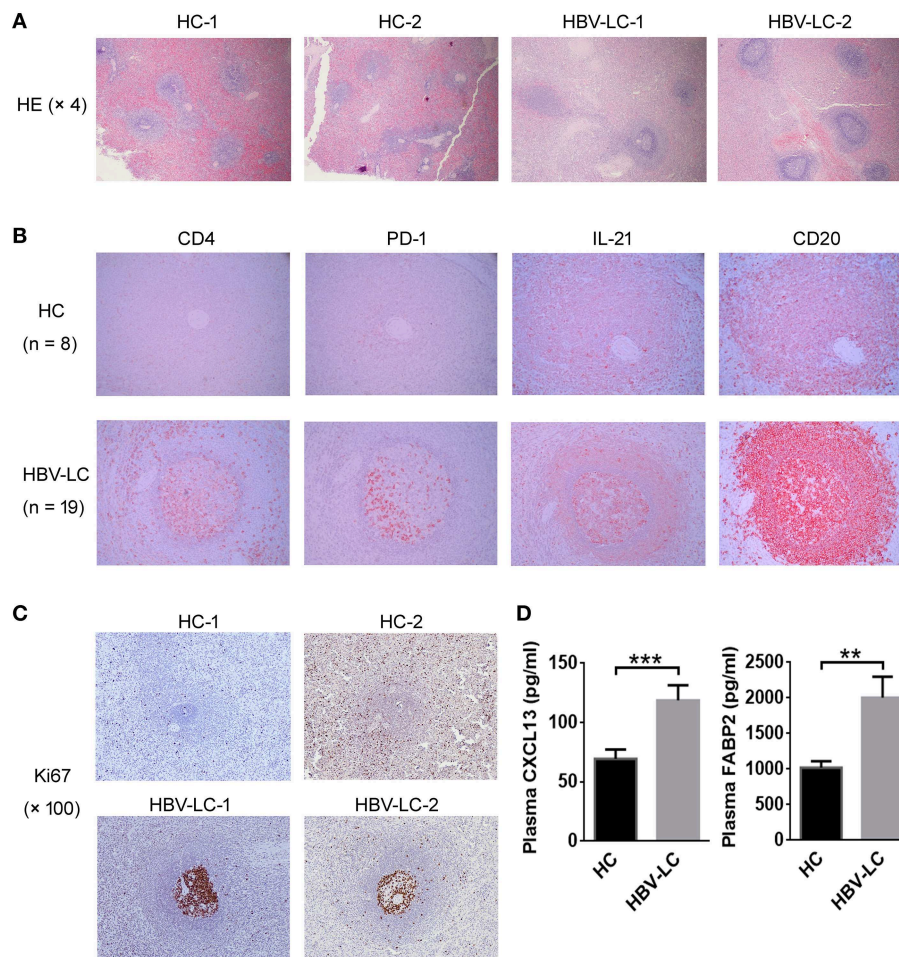


FIGURE 4 | More splenic Tfh cells are migrated in the MZ region in LC patients. **(A)** Representative HE staining indicated obvious follicular-like structure in the spleen of 2 LC patients as compared to 2 HC subjects. **(B)** Immunohistochemical staining detected the expression of CD4, PD-1, IL-21, and CD20 in the continuous tissue sections from an HC subject and an HBV-LC patient (magnification, 200 $\times$). Obvious CD4⁺, PD-1⁺, and IL-21⁺ cells infiltrated into B cell MZ region in LC patients. Similar data were also found in more LC patients ($n = 19$) and HC donors ($n = 8$). **(C)** Representative Ki67 staining indicated obvious proliferation of GC B cells in the spleen of 2 LC patients as compared to 2 HC subjects. **(D)** Plasma CXCL13 and FABP2 concentrations in HBV-LC ($n = 27$) and HC subjects ($n = 22$). The data are shown as means and standard deviations. ** $P < 0.01$, *** $P < 0.001$, Mann-Whitney U -test.

of ALB, PTA, and TBIL (**Figures 7B–D**). These data indicated that expanded splenic Tfh cells are positively correlated with cirrhotic-complications in HBV-LC patients.

DISCUSSION

Little is known about the immune characteristics of CAID in the SLO such as spleen in cirrhotic patients, especially for Tfh and B cells acting as two important arms in the human immune system. Here, we demonstrated that splenic Tfh cells were expanded in B cell follicular region of cirrhotic patients, where they expressed higher levels CXCR3 and produced more IL-21. Thus, splenic Tfh cells induced B cells to robustly differentiate into plasma cells through IL-21, and correlated with cirrhotic severity in LC patients. This study revealed novel immune properties of CAID

and highlighted the immune consequences of the interaction between Tfh and B cells in LC patients.

Our data provides novel insights to the splenic Tfh cell compartments in cirrhosis. We examined the splenic and peripheral CD4 and CD8 T cell subsets based on CXCR5, CXCR3, and CCR6 expression from LC and HC subjects. In the spleen, CXCR5⁺CD4⁺ Tfh-like cells were significantly increased in LC patients as compared to those in HC subjects. More important, PD-1^{high}ICOS⁺ Tfh cells are specifically accumulated within the splenic Tfh-like cell subset rather than other Th and Tc subsets including the CXCR5⁺ CD8 T cells which play antiviral roles in SLOs (26). Although these splenic PD-1^{high}ICOS⁺ Tfh cells showed substantial heterogeneity, they preferentially expressed higher levels of CXCR3. In addition, these splenic Tfh cells are largely expanded and produced more IL-21 in LC patients than those from HC subjects regardless of cirrhosis-associated

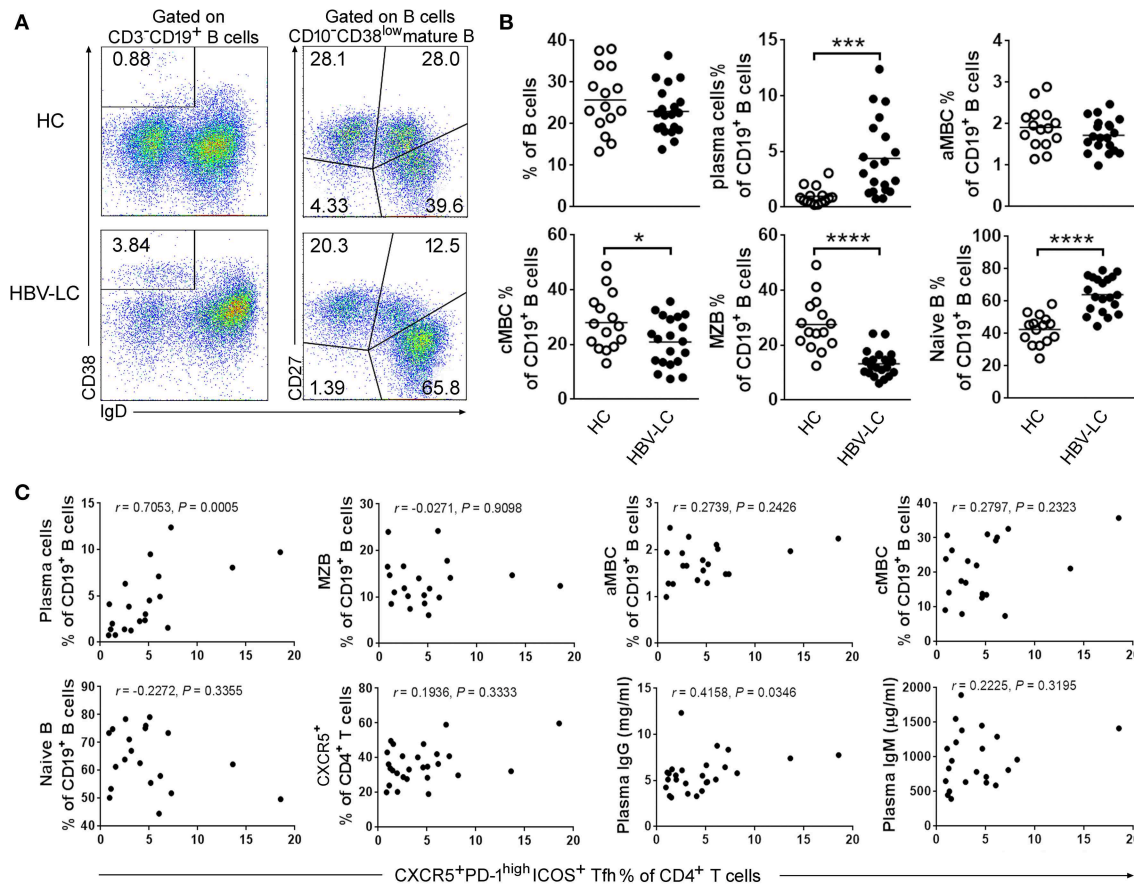


FIGURE 5 | Disruption of B cell subset distribution in LC patients. **(A)** Representative dot plots show the gating strategy of B cell subsets in spleen from an LC patient. Values in the quadrants represent the percentages of each subset. Within CD3⁺CD19⁺ B cells, IgD⁺CD38^{high} plasma cells, IgD⁺CD27⁺ cMBC cells, IgD⁺CD27⁺ MZB cells, IgD⁺CD27⁺ naive B cells, and IgD⁺CD27⁺ aMBC cells were defined, respectively. **(B)** Summarized data show the percentages of B cell subsets in the spleen from HC ($n = 15$) and HBV-LC groups ($n = 20$). Each dot represents one subject, and line represents mean value. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$, Mann-Whitney U -test. **(C)** Correlation analysis between splenic Tfh cells and B cell subsets. The percentages of splenic Tfh cells were significantly positively with plasma cell proportion and plasma IgG levels but not with MZB, aMBC, cMBC, naive B, splenic CXCR5⁺ CD4⁺ T cell subsets, and plasma IgM levels in HBV-LC patients ($n = 20$). Each dot represents one subject. Spearman rank correlation test was used: r , correlation coefficient; P -values are shown.

etiology. This comprehensive analysis highlights splenic Tfh cells from LC patients are expanded and hyper-activated.

The immunological consequences of the increased splenic Tfh cells remained unclear in LC patients. Previous studies have indicated that the decreased T cell immunity was generally accompanied by enhanced humoral immune responses in various chronic inflammatory conditions (18). Here, our data support the notion that the expanded splenic Tfh cells may promote B cell differentiation in LC patients. We provide several evidences including the extensive co-localization and the significant positive correlation between splenic Tfh cells and plasma cell subsets as well as the potential of Tfh cells inducing B cell differentiation *in vitro*. The contribution of hyperactive Tfh cells to plasma cell differentiation may lead to the increased levels of non-specific antibodies and autoimmune antibodies during cirrhosis. It is reported that cirrhotic patients manifested hyper-immunoglobulinemia (9) and had more anti-microbial antibodies than control subjects (27). Therefore, the

enhanced systemic inflammation and Tfh cell immunity in cirrhotic patients may break the barrier tolerance and facilitate the induction of anti-microbial antibodies, which consequently affect the normal symbiosis of gut flora and lead to the increased microbial translocation. The reported dominant elevation of IgA type antibodies over IgG and IgM also suggested that compromised mucosal immunity could play roles in inducing anti-microbial antibodies during cirrhosis (27). Enhanced Tfh cell responses may also result in increased autoantibodies production, and HBV-infected patients with autoantibodies also showed more activated Tfh cell phenotypes than the patients without autoantibodies (20). Whether the increased non-specific and autoantibodies have detrimental impact on cirrhosis associated pathophysiology needs future studies.

Enhanced Tfh immune responses may deplete memory B cell pool through inducing more B cells differentiating into plasma cells. Recently, extra-follicular Tfh-like cells located at the border of the T cell zone and B cell follicle in human

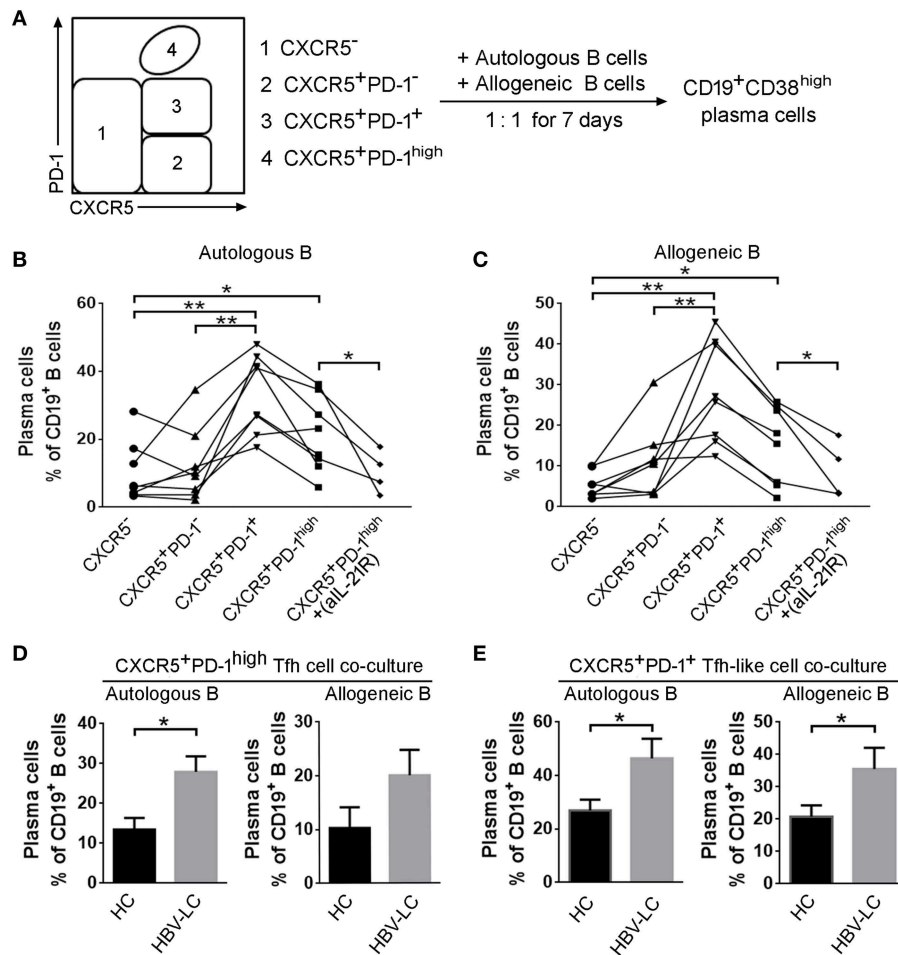


FIGURE 6 | Splenic Tfh cells from HBV-LC patients have more potential to induce B cell differentiation into plasma cells *in vitro*. **(A)** Flowchart indicated that splenic CXCR5⁻CD4⁺ T cells, CXCR5⁺PD-1⁺CD4⁺ T cells, CXCR5⁺PD-1⁻CD4⁺ T cells, and CXCR5⁺PD-1^{high} Tfh cells were sorted and co-cultured with allogeneic or autologous CD19⁺ B cells at the ratio of 1:1 for 7 days, respectively. Then the percentage of CD19⁺CD38^{high} plasma cells was detected. **(B,C)** Summary data indicated that the proportion of plasma cells induced by each type of CD4⁺ T cell subsets from HBV-LC patients ($n = 5$) and HC subjects ($n = 4$) in autologous **(B)** or allogeneic **(C)** CD19⁺ B cell co-culture. * $P < 0.05$, ** $P < 0.01$, Wilcoxon's matched-pairs test. **(D,E)** Pool data showed that the proportion of plasma cells induced by Tfh cells **(D)** and CXCR5⁺CD4⁺ Tfh-like cells **(E)** and in autologous or allogeneic CD19⁺ B cell co-culture in HBV-LC patients ($n = 5$) and HC subjects ($n = 4$). The data are shown as means and standard deviations. * $P < 0.05$, Mann-Whitney U -test.

tonsils were reported to promote memory B cells to produce antibodies via CD40L and IL-21 (17). Here, we observed that Tfh cells in both follicular and extra-follicular regions in spleen from cirrhotic patients are significantly expanded and may hasten the B cell differentiation process. This finding was reminiscent with the observations in other chronic autoimmune diseases. For instance, patients with primary Sjogren's syndrome and systemic lupus erythematosus had an expansion of peripheral Tfh-like cells with the increased IL-21 production, along with decreased CD27⁺ memory B cells and increased plasma cells, indicating prominent role of Tfh cells in aberrant distribution of B cell subsets (28, 29). These data suggest that splenic functionally enhanced Tfh cells may expedite memory B cell differentiating into plasma cells, deplete memory B cell pool, and induce non-specific antibody production.

The factors leading to functional activation of Tfh cells in cirrhotic spleen are unclear, however, it likely relates to systemic inflammatory responses in LC. Recently, the bacterial mRNA recognition is reported to increase Tfh cell differentiation, and enhances antibody responses (30, 31). The activation of the MyD88-dependent TLR8 signaling on human monocytes initializes the expression of IL-12, a main cytokine inducing Tfh cell differentiation. Human IL-1 β is produced in response to bacteria and affects Tfh differentiation (32). Therefore, sensing viable bacteria by monocytes may serve as a trigger to induce Tfh cell differentiation, subsequent class-switch of B cells and antibody production. During cirrhosis, there is episodic bacterial translocation and persistent immune cell activation, as indicated by the increased FABP2 and CXCL13 levels in our study. In addition, the expansion of the Tfh cells could also be driven

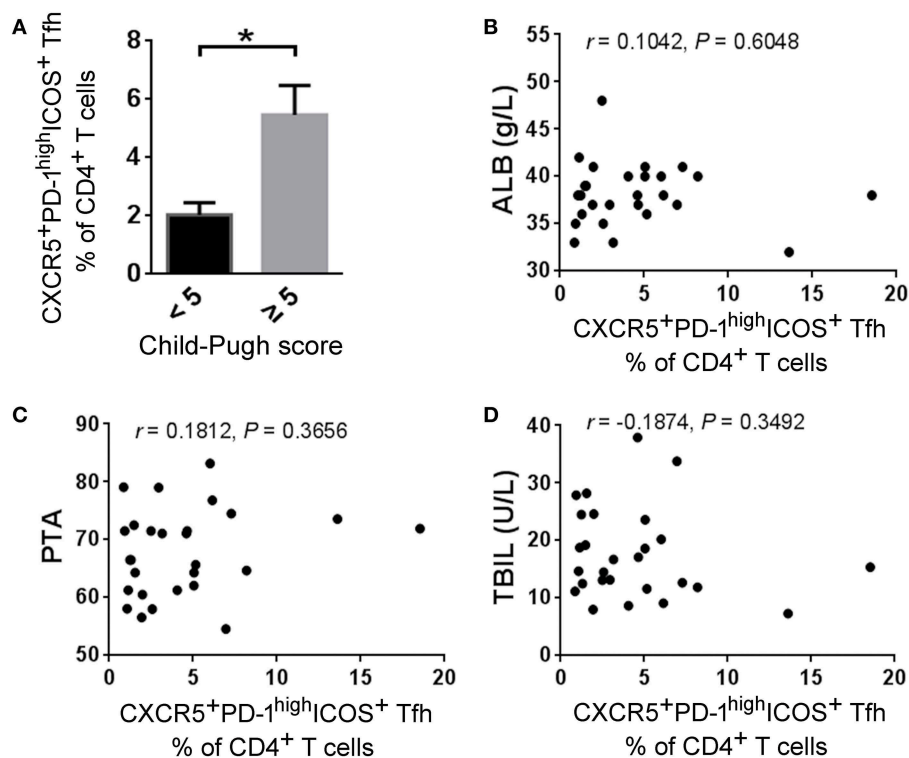


FIGURE 7 | Splenic Tfh cells correlate with disease severity in HBV-LC patients. **(A)** Higher frequencies of splenic Tfh cells were present in HBV-LC patients with child score more than 5 ($n = 19$) than in those with child score <5 ($n = 8$). The data are shown as means and standard deviations. * $P < 0.05$, Mann-Whitney U -test. **(B–D)** The correlation analysis between the percentages of splenic Tfh cells and clinical markers such as ALB **(B)**, PTA **(C)**, and TBIL **(D)** levels in HV-LC patients ($n = 27$). Spearman rank correlation test was used: r , correlation coefficient; P -values are shown.

by IL-6 signaling derived from follicular dendritic cells and the persistent viral antigen in the host environment (33). Thus, the activation of innate immune cells including activated dendritic cells, increased inflammatory cytokines and viable microbes are all present during liver cirrhosis. We think that these combined effects may eventually promote an enhanced Tfh cell response in LC patients, especially during advanced diseases.

This study is limited by several aspects. First, we have not performed the functional and clinical analysis of splenic Tfh cells from non-HBV-LC patients although splenic Tfh cells were significantly increased in both HBV-LC patients and non-HBV-LC patients. Second, it is unclear whether the increased Tfh cells correlated with the levels of autoantibodies in LC patients, which may generate a novel understanding on CAID. Third, clinical significance has not been fully addressed in the study. Nonetheless, we reported that the hyperactive Tfh cells contribute to dysregulated humoral immunity in LC patients. Our study indicated that targeting Tfh cells may provide a way to ameliorate CAID.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JZ, SZ, and ZZ designed the study. JZ, JS, MQ, and ZZ did the experiments and analyzed the flow cytometry data. MH provided help with IHC experiments. XZ checked case history. HW, ZLiu, QH, and ZLi provided human samples. SZ and ZZ wrote the manuscript.

FUNDING

This work was supported in part by grants from the National Science and Technology Major Project of the Infectious Diseases (2018ZX10301404 to ZZ), the National Natural Science Foundation of China (81672037 to JZ), the Science and Technology Innovation Committee of Shenzhen Municipality (JCYJ20170412151722110 to ZZ and JCYJ20170412151650600 to HW), and the National Program on Key Basic Research Project (2015CB554300 to SZ).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.01915/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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HBV-Induced Immune Imbalance in the Development of HCC

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Chronic hepatitis B virus (HBV) infection is one of the high-risk factors for human HCC. Despite the integration of virus DNA and the oncoprotein HBx, chronic necroinflammation and hepatocellular regeneration account for hepatocarcinogenesis. As a non-cytopathic virus, HBV is extensively recognized to mediate chronic liver damage through abnormal immune attack. However, the mechanisms driving HBV infection to HCC are poorly understood. During chronic HBV infection in humans, the adaptive immunity changes from immune tolerance to progressive immune activation, inactivation, reactivation and exhaustion, all of which may be the immune pathogenic factors for the development of HCC. Recently, the immunopathogenic mechanisms were described in mouse HBV-induced HCC models, which is absolutely dependent on the presence of HBV-specific T cell response and NK cell-derived IFN- γ , findings which are consistent with the observations from CHB and HCC patients. In this review, we summarize recent research progression on the HBV-specific CD8⁺ T cells, and also CD4⁺ T cells, B cells and non-specific immune cells and molecules underlying chronic HBV infection and eventual HCC development to demonstrate the pathogenesis of HBV-induced immune imbalance. Based on the progression, we discussed the potential of immune-based therapies and their challenges in the treatment of HBV-related HCC, including the checkpoint inhibition, genetically modified T cell transfer, therapeutic vaccines and metabolic modulation.

Keywords: HBV, immune dysfunction, HCC, adaptive immunity, innate immunity

OPEN ACCESS

Edited by:

Yuan Quan,
Xiamen University, China

Reviewed by:

Cai Zhang,
Shandong University, China
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Nankai University, China

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Specialty section:

This article was submitted to
Viral Immunology,
a section of the journal
Frontiers in Immunology

Received: 13 June 2019

Accepted: 13 August 2019

Published: 27 August 2019

Citation:

Chen Y and Tian Z (2019)
HBV-Induced Immune Imbalance in
the Development of HCC.
Front. Immunol. 10:2048.
doi: 10.3389/fimmu.2019.02048

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is one of the high-risk factors for human HCC, responsible for 50~80% of HCC cases worldwide. As one of the leading causes of cancer death, HCC represents an important human health problem (1). It has been an urgent issue how chronic HBV infection promotes hepatocarcinogenesis. In adults, HBV infection causes a rapid immune response, typically resulting (more than 95% patients) in life-long immunity with acute self-limited infection; while in infants and children, HBV infection has the potential to become chronic with life-long HBV persistence (2).

Despite the direct gene activation and transactivation by the integration of HBV DNA into hepatocyte genome and the oncoprotein HBx and preS/S, HBV-inflicted DNA damage due to hepatocellular regeneration associated with chronic necroinflammation accounts for hepatocarcinogenesis (3–6). In the cytoplasm of infected hepatocytes, HBV has its own nucleocapsids in which HBV completes its replication as a stealth virus without type I IFN-mediated responses (2). As a non-cytopathic virus, HBV-induced

liver damage is extensively recognized to be mediated by abnormal immune attack. A dynamic balance between immune clearance and immune tolerance accounts for the outcome in patients with chronic HBV infection. It has been increasingly accepted that immunopathogenesis plays a critical role in HBV-related HCC development; however, the precise mechanisms by which HBV drives to HCC remain poorly understood. Here, the recent progress was summarized in understanding the adaptive and innate immune responses underlying chronic HBV infection that can lead to HCC development and examine the critical pathogenesis of HBV-induced immune imbalance. Further, the cross-talk and network regulation among these immune cells and their released key factors was discussed. Additionally, we discuss the potential of immune-based therapies and their challenges for HBV-related HCC.

Immune Response Is Related to Disease Progress During HBV Infection

During self-limited acute HBV infection, the efficient HBV-specific immune response is essential. A vigorous response of CD4⁺ T and CD8⁺ T cells was generated to control and clear HBV. HBV-specific CD8⁺ T cells exhibit antiviral activity by producing IFN- γ and TNF- α (7, 8) or by directly killing the infected hepatocytes (9–11). B cells are co-stimulated by T cells and subsequently produce antibodies to HBV surface antigen (HBsAg), HBV e

antigen (HBeAg) and HBV core antigen (HBcAg). These antibodies act to clear antigens and HBV virus from the circulation, preventing or limiting HBV reinfection (12). In addition, NK cells and NKT cells efficiently control HBV, the activities of which peak earlier than that of HBV-specific T cells (12).

During chronic HBV infection, the early phase termed “immune tolerant” stage with a high-replication of HBV-DNA and low-inflammation during childhood (13). The progressive loss of immune tolerance leads to the “immune active” stage with HBV-specific CD8⁺ T cell responses during adolescence, which results in chronic liver injuries, inflammation and liver regeneration. Patients may subsequently enter an “immune inactive” stage with low level of HBV replication and limited inflammation. Particularly, approximately 20–30% of patients in the inactive carrier stage are subject to a viral relapse, displaying replicative HBV and thus enter the “immune reactive” stage with chronic hepatitis that progress to liver fibrosis, cirrhosis and HCC. In the late stage, a series of oncogenic signaling pathways activated by HBV result in immune escape, and promotes the finally developing HCC (14). More recently, studies show that HBV-immunotolerant patients develop HCC (~12% in 10 years), while treated “immune active” patients develop HCC (~6% in 10 years) with a lower rate. Notably, patients with more cumulative immune-mediated hepatocyte damage would be more susceptible to

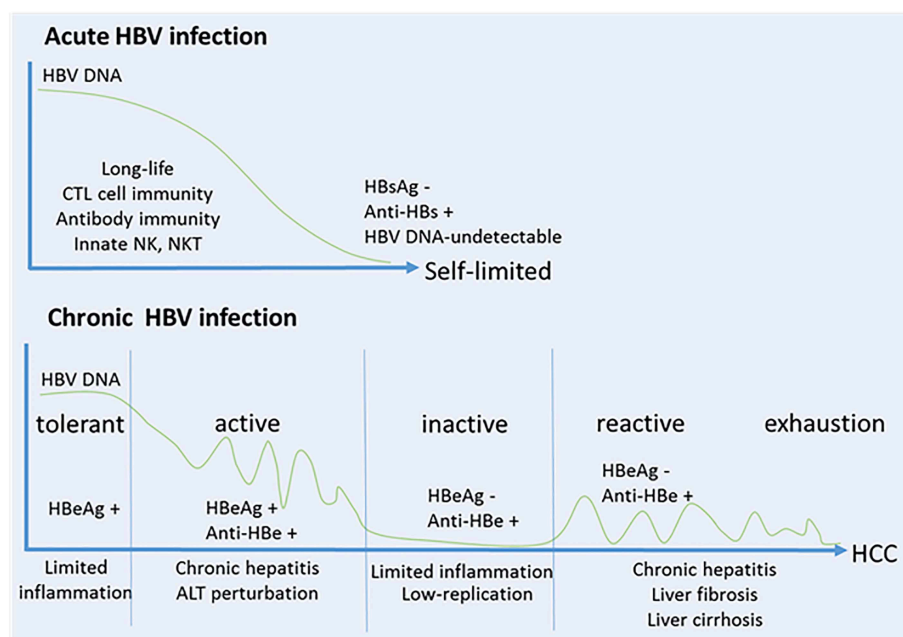


FIGURE 1 | HBV infection relates to the magnitude and quantity of anti-viral immune response. **(A)** Self-limited acute HBV infection. NK cells and NKT cells play important roles in early control of HBV, and then a robust response of CD4⁺ T cells and CD8⁺ T cells is generated to control and eliminate HBV. B cells co-stimulated by T cells produce anti-HBs, anti-HBe and anti-HBc. These protective antibodies clear HBV antigens and virus from the circulation, and prevent or limit HBV reinfection. **(B)** Chronic HBV infection. Five stages are identified including “immune tolerant” stage with a high-replication of HBV-DNA and low-inflammation, “immune active” stage with HBV-specific CD8⁺ T cell response and antibody production which results in chronic liver injuries, inflammations and liver regeneration, “immune inactive” stage with low-replication of HBV and limited inflammation, “immune reactive” stage with chronic hepatitis progressed to liver fibrosis, cirrhosis and HCC, and in the late stage of “immune exhaustion.”

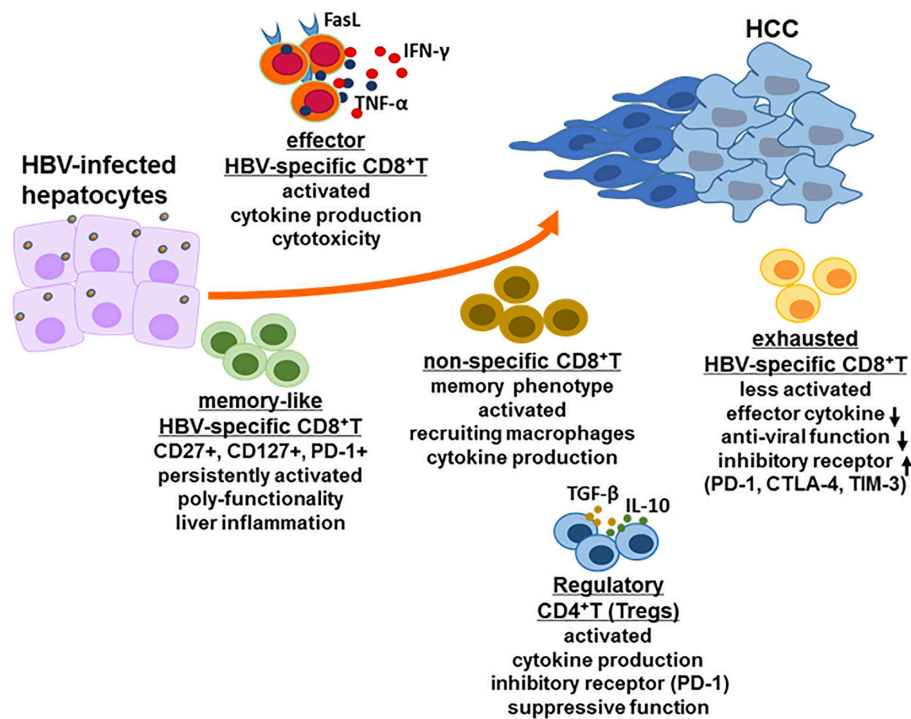


FIGURE 2 | CD8⁺ T cell response in HBV-related HCC. In the HBV-infected individuals, there are several kinds of CD8⁺ T cell populations including exhausted HBV-specific CD8⁺ T cells, HBV core-specific and polymerase-specific CD8⁺ T cells with long-term memory-like phenotype, effector CD8⁺ T cells and HBV non-specific CD8⁺ T cells. The function of each CD8⁺ T cell population is different but possibly promote the development of HCC together. Additionally, regulatory CD4⁺ T cells (Tregs) also play a critical role in the stage of HCC for immune escape, for example inducing CD8⁺ T cell exhaustion.

HCC (15, 16). The patient consequences of HBV infection relate to the magnitude and quantity of anti-HBV immune responses (**Figure 1**). As one of the hallmarks of cancer, chronic inflammation is considered to be an important element and contributes to changing the tumor microenvironment during this process (17).

HBV-Specific CD8⁺ T Cell Response in HBV-Related HCC

During acute HBV infection, potent HBV-specific CD8⁺ T cell responses control HBV, typically reducing its titer to undetectable levels. Furthermore, HBV-specific memory T cells with an activated phenotype was persistent after resolving acute HBV infection (18). However, during chronic HBV infection, HBV replication levels as ranging from 10^3 to 10^9 HBV DNA copies/mL in peripheral blood of patients with chronic HBV, significantly affected HBV-specific CD8⁺ T cell responses. HBV DNA load of 10^7 copies/ml was suggested as a threshold, below which multiple HBV-specific CD8⁺ T cells, including env-specific, pol-specific and core-specific CD8⁺ T cells are consistently detected in the peripheral blood; while above the threshold of HBV replication level, env-specific CD8⁺ T cells and pol-specific CD8⁺ T cells can occasionally be found (19). The role and function of highly heterogeneous HBV-specific CD8⁺ T cell are diverse in the development of HBV-related HCC (**Figure 2**).

The decrease of HBV-specific CD8⁺ T cell functions in CHB patients is mainly shown by the low frequency due to antigen-specific deletion and restricted proliferation, and also the high expression levels of inhibitory receptors such as CTLA-4, PD-1, and TIM-3 (12, 20–23). The higher expression levels of TIM-3 in active CHB patients compared to inactive CHB patients suggest that CD8⁺TIM-3⁺ T cells are functionally exhausted during the active state of chronic HBV infection (24). Oncofetal gene SALL4 reactivation by HBV-induced STAT3 signal in adulthood counteracts miR-200c, which accounted for PD-L1-induced CD8⁺ T cell exhaustion (25). Exhausted CD8⁺ T cells were unable to effectively produce cytokines and exert anti-viral activity when re-exposure to HBV in an acute immune active state, indicating that exhausted CD8⁺ T cells displaying dysfunctional differentiation are associated with chronic HBV replication and the resulting disease progression (26). Furthermore, CD8⁺ resident memory T cells (TRM) were enriched with higher expression levels of PD-1 in the tumor tissue of HBV-related HCC, which were functionally more suppressive and exhausted (27). Single-cell RNA-sequencing demonstrated that there was higher frequency of exhausted CD8⁺ T cells and regulatory T cells (Tregs) with clonally expansion in HCC patients, and layilin (LAYN) was related to the suppressive function of Tregs and exhausted CD8⁺ T cells in HCC (28).

In addition to the inhibitory pathways, a more complex mechanism of energetic and metabolic impairment accounted for CD8⁺ T cell exhaustion during HBV infection. In patients with chronic HBV infection, exhausted HBV-specific CD8⁺ T cells with high ROS level showed extensive downregulation of mitochondrial functions including electron transport, mt DNA transcription and translation, membrane transport and metabolism, and marked downregulation of proteasome subunits and proteins involved in DNA repair (29). Additionally, HBx protein dysregulated glucose metabolism with increased lactate production, which impaired the migration of T cells in the liver and their cytolytic activity (30).

Recently, numerous HBV-specific T cell populations including HBV core-specific and HBV polymerase-specific CD8⁺ T cells were detected in the circulation of CHB patients, and these cells exhibited a long-term memory-like phenotype and poly-functionality, which was not terminally exhausted (31–34). Clinical observation demonstrated that when residual antigen-specific CD8⁺ T cells were persistently activated but unable to control HBV replication, they might contribute to sustain liver inflammations predisposing patients to HCC development (11, 13, 15, 16, 19).

To reveal the exact immunopathogenic mechanisms of CD8⁺ T cells in HBV-related HCC, murine HBV-induced HCC models were generated for analysis. In 1998, Nakamoto et al. (35, 36) demonstrated that HBsAg-specific cytotoxic T lymphocytes (CTLs) constantly attacked HBsAg-expressing hepatocytes, eventually triggering HCC in HBV transgenic mice *via* thymectomy, bone marrow reconstruction and adoptive transfer of splenic HBsAg-specific CD8⁺ T cells from HBsAg-immunized mice. Using this model, they further demonstrated that use of an anti-FasL neutralizing antibody could attenuate the hepatotoxicity of HBsAg-specific CTLs and prevented the chronic hepatitis and eventual HCC (36). Studies in our lab have also illustrated that breakdown of adaptive immune tolerance by blockade of TIGIT (T cell immunoglobulin and ITIM domains, a checkpoint receptor involved in mediating T cell exhaustion in tumors) combined with HBsAg vaccination is able to recover the anti-HBV function of autologous HBsAg-specific CTLs including IFN- γ and TNF- α production, which was responsible for mediating HCC progression in HBs-Tg mice (37). To mimic naturally occurring anti-HBV immunity and immunopathology, we generated a novel HBV mouse model by transferring HBsAg⁺ hepatocytes from HBs-Tg mice into an immunocompetent recipient mouse (Fah^{-/-} mouse) with the same genetic background. In this mouse model, HBsAg-specific CD8⁺ T cells were naturally generated and responsible for mediating hepatocyte apoptosis and chronic hepatitis, eventually leading to HCC (unpublished data).

Additionally, non-specific CD8⁺ T cells with memory phenotypes secreted IFN- γ when activated by anti-CD137 mAb in HBV transgenic mice, and played a central role in the subsequent development of chronic inflammation, fibrosis, cirrhosis and HCC progression. During this process, non-specific CD8⁺ T cells preferentially recruited hepatic macrophages, which promoted the

development of HCC through secreting TNF- α , IL-6, and MCP-1 (38). In patients with chronic HBV infection, circulating CD14⁺ monocytes with elevated expression of the natural ligand of CD137 might contribute to the sustained CD137 stimulation of CD8⁺ T cells for the liver immunopathology (38).

HBV-Specific CD4⁺ T Cell Response in HBV-Related HCC

CD4⁺ T cells are considered to contribute to anti-viral and anti-tumor immune responses by producing cytokines that activate CD8⁺ T cells and B cells. Patient circulating and liver-infiltrating CD4⁺ CTLs were increased in the early stage of HCC, which was significantly higher than that of CHB patients (39). This finding indicated that chronic HBV infection may not be the principal element accounting for the observed increase in CD4⁺ CTLs in HBV-related HCC. Both CD4⁺ CTL number and activity decreased in progressive stages of HCC due to the increased Tregs, and the progressive deficit in CD4⁺ CTLs was linked to the high recurrence and poor survival of HCC patients (39).

Tregs are known to exert their suppressive function via cell-to-cell contact or through cytokines such as IL-2, IL-10, TGF- β , and IL-35 (40). Noticeably, in HBV-related HCC patients, Tregs were enriched and showed greater expression of PD-1 with increased suppressive function, which accounted for the more immunosuppressive and exhausted microenvironment of HBV-related HCC compared to the non-virus-related HCC (27). Increased Tregs in HBV-related HCC patients have also been implicated in the reduction of the function of CD8⁺ T cells, as demonstrated by the inhibited proliferation and activation of CD8⁺ T cells and attenuated cytotoxicity of CD8⁺ T cells with less production of granzymeA/B and perforin (41). Persistent presence of HBV led to elevated TGF- β which suppressed miR-34a expression and enhanced CCL22 expression, thus recruiting Tregs in the liver tissue (42). Tregs facilitated the immune escape of HBV⁺ HCC, resulting in the development of portal vein tumor thrombus in HCC patients (42). The increased Tregs not only suppressed HBV antigen-specific immune responses, but also suppressed HCC tumor antigen-specific immune responses (43). Further, it was found that compared with the healthy donors and patients of chronic HBV infection, the frequency of circulating CD4⁺CD25⁺CD127⁻ Tregs was much lower in HCC patients, but surgery resulted in significantly increasing the frequency of circulating CD4⁺CD25⁺CD127⁻ Tregs in HCC patients, correlating with tumor aggressiveness (44). These results suggest a therapy targeted to reduce Treg activity may prove beneficial for HCC patients (45).

The frequency of circulating CD4⁺ T follicular helper cells (CXCR5⁺CD4⁺ Tfh) decreased and their function was impaired with disease progression in HBV-related HCC patients (46). Further, the infiltrated CXCR5⁺CD4⁺ T cells was demonstrated to be significantly less in HCC tumor regions than that of non-tumor regions (46). These Tfh cells from HCC patients resulted in less effective induction of the differentiation of plasmablasts from naive B cells, since they reduced ICOS expression and the ability to produce IL-10 and IL-21 with less proliferation activity (47).

HBV-Specific B Cell and Antibody Responses in HBV-Related HCC

In patients of HBV infection, serological biomarkers changed from HBsAg⁺ and HBeAg⁺ to anti-HBs⁺ and anti-HBe⁺ are used to describe the recovery of HBV infection. HBV is never completely eliminated from the patients system, due to the persistence of a little of covalently closed circular DNA (cccDNA) and integrated DNA of HBV in infected individuals (18). The integrated DNA can express HBV antigens including HBsAg. It is increasingly recognized that humoral immune responses involving anti-HBs antibody production exhibit an important activity in the process of controlling HBV (48, 49). Lack of effective B cell and neutralizing antibody responses promote the disease progression of chronic HBV infection (48). Recently, fluorochromes-labeled HBsAg was utilized as “baits” to specifically detect HBsAg-specific B cells *in vitro* by using a dual-staining method. Studies by using this method demonstrated that there was no significant difference in the number of circulating HBsAg-specific B cells among patients with chronic HBV infection, patients with acute HBV infection and vaccinated subjects. Furthermore, the frequency of HBsAg-specific B cells did not correlate to the quantity of HBsAg or HBV-DNA during chronic HBV infection (50). B cells from CHB patients exhibit an atypical phenotype (CD21[−] CD27[−]) and show functional alterations with PD-1 expression, resulting in the reduction of antibody production (50, 51). When supplemented with cytokine (IL-2 and IL-21) signals and costimulatory signals-derived from CD40L-expressing feeder cells, the maturation of these HBsAg-specific B cells from CHB patients could be partially restored; and functional blockade of the PD-1 expressed on these HBsAg-specific B cells could partially rescue their functions (50, 51). Depletion of B cells by antibody treatment reactivated HBV in patients with chronic HBV infection with a high rate to 60%, even in the subjects with resolved infection years earlier, and this reactivation may lead to severe disease (52).

Beyond the production of HBV-specific antibodies, significantly higher frequencies of IL-10-expressing B cells (Bregs) were observed in HCC patients than that of healthy controls. Furthermore, these Bregs preferentially expressed TIM-1, negatively correlating with the expression of granzyme A/B and perforin in CD4⁺ T cells (53). The different immune phases of patients with chronic HBV infection changes in the Breg frequency, with elevated serum levels of IL-10 observed in the immune active patients when compared to the immune tolerant patients (54). Additionally, the frequency of circulating Bregs was significantly increased after surgery, which was associated with the levels of HBeAg and HBV-DNA copy number (44). These results suggest a potential therapy against Bregs may offer improved outcomes for HCC patients.

Innate Immune Responses in HBV-Related HCC

The liver as a lymphoid organ has an overwhelming innate immune system, including several kinds of innate immune cells with high frequency such as NK and NKT cells (55–57). Among

total intrahepatic lymphocytes, the frequency of NK cells is as high as 25–40 and 10–20% in human and mouse livers, respectively. It is well-known that NK cells exhibit early anti-viral and anti-tumor activities (58); however many mechanisms are involved in the alterations of NK cell functions during the progression of HBV infection.

NK cells participate in controlling HBV replication in mice (59), but within patients with chronic HBV infection their anti-viral activity is suppressed in the presence of IL-10 and transforming growth factor β (TGF- β) (60). Further, in NK cells from patients with chronic HBV infection, inhibitory receptors NKG2A, TIM-3, and PD-1 expression was up-regulated and the ability of IFN- γ and TNF- α secretion was reduced, which was involved in the HCC progression (61–63). Additionally, HBV was reported to secrete exosomes, which play a critical role in mediating HBV transmission in NK cells and consequently impairing NK cell functions, proliferation and survival (61). Furthermore, compared with healthy individuals, NK cells from CHB and HCC patients show significant increases in the expression of microRNA (miR)-146a, which related to the downregulation of NK cell function including reduced cytotoxicity and decreased IFN- γ and TNF- α production (64). In HCC patients, the presence of infiltrating CD11b[−]CD27[−] NK subsets in the tumor origins was positively correlated with the clinical outcomes, since a substantial proportion of these cells exhibited inactive and immature phenotypes with weak cytotoxicity and poor IFN- γ production (63). In addition to their anti-viral and anti-tumor activities, activated NK cells may also mediate HBV-associated hepatocyte damage (56, 65). Recently, our study demonstrated that NK cells mediate liver inflammation by secreting IFN- γ , which promote the development of HCC through the epithelial cell adhesion molecule (EPCAM)-epithelial cell to mesenchymal transition (EMT) axis in HBs-Tg mice (66). In HCC patients, increased levels of IFN- γ also mediated liver dysfunction and associated with HCC progression (67).

Activation of non-virus-specific cells may result in widespread inflammation, promoting HCC development. NKT cells promoted hepatic stellate cells (HSCs) activation in liver fibrogenesis through producing the inflammatory cytokines IL-4 and IL-13, accounting for the spontaneous liver fibrosis in HBV-Tg mice (68). HSCs upregulated the level of Tregs in the liver, which was involved in the occurrence of HCC following fibrosis and cirrhosis (69). Activation of NKT cells could be augmented by CD205⁺ Kupffer cells through IL-12 production during HBV infection (70). Hepatic macrophages recruited by CD8⁺ T cell-derived IFN- γ subsequently produced TNF- α , IL-6 and MCP-1 to mediate chronic hepatitis and HCC progression (38). Circulating CD14⁺ monocytes may contribute to the activation of CD8⁺ T cells through CD137 ligand upregulation in patients with chronic HBV infection (38). Ly6C⁺ monocytes-secreted TNF- α also played the critical role in enhancing CD8⁺ T cell response for HBV clearance (71). Although hepatic macrophages promote the development of HCC during chronic HBV infection, they have also shown opposite roles. Kupffer cell-derived IL-10 operates to maintain humoral immune tolerance and induce anti-HBV CD8⁺ T

cell exhaustion in chronic HBV carrier (72, 73). Additionally, maternal HBeAg-predisposed hepatic macrophages may mediate immune tolerance to HBV (74).

As an important population of liver-resident innate immune cells, $\gamma\delta$ T cells-driven myeloid-derived suppressor cell (MDSC) accumulation in the HBV-tolerant liver strongly suppressed CD8⁺ T cell function and promoted systemic CD8⁺ T cell exhaustion (75). Recent studies demonstrated the circulating $\gamma\delta$ T cells showed distinct phenotypes and functions with higher frequency of T-bet^{hi} Eomes^{dim} V δ 2⁺ $\gamma\delta$ T cells in patients of chronic HBV infection compared with uninfected control subjects (76). Further, IFN γ /TNF responses were weaker in V δ 2⁺ $\gamma\delta$ T cells from chronic HBV infected patients with hepatitis flare when compared to those without hepatitis flare (76).

Complement system is a vital part of the innate immune system, comprising a variety of different proteins including complements, complement receptors and the regulatory protein. In CHB patients, elevated C5a promoted HSC activation and inhibited HSC apoptosis, which positively correlated with disease severity demonstrated by the clinical parameters of liver fibrosis (77). Gene polymorphisms of complement receptor 1 (CR1) contributed to the risk of HBV-related HCC in males (78). Additionally, HBx increased the expression of complement regulatory protein CD59, which prevented the formation of terminal membrane attack complex C5b-9 on the hepatoma cells (79). Thus, the dysregulation of complement system induced by chronic HBV infection promoted the development of HCC in several manners.

Cross-Talk and Regulation Among Different Immune Cells in HBV-Related HCC

HBV-induced immune imbalance leads to the development of HCC as described above. We further summarized the HBV-induced changed immune cells and their released cytokines (Table 1). Complicated cross-talk and regulation among these immune cells further makes it difficult to control and regulate the immune disorders. A variety of cytokines and other molecules are involved in this process (Figure 3). When these immune cells including CD4⁺T, CD8⁺T, NK, NKT, monocytes/macrophages, and HSCs are activated, they participate in mediating the liver inflammation during chronic HBV infection, which eventually promote the development of HCC. Moreover, interactions among these activated cells through producing cytokines such as TNF- α , IFN- γ , IL-12, IL-4, and IL-13 aggravate the chronic hepatitis (38, 68, 70). On the other hand, several kinds of immunosuppressive cells including Treg, Breg, MDSC, and Kupffer cells negatively regulate those activated immune cell especially by producing cytokines such as TGF- β and IL-10 (27, 41, 53, 54, 60, 75). Noticeably, the negative regulation is also a key factor in inducing the exhaustion of CD8⁺ T and NK cells, resulting in the immune escape of HBV and HCC tumor cells (26, 40, 63, 73). Additionally, the protective antibody production of B cells was also inhibited by Kupffer cell-derived IL-10 (72). How to control and maintain the immune balance is a key issue in the treatment of HBV-related HCC.

Immune-Based Approaches to HBV-Related HCC Therapy

When the disease cycle of inflammation, progressive fibrosis, cirrhosis and regeneration is broken, the pathways to HCC development are interrupted. It has been recognized that immune balance is important to the outcome of HBV infection, and the effective immune responses with sufficient magnitude and quality of the HBV-specific immune cells is able to adequately control HBV (12). Two clinical cases have further confirmed this speculation. HBV can be eliminated from the infected liver in the recipients with pre-existing HBV immunity when transplanted with a liver from the donor of chronic HBV infection (91). Moreover, after bone marrow transplantation, patients with chronic HBV infection develop an effective anti-HBV immune response in their body and clear HBV (92, 93). Based on these increasing understanding of the mechanisms of immune pathogenesis in patients with chronic HBV infection and HBV-associated HCC, immune-based therapies able to restore immunity and consequently eliminate HBV has been proposed and promoted. Such strategies include checkpoint inhibition, genetically modified T cell transfer, therapeutic vaccines and metabolic modulation.

Checkpoint Inhibition

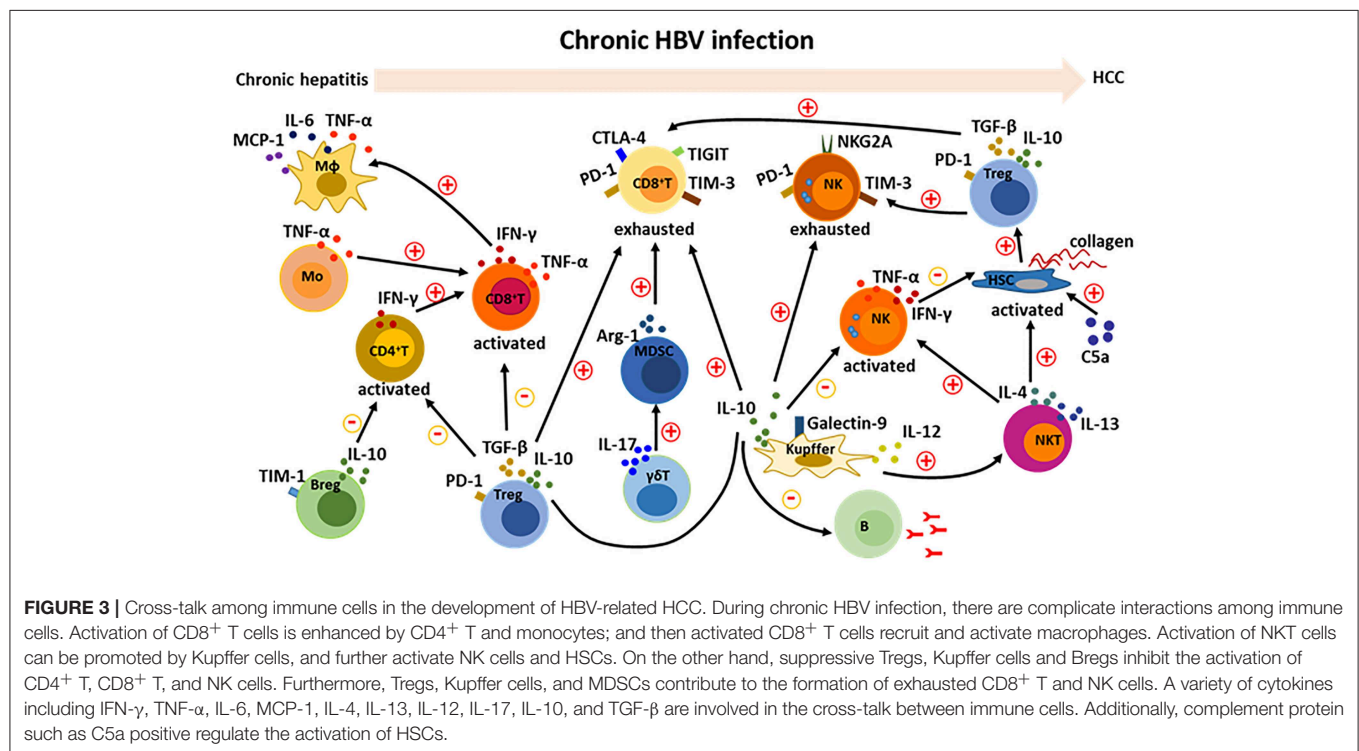
In HBV-infected patients, the function of PD-1⁺ HBV-specific CD8⁺ T cells and PD-1⁺ HBV-specific CD4⁺ T cells were suppressed by the expression of inhibitory ligands on several kinds of immune cells including HSCs, liver endothelial sinusoidal cells, liver-resident macrophages and dendritic cells. By using checkpoint inhibitors to block PD-1/PD-L1 function, HBV-specific T cells were reactivated and their functions were improved (21, 22). Furthermore, a combination therapy involving simultaneous stimulation of OX40 and blockade of PD-L1 functionally augmented HBV-specific CD4⁺ T cells to produce IFN- γ and IL-21 (84). HBV-specific B cell response would be promoted by the activated CD4⁺ T cells in patients with chronic HBV infection (50, 51). CTLA-4 and TIM-3 may offer alternative potential immunotherapeutic targets for patients with HBV chronic infection, since these immune inhibitory receptors also correlated to the inactivation of HBV-specific T cells (94). Higher TIGIT expression was observed in the tumor region of HCC patients and was potentially linked to tumor progression. However, prior to the appearance of a tumor in the liver tissue, TIGIT maintains hepatic adaptive immunotolerance and delays tumor initiation (37). Consequently, the distinct roles of TIGIT in the various stages of tumor initiation and progression should be accounted for the use of checkpoint inhibitors.

Genetically Modified T Cell Therapy

In HBV-related HCCs, the HBV DNA integration into the host genomes leads to the expression of HBV antigens on HCC cells, which can be presented by specific MHC molecules and subsequently recognized by HBV-specific CD8⁺ T cells. Autologous T cells that are genetically modified to express HBV antigen-specific T cell receptor (TCR) were used to control HBV-related HCC in patients, such as HBsAg-specific TCR-redirectioned T cells targeted HBsAg⁺ HCC tumor cells and reconstitute

TABLE 1 | HBV-induced changed immune cells and released cytokines.

Immune cells	Released cytokines	References
CD8 ⁺ T cells	IFN- γ , TNF- α , IL-2, IL-10, IL-17, IL-21	(7, 8, 37, 38, 80–83)
CD4 ⁺ T cells	IFN- γ , IL-21, IL-17	(39, 84, 85)
NK cells	IFN- γ , TNF- α , IL-10	(12, 66, 86)
NKT cells	IFN- γ , IL-4, IL-13	(12, 68, 70)
Macrophages	IFN- γ , IL-6, MCP-1, IL-1 β , TNF- α , CXCL10	(38, 87)
Monocytes	TNF- α , IL-10, TGF- β	(71, 86)
Hepatic stellate cells (HSCs)	IL-1 β , IL-6, TGF- β	(85)
$\gamma\delta$ T cells	IL-17, TNF- α	(75, 88)
Kupffer cells	IL-10, TGF- β , IL-12, IL-6, TNF- α	(70, 72, 73, 89)
Regulatory T cells (Tregs)	IL-10, TGF- β , IL-35	(27, 40, 41)
Regulatory B cells (Bregs)	IL-10	(54, 90)



anti-tumoral immune responses (95, 96). HLA-A*02-restricted HBV envelope- or core-specific TCRs cloned from patients with acute or resolved HBV infection were used to genetically modify T cells with high functional avidity, demonstrating these envelope- or core-specific TCR-transduced T cells could effectively kill hepatoma cells replicating HBV (97). Further work showed that persistently infected hepatocytes carrying HBV cccDNA could be eliminated by redirecting cytolytic T cells against HBsAg-producing cells (98). The first successful clinic trial of HBV-TCR redirected T cell therapy was from a liver transplant patient with extrahepatic HCC metastasis which produced HBsAg due to the HBV DNA integration of tumor cells (96). Since HLA mismatched in the transplant liver, the HBV-TCR redirected T cells only attacked the extrahepatic tumor cells but not the normal hepatocytes. However, in HBV-related HCC

patients there are HBsAg⁺ normal hepatocytes and HBsAg⁺ transformed cells, thus HBV-TCR redirected T cells may induce sever liver damage by targeting HBsAg⁺ normal hepatocytes. How to reduce this risk and overcome the drawback deserves further investigation (99). Additionally, non-lytic lymphocytes engineered to express HBV-specific TCR could reduce HBV replication and limit HBV infection by activating apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3 (APOBEC3) (100). These findings suggest that redirecting T cells genetically modified with high functional TCRs may be therapeutic potential in the treatment of CHB and HBV-related HCC.

Therapeutic Vaccines

In chronic HBV infection, a therapeutic vaccine against HBV is primarily able to break HBV-specific immune tolerance and elicit

an effective immune response. The combination of a therapeutic vaccine (GS-4774) that HBV antigens were engineered to express in yeast and tenofovir disoproxil fumarate (TDF) was shown to improve HBV-specific CD8⁺ T cell responses, strongly with increased cytokine production of IFN- γ , TNF- α and IL-2 to boost the anti-virus immune responses in CHB patients (80). Treatment of GS-4774 resulted in the reduction of Treg numbers in these patients, potentially offering beneficial effects from both single and combination therapy for CHB patients (80).

Metabolic Modulation

In tumor microenvironment, the suppression of T cell metabolism by lack of nutrients or accumulation of lactate, lactic acid and kynurenine resulted in the inhibited effector T cell activity and the promoted suppressive Treg cell function (101). Consistent with the progression on the metabolic dysregulation on immune cells by chronic HBV infection, a novel reconstitution therapy by metabolic modulation might be promising for HBV-related HCC. Targeting exhausted CD8⁺ T cells by mitochondrion-targeted antioxidants such as mitoquinone (MitoQ) and piperidine-nitroxide (Mito Tempo) rescued their anti-viral activity demonstrated by significantly enhanced production of IFN- γ and TNF- α , especially the presence of double positive IFN- γ ⁺TNF- α ⁺ CD8⁺ T cells (29). Similar effects of the metabolic modulation were also observed in CD4⁺ T cells. Regarding the restoration, metabolic modulation by mitochondrion-targeted antioxidants was effective on exhausted T cells in patients with chronic HBV infection, but not on function-competent T cells, which will reduce the risk of indiscriminate T cell amplification and antoimmune reactions *in vivo* when patients were treated. Additionally, the metabolic status of the T cells significantly affects their anti-viral and anti-tumor activity, such as in adoptive transfer of genetically

modified T cells. Thus, the effects of immunotherapy may be improved by combination of these two strategies.

SUMMARY

During chronic HBV infection, the immune imbalance at a cellular and molecular level is highly complex. Although our understanding of HBV immunopathogenesis has improved in recent years, the precise mechanisms governing this disease progression require further investigation. HBV-specific CD8⁺ T cells, HBV-non-specific CD8⁺, CD4⁺T, B, NK/NKT, Kupffer cells, and HSCs are all involved in the development of HBV-related HCC. Furthermore, cell-to-cell interactions and the regulations between these immune cells increase the complexity of HBV immunopathogenesis. Based on these progression, the potential strategies for the intervention and treatment of HBV-related HCC operate to boost the magnitude and quality of the HBV-specific immune responses with the aim of eliminating HBV and maintaining immune homeostasis in patients.

AUTHOR CONTRIBUTIONS

YC prepared and wrote the manuscript. ZT directed the content and revised the manuscript.

FUNDING

This work was supported by the Chinese Academy of Sciences (XDB29030201), National Natural Science Foundation of China (Grant No. 81788101, 81671554, 81821001, 91542000), and the Ministry of Science & Technology of China (2017ZX10202203-002-001, 2017ZX10202203-009-002).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Heterogeneity of HBV-Specific CD8⁺ T-Cell Failure: Implications for Immunotherapy

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OPEN ACCESS

Edited by:

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Catholic University of Korea,
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Reviewed by:

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University College London,
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Alexandre P. Bénéchet,
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Specialty section:

This article was submitted to
Viral Immunology,
a section of the journal
Frontiers in Immunology

Received: 22 July 2019

Accepted: 04 September 2019

Published: 20 September 2019

Citation:

Heim K, Neumann-Haefelin C,
Thimme R and Hofmann M (2019)
Heterogeneity of HBV-Specific CD8⁺
T-Cell Failure: Implications for
Immunotherapy.
Front. Immunol. 10:2240.
doi: 10.3389/fimmu.2019.02240

Chronic hepatitis B virus (HBV) infection is a major global health burden affecting around 257 million people worldwide. The consequences of chronic HBV infection include progressive liver damage, liver cirrhosis, and hepatocellular carcinoma. Although current direct antiviral therapies successfully lead to suppression of viral replication and deceleration of liver cirrhosis progression, these treatments are rarely curative and patients often require a life-long therapy. Based on the ability of the immune system to control HBV infection in at least a subset of patients, immunotherapeutic approaches are promising treatment options to achieve HBV cure. In particular, T cell-based therapies are of special interest since CD8⁺ T cells are not only capable to control HBV infection but also to eliminate HBV-infected cells. However, recent data show that the molecular mechanisms underlying CD8⁺ T-cell failure in chronic HBV infection depend on the targeted antigen and thus different strategies to improve the HBV-specific CD8⁺ T-cell response are required. Here, we review the current knowledge about the heterogeneity of impaired HBV-specific T-cell populations and the potential consequences for T cell-based immunotherapeutic approaches in HBV cure.

Keywords: chronic HBV infection, CD8⁺ T cells, exhaustion, T-cell heterogeneity, viral antigen

INTRODUCTION

Infection with Hepatitis B virus (HBV) represents one of the major causes of morbidity and mortality worldwide. Despite the availability of a prophylactic vaccine for over 30 years, the number of infections remains dramatically high. Approximately, 257 million people globally suffer from chronic HBV infection (1). Current antiviral treatments such as nucleos(t)ide analogous (NUCs) can effectively inhibit HBV polymerase activity and decelerate the disease progression (2). However, there is a low prevalence of HBs antigen loss and anti-HBs seroconversion in patients undergoing NUC therapies and therefore a continuous clinical follow-up is necessary (3). In the clinical setting, HBs antigen persistence is a key marker for the diagnosis of chronic HBV infection. Anti-HBs seroconversion in turn is a marker for sustained viral control by the cellular and humoral immune response and thus is defined as “functional cure”. Identification of new therapeutic targets and development of additional therapeutic approaches are urgently needed. Antibody-mediated depletion studies in acutely HBV infected chimpanzees highlighted HBV-specific CD8⁺ T cells as the main effector cells that contribute to immunological control. In fact, in this study, a prolonged viremia was observed until the reappearance of HBV-specific CD8⁺ T cells in the blood and liver (4). In contrast, the development of a persistent HBV infection is associated with a compromised HBV-specific CD8⁺ T-cell response (5). The mechanisms underlying the impaired HBV-specific CD8⁺ T-cell dysfunction are still not completely understood. Recently, it was shown

that the dysfunctional HBV-specific CD8⁺ T cells differ with respect to the targeted HBV antigens. Therefore, different strategies may be required to improve the HBV-specific CD8⁺ T-cell response. In this article, we focus on the heterogeneity of HBV-specific CD8⁺ T cells and the potential for reinvigoration of these populations during chronic HBV infection.

MECHANISMS OF T-CELL EXHAUSTION IN CHRONIC HBV INFECTION

Chronic HBV infection is associated with an impaired HBV-specific CD8⁺ T-cell response. Although the HBV-specific CD8⁺ T-cell response is initially induced, several phenotypic and functional deficiencies can be observed under the conditions of antigen persistence. These so-called exhausted CD8⁺ T cells represent a unique immune cell differentiation stage that was first described in mice chronically infected with the lymphocytic choriomeningitis virus (LCMV). In chronic HBV, early studies analyzing HBV-specific CD8⁺ T cells during persistent HBV infection also showed a compromised functionality of HBV-specific CD8⁺ T cells including reduced production of the antiviral cytokine interferon γ (IFN γ) and the immunomodulatory cytokine interleukin 2 (IL-2), as well as the loss of cytotoxicity and impaired proliferative capacities (6–8). Further studies showed that these functionally exhausted HBV-specific CD8⁺ T cells displayed a high expression of multiple inhibitory receptors like programmed cell death protein 1 (PD1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG3), T-cell immunoglobulin mucin domain-3 (TIM3), CD244/2B4, or CD160. The co-expression of these markers contributes to T-cell dysfunction by excessive T-cell suppression (6, 9–14). Moreover, HBV-specific CD8⁺ T cells are characterized by an impaired T-cell homeostasis. In particular, the intracellular expression of the pro-apoptotic protein BCL2-interacting mediator (BIM) has been shown to be increased in chronically HBV-infected patients and to drive the decline of HBV-specific CD8⁺ effector T cells (15). A specific transcriptional profile has also been ascribed to the exhausted HBV-specific CD8⁺ T-cell phenotype (8, 16). In fact, the expression patterns of T-bet and Eomesodermin (Eomes) have been shown to tightly regulate differentiation of exhausted HBV-specific CD8⁺ T cells. Specifically, a dysregulated T-bet expression was found in exhausted HBV-specific CD8⁺ T cells, whereas Eomes was shown to compensate for the lack of T-bet (8). Recently, *ex vivo* transcriptome analysis of exhausted HBV-specific CD8⁺ T cells in chronically infected patients also revealed substantial mitochondrial dysfunction and impaired metabolism (16). These mitochondrial alterations contribute to the functional exhaustion in these patients (16, 17). Noteworthy, *in vitro* manipulation reinvigorated the antiviral activity of exhausted HBV-specific CD8⁺ T cells in short-term cultures. In these experiments, the addition of mitochondrion-targeted antioxidants or cytokines partly restored the cytokine production of these cells (16, 17).

Although excessive antigen triggering seems to be a main driver of T-cell exhaustion, several other factors may also play

an important role (18). These include limited CD4⁺ T-cell help (19–23), the induction of suppression by regulatory T cells (Tregs) (24–27) and an immunosuppressive liver environment which is also characterized by the action of immunosuppressive cytokines such as IL-10 and transforming growth factor β (TGF β) (23, 28).

Taken together, HBV-specific CD8⁺ T cells clearly show phenotypic and functional evidence of T-cell exhaustion in chronically infected patients. Noteworthy, recent studies demonstrated that exhausted CD8⁺ T cells do not represent a homogeneous T-cell population but are rather heterogeneous in phenotype and function.

T-CELL HETEROGENEITY—LESSONS FROM LCMV MOUSE MODEL

The LCMV mouse model first strongly contributed to dismiss the initial view about exhausted T cells to be a homogeneous dysfunctional population. Early studies have reported different subsets of exhausted LCMV-specific CD8⁺ T cells with distinct phenotypic and functional characteristics (**Figure 1**). The classification of these subsets is based on distinct expression patterns of the inhibitory receptors PD1 and CD44. In fact, two distinct exhausted LCMV-specific CD8⁺ T-cell subpopulations can be distinguished: the less functionally exhausted PD1^{int}CD44^{hi} T-cell subset and the terminally exhausted PD1^{hi}CD44^{int} counterpart (29, 30). Subsequently, by studying co-expression of the two T-box transcription factors T-bet and Eomes (31), it could be shown that the PD1^{int} T-cell subset was largely T-bet^{hi} and Eomes^{lo}. This exhausted CD8⁺ T-cell subset functions as a progenitor population with improved proliferative capacity and cytokine production. In contrast, the terminally exhausted PD1^{hi} T-cell population has a quite unique expression pattern with a particularly high expression of Eomes and low expression of T-bet. Interestingly, some functionality was also retained from this PD1^{hi}T-bet^{int}Eomes^{hi} T-cell subset indicating that both exhausted CD8⁺ T-cell subsets are required to sustain viral control (31). Additional studies provided further evidence that these two exhausted CD8⁺ T-cell subsets are in a progenitor/progeny relationship. For example, the transcription factor T-cell factor 1 (TCF1) plays a central role (32, 33), as it is important for the establishment of CD8⁺ T-cell memory and for T-cell proliferation (34). Thereby, TCF1⁺PD1^{int} LCMV-specific CD8⁺ T cells represent a circulating T-cell subpopulation that sustains the LCMV-specific CD8⁺ T-cell pool during chronic viral infection (32, 33). Additionally, in lymphoid tissue, a population of chemokine receptor CXCR5 expressing TCF1⁺PD1^{int} LCMV-specific CD8⁺ T cells has been described that gives rise to the terminally exhausted T-cell pool in the periphery (35, 36). Overall, these combined findings revealed the functional T-cell heterogeneity within exhausted LCMV-specific CD8⁺ T cells. The biological importance of this T-cell heterogeneity in chronic infections was demonstrated by immunotherapeutic interventions, where the proliferative burst upon PD1 pathway blockade was almost exclusively restricted to the less differentiated progenitor/memory-like populations. In

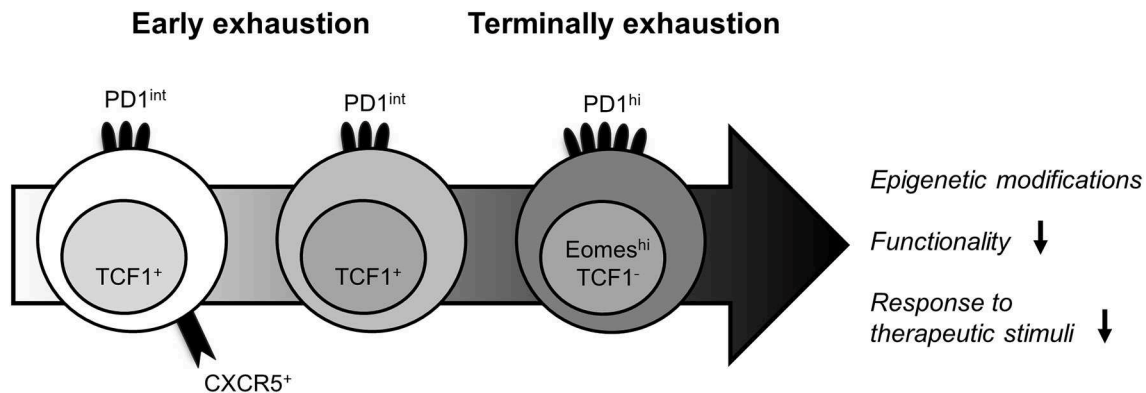


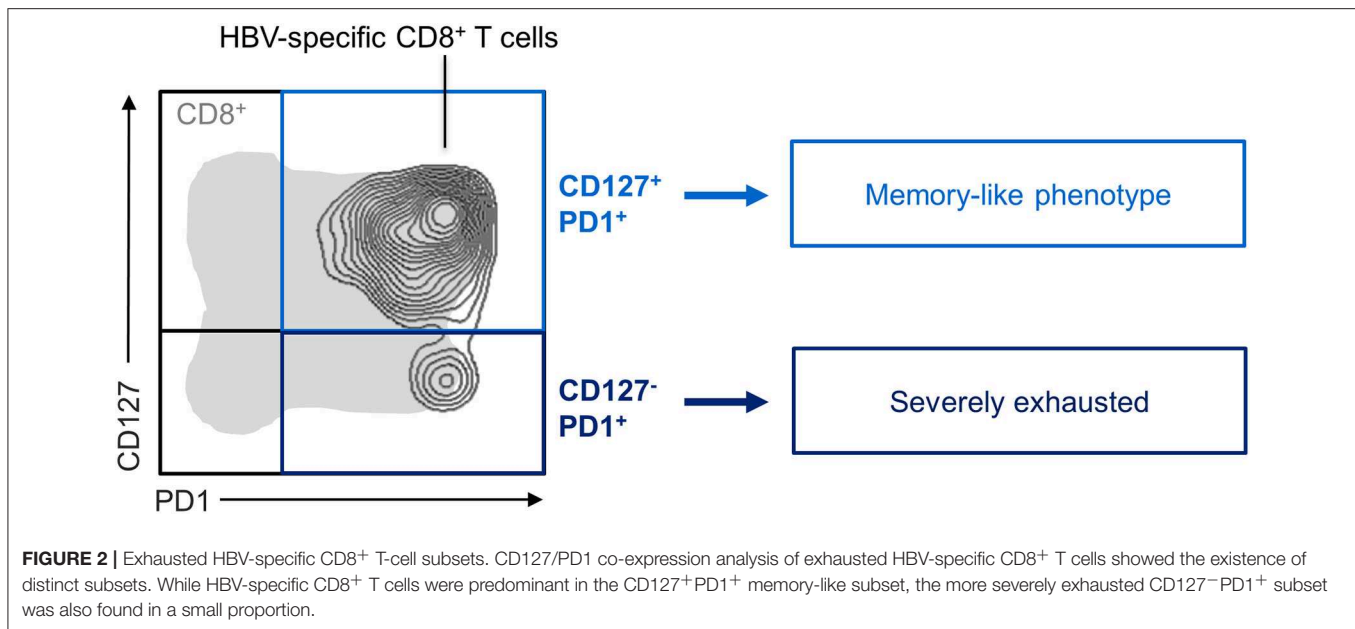
FIGURE 1 | Heterogeneity of exhausted LCMV-specific CD8⁺ T cells. The identification of functionally distinct T-cell subpopulations within exhausted LCMV-specific CD8⁺ T cells has enabled the definition of their lineage dynamics. Despite the epigenetic fingerprint of T-cell exhaustion, the expression patterns of several phenotypical and transcriptional markers can discriminate between less exhausted and terminally exhausted LCMV-specific CD8⁺ T cells. While the early differentiated LCMV-specific CD8⁺ T-cell subpopulation is defined by co-expression of PD1, CXCR5, and TCF1 and provides a strong response to therapeutic stimuli, the more differentiated PD1^{int} TCF1⁺ LCMV-specific CD8⁺ T-cell subpopulation still harbors some effector function. In contrast, the terminally exhausted PD1^{hi}, Eomes^{hi} LCMV-specific CD8⁺ T-cell population exhibit a more severely impaired functionality and is unresponsiveness to immunotherapies.

contrast, the terminally differentiated subset of exhausted LCMV-specific CD8⁺ T cells showed only a slight improvement in the T-cell response to PD1 pathway blockade that was associated with protective immunity (29, 31, 35, 36). However, PD1 pathway blockade does not fully restore exhausted CD8⁺ T cells due to an epigenetic imprinting of T-cell exhaustion (37–39). In fact, exhausted LCMV-specific CD8⁺ T cells differ from effector and memory CD8⁺ T cells by ~6,000 open chromatin regions. The comprehensive characterization of the genomic profile revealed significant alterations in the expression of genes encoding inhibitory receptors as well as transcription factors and genes controlling TCR signaling pathways, costimulatory and cytokine signaling, and cellular metabolism (38, 39). Furthermore, in several recent studies, the HMG box transcription factor TOX was identified as master regulator of T-cell exhaustion (40–42). In particular, a robust expression of TOX induces the fate commitment of an exhausted and dysfunctional phenotype in CD8⁺ T cells by driving epigenetic remodeling events at key gene loci (40, 41). Of note, despite this TOX-mediated epigenetic fingerprint of T-cell exhaustion, there are still differences in the epigenetic landscape of TCF1⁺PD1^{hi} vs. TCF1⁺PD1^{int} cells pointing toward stem-cell like characteristics of the latter subset within the exhausted LCMV-specific CD8⁺ T-cell population (43). In sum, these data clearly show that CD8⁺ T-cell exhaustion represents a distinct and stable differentiation lineage comprising different subsets that respond differently to therapeutic stimuli.

HETEROGENEITY OF EXHAUSTED HBV-SPECIFIC CD8⁺ T CELLS

Recently, the co-existence of heterogeneous and distinctly differentiated exhausted virus-specific CD8⁺ T-cell subsets in human chronic viral infections such as human immunodeficiency virus (HIV) (44, 45) and chronic Hepatitis C virus (HCV) infection (46) have also been reported.

Specifically, HCV-specific CD8⁺ T cells can be divided in distinct subsets based on the CD127/PD1 co-expression patterns. The CD127⁺PD1^{hi} HCV-specific CD8⁺ T-cell subpopulation clearly showed hallmarks of terminal differentiation, such as high levels of inhibitory receptors, indicating severe exhaustion in this subset. In contrast, the CD127⁺PD1⁺ HCV-specific CD8⁺ T-cell subset remarkably shared phenotypic and molecular properties with conventional memory CD8⁺ T cells. However, this subset also displayed characteristics of T-cell exhaustion such as an expression of PD1 and low functionality in terms of proliferation and cytokine production. Therefore, this population is referred to as memory-like (46). In HBV, previous studies on the phenotype of HBV-specific CD8⁺ T cells were often hampered by the low frequency of CD8⁺ T cells present in the peripheral blood of chronically HBV-infected patients (14). Recently, we have deployed pMHC I tetramer-based magnetic bead enrichment approaches to increase the number of detectable HBV-specific CD8⁺ T cells and thereby improving the potential for analyzing their phenotype, function, and subset distribution. By using this approach, we were able to detect HBV-specific CD8⁺ T cells in the majority of chronically HBV-infected patients with low viral load. Subsequently performed in-depth analyses revealed that HBV-specific CD8⁺ T-cell populations are indeed also heterogeneous on a subset level (Figure 2). Based on the CD127/PD1 co-expression analyses, we were able to ascribe the existence of distinct HBV-specific CD8⁺ T-cell subsets including less differentiated memory-like CD127⁺PD1⁺ and terminally exhausted CD127⁺PD1^{hi} subpopulations. Interestingly, in contrast to HCV-specific CD8⁺ T cells, the memory-like CD127⁺PD1⁺ subset predominates in chronically HBV-infected patients with low viral load. This subset is further defined by markers characteristic for memory T cells like BCL2 and TCF1. Importantly, in contrast to conventional memory CD8⁺ T cells, HBV-specific CD8⁺ T cells also uniformly express PD1. Altogether, HBV-specific CD8⁺ T cells exhibit a distinct subset distribution in the setting of chronic HBV infection (47).



Heterogeneity of HBV-specific CD8⁺ T cells is also existent on the level of the targeted antigens. Indeed, immunological characterization of transgenic mice has already shown a hierarchy of dominant and subdominant HBV antigens with various frequencies and antiviral activity (48–50). Recently, studies in chronically HBV-infected patients also observed different properties of HBV-specific CD8⁺ T cells targeting different HLA-A*02:01 restricted epitopes located in the core (core_{18–27}: FLPSDFFPSV), envelope (env_{183–191}: FLTRLITI), and polymerase (pol_{455–463}: GLSRYVARL) proteins (Table 1). First, the frequencies of HBV-specific CD8⁺ T cells targeting the different epitopes varied significantly. Core₁₈-specific CD8⁺ T cells were present in a higher frequency compared to pol₄₅₅-specific CD8⁺ T cells, whereas env₁₈₃-specific CD8⁺ T cells were rarely detectable in patients with chronic HBV infection (47, 51). The low frequency of env₁₈₃-specific CD8⁺ T-cell responses was generally related to the high levels of HBs antigen and is thus most likely caused by deletion as a consequence of hyperactivation. Second, differences within the CD127/PD1-based subset distribution were observed between core₁₈- and pol₄₅₅-specific CD8⁺ T cells in chronically HBV-infected patients. Precisely, pol₄₅₅-specific CD8⁺ T cells showed a diminished proportion of the memory-like CD127⁺PD1⁺ subset compared to core₁₈-specific CD8⁺ T cells (47). This finding together with the distinct expression of killer cell lectin like receptor G1 (KLRG1), Eomes and CD38 on pol₄₅₅-specific CD8⁺ T cells reflected a higher degree of terminal T-cell exhaustion compared with core₁₈-specific CD8⁺ T cells (47, 51). The different exhaustion profile of both HBV-specific CD8⁺ T-cell subpopulations was further underpinned by the functional analyses revealing decreased expansion capacity of pol₄₅₅-specific CD8⁺ T cells which was linked to a dysregulated TCF1/BCL2 expression (47). Thus, these findings give a first hint that T-cell failure of HBV-specific CD8⁺ T-cell populations may occur due to different molecular mechanisms. Additionally,

in both studies (47, 51), phenotypic and functional differences of HBV-specific CD8⁺ T cells targeting core vs. polymerase epitopes were also evident in the context of non-HLA-A*02 alleles (HLA-A*01:01: core_{30–38}: LLDTASALY; HLA-A*11:01: core_{88–96}: YVNVNMGLK; core_{141–150}: STLPETVVRR; HLA-A24:02: core_{117–125}: EYLVSGVW, pol_{756–764}: KYTSFPWLL; HLA-B*08:01: core_{123–130}: GLKILQLL; HLA-B*35:01: core_{19–27}: LPSDFFPSV, pol_{173–181}: SPYSWEQEL; HLA-B51:01: core_{19–27}: LPSDFFPSV; HLA-B*40:01: pol_{40–48}: AEDLNLGNL) indicating an antigen-related phenomena. In line with this observation, another recent study also highlighted different exhaustion profiles of HBV-specific CD8⁺ T cells targeting different HLA-A*11:01 restricted epitopes within the core and polymerase antigens (core_{169–179}: STLPETAVVRR and pol_{387–396}: LVVDFSQFSR) (52). Furthermore, Cheng et al. showed that HBV-specific CD8⁺ T-cell heterogeneity is also associated with the status of HBV infection (52). Still, a more comprehensive study is required to precisely dissect the impact of epitope, HLA-restriction, antigen, and disease status on HBV-specific CD8⁺ T-cell heterogeneity. Another open point that needs to be addressed in future studies is the HBV-specific CD8⁺ T-cell heterogeneity within the liver since current studies have focused on circulating lymphocytes within the blood and thus the effect of a possible compartmentalization has not been taken into account. This knowledge might be of particular importance for immunotherapeutic approaches since different strategies have potentially to be applied to boost the heterogeneous HBV-specific CD8⁺ T-cell populations.

The mechanisms responsible for different exhaustion profiles of circulating HBV-specific CD8⁺ T cells specific for distinct HBV antigens are still unclear (Figure 3). Nevertheless, one hypothesis is that different quantities of HBV antigens produced in infected hepatocytes may play a role in this process. In particular, in the infected liver, HBc antigen is present in higher amounts compared to polymerase antigen (53). This seems

TABLE 1 | Different facets of exhausted HBV-specific CD8⁺ T cells targeting different antigens.

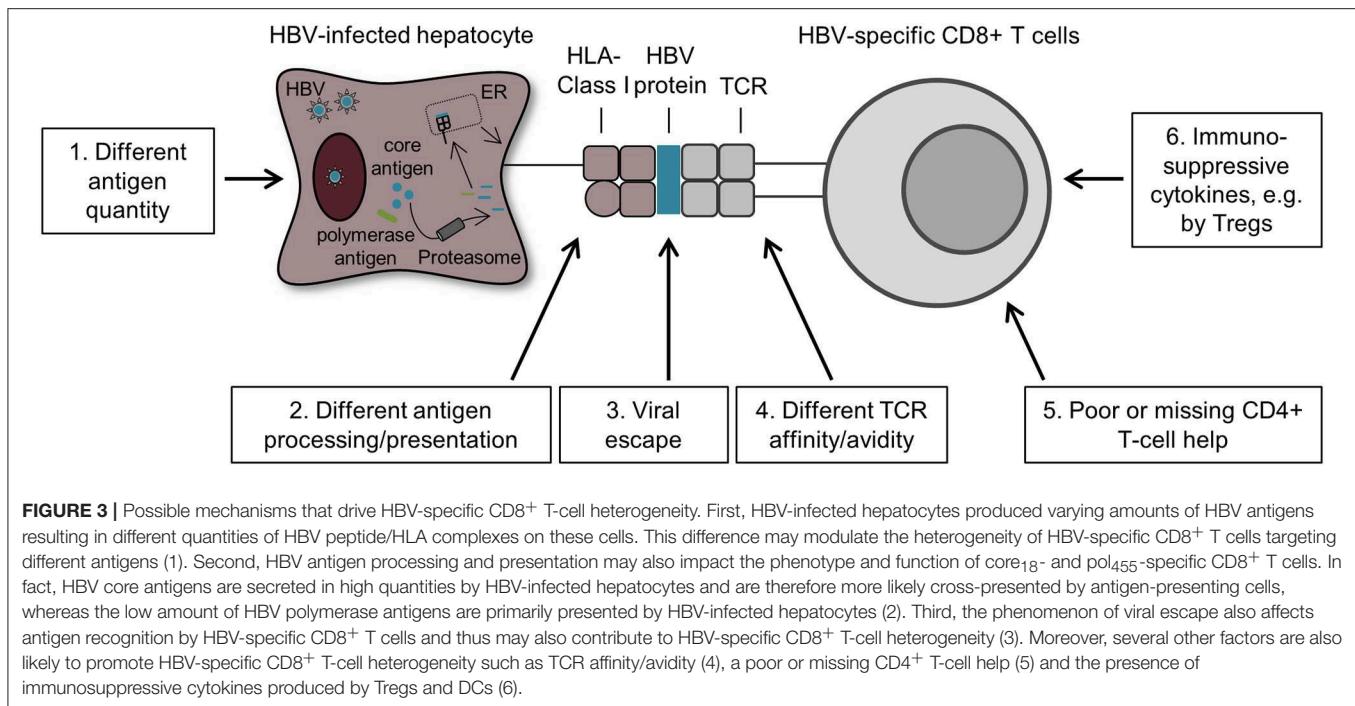
	Core	Pol
Frequency	+++	++
Memory-like subset	TCF1↑ BCL2↑	TCF1↑ BCL2↓
Expansion capacity	↑	↓
Degree of T-cell exhaustion	↓	↑

contradictory to the less exhausted phenotype of core₁₈- vs. pol₄₅₅-specific CD8⁺ T cells (47, 51). Since antigen recognition is one of the main drivers of T-cell exhaustion, one possible explanation for this phenomenon is that the processing and presentation of core peptides is altered which may affect the core₁₈-specific CD8⁺ T-cell activation. Thus, HBV antigen presentation rather than solely the antigen level may contribute to HBV-specific CD8⁺ T-cell heterogeneity. A recent study showed that HBV antigen presentation is also heterogeneous within an HBV-infected liver. Indeed, HBV-infected hepatocytes expressed antigens in different quantities and localizations and from different sources resulting in a non-uniform HLA class I/HBV epitope presentation in the liver and thereby triggering different level of HBV-specific CD8⁺ T-cell activation (54). Moreover, antigens produced and secreted in high quantities by HBV-infected hepatocytes such as HBe antigen may be presented either by hepatocytes themselves or cross-presented by other professional antigen-presenting cells (55). Thus, the different initial CD8⁺ T-cell priming process along with differences in quality and dynamics of epitope processing may lead to different antiviral efficacies of HBV-specific CD8⁺ T cells targeting different specificities. Additionally, viral escape also affects antigen recognition and thus may contribute to HBV-specific CD8⁺ T-cell heterogeneity. Of note, in contrast to the previous studies on escaped epitopes in chronic HCV (56), the presence of HBV sequence variations within the core₁₈ epitope did not alter the functional or phenotypic fingerprints of core₁₈-specific CD8⁺ T cells at all (47, 51). Several other mechanisms probably may also favor HBV-specific CD8⁺ T-cell heterogeneity such as different TCR affinity/avidity, a poor or missing CD4⁺ T-cell help or immunosuppressive cytokines produced by Tregs and dendritic cells (DCs) (57). Hence, further studies are required to identify and dissect the determinants of HBV-specific CD8⁺ T-cell heterogeneity. Overall, these new findings showed that circulating HBV-specific CD8⁺ T cells are not equal and in fact are differentially characterized depending on their antigen specificity.

APPLICATION OF EXHAUSTED HBV-SPECIFIC CD8⁺ T-CELL HETEROGENEITY TO IMMUNOTHERAPY

Since the discovery of T-cell exhaustion, researchers are investigating the potential for functional restoration of exhausted T-cell populations. Such approaches aim to restore an endogenous dysfunctional antiviral immune response. Indeed, great efforts have been made to boost HBV-specific CD8⁺

T-cell responses by blocking the interactions of inhibitory receptors with their ligands (e.g., PD1, CTLA-4, or CD244/2B4 pathway blockade). *In vitro* studies have shown an at least partial restoration of HBV-specific CD8⁺ T cells including an enhanced proliferative potential and increased cytokine production upon checkpoint pathway blockade (6, 9, 11–14). Recently, a pilot study performed in HBeAg negative chronically HBV-infected patients treated with nivolumab (PD-1 pathway blockade) showed a modest decline of HBsAg in most of the patients, while one patient even achieved a sustained HBs antigen loss and anti-HBs seroconversion (58). However, treatment of patients with advanced hepatocellular carcinoma and HBeAg negative chronic HBV infection with nivolumab in a phase 1/2 clinical trial had no effect on antiviral immune response and caused no anti-HBs seroconversion (59). The above reviewed recent research offers a novel perspective on the complexity of the T-cell response present in chronically HBV-infected patients that needs further investigation in light of immunotherapy, in particular with respect to responsiveness and antiviral efficacy. So far, it is still not understood which HBV-specific CD8⁺ T-cell subpopulation might endow with a better antiviral activity. The current findings suggest that core₁₈-specific CD8⁺ T cells have a less impaired functionality than pol₄₅₅-specific CD8⁺ T cells reflected by their mildly exhausted phenotype. Thus, this finding indicates that core₁₈-specific CD8⁺ T cells may show an improved responsiveness to PD1 pathway blockade. In contrast, pol₄₅₅-specific CD8⁺ T cells which exhibit a terminally exhausted phenotype may only poorly respond to PD1 pathway blockade as already described in the chronic LCMV mouse model (29, 31, 35, 36). However, reinvigoration of the pol₄₅₅-specific CD8⁺ T-cell response might be an efficient approach to achieve HBV cure. It is known that the pol₄₅₅-specific CD8⁺ T-cell response is particularly impaired in chronic HBV infection compared to acutely resolved infection, whereas core₁₈-specific CD8⁺ T cells are similar in their expansion capacity in both HBV infection settings (47). Moreover, metabolic alterations of HBV-specific CD8⁺ T cells highlight the difficulty of boosting HBV-specific CD8⁺ T cells. Interestingly, a marked recovery of antiviral capacity was achieved by treating core₁₈-specific CD8⁺ T cells with mitochondrion-targeted antioxidant compounds *in vitro* (16). Further studies are now needed to investigate the effect of metabolic reprogramming on pol₄₅₅-specific CD8⁺ T-cell responses since the combination of compounds targeting both, the impaired metabolism together with checkpoint blockade, provides a promising option in HBV treatment. In light of the recent finding that TOX represents a master regulator of T-cell exhaustion (40–42), it is tempting to speculate that TOX-mediated epigenetic changes are also involved in HBV-specific CD8⁺ T-cell dysfunction. Hence, to boost the defective pol₄₅₅-specific CD8⁺ T-cell response, combining the PD1 pathway blockade with epigenetic modifications might be necessary. Early studies on the manipulation of epigenetic pathways have shown promising results to overcome T-cell exhaustion in chronic viral infections. Indeed, treatment of exhausted LCMV-specific CD8⁺ T cells with either histone deacetylase inhibitors (60) or the blockade of *de novo* DNA methylation (61) rescued LCMV-specific CD8⁺ T cells from functional exhaustion. Thus, attempts to manipulate the



epigenetic signature could have important clinical implications. However, such studies investigating therapeutic manipulations of epigenetic changes have not been performed yet in chronic viral hepatitis and probably face safety issues due to the broadly acting characteristics of currently available reagents. As a consequence of the terminally exhausted phenotype of pol₄₅₅-specific CD8⁺ T cells and the complexity in restoring pol₄₅₅-specific CD8⁺ T-cell immunity, another immunotherapeutic strategy is based on adoptive transfer of engineered T cells. Recently, it was demonstrated by Kah et al. that engineered T cells that transiently express HBV-specific T-cell receptors exert a great antiviral effect in HBV-infected humanized chimeric mice (62). The extreme rarity of env₁₈₃-specific CD8⁺ T cells might be caused by the induction of T-cell tolerance which results in reduced T-cell expansion and elevated T-cell apoptosis (63). Thus, for env₁₈₃-specific CD8⁺ T cells in chronic HBV patients, the usage of adoptive transfer is also implicated. However, the induction of novel env₁₈₃-specific CD8⁺ T-cell responses by therapeutic vaccines should additionally be considered. So far, different therapeutic vaccines are already used in several clinical trials with limited effect on chronic HBV infection (64–67). In sum, the heterogeneity of HBV-specific CD8⁺ T cells suggest that HBV cure can probably not be achieved by only one single approach. Therefore, a combination of different immunotherapeutic interventions might be necessary to induce viral elimination or at least T cell-based control in chronic HBV infection.

CONCLUSION

Exhausted T cells consist of functionally diverse T-cell subpopulations that co-exist during chronic HBV infection.

These findings revealed that HBV-specific CD8⁺ T cells specific for different HBV proteins harbor distinct phenotypical and more importantly functional features in chronically HBV-infected patients. This knowledge allows specializing future immunotherapeutic approaches to target the specific T-cell subpopulation and its molecular pathway that is suitable for the desired kind of immunomodulation. However, the current state of immunotherapeutic treatments suggests that the task of controlling chronic HBV infection is quite difficult. Further characterization of the recently discovered HBV-specific CD8⁺ T-cell subpopulations are needed to uncover new molecular pathways that could be additionally targeted by immunotherapeutic interventions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by CRC 1160/IMPATH (Project A02 and A06) of the German Research Foundation (DFG) to RT, MH, and CN-H. MH was furthermore supported by a Margarete von Wrangell fellowship from the Ministry of Science, Research and Arts of Baden-Württemberg.

ACKNOWLEDGMENTS

We thank all members of the Thimme/Hofmann lab for fruitful discussions.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Toward a Functional Cure for Hepatitis B: The Rationale and Challenges for Therapeutic Targeting of the B Cell Immune Response

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OPEN ACCESS

Edited by:

Seung Kew Yoon,
Catholic University of Korea,
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Specialty section:

This article was submitted to
Viral Immunology,
a section of the journal
Frontiers in Immunology

Received: 19 July 2019

Accepted: 12 September 2019

Published: 24 September 2019

Citation:

Ma Z, Zhang E, Gao S, Xiong Y and
Lu M (2019) Toward a Functional Cure
for Hepatitis B: The Rationale and
Challenges for Therapeutic Targeting
of the B Cell Immune Response.
Front. Immunol. 10:2308.
doi: 10.3389/fimmu.2019.02308

The central role of the cellular immune response in the control and clearance of the hepatitis B virus (HBV) infection has been well-established. The contribution of humoral immunity, including B cell and antibody responses against HBV, has been investigated for a long time but has attracted increasing attention again in recent years. The anti-HBs antibody was first recognized as a marker of protective immunity after the acute resolution of the HBV infection (or vaccination) and is now defined as a biomarker for the functional cure of chronic hepatitis B (CHB). In this way, therapies targeting HBV-specific B cells and the induction of an anti-HBs antibody response are essential elements of a rational strategy to terminate chronic HBV infection. However, a high load of HBsAg in the blood, which has been proposed to induce antigen-specific immune tolerance, represents a major obstacle to curing CHB. Long-term antiviral treatment by nucleoside analogs, by targeting viral translation by siRNA, by inhibiting HBsAg release via nucleic acid polymers, or by neutralizing HBsAg via specific antibodies could potentially reduce the HBsAg load in CHB patients. A combined strategy including a reduction of the HBsAg load via the above treatments and the therapeutic targeting of B cells by vaccination may induce the appearance of anti-HBs antibodies and lead to a functional cure of CHB.

Keywords: hepatitis B virus, functional cure, B cell response, therapeutic vaccine, chronic hepatitis B

INTRODUCTION

It is estimated that more than 250 million people worldwide are chronically infected with the hepatitis B virus (HBV), and over 887,000 deaths are caused by HBV infection annually due to the disease's complications, such as cirrhosis and hepatocellular carcinoma (HCC) (1, 2). Up to 95% of adults are spontaneously cleared of the virus after an acute self-limiting HBV infection, while 90% of newborns develop a chronic infection. The outcome of acute HBV infection is mainly determined by the strength and breadth of the host's adaptive immune responses against the virus (3). An acute resolving HBV infection is always associated with multi-specific and vigorous HBV-specific CD8⁺ T cell responses (4–6), which are functionally exhausted during chronic HBV infection partly due to the high load of viral antigens in the blood (7–9). While the central role of the CD8⁺ T cell response in the control of HBV infection is well-established, the contribution

of humoral immunity (including B cell and antibody responses) has largely been neglected. Recently, several groups have addressed this question and highlighted that HBV-specific B cells are present in the blood and liver but functionally impaired during chronic HBV infection (10–12). Moreover, the level of the serum hepatitis B core antigen (HBcAg) antibody (anti-HBc) was to be found positively correlated with HBV-induced liver disease or inflammation (13, 14) and predicted the therapeutic efficacy of peginterferon (Peg-IFN) and nucleos(t)ide analogs (NAs) in hepatitis B e antigen (HBeAg)-positive chronic hepatitis B (CHB) patients (15–17).

Currently, Peg-IFN and NAs are approved and recommended as first-line therapies for CHB in clinical guidelines (18). However, it is difficult to cure CHB with these drugs, which means achieving sustained undetectable HBV surface antigen (HBsAg) and HBV DNA levels in serum, with or without the appearance of antibodies to the HBsAg (anti-HBs) (19). Anti-HBs antibodies were initially recognized as a diagnostic marker, indicating protective immunity after acute-resolving HBV infection or vaccination. Now, they are considered a biomarker for a functional cure of CHB. Thus, the therapeutic targeting of HBV-specific B cells and the induction of an anti-HBs antibody response are essential parts of a rational strategy to cure CHB. Knowledge of the features of B cell responses against HBV during acute and chronic viral infections is needed to rationally design strategies that target B cells in CHB patients. In this review, we summarize the currently available information about host B-cell and antibody responses in HBV infections, as well as their relationship with HBV pathogenesis, the pivotal role of HBsAg in HBV pathogenesis, and immunotherapeutic approaches to induce HBV-specific immunity and anti-HBs antibodies in chronic HBV infection.

DOES B CELL-MEDIATED HUMORAL IMMUNITY PLAY A KEY ROLE IN HBV CONTROL AND CLEARANCE?

Unlike HBV-specific CD8⁺ T cells, which can be detected by tetramer staining *ex vivo* or after *in vitro* expansion, the specific B cells that target HBV have only been studied recently by using fluorochrome-labeled HBV proteins (10–12). However, several clinical observations suggest that B cell response may have an important role in the control of HBV infection. Rituximab, an antibody of CD20, is widely used to deplete B cells during chemotherapy in B cell lymphomas or autoimmune diseases. If these patients have a previously controlled HBV infection, the clinical application of rituximab may cause HBV reactivation, indicating that B cell responses against HBV are essential to maintaining effective host immune control over HBV (20–22). Moreover, anti-HBs positivity is associated with a decreased risk of HBV reactivation in these patients, suggesting that anti-HBs may also prevent the HBV reactivation (23). Indeed, anti-HBs are known as protective antibodies, which block HBV entry into host hepatocytes and clear infectious HBV particles *in vivo* (24). Several B cell epitopes have been identified on

three large, middle, and small HBV surface proteins. One well-defined region called the a-determinant comprises a number of conformation-dependent epitopes, which are located within the first loop amino acid (aa)124–137 and the second loop aa139–147 of HBsAg. The majority of anti-HBs antibodies developed by vaccination recognize the a-determinant. Another important region is located within the sequence aa21–47 of the HBV large surface protein, which contains the binding site of the HBV cellular receptor, sodium-taurocholate co-transporting polypeptide (NTCP). Thus, the antibodies specific to this region may have potent neutralization activities (24, 25). These studies imply that the B cell-mediated humoral immune response represents an important component in the sustained control of HBV infection.

However, whether the B cell-mediated immune response directly influences the HBV immunopathogenesis remains unclear. In patients with HBeAg-positive CHB, the appearance of the HBeAg antibody (anti-HBe) has been known to be an indicator of a low level of hepatic HBV replication, which is usually associated with the clinical remission of liver disease and a favorable outcome (2). Recently, the level of the hepatitis B core antigen (HBcAg) antibody (anti-HBc) in CHB patients was found to be positively correlated with HBV-induced liver disease or inflammation in CHB patients (13, 14). Consistently, the baseline anti-HBc level was found to be a useful predictor of Peg-IFN and NAs therapy efficacy in HBeAg-positive CHB patients (15–17). In a recent study, a higher baseline anti-HBc titer could predict a higher rate of spontaneous HBeAg seroconversion in HBeAg-positive children with a normal alanine aminotransferase (ALT) level. The anti-HBc level could reflect the strength of the anti-HBV immune response in the HBeAg-positive normal ALT phase of CHB (26). A study using liver samples from different phases of CHB was performed to determine the intrahepatic gene signatures. The results demonstrated that a high up-regulation of immunoglobulin-encoding genes and B-cell function-related genes was found in the immune active phase compared with the immune tolerant and inactive carrier phases (27). Moreover, a powerful B cell response with a massive accumulation of plasma cells secreting IgG and IgM to HBcAg was found in the liver of two patients with HBV-related acute liver failure (28, 29). In a very recent study, Le Bert et al. showed that the frequencies of HBcAg-specific B cells, but not HBsAg-specific B cells, were temporarily increased in the blood during hepatic flares in four CHB patients (12), again indicating a possible link between HBcAg-specific B cells and liver damage in CHB patients. These data suggest that humoral immunity may participate in the pathogenesis of HBV infection. It remains to be investigated whether the anti-HBc antibody response is, indeed, the initial trigger of the host's immune attack against HBV in the liver, thereby leading to inflammation and immunopathological processes in chronically HBV infected patients.

Given the importance of anti-HBs in preventing and curing HBV infection, several groups have attempted to detect the HBsAg-specific memory B cell response using different methods in vaccinated people and CHB patients. An HBs-ELISPOT assay has been developed to identify anti-HBs secreting B cells by culturing enriched CD19⁺ cells with stimulation of

CD40-CD40L for 5 days (30, 31). Another group directly detected HBsAg-specific memory B cells by FACS analysis *ex vivo* utilizing HBsAg-conjugated microbeads in an enriched CD19⁺ cell population (32). Apparently, the frequency of HBsAg-specific memory B cells was extremely low. These two methods efficiently detected HBsAg-specific B cells in HBsAg-vaccinated individuals but not in patients with chronic HBV infection. Recently, two groups utilized fluorochrome-labeled recombinant HBsAg as “bait” to detect and characterize HBsAg-specific B cells by flow cytometric analysis *ex vivo* in HBV-infected patients (10, 11). Similar results were obtained from the two studies, demonstrating that the frequencies of HBsAg-specific B cells in the blood were similar in acute, chronic, and resolved HBV infection, and have no correlation with the serum levels of HBsAg, HBV DNA, and ALT. The phenotype of HBsAg-specific B cells from CHB patients resembled “atypical memory B cells” that are characterized by a low expression of CD21 and CD27 but a high expression of inhibitory markers, such as programmed cell death receptor-1 (PD-1) and the transcription factor, T-bet. Moreover, HBsAg-specific B cells from CHB patients were unable to mature into anti-HBs-secreting cells *in vitro*. However, their function could be partially restored by specific culture conditions, such as a PD-1 blockade or the addition of IL-2, IL-21, and CD40L-expressing feeder cells (10, 11, 33). The dysfunction and phenotype change of B cell responses during chronic HBV infection were also confirmed by other groups (34–36). Le Bert et al. conducted a more comprehensive characterization of B cell responses against HBsAg and HBcAg in CHB patients (12). They found that HBcAg-specific B cells are present at a higher frequency than HBsAg-specific B cells in CHB patients. Furthermore, nearly all HBcAg-specific B cells are the IgG⁺ memory B cell phenotype and mature efficiently into antibody-secreting cells *in vitro*. The differences in the phenotype and function between HBsAg- and HBcAg-specific B cells in the same patient suggest that a high level of HBsAg might cause dysfunctional programming of HBsAg-specific B cells through persistent stimulation (12). The characterization of the HBsAg-specific B cell response in chronic HBV infection facilitates the development of strategies that target B cell responses to induce anti-HBs antibodies for the functional cure of CHB.

Using highly sensitive immunoassays, previous studies demonstrated the existence of anti-HBs antibodies in CHB patients, but they are mainly detected as being complexed to HBsAg (37, 38). This result may suggest that anti-HBs antibodies are produced by HBV-specific B cells in chronic HBV infection but masked by the high level of circulating HBsAg. This may lead to the coexistence of HBsAg and anti-HBs antibodies in CHB patients when their specificity does not match, as shown by several studies (39, 40). In some cases, the appearance of anti-HBs antibodies alone may clear HBsAg in the peripheral blood, but they do not terminate chronic HBV infection in the liver (41). Thus, the HBV-specific B cell response is an integrative part of the host's defense and contributes to HBV pathogenesis, clearance, and protective immunity but is not sufficient to control HBV infection alone.

THE EFFICACY OF ANTIBODY-MEDICATED IMMUNOTHERAPY OR TRIGGERING B-CELL RESPONSE TO CURE CHRONIC HEPATITIS B: RESULTS FROM CLINICAL STUDIES

Polyclonal hepatitis B immunoglobulins (HBIG) are prepared from the pooled plasma of vaccine recipients with a high titer of anti-HBs antibodies. HBIG has been widely used in clinical environments for post-exposure prophylaxis against HBV infection in neonates born to HBsAg carriers (42), as well as in HBV infected liver transplant patients (43). The success of HBIG in these conditions raises the possibility of antibody-mediated immunotherapy against chronic HBV infection (25). Indeed, three pioneering studies tested the efficacy of anti-HBs monoclonal antibodies or HBIG in CHB patients, alone or in combination with alpha-interferon (44–46). All these studies demonstrated the temporary reduction of HBsAg and HBV DNA after a high dose of anti-HBs antibody treatment, though the long-term clearance of HBsAg was not observed. Particularly, in a phase I clinical study, a mixture of two monoclonal antibodies, named HBV-AB^{XTL}, was well-tolerated and led to a significant reduction of serum HBsAg and HBV-DNA after repeat administration (45). A phase II clinical study was also conducted to evaluate the therapeutic efficacy of a combination of HBV-AB^{XTL} with antiviral treatment in chronic HBV-infected patients. However, the results have not yet been released. In a recent pilot study, eight lamivudine-treated CHB patients received monthly HBIG injections followed by an HBV vaccination, which led to a significant decrease of serum HBsAg in half of the patients after 1-year of treatment (47). Importantly, three patients became anti-HBs positive, thereby achieving the goal of a functional cure (47). This study implied that a combination strategy with antiviral treatment and antibody mediated immunotherapy, followed by triggering a B cell response through vaccination may lead to a sustained loss of HBsAg and a functional cure for CHB. However, further clinical trials are needed to test and confirm the therapeutic efficacy of this combination strategy.

As mentioned above, the majority of patients with persistent HBV infections are always linked with the dysfunction of HBV-specific T-cell and B-cell responses (3). During the past two decades, repeated attempts have been made to restore efficient HBV-specific T-cell and B-cell responses in such patients, using conventional or modified HBV vaccines. These attempts have been called “therapeutic vaccination,” with the goal of stopping chronic HBV infection. Using a mammalian cell expressed recombinant Pre-S2/S protein, Pol et al. were the first to demonstrate that vaccination with this protein leads to a significant decrease, or the disappearance, of serum HBV DNA in 50% of treated patients in a pilot study (48). However, the treatment's therapeutic efficacy was not confirmed in a multicenter study including 118 CHB patients (49). Six immunizations with a vaccine consisting of recombinant HBsAg with anti-HBs immune complexes (HBsAg-IC) induced a higher

rate of HBeAg seroconversion in HBeAg-positive CHB patients compared to the control group (50). However, in a larger scale phase III study, the therapeutic efficacy of HBsAg-IC was not confirmed, though the vaccination was performed at a high dose and given 12 times (51). Antiviral therapy with NAs could partially restore the HBV-specific T cell response in CHB patients (52–54), so it is rational to enhance the efficacy of therapeutic vaccination through combination with antiviral therapy.

Co-administration of the HBsAg/AS02B adjuvant candidate vaccine with lamivudine induced a vigorous HBsAg-specific T cell response in patients with HBeAg positive CHB. However, this vaccination strategy did not demonstrate superior clinical efficacy to improve the HBeAg seroconversion rate when compared to treatment with lamivudine alone (55). In another study, 180 HBeAg positive patients were randomly assigned into three groups to receive a mammalian cell-derived vaccine containing PreS1/PreS2/S proteins (named Sci-B-VacTM), lamivudine monotherapy, or a combination treatment. The PreS1/PreS2/S vaccine showed improved immunogenicity and could rapidly induce higher levels of anti-HBs antibodies than the conventional HBsAg vaccines in previous studies (56, 57). The results demonstrated that a combination treatment with the PreS1/PreS2/S vaccine and lamivudine led to enhanced efficacy in viral inhibition, although the HBeAg seroconversion rate was not different. Moreover, anti-HBs antibodies were detected in 55/120 vaccine recipients. The appearance of anti-HBs was associated with significantly higher HBeAg seroconversion rates and a greater suppression of HBV DNA levels in these patients (58). This study highlighted that anti-HBs antibodies could be induced by therapeutic vaccination and might play an important role in the suppression of viral replication.

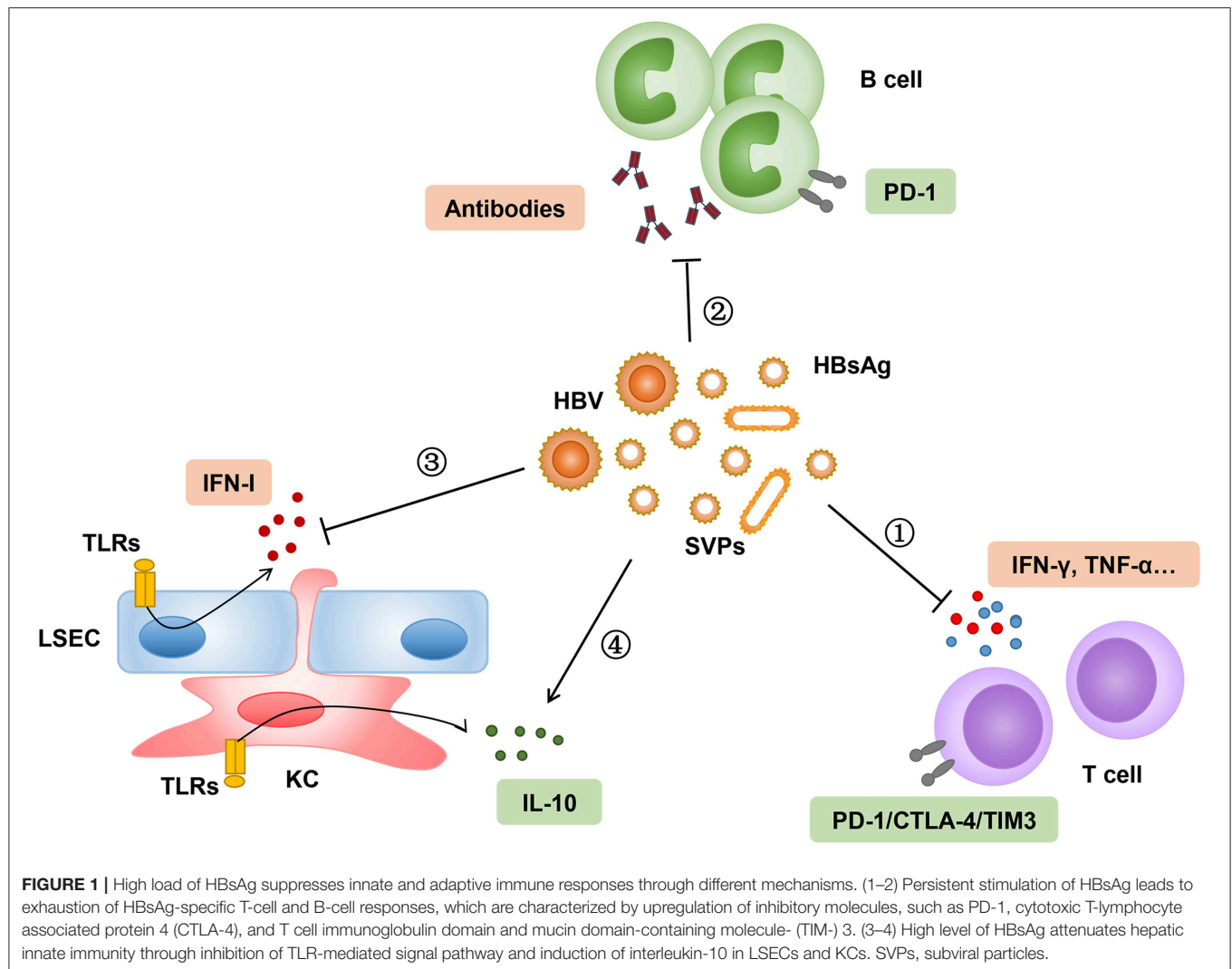
Taken together, these various clinical trials mentioned suggest that antibody-mediated immunotherapy or targeting B cells by therapeutic vaccination may reconstitute the HBV-specific immune response and lead to a reduction of HBV replication in some CHB patients. However, the efficacy of these treatments is low, and further efforts are needed to optimize the therapeutic efficacy.

THE PIVOTAL ROLE OF HBSAG IN THE PATHOGENESIS OF CHRONIC HBV INFECTION AND THE SUPPRESSION OF EFFECTIVE HOST IMMUNITY

Following viral entry into the hepatocytes, the HBV relaxed circular DNA (rcDNA) is converted into a covalently closed circular DNA (cccDNA) minichromosome, which is serviced as a template for subsequent transcription and translation of viral proteins. Many viral proteins were synthesized and secreted into the serum, including three HBV surface proteins and the hepatitis B core-related antigen (HBcrAg), which contains HBeAg (59). The serum level of HBcrAg has been shown to correlate with HBV cccDNA transcriptional activity in CHB patients (60, 61). Furthermore, CHB patients with a persisting high level of HBcrAg during antiviral therapy have an increased

risk to develop HCC despite sustained viral suppression via long-term NAs treatment (62, 63). The clinical significance of HBsAg quantification during chronic HBV infection and antiviral treatment has been studied extensively (64–66). The HBsAg level is the highest in the immune tolerance phase and starts to decline slowly during the immune clearance phase. The HBsAg level progressively decreases after spontaneous or antiviral therapy induced HBeAg seroconversion. Accumulating evidence suggests that high levels of HBsAg may have an immunosuppressive role on both innate and adaptive immunity against HBV (67–69). Indeed, HBsAg has been shown to suppress innate hepatic immunity by inhibiting the toll-like receptor (TLR) mediated signal pathways in Kupffer cells (KCs) and sinusoidal endothelial cells (LSECs) (70, 71). The function of myeloid dendritic cells (DCs) was also impaired to stimulate T cell responses in the presence of HBsAg *in vitro* (72). However, in another study, the DCs isolated from CHB patients showed a normal ability to stimulate the expansion of autologous HBV-specific T cells through the cross presentation of circulating HBsAg (73). The available results about the HBsAg-mediated suppression of innate immunity need to be carefully interpreted, because the majority of experiments were performed *in vitro*. In general, CHB patients are immunologically intact and do not show a higher susceptibility to bacterial or other opportunistic infections. The dysfunction and exhaustion of HBV-specific CD8⁺ T cell responses is a hallmark of chronic HBV infection. The high load of circulating and hepatic HBsAg may contribute to the impairment of HBsAg-specific CD8⁺ T cell response through persistent antigen stimulation (74–76). Moreover, HBsAg may suppresses T cell responses by promoting the differentiation of monocytes into myeloid-derived suppressor cells (MDSCs) and enhance the regulatory T cell response (77, 78). In a woodchuck hepatitis virus (WHV) transgenic mouse model, the high level of virus replication and protein expression in male mice induced the expansion of intrahepatic regulated T cells, leading to the impairment of WHV-specific CD8⁺ T cell responses and gender-related differences in the outcomes of viral infection (79). As mentioned above, a high level of HBsAg might lead to the dysfunctional differentiation of HBsAg-specific B cells, but not HBcrAg-specific B cells in CHB patients (12). These studies demonstrated that the high HBsAg load may induce HBsAg-specific immune tolerance through different mechanisms and may represent a main obstacle in curing CHB (**Figure 1**).

Several studies demonstrated that quantitative baseline HBsAg levels and their changes during the early phase of antiviral therapy could predict therapeutic efficacy, as well as the clearance of HBsAg in CHB patients (64, 65, 80–83). Moreover, lower levels of serum HBsAg at the end of antiviral therapy were associated with a higher rate of HBsAg loss in HBeAg-negative CHB patients after the cessation of long term NAs treatment (84, 85). Although NAs treatment efficiently inhibits HBV replication, it has little effect on the secretion and clearance of HBsAg (86, 87). Peg-IFN therapy may increase the rate of HBsAg loss in low level HBsAg patients, which, in previous studies, has been highlighted in both switch to or add-on strategies combining IFN and NAs to cure CHB (88–90). In patients experiencing HBsAg seroclearance, the Peg-IFN therapy or a cessation of

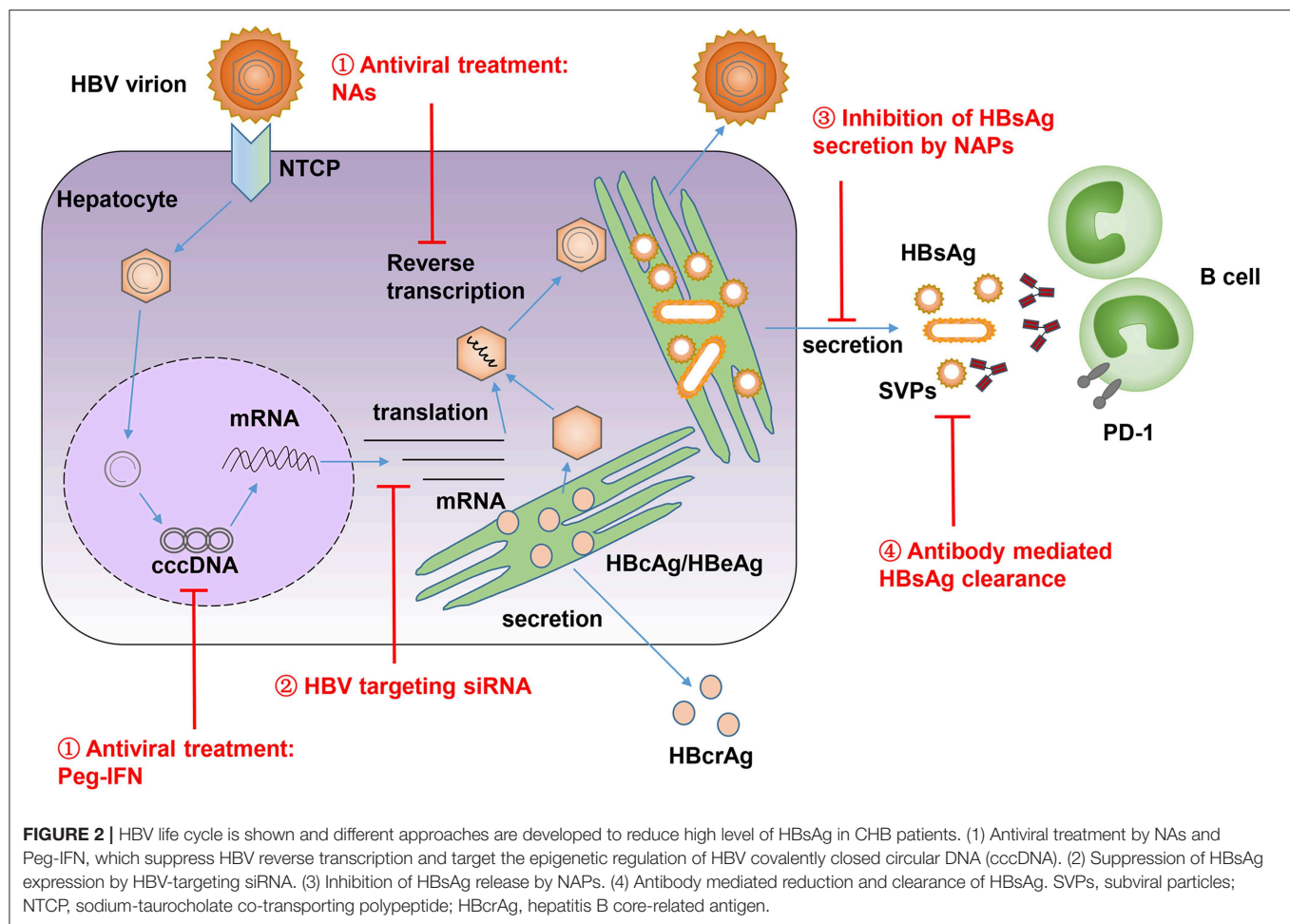


NAs treatment enhanced the natural killer cell's functionality and increased HBV-specific T cell responsiveness (90–94). These results suggest that a sequential combinatorial therapy should ideally cause a precipitous decrease in HBsAg that, when followed by an immunomodulatory therapy, leads to sustained HBsAg loss (95).

HOW TO OPTIMIZE THE THERAPEUTIC EFFICACY OF STRATEGIES TARGETING B CELL RESPONSES IN CHB PATIENTS

Due to the low efficacy of therapeutic vaccination targeting B cell responses in chronic HBV infection, we need to optimize the efficacy of this strategy to achieve a functional cure for CHB. Several approaches, including the reduction of circulating HBsAg, improvement of B-cell targeting vaccines, a combination with immunomodulation, and a rational selection of patients could be employed to boost the therapeutic efficacy of B cell targeting vaccination in CHB patients.

Currently, long-term NAs treatment in CHB patients can lead to a gradual decrease in the serum level of HBsAg. However, only a few patients can reach a significant decrease in serum HBsAg (86, 87). Thus, we need more potent strategies to reduce the serum level of HBsAg. Several approaches were developed to allow a temporary reduction of circulating HBsAg, including targeting viral translation via siRNA, inhibiting HBsAg release via nucleic acid polymers (NAPs), or neutralizing HBsAg with specific antibodies (Figure 2) (68). Recently, an RNA interference based therapeutic agent ARC-520 has been developed. ARC-520 shows a strong efficacy in reducing HBsAg levels in treatment-naïve CHB patients who are positive for HBeAg. However, their therapeutic efficacy is compromised in patients who are HBeAg-negative or have received long-term therapy with NAs (96, 97). Moreover, the clinical development of ARC-520 was put on hold by the U.S. Food and Drug Administration (FDA) due to the delivery vehicle EX1, which probably led to the deaths of non-human primates in another study (98). NAPs are single stranded phosphorothioate oligonucleotides that can block viral entry in many viruses, including the hepatitis B and hepatitis D virus



(HDV), and also have the unique ability to inhibit the release of HBsAg from HBV-infected hepatocytes (99, 100). One NAP named REP 2139 was selected and evaluated in clinical studies for its therapeutic efficacy in CHB patients (101, 102). In an open label, non-randomized study, weekly intravenous administration of REP 2139 led to a dramatic decrease of serum HBsAg and HBV DNA in 12 HBeAg-positive CHB patients, accompanied by HBsAg seroconversion (101). In another study with 12 HBV and HDV co-infection patients, the combination of REP 2139 and peg-IFN led to a reduction of HBsAg by 2–7 logs and undetectable HDV RNA in 11 patients during therapy. One year after treatment, five patients maintained negative HBsAg, five patients had high titer anti-HBs, and seven patients remained HDV RNA negative (102). These results suggest that REP 2139 has a strong ability to decrease circulating HBsAg. However, the treatment's efficacy and side effects should be further evaluated in larger scale, blind, and randomized or real-world studies. The efficacy of specific antibody mediated reduction of HBsAg has been described in a previous section of this review. It is shown that using HBIG or monoclonal antibodies against HBsAg can efficiently decrease the serum level of HBsAg in CHB patients (44–47). In addition, high affinity human anti-HBs and anti-Pre-S1-monoclonal antibodies have been developed recently, and

these antibodies have shown an excellent ability to efficiently clear circulating HBsAg in different mouse models (103–106). These studies emphasize the new interest in developing neutralizing antibodies as a therapeutic strategy to reduce circulating HBsAg.

Besides the reduction of circulating HBsAg to overcome immune tolerance in CHB patients, the improvement of B-cell targeting vaccines is the second step to optimize the therapeutic efficacy of this combination strategy. Therapeutic vaccination with conventional yeast derived HBsAg vaccines showed limited efficacy for HBsAg seroclearance in CHB patients, even when combined with antiviral treatment (55, 107). New generation HBV vaccines containing one (PreS2) or two (PreS1 or PreS2) additional envelope proteins have been developed in transfected mammalian cells. Compared with conventional vaccines, new generation vaccines display more immunogenic properties and rapidly induce higher levels of anti-HBs antibodies both in healthy individuals and in non-responders to yeast-derived vaccines (49, 57, 108). As described in the previous section of this review, the PreS2 containing HBV vaccine had limited therapeutic efficacy in HBeAg positive CHB patients in the absence of antiviral treatment (49, 109, 110). In another study, a PreS2-S vaccine combined with IFN- α -2b showed a better potential benefit in children with CHB than IFN- α -2b

monotherapy (111). In contrast, the PreS1-PreS2-S vaccine Sci-B-VacTM induced anti-HBs antibodies in nearly 50% of vaccine recipients in HBeAg positive CHB patients, even though these antibodies had a poor ability to neutralize the circulating HBsAg (58). However, the appearance of anti-HBs was associated with significantly higher HBeAg seroconversion rates and a greater suppression of HBV DNA levels in these patients (58). A nasal vaccine candidate (NASVAC), comprising HBsAg and HBeAg, has been shown to be safe and highly immunogenic in healthy volunteers (112). The administration of NASVAC through intranasal and subcutaneous routes led to sustained negative HBV DNA in 50% patients with CHB (113). In a phase III clinical study, the therapeutic efficacy of NASVAC was comparable to Peg-IFN in reducing HBV DNA under the limits of detection (114). It is necessary to further confirm the therapeutic efficacy of NASVAC on a large scale and in multicenter studies. Current studies have demonstrated the importance of improving the therapeutic efficacy of B-cell targeting vaccines.

The crosstalk of TLR7/9 and the B cell receptor (BCR) signal pathway in B cells has been previously described; this crosstalk may modulate the B cell's response to foreign or endogenous antigens (115, 116). The stimulation of B cells with a TLR7 ligand and antigen induced a more robust increase in germinal center B cells, plasmablasts, plasma cells, and serum antibodies, compared to their cohorts who received antigen alone (117). A similar intrinsic signal pathway via TLR9 in B cells was also described. This pathway increased production of IgG, in particular, with a shift to the IgG2a subclass (118, 119). These results support the use of TLR7/9 ligands as adjuvants to enhance the therapeutic efficacy of B-cell targeting vaccination (120). Indeed, the immunogenicity of HBsAg was enhanced by a mixture of TLR9 agonists CPG 7909 and 1018 ISS. New vaccines induced a higher titer of anti-HBs accompanied by increased avidity through modulation of the late affinity maturation process (121–124). Importantly, this new vaccine formula proved more effective in immune-suppressed populations (125–127). Further studies are needed to explore the therapeutic efficacy of these improved vaccines on CHB patients.

The lessons from clinical studies suggest that patients with low levels of HBsAg easily reach the goal of HBsAg loss after ceasing long-term NAs treatment (84, 85) or switching to Peg-IFN therapy (82, 83). Thus, the rational selection of patients with low levels of serum HBsAg after long term NAs treatment could be employed to optimize the therapeutic efficacy of B cell targeting vaccination. Indeed, in a recent study, conventional HBsAg-based vaccination in 20 HBeAg-negative patients with HBsAg <1,000 IU/ml, resulted in a significant HBsAg decline in 14 patients and HBsAg loss in 2 patients (128). In another study, the switch from long-term entecavir (ETV) treatment to a combined therapy with IFN- α -2b, HBsAg-based vaccination, and IL-2 resulted in a higher HBsAg loss rate (9.38%) compared to IFN- α -2b (3.03%) alone or continued entecavir (3.70%) therapy in HBeAg-negative patients. Moreover, among patients with baseline HBsAg titers ranging from 100 to 1,500 IU/mL,

the HBsAg loss rate was 27.3% in the combination therapy group (90). These studies suggest that in long-term NAs treated CHB patients, particularly those with low baseline HBsAg levels, therapeutic vaccination with conventional HBsAg may enhance HBsAg loss. In a recent study, CHB patients under NAs treatment (and with low HBsAg levels) were selected for a pilot immunization study with Sci-B-VacTM (129). Three vaccinated patients developed anti-HBs and were cleared of HBsAg after the vaccination. Though this study only involved a few patients, the result is encouraging and hints at the potential usefulness of this approach.

CONCLUSIONS

HBV affects more than 250 million people worldwide and represents a major global public health concern. Current antiviral therapies with Peg-IFN and NAs can suppress HBV replication and improve the prognosis of CHB, but they can hardly clear HBsAg to achieve a functional cure of CHB. During chronic HBV infection, HBV-specific T-cell and B-cell responses are functionally impaired, which leads to a limited efficacy of B-cell targeting therapeutic vaccination in CHB patients. A high level of circulating and hepatic HBsAg may contribute to HBV-specific immune tolerance and represents a main obstacle in curing CHB. Declining HBsAg levels were found to be associated with a higher chance to achieve a sustained response in CHB patients under IFN treatment (130). Moreover, a lower level of serum HBsAg at the end of the NAs treatment was associated with a higher rate of HBsAg loss in HBeAg-negative CHB patients after cessation of NAs treatment or a switch to Peg-IFN therapy. The reduced HBsAg level in these patients is attributed to the effective removal of HBV cccDNA in patients and considered to be a useful biomarker. At the same time, a reduced serum HBsAg level may also facilitate the recovery of the host's immune system and help control HBV. Thus, the reduction of HBsAg by siRNA, NAPs, antibody-mediated neutralization, or long-term NAs treatment may overcome HBsAg-specific immune tolerance and optimize the therapeutic efficacy of B-cell targeting vaccination in CHB patients. Therefore, a sequential combination therapy strategy with antiviral treatment, a reduction of HBsAg, and therapeutic vaccination against envelope proteins may induce the appearance of anti-HBs antibodies and lead to a functional cure for CHB.

AUTHOR CONTRIBUTIONS

ZM, EZ, and ML designed and wrote the paper. EZ designed and drew the figure. ML, SG, and YX carefully revised the paper.

FUNDING

This work was supported by grants from the National Natural Science Foundation, China (81401663 and 81771688) and Deutsche Forschungsgemeinschaft (TRR60 and GK1949).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Human Monoclonal Antibodies as Adjuvant Treatment of Chronic Hepatitis B Virus Infection

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OPEN ACCESS

Edited by:

Seung Kew Yoon,
Catholic University of Korea,
South Korea

Reviewed by:

Robert Thimme,
University of Freiburg, Germany
Eui-Cheol Shin,
Korea Advanced Institute of Science &
Technology (KAIST), South Korea

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Specialty section:

This article was submitted to
Viral Immunology,
a section of the journal
Frontiers in Immunology

Received: 03 July 2019

Accepted: 10 September 2019

Published: 25 September 2019

Citation:

Cerino A, Mantovani S, Mele D,
Oliviero B, Varchetta S and
Mondelli MU (2019) Human
Monoclonal Antibodies as Adjuvant
Treatment of Chronic Hepatitis B Virus
Infection. *Front. Immunol.* 10:2290.
doi: 10.3389/fimmu.2019.02290

Despite the availability of an effective prophylactic vaccine leading to sterilizing immunity, hepatitis B virus (HBV) is responsible for chronic liver disease in more than 250 million individuals, potentially leading to cirrhosis and hepatocellular carcinoma. Antiviral drugs able to completely suppress virus replication are indeed available but they are, by and large, unable to eradicate the virus. Several alternative new treatment approaches are currently being developed but none have so far captured the interest of clinicians for possible clinical development. A constant feature of chronic HBV infection is T-cell exhaustion resulting from persistent exposure to high antigen concentrations as shown by the high expression of programmed cell death protein 1 (PD-1) by HBV-specific CD8 T cells. One way of tackling this problem is to develop HBV-specific neutralizing antibodies that would clear excess envelope proteins from the circulation, allowing for nucleos(t)ide analogs or other antiviral drugs now in preclinical and early clinical development to take advantage of a reconstituted adaptive immunity. Several fully human monoclonal antibodies (mAb) have been developed from HBV-vaccinated and subjects convalescent from acute hepatitis B that show different properties and specificities. It is envisaged that such neutralizing mAb may be used as adjuvant treatment to reduce viral protein load, thus rescuing adaptive immunity in an effort to optimize the effect of antiviral drugs.

Keywords: human monoclonal antibody, HBV—hepatitis B virus, B cells, immune system, adaptive immunity

ADAPTIVE IMMUNITY: THE MAIN PLAYER IN HBV CONTROL

Hepatitis B virus (HBV) is responsible for acute and chronic hepatitis, potentially leading to cirrhosis and hepatocellular carcinoma [reviewed in (1)]. Hepatitis B can be effectively prevented by a prophylactic vaccine (2), whereas, antiviral drugs are virtually unable to eradicate the virus in chronically infected individuals despite efficient suppression of HBV DNA replication (3). Adaptive immunity plays a major role to provide long-term control of infection; however, the very low frequency of circulating HBV-specific T cells in chronic infection contributes to the inability to clear the virus (4). Indeed, HBV may settle for life in occult form in the nuclei of hepatocytes as minichromosome (covalently closed circular DNA, cccDNA), despite apparent recovery, potentially reactivating in case of immune suppression (5). The persistence of cccDNA in hepatocytes is the main hurdle to eradicate HBV infection. The problem is further compounded by the rapid decline of T-cell and B-cell responses as a result of exhaustion induced by production of large amounts of excess HBV envelope proteins (6), largely resulting from integration of HBV

DNA sequences into the host genome particularly in the HBeAg-negative chronic HBV infection [reviewed in (7)]. Of note, there is evidence that increased levels of HBsAg may contribute the CD8⁺ T cell dysfunction (8), and that HBsAg induces disruption of TLR9-mediated Interferon- α production by circulating plasmacytoid dendritic cells (9, 10). Moreover, HBV-specific T cells are mainly concentrated in the intrahepatic compartment together with a large number of HBV non-specific T cells (11), which may contribute to maintain liver inflammation via antigen-independent by-stander activation (12). Exhaustion caused by persistent exposure to high antigen concentrations provides the basis for T cell dysfunction, and results in up-regulation of programmed cell death protein 1 (PD-1) and other check-point molecules by HBV-specific CD8⁺ T cells (13, 14). In line with this interpretation, the intensity of the T cell response appears inversely correlated with HBV DNA levels, with more intense HBV-specific responses detectable in patients with lower viral loads.

B-cell responses also play a fundamental role in HBV infection. Antibodies specific for HBsAg are critical for the neutralization of free extracellular HBV, thus preventing viral entry into susceptible hepatocytes (15). However, antibodies are unable to eradicate intracellular virus, a task fulfilled by MHC class I-restricted virus-specific CD8⁺ T cells by lytic and non-lytic mechanisms (16). Anti-HBs antibodies are also produced during the chronic phase of the infection in minimal amount, most likely complexed by the large amount of the HBV envelope proteins present in the serum. Antibodies to the viral nucleoprotein, anti-HBc and anti-HBe, instead persist during chronic infection and detection of anti-HBe is linked to the emergence of the e-minus variant. The finding of undetectable HBV DNA by standard assays and anti-HBc in the absence of HBsAg is taken as surrogate evidence of occult HBV infection (17).

Current evidence supports the view that a coordinated activation of both T and B cells is critical for eradication of infection. Of note, CD4⁺ T cells control CD8⁺ T cell activity and antibody production (18), and the inability of the infected host to mount robust virus-specific antibody responses correlates with a premature loss of CD4⁺ T cell responsiveness during the establishment of viral persistence in a chimpanzee model of HBV infection (19, 20). Interestingly, sera containing high-titer anti-HBs antibodies provide protection upon exposure to HBV (21), the eradication of which is closely related to the development of neutralizing anti-HBs antibodies. The discovery of the sodium taurocholate cotransporting polypeptide (NTCP) as the receptor used by HBV to bind and infect liver cells allowed identification of the preS1 sequence which is used by HBV to bind liver cells and to characterize the role of anti-preS1 antibodies in the inhibition of HBV entry and infectivity (22). The potency and efficiency of anti-hepatitis B surface antibodies in controlling HBV also comes from early evidence showing that hepatitis B vaccines may be efficacious even when given after exposure (2) and that administration of anti-HBs immunoglobulin can prevent reinfection of liver grafts (23).

THE NEGLECTED ROLE OF B-CELL RESPONSES IN CHRONIC HBV INFECTION

Humoral immunity seems to play an important, but only partially understood role in the control of HBV infection (24). This is also exemplified by the observation that treatment of individuals with manifest or occult chronic HBV infection with specific anti-B cell therapy, e.g., the anti-CD20 monoclonal antibody rituximab, to treat autoimmune disease or non-Hodgkin lymphomas, may carry a substantial risk of HBV reactivation, even those who achieved HBsAg clearance and seroconversion to anti-HBs. Indeed, the rate of reactivation after rituximab is higher than for similarly matched patients treated with T cell immunosuppressive agents (25, 26), pointing to the importance of B cell responses in the control of HBV.

In contrast with the wealth of studies on antibody responses to HBV proteins, there is limited information on the B-cell phenotypic and functional features during HBV infection, particularly during the chronic phase. Of note, and in contrast with evidence of exhausted T cells in the peripheral blood of patients with chronic HBV infection, bulk peripheral blood B cells express several activation markers and produce large amounts of immunoglobulin in response to innate and adaptive immunity signals, significantly more than B cells from patients with chronic HCV infection (27). In agreement with this vigorous polyclonal B-cell activation there was no evidence of upregulation of B-cell exhaustion markers, i.e., FcRL4, adding further support to the concept that the bulk B cell population apparently remains functionally intact in this setting. This would explain why in common practice patients with chronic HBV infection maintain the ability to produce antibodies to recall antigens and are able to respond to soluble protein vaccines. However, recent evidence indicates that, after enrichment, HBsAg-specific B cells show a phenotype of atypical memory B cells, a functionally defective subset which lack expression of CD21 and CD27 resulting from chronic pathogen exposure, particularly HIV infection (28). These B cells demonstrated altered signaling, homing, differentiation into antibody-producing cells, survival and antiviral/proinflammatory cytokine production, that could be partially rescued by PD-1 blockade (29). These findings suggest that persistent HBV infection drives an accumulation of atypical antigen-specific B cells with impaired antiviral capacity. These findings were akin to a simultaneously published study (30), which suggested that B cell alterations were not limited to HBsAg-specific B cells but affected the global B cell population. However, this last finding would apparently be difficult to reconcile with previous evidence of conserved global B-cell function in chronic HBV infection (27). Recent, interesting work from one of these two highly reputed laboratories shed light on the matter. Indeed, a comparative characterization of B cells specific for the HBV nucleocapsid and envelope proteins showed that HBcAg-specific B cells prevailed over HBsAg-specific in patients with chronic HBV infection and were able to efficiently differentiate into immunoglobulin-secreting cells, compared with HBsAg-specific B cells. Moreover, HBcAg-specific B cells have a classical phenotypic and transcriptome

profile of IgG+ memory B cells, whereas HBsAg-specific B cells were confirmed to have an atypical memory B cell profile. This is one of the first reports of dichotomous B-cell differentiation according to antigenic specificity in viral infections and provides a plausible explanation for the higher anti-HBc titers, relative to low anti-HBs titers observed in sera from patients with chronic hepatitis B (31).

A recently identified family of regulatory cells, regulatory B cells (Bregs), was reported to control immune responses at the innate and the adaptive levels, and only a few studies have investigated the role of Bregs in chronic hepatitis B. Bregs classically suppress immune function through secretion of the inhibitory cytokine IL-10, which inhibits the production of pro-inflammatory cytokines and supports regulatory T cell differentiation. One study reported increased Breg cells in PBMC of patients with chronic HBV infection during spontaneous hepatic flares, demonstrating suppressive IL-10 mediated activity of Bregs on HBV-specific CD8 cells *in vitro* (32). Enhanced serum levels of IL-10 have indeed been described in chronic hepatitis B, particularly in the so-called immune activation phase (33). However, such temporal association with necro-inflammatory flares may either suggest a direct Breg responsibility via suppression of HBV-specific CD8 T cells, therefore diminishing viral control, or a secondary Breg-mediated modulation of the inflammatory flare induced by a virus-specific CD8 T cell response, to switch-off the exaggerated immune activation. Both scenarios probably apply to most chronic infections but further data are needed to better understand the role of Breg cells in this clinical setting.

HUMAN MONOCLONAL ANTIBODIES TO TREAT HBV INFECTION?

Human memory B cells and plasma cells are a major source of antibodies. Indeed, B cells undergo somatic mutations and selection by antigen and T-cell help in response to pathogens and persist for life as memory cells in a given individual, rapidly responding to booster immunization to yield a large number of plasma cells. Even though long-lived plasma cells are the main source of serum antibodies, these terminally differentiated cells are certainly not ideal for long-term culture. Several methods have been developed to isolate human monoclonal antibodies (humAbs). The original relatively simple approach consists of the isolation of single antigen-specific Epstein-Barr virus (EBV)-immortalized B cells from donors with high-titer specific immunoglobulin (Ig) after labor-intensive, time-consuming rounds of cloning steps (34). This approach is limited by the instability of the cell lines, the low level of specific antibody secretion, and the poor cloning efficiency, yielding low numbers of Ag-specific clones, usually <0.5% of the initially seeded wells. Improvements of the original methodology are represented by enrichment of antigen-specific B cells using fluorochrome-conjugated antigen to capture B cells of interest, thus increasing the chances of obtaining specific antigen-specific B-cell clones [reviewed in (35)]. This method has been successfully used, but potential technical issues pertaining to the antigen used may

represent an unsurmountable hurdle. Another technique that has been extensively used includes phage-displayed antibody libraries with random pairs of antibody heavy and light chains, enabling the use of appropriate target antigens to screen specific antibodies with high affinity to the antigen (35). Humanized transgenic mice may also provide an alternative option to obtain fully human therapeutic antibodies. In humanized transgenic mice, the endogenous murine antibody gene is replaced by human Ig loci (36). Transgenic mice are then immunized with Ag to elicit specific human antibodies. Alternative approaches to improve the cloning efficiency of the original methodology have been considered, including selection of memory (CD27+) B cells and EBV immortalization and delivering an innate immunity signal, with a TLR9 agonist, such as CpG oligodeoxynucleotides, for potent activation of memory B cells (37). EBV-transformed B-cells can then be further stabilized by fusion with non-Ig secreting mouse-human myeloma heterohybrids (34). Using this approach, we have generated a number of humAbs specific for hepatitis C virus (HCV) (38–40) and more recently for HBV (41). Among the HBV-specific humAbs developed, one derived from a vaccinated individual showed extremely potent neutralizing activity in a *Tupaia belangeri* model of HBV infection *in vivo*, whereas others apparently did not or had a low neutralization titer (41). Interestingly, the humAb showing the highest neutralizing activity recognized a conformational epitope within the common “a” determinant in the major envelope protein (S), whereas, the remainder obtained from acute hepatitis B convalescents recognized linear epitope(s) in the same envelope polypeptide (41). The data were not entirely superimposable to those obtained with a NTCP-expressing HepG2 cell line, as some of the *in vivo* poorly neutralizing humAbs were, instead, efficiently neutralizing in the NTCP-expressing cell line *in vitro* system (unpublished data). The anti-HBV humAbs were originally developed to obtain sustainable reagents for immunoprophylaxis of HBV re-infection in liver transplant recipients and other settings, such as treatment of accidental needle-sticks and in the prevention of vertical-perinatal HBV transmission. Anti-HBs antibodies are usually administered as an additional prophylactic measure while waiting for the appearance of neutralizing anti-HBs antibodies. However, it has not escaped our attention that another potentially important application may emerge in the future as an adjuvant treatment of chronic HBV infection.

PERSPECTIVE FOR A COMBINED USE OF HUMABS AND NUCLEOS(T)IDE ANALOGS FOR HBV TREATMENT

Antibodies are usually effective in prophylaxis if given shortly after viral exposure, their efficiency declining after infection. However, highly potent and/or broadly cross-reactive human monoclonal antibodies may counteract viral escape mechanisms and open new avenues for intervention. For instance, potent HIV-1 envelope-specific broadly neutralizing antibodies (super-antibodies) have been identified that may have potential therapeutic applications (42). In chronic hepatitis B, the potential

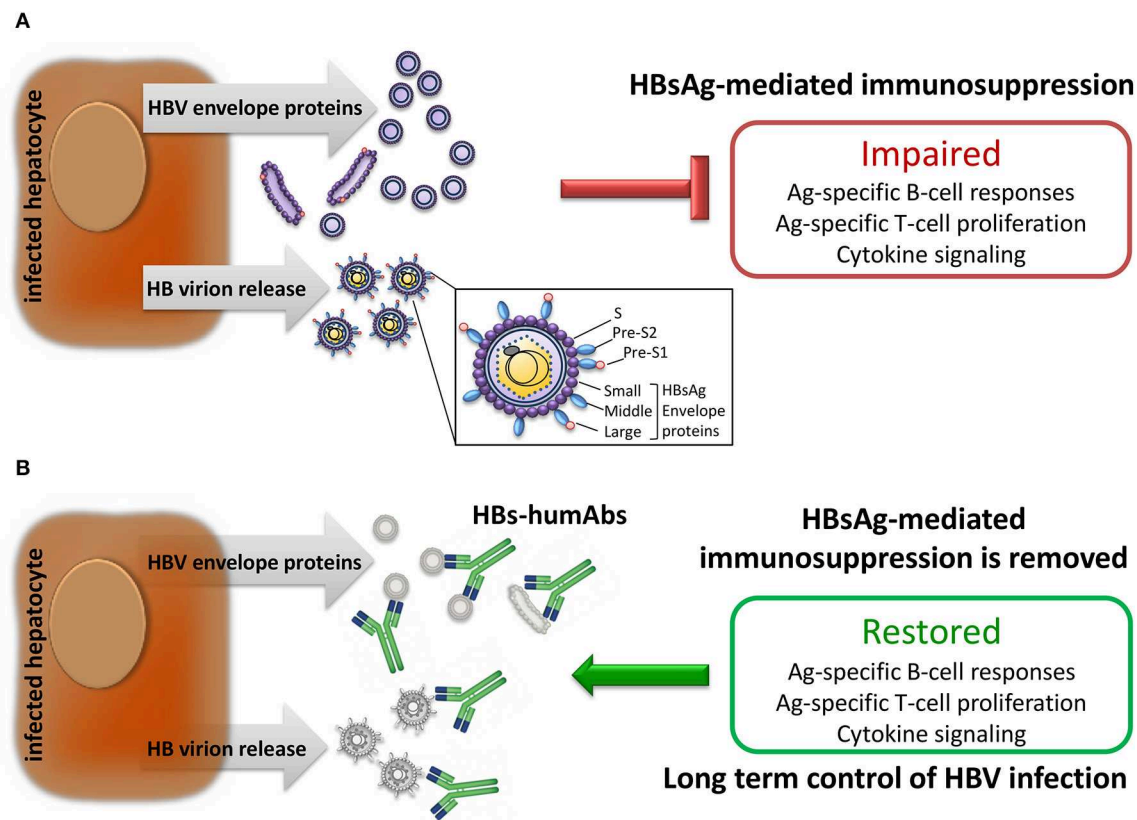
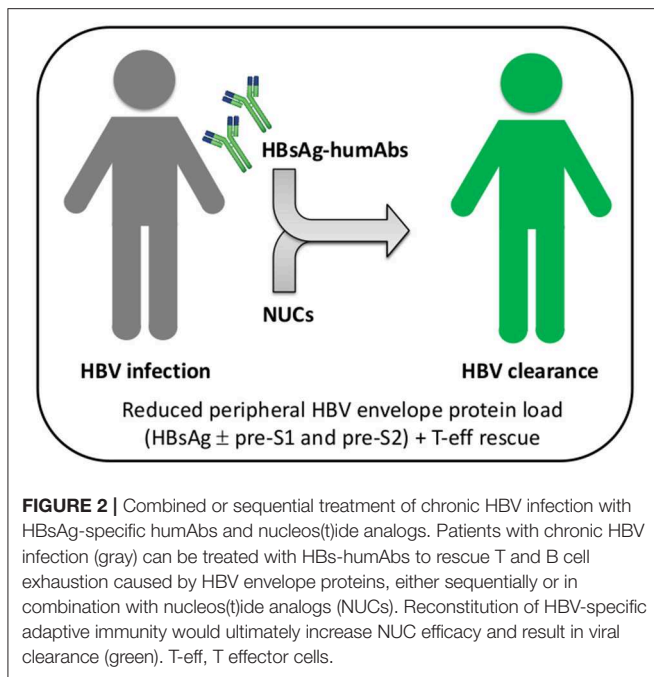


FIGURE 1 | Effect of HBV infection on immune responses. **(A)** Infected hepatocytes produce and release large amounts of HBV envelope polypeptides also expressed on HB virions which suppress adaptive immune responses. **(B)** Human monoclonal antibodies specific for HBV envelope proteins (HBs-humAbs) may reduce peripheral protein load allowing restoration of B and T cell-mediated immune responses.

advantage of anti-HBs humAb therapy relies on the possibility to regenerate effective T cell responses in patients with chronic HBV infection whose T cells are dysfunctional as a result of exposure to very large amounts of HBV envelope proteins produced in excess during viral replication. Indeed, removal of circulating HBsAg by neutralizing anti-HBs mAb was reported to rescue the exhausted adaptive immune responses to hepatitis B vaccination, eventually leading to anti-HBs seroconversion in a murine model (43). It may, thus, be possible to apply this concept to treatment of chronic hepatitis B (**Figure 1**). Notably, a murine anti-HBs mAb was employed to treat hypogammaglobulinemic HBV-infected individuals yielding reduced HBV DNA levels (44). Moreover, a human anti-HBs mAb, alone or in combination with interferon- α , was used in patients with chronic HBV infection, resulting in a substantial decrease in HBsAg titers (45). Interestingly, combination therapy with interferon- α successfully cleared HBsAg. Therefore, combined use with HBV nucleos(t)ide analogs can be definitely envisaged (**Figure 2**). It is important to emphasize that a potentially dangerous effect of immune complex formation following intravenous injection of monoclonal or polyclonal anti-HBs antibodies in HBsAg positive mice, chimpanzees and human subjects has so far not been convincingly demonstrated. Indeed, no renal

complications or immune complex disease has been described in this setting. Similarly, no untoward effects were reported in HBV-infected individuals receiving high doses of polyclonal hepatitis B immunoglobulin (46). It is anyway important, in principle, that anti-HBs humAbs be administered in molar excess to ensure the presence of excess uncomplexed humAbs for effective neutralization and elimination of circulating HBV.

Cooperation between the humoral and cellular arms of adaptive immunity is required for efficient elimination of pathogens. To this end, antiviral humAbs can be exploited to mediate antibody-dependent cell-mediated cytotoxicity (ADCC), which takes place via specific receptors recognizing the Fc fragment of IgGs (Fc γ R) expressed on the membrane of several cells involved in innate and adaptive immune responses. The Fc γ R family includes several classes of receptors that show different affinity for the Fc fragment. One of these, the low affinity Fc γ RIII (CD16), is the most potent activating receptor of NK cells mediating ADCC (47). To evaluate the potential to mediate ADCC, an assay has been developed to measure the ability of antiviral IgG triggering activation of cells expressing Fc γ receptors (48). This is obtained by the interaction of chimeric Fc γ receptors with immune complexes formed on the surface of IgG-coated target cells resulting in Fc γ R-mediated activation,



which makes use of IL-2 secretion by a mouse hybridoma as a surrogate readout. It is thus possible to screen different humAbs for their potential to efficiently mediate ADCC. Whether ADCC plays a role in the setting of HBV infection is presently unclear, but would also depend on the ability of NK cells and other innate lymphoid cells to perform this function, as well as on the affinity of FcγRIII (CD16). Of note, studies using a mAb targeting a linear epitope (aa 119–125 of HBsAg on the first loop N-terminus located at the major hydrophilic region of HBsAg) promoted HBV clearance and blockade of viral infection (49). Even more

interestingly, other humAbs targeting the NTCP-binding site of preS1 (50) were able to reduce HBV DNA and HBsAg levels in murine models of established HBV infection and efficiently mediated ADCC, which was abolished when these antibodies were engineered in the Fc to prevent binding to FcγRs.

The findings discussed above suggest that humAbs targeting HBV envelope epitopes might indeed have a potential for combination therapies in chronically infected individuals. Phase I clinical trials are therefore eagerly awaited in this setting.

AUTHOR CONTRIBUTIONS

MM wrote and conceived the manuscript. AC, DM, and SM contributed to conception and generated data. BO, SV, and SM revised the manuscript, read, and approved the submission.

FUNDING

This publication is part of a project entitled CurB—Sviluppo di nuove molecole candidate alla cura di HBV, funded by Regione Lombardia, Programma Operativo Regionale 2014–2020, Obiettivo Investimenti in favore della crescita e dell'occupazione (cofinanziato con il FESR), Asse Prioritario I—Rafforzare la Ricerca, lo Sviluppo e l'Innovazione, Azione I.1.b.1.3—Sostegno alle attività collaborative di R&S per lo sviluppo di nuove tecnologie sostenibili, di nuovi prodotti e servizi—R&S Per Aggregazioni. Call per l'Attivazione di un percorso Sperimentale Volto alla Definizione degli Accordi per la Ricerca, Sviluppo e Innovazione. art. 11 della Legge 241/1990¹.

¹English translation: This publication is part of a project entitled CurB—Development of New Candidate Molecules for HBV Cure, funded by Regione Lombardia, Regional Operative Programme 2014–2020, co-financed by the European Fund for Regional Development, supporting collaborative R&D actions for novel sustainable technologies, products and services.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Liver-Mediated Adaptive Immune Tolerance

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The liver is an immunologically tolerant organ that is uniquely equipped to limit hypersensitivity to food-derived antigens and bacterial products through the portal vein and can feasibly accept liver allografts. The adaptive immune response is a major branch of the immune system that induces organ/tissue-localized and systematic responses against pathogens and tumors while promoting self-tolerance. Persistent infection of the liver with a virus or other pathogen typically results in tolerance, which is a key feature of the liver. The liver's immunosuppressive microenvironment means that hepatic adaptive immune cells become readily tolerogenic, promoting the death of effector cells and the "education" of regulatory cells. The above mechanisms may result in the clonal deletion, exhaustion, or inhibition of peripheral T cells, which are key players in the adaptive immune response. These tolerance mechanisms are believed to be responsible for almost all liver diseases. However, optimal protective adaptive immune responses may be achieved through checkpoint immunotherapy and the modulation of hepatic innate immune cells in the host. In this review, we focus on the mechanisms involved in hepatic adaptive immune tolerance, the liver diseases caused thereby, and the therapeutic strategies needed to overcome this tolerance.

Keywords: liver tolerance, T cell dysfunction, innate cell dysfunction, immune regulation, liver-draining lymph node, liver diseases

OPEN ACCESS

Edited by:

Yuan Quan,
Xiamen University, China

Reviewed by:

Robert Thimme,
University of Freiburg, Germany
Cai Zhang,
Shandong University, China

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Specialty section:

This article was submitted to
Viral Immunology,
a section of the journal
Frontiers in Immunology

Received: 24 June 2019

Accepted: 10 October 2019

Published: 05 November 2019

Citation:

Zheng M and Tian Z (2019)
Liver-Mediated Adaptive Immune
Tolerance. *Front. Immunol.* 10:2525.
doi: 10.3389/fimmu.2019.02525

INTRODUCTION

As the largest organ in the body, the liver has a rich circulatory supply, receiving blood from both the hepatic artery and the portal vein. As a result, the liver comes into contact with a large proportion of microbial products, as well as harmless food-derived antigens, via the intestines. A high level of exposure to these antigens endows the liver with a distinctive form of immune privilege. This so-called immunotolerance ensures that the liver does not mount a strong immune response against gastrointestinal tract-derived molecules and pathogens. This tolerance effect is also evidenced by the fact that the liver readily accepts allografts, despite a major histocompatibility complex (MHC) mismatch, as seen early on in the pig model of transplantation (1).

Later studies have shown that the liver can accept subsequent non-hepatic allografts from the same donor by inducing systemic immune tolerance (2). Similarly, the tolerance induced via the liver-mediated expression of exogenous proteins is used in gene therapy for hemophilia, metabolic disorders, lysosomal storage disorders (3), and even autoimmune diseases (4). However, the hepatic immune tolerogenic environment is also exploited by hepatitis viruses, parasites, and tumors and can lead to persistent infection and rapid cancer progression.

Adaptive immunity plays a key role in defending the host against pathogens and tumors. The liver determines organ/tissue-localized and systematic adaptive immune responses, highlighting the link between adaptive immune responses and the hepatic microenvironment (5). Evidence also suggests that relationships exist between adaptive immune responses and the hepatic tolerogenic microenvironment (6). This tolerogenic microenvironment leads to liver T cell dysfunction, including clonal deletion, anergy, senescence, deviation, and exhaustion. The liver is home to large numbers of hepatocytes, nonparenchymal cells, and lymphocytes (7). This means that complex interactions between these cells contribute to the induction of adaptive immune tolerance in the liver. For example, parenchymal and nonparenchymal cells suppress the adaptive immune response, leading to hepatic T cell dysfunction, partially as a result of inhibitory receptor and anti-inflammatory cytokine expression (8).

Here, we describe hepatic adaptive immune cell-related dysfunction in the context of liver-mediated adaptive immune tolerance. We focused on: (i) T cell dysfunction, including anergy, exhaustion, and apoptosis, (ii) the regulatory mechanisms involved in the induction of T cell dysfunction, (iii) the current understanding of the role of T cell dysfunction in liver disease, and (iv) the therapeutic strategies developed to counteract adaptive immune tolerance, to illustrate the complexity of and challenges related to liver-mediated adaptive immune tolerance.

THE ADAPTIVE IMMUNE TOLERANCE MECHANISMS

How does the liver tolerize adaptive immune cells? Since adaptive immune cells are easier to render tolerant in the liver than in other organs, the liver has been classically referred to as a “graveyard” for effector T cells and a “school” for regulating cells. In this regard, several reports demonstrate that local antigen presentation in the liver results in T cell apoptosis (9, 10).

The Liver Acts as a T Cell “Graveyard”

The classic hypothesis that the liver functions as a “graveyard” for T cells suggests that the liver represents a specific site for the apoptosis of activated T cells (11) that become trapped and eventually destroyed in the liver by clonal deletion, clonal anergy, clonal deviation, and T cell exhaustion.

Clonal deletion is a process whereby T and B cells expressing antigen-specific receptors with self-reactive specificities are deleted during their development. Huang and colleagues used T cell receptor (TCR) transgenic mice to show that activated T cells could be programmed to undergo apoptosis in the liver through peptide injection (12). Another study suggested that the

liver trapped and passively sequestered activated CD8⁺ T cells (13). In line with these findings, another transgenic mouse model indicated that hepatocyte-activated CD8⁺ T cells with increased expression of Bim were associated with premature death (14). A landmark study by the Bertolino group demonstrated that CD8⁺ T cells undergoing emperipolesis were endocytosed and deleted by hepatocytes, suggesting that “suicidal emperipolesis” is a unique mechanism of peripheral deletion (15). Thus, this “suicidal emperipolesis” plays an important role in liver-activated autoreactive CD8⁺ T cell clearance and immune homeostasis in the liver (16).

Clonal anergy refers to a state of inactivation experienced by self-reactive lymphocytes. Anergic lymphocytes cannot induce strong immunity in healthy individuals. Liver sinusoidal endothelial cells (LSECs), acting as antigen-presenting cells, in the absence of accessory signals reportedly induce anergy in T cells within the hepatic microenvironment (17). Another study supports the idea that plasmacytoid dendritic cells (pDCs) may also lead to the inhibition of T cell activity in the liver, resulting in the anergy or deletion of antigen-specific T cells (18).

Clonal deviation is the process whereby naïve CD4⁺ T cells preferentially assume the Th2 but not the Th1 or the Th17 phenotype during differentiation in the liver. The priming of naïve CD4⁺ T cells by liver sinusoidal endothelial cells (LSECs) fails to promote their differentiation into Th1 cells, even with the exogenous administration of the cytokines IL-1 β , IL-12, and IL-18 (19). Thus, LSECs suppress the IFN- γ -producing Th1 cells in favor of the IL-4-expressing Th2 cells, contributing to the process of immune T cell deviation in the liver (20).

Another form of T cell dysfunction, T cell exhaustion, is often associated with chronic infection and tumorigenesis (21). An exhausted T cell is characterized by impaired effector functions and proliferative capacity, as well as altered transcriptional, epigenetic, and metabolic signatures, including the overexpression of inhibitory receptors and a dysregulated cytokine milieu (22, 23). The first report of T cell exhaustion occurred in a mouse model of noncytopathic lymphocytic choriomeningitis virus (LCMV) infection, in which exhausted CD8⁺ T cells displayed impaired effector functions compared to functional CD8⁺ T cells (24). This begs the question of what causes T cell exhaustion in the first place.

Firstly, persistently high levels of antigen contribute to T cell exhaustion (25). A threshold of intrahepatic antigen levels tunes the fate of cytotoxic T lymphocyte (CTL) function, and high levels of antigen maintain an exhausted T cell phenotype (26). Secondly, altered inflammatory and tissue microenvironments play an important role in inducing T cell tolerance (22). In such circumstances, T cells lose their robust effector functions, accompanied by an increase in the expression of multiple inhibitory receptors, such as PD-1, CTLA-4, LAG-3, and TIM-3.

In addition, T cells receive inhibitory signals from various immunosuppressive cytokines. The phenomenon of T cell exhaustion has been reported both in chronic infections and cancer of the liver. Exhausted hepatic T cells are closely related to inefficient clearance of persisting pathogens and tumorigenesis in chronic liver diseases, including hepatitis

Abbreviations: TCR, T cell receptor; LSECs, Liver sinusoidal endothelial cells; pDCs, Plasmacytoid dendritic cells; LCMV, Lymphocytic choriomeningitis virus; HCV, Hepatitis C virus; APCs, Antigen presenting cells; HSCs, Hepatic stellate cells; MHC, Major histocompatibility complex; Tregs, Regulatory T cells; NKT, Natural killer T; HBV, Hepatitis B virus; LNs, Lymph nodes; HCC, Hepatocellular carcinoma; (m)DCs, Myeloid; iMATES, Intrahepatic myeloid-cell aggregates for T cell expansion.

B and C, malaria, schistosomiasis, and liver cancers. Thus, T cell exhaustion is considered to be associated with hepatic tolerogenic characteristics in liver diseases. Recently, the signal-regulatory protein α was shown to act as an inhibitory receptor when expressed on CD8⁺ T cells during chronic exhaustion in chronic hepatitis C virus (HCV) infection (27).

The Liver Acts as a School to Educate T Cells

The coordination between innate and adaptive immune cells often occurs when confronting liver disease, as the unique structure of this organ facilitates interactions between these cells. There are several hepatic antigen presenting cells (APCs) including resident hepatocytes and non-parenchymal cells like DCs, LSECs, Kupffer cells, and hepatic stellate cells (HSCs) involved in antigen presentation, which facilitate adaptive immune tolerance in the liver (28). During the induction of liver immune tolerance, cytokines like IL-10, TGF- β , and IFN- γ are thought to be involved in the development of chronic liver disease and T cell dysfunction (29–31). In the liver environment, multiple factors, including APCs, the site of primary T cell activation, and altered inflammation, dictate the immune outcomes of intrahepatic T cells (32, 33).

Hepatocytes, which do not normally express MHC class II molecules, acquire the ability to express MHC II and activate CD4⁺ T cells during hepatitis (34). Under specific circumstances, however, antigen presentation by hepatocytes can promote immune tolerance. For instance, MHC II-expressing hepatocytes seem to be associated with defective CD4⁺ and CD8⁺ T cell function and higher LCMV titers in class II transactivator molecule (CIITA)-transgenic mice compared with nontransgenic mice (35). Furthermore, the adeno-associated viral vector-mediated expression of a single MHC I allele in hepatocytes induced tolerance toward an allogeneic graft in a transfer experiment involving liver-generated CD8⁺ regulatory T cells (Tregs) (36).

A recent study showed that Qa-1 expression in hepatocytes with NKG2A⁺ natural killer (NK) cells induced CD8⁺ T cell exhaustion and persistent HCV infection in humanized C/O^{Tg} mice (37). Interestingly, hepatocytes are also capable of converting CD4⁺ T cells into Foxp3⁺ Tregs *in vitro*, resulting in the Treg-mediated suppression of the CD4⁺ T cell response via the Notch signaling pathway (38). Together, these observations indicate that hepatocytes mediate T cell dysfunction in the liver.

The DCs are professional antigen-presenting cells (APCs) that migrate to the draining lymph node and present antigens to T cells (39, 40). Hepatic DCs exhibit an immature phenotype, thus maintaining liver tolerance (41). More importantly, tolerogenic DCs, associated with low MHC class I and II levels and a high expression of T cell coinhibitory ligands, mediate tolerogenic effects, including T cell deletion, anergy, Th2 polarization, and the induction of Tregs (42). Tolerogenic DCs also show considerable promise in the control of autoimmune diseases and allograft rejection (43, 44) by promoting tolerance within the hepatic microenvironment. Liver DCs secrete IL-10 and

are associated with reduced T cell proliferation and function compared to blood DCs (45).

Kupffer cells account for the largest population of macrophages in the liver. Under many circumstances, Kupffer cells play an important role in antigen uptake and pathogen clearance. However, during homeostasis, Kupffer cells secrete anti-inflammatory soluble factors, such as IL-10, to maintain hepatic tolerance (46, 47). In addition, Kupffer cells reportedly mediate T cell suppression, without the need for cytokines like IL-10, TGF- β , and nitric oxide (48).

The hepatic stellate cells (HSCs) and LSECs are well-characterized liver-resident APCs that are capable of tolerizing T cells. For example, TGF- β 1 produced by HSCs inhibits T cells via glycoprotein A repetitions predominant (GARP)-dependent expression on HSCs (49). Moreover, the expression of B7-H1 on HSCs contributes to the regulation of T cell responses by promoting their apoptosis (50). Within the hepatic microenvironment, LSECs can also tolerize both CD4⁺ and CD8⁺ T cells. Furthermore, while LSECs can prime CD4⁺ T cells, these CD4⁺ T cells do not acquire a Th1 phenotype (19). Antigen cross-presentation by LSECs to CD8⁺ T cells also leads to tolerance rather than CD8⁺ T cell activation (51).

The NK cells, which belong to a major group of innate immune cells in the liver, contribute to host defense against virally infected cells and tumors. Mice reportedly contain two liver NK cell subsets, which are referred to as conventional NK cells (which enter the circulation) and tissue-resident NK cells (52, 53). The markers CD49a and DX5 can be used to subdivide murine NK cells into conventional (CD49a⁺DX5⁻) and liver-resident (CD49a⁺DX5⁺) NK cells (54). Similarly, human livers are also populated with two overlapping NK cell subsets (55).

Generally, NK cell function is controlled by a diverse set of activating and inhibitory receptors, the balance between which also contributes to the regulation of T cells (56, 57). For example, hepatic conventional NK cells contribute to effective anti-hepatitis B virus (HBV) T cell responses, while liver-resident NK cells directly suppress T cell responses through the programmed cell death-1 ligand-receptor (PDL1-PD1) axis (58, 59). Impaired NK cell function is accompanied by weakened cytotoxic CD8⁺ T cell activity in persistent viral infections (60). Indirectly, NK cells also diminish CD8⁺ T cell responses during chronic infection by interacting with DCs (61).

Interestingly, the hepatic NK cell-associated modulation of the effector T cell response is, in turn, regulated by the liver microenvironment, such as the presence of IL-10 (62). In addition, HBV-specific CD8⁺ T cells become susceptible to TNF-related apoptosis-inducing ligand (TRAIL)-expressing NK cell-mediated killing by upregulated TRAIL-R2 expression in patients with chronic HBV infection (CHB), indicating that NK cells downregulate HBV-specific CD8⁺ T cell responses (63, 64). In this scenario, upon TRAIL and NKG2D blockade, NK cell-mediated HBV-specific T cell function is also enhanced in patients with CHB who are treated with a nucleos(t)ide analog (65).

Also residing in the liver are natural killer T (NKT) cells, innate-like T cells that modulate the hepatic immune response by producing pro- and anti-inflammatory cytokines upon

activation. There are two types of NKT cells, type I and type II NKT cells. Type I NKT cells express a semi-invariant TCR and is also referred to as invariant (i) NKT cells. By contrast, type II NKT cells express a relatively diverse TCR repertoire. Type II NKT cells conversely appear to be more abundant than type I NKT cells in humans, but in liver diseases, they are similar to type I NKT cells in phenotype and function (66).

By bridging the innate and adaptive responses, NKT cells act as immunoregulators during immunological liver disease. Lan et al. (67) revealed that the pyroptosis of iNKT cells through OX40 signaling can lead to liver inflammation and damage, suggesting that NKT cells play an important role in liver homeostasis. On the other hand, activated NKT cells contribute to the recruitment of Tregs via the CXCR3-CXCL10 pathway (68). The NKT cells also reportedly promote the priming of IL-10-producing CD8⁺ T cells by hepatocytes in order to limit liver injury (69).

Similar to NKT cells, mucosal-associated invariant T (MAIT) cells are the T cell subpopulation restricted to the MHC-I-related (MRI) molecule MRI populated in humans that produces a Th1 and Th17 cytokine milieu (70). The presence of highly enriched MAIT cells in the human liver suggests the importance of these innate cells in the control of liver infections (71, 72). However, in patients with chronic HCV infections, CD8⁺CD161⁺⁺TCRVa7.2⁺ MAIT cells exhibit exhausted features, thereby contributing to HCV persistence (73). In line with these findings, MAIT cells from patients with chronic hepatitis delta virus (HDV) are functionally impaired and subsequently lost during HDV infection (74). In patients with hepatocellular carcinoma (HCC), tumor-educated MAIT cells upregulate inhibitory receptors and display functional impairment, both of which correlate with HCC progression (75).

The Tregs negatively regulate effective T cell immune responses via the production of immunosuppressive cytokines (including IL-10 and TGF- β) during chronic infection and are considered to be a potential target for the treatment of patients with CHB (7). Through upregulated Tregs, IL-33 exerts a negative effect on CD4⁺ T cell proliferation and alleviates hepatitis (76). Similarly, it was found that Tregs orchestrate CD8⁺ T cell exhaustion by engaging the PD-1 inhibitory pathway during LCMV infection (77). However, circulating CD4⁺CD25⁺ regulatory T cells exist in patients with resolved HBV infection (78). Furthermore, the numbers of these regulatory cells are increased and correlate with hepatic inflammation in patients with hepatitis B (79). Therefore, Tregs might play a role in anti-inflammatory activity and need to be more thoroughly assessed (80).

In contrast to T cell tolerance, antibody response to HBV proteins does not provide evidence for B cell tolerance during HBV infection. For example, antibodies specific to the HBV core antigen (anti-HBc) are clearly detectable during acute HBV infection (81). Interestingly, anti-HBc antibodies can be elicited in patients with CHB and are more abundant in CHB infection compared with in patients with self-limited infections (82, 83). Furthermore, highly active B cell responses are indicated during chronic HBV infection through gene expression profiling (84). In contrast, hepatitis B surface antibodies (anti-HBs)

are considered to be protective and are commonly associated with viral control and the resolution of clinical disease. A recent study demonstrated that HBcAg-specific B cells and HBsAg-specific B cells were different in phenotype and function but shared an increased mRNA expression of genes linked with the role of cross-presentation and innate immunity in patients with CHB (85). Overall, the above results indicate that HBV-specific humoral responses are apparently not suppressed in the liver.

The Role of Liver-Draining Lymph Nodes (LNs) in the Induction of Hepatic Immune Tolerance

Although the “graveyard” and “school” models are adequate under certain circumstances, some argue that T cell tolerance is not the direct consequence of local antigen-presentation (86), and that the “graveyard” theory cannot account for the existence of efficient immune responses under different conditions (87). Since many other factors are involved, the two models may not present full explanations of the tolerogenic mechanisms at play in the liver, and additional hypotheses may be required.

The liver produces considerable amounts of lymphatic fluid, which is one of the two major sources of abdominal lymph. Hepatic lymph is thought to originate from the filtration of the sinusoids into the space of Disse, even before the lymph drains from the liver through the lymphatic vessels to the draining LN (88). Although the liver-draining LNs are well-reported in humans, the portal and celiac liver-draining LNs in the mouse have only recently been clearly described in studies that used Evans blue dye or infection with an adenovirus vector carrying the enhanced green fluorescent protein gene (Ad-EGFP) to track hepatic lymphatic draining (89, 90). These studies also show that DCs exit the liver and migrate to the liver-draining LNs, where they prime and facilitate specific T cell responses.

Interestingly, portal and celiac LNs appear to be independent liver-draining LNs, with different cellular compositions and modes of organogenesis. Furthermore, the portal LN participates in oral tolerance via Treg induction, while the celiac LN facilitates effective T cell responses (91). The immune response that occurs in liver-draining LNs is associated with the liver microenvironment, which is considerably different from that of the spleen. Importantly, liver-draining LNs are implicated in chronic human disease (92, 93). Recent progress in research studies related to the association between the liver and human portal LNs indicates that a paucity of DCs in human portal LNs contributes to hepatic immune tolerance (94). In addition, the regional immunity implicated in liver homeostasis and disease is associated with tissue-specific immune cell subsets and their interactions with the liver (7). Thus, a major aspect of liver function is dependent on specific hepatic immune cell subsets, which may, in turn, be influenced by the immune responses modulated by liver-draining LNs.

Moreover, studies indicate that liver inflammation is also involved in liver tolerance. Patients with chronic hepatitis B have fewer signs of inflammation than those with acute hepatitis B who clear the viral infection and display significant inflammation (95).

Furthermore, circulating monocytes under inflammatory stimuli can activate autologous HBV-specific T cells during chronic HBV infection, suggesting that inflammatory conditions might have an impact on intrahepatic HBV-specific T cells (80, 96).

In a study conducted in chimpanzees with chronic infections, agonists of toll-like receptor (TLR) 7 activated TLR-7 signaling and reversed immune tolerance associated with significant intrahepatic inflammation (97). Similarly, TLR 7 agonists appear to enhance T cell and NK cell activities in patients with CHB who are subjected to nucleos(t)ide therapy (98). The above results, taken together, support the hypothesis that inflammatory events in the liver might alter the features of liver tolerance. However, liver tolerance is not absolute during viral hepatitis infection. For example, patients with acute hepatitis elicit an effective adaptive immune response but lack immune tolerance to hepatitis A, B, and C (99–101).

In summary, several mechanisms are involved in the induction of T cell dysfunction in the liver. On the one hand, the liver is seen as a “graveyard” or killing field for activated T cells, because it can induce T cell dysfunction in the local microenvironment. On the other hand, the large population of liver APCs, and cytokines like IL-10, TGF- β , and IFN- γ lead to the negative regulation and further dysfunction of T cells. Additionally, theeliac and portal liver-draining LNs apparently play key roles in promoting liver-mediated adaptive immune tolerance through the induction of Tregs and paucity of DCs. Moreover, a lack of inflammatory events under certain circumstances is also associated with T cell dysfunction (Figure 1).

ADAPTIVE IMMUNE TOLERANCE IN LIVER DISEASE

Under certain pathological circumstances, pathogens, including HBV, HCV, malaria, and schistosomes, exploit the liver's tolerogenic mechanisms to establish persistent infections. For the same reason, the hepatic immunotolerant microenvironment further facilitates the progression of chronic infection to liver fibrosis, cirrhosis, and cancer. Based on the mechanisms involved in liver tolerance, the presence of dysfunctional adaptive immune cells and immunosuppressive regulatory cells is a hallmark of chronic liver disease, including chronic infections and HCC.

Chronic Liver Infection

Effective T cell responses mediate viral clearance in murine models of HBV infection (58). Intrahepatic HBV-specific CD8⁺ T cells contribute to viral elimination and disease pathogenesis in chimpanzees acutely infected with HBV (102). Similarly, patients with acute HBV infection reportedly have enhanced HBV-specific CD8⁺ T cell responses, which are associated with viral control (103, 104).

Conversely, patients with chronic HBV exhibit HBV-specific CD8⁺ T cell dysfunction, with increased frequencies and intensities of PD-1 expression (105). These findings were reported in the first study to show that HBV-specific CD8⁺ T cells in humans can be exhausted. A recent study using peptide-loaded MHC I tetramers suggests that the phenotypic and functional

differences of HBV-specific CD8⁺ T cells can be detected by targeting core vs. polymerase antigen epitopes in patients with CHB, indicating that the molecular mechanisms underlying dysfunctional CD8⁺ T cell populations are not homogeneous in patients with CHB patients (106).

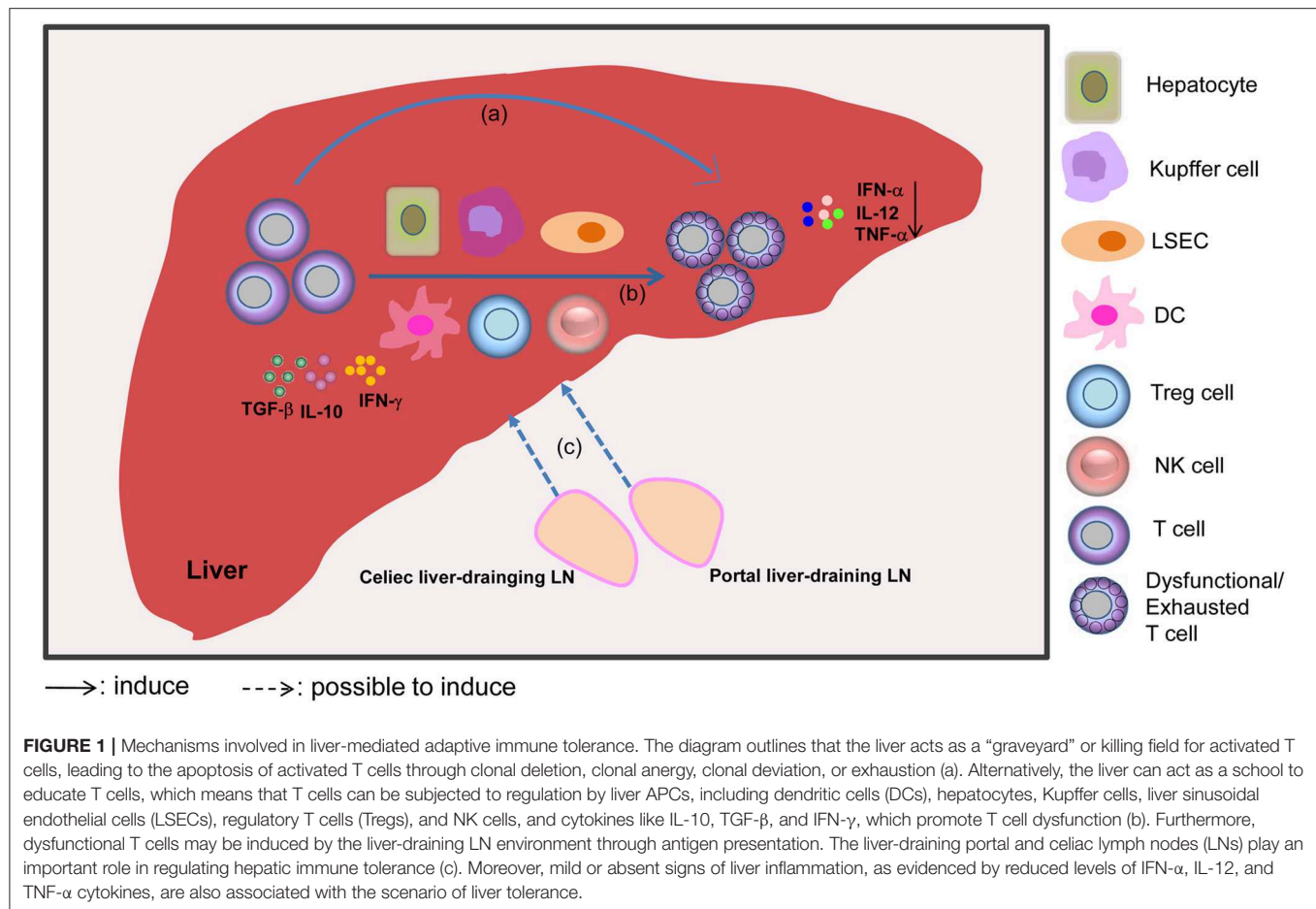
Intrahepatic HCV-specific CD8⁺ T cells have an impaired ability to produce IFN- γ , resulting in a failure to control HCV infection in patients in whom the infection is chronic (107). In addition, it is found that HBV clearance can be achieved by the reconstitution of HBV-specific CD8⁺ T cells, thereby reestablishing adaptive immune responses and reversing HBV-specific tolerance (108). The upregulation of inhibitory receptors on T cells in chronic infection is indicative of T cell exhaustion during viral persistence. For instance, T cell dysfunction is associated with the increased expression of PD-1 and CTLA-4 in patients with CHB compared with in healthy controls (109). Furthermore, in chronic HCV infection, HCV-specific CD8⁺ T cell exhaustion is associated with high expression of inhibitory receptors, while the population of PD-1⁺TIM-3⁺HCV-specific CD8⁺ T cells outnumbers the frequency of PD-1⁺TIM-3⁺T cells in acute resolving HCV infection (110).

Regarding parasitic infections, malaria, and schistosomiasis also establish pathogen persistence and liver tolerance. The CD8⁺ T cells generated in the liver fail to eliminate malaria-causing sporozoites owing to hepatic immune tolerance (111). Moreover, the poor effector functions of exhausted parasite-specific T cells during malaria infection are also linked to PD-1 expression (112). During hepatic schistosomiasis, Th2 cells and Tregs dominate the immune response and release immunosuppressive cytokines, including IL-10 and TGF- β in the liver (113, 114). In addition, many other factors, including the ligands of inhibitory receptors expressed on APCs, account for the failure of dysfunctional T cells to eliminate pathogenic infections in the liver. For example, owing to the selective overexpression of PD-L1 on the surface of macrophages, both CD4⁺ T and CD8⁺ T cells become anergized by the *Schistosoma mansoni* parasite (115).

Liver Cancer

Antigen-specific T cells play a key role in controlling cancer, but similar to chronic viral infections, persistent tumor cell stimulation causes T cell exhaustion (25). A single T cell database revealed that exhausted tumor-infiltrating CD8⁺ T cells preferentially accumulate in the HCC tumor microenvironment (116). In addition, the epigenetic profile of exhausted T cells is distinct from that of functional effector and memory T cells (117). In the context of the tumor microenvironment, exhausted CD8⁺ T cells exhibit reduced effector functions and proliferative capacity. Furthermore, in HCC tissue, CD4⁺ and CD8⁺ T cells display increased expression of inhibitory receptors such as PD-1, TIM-3, LAG-3, and CTLA-4 (118).

Moreover, HCC specimens reportedly harbor exhausted CD8⁺ T cells with varying levels of PD-1 expression. The PD-1^{High} CD8⁺ T cell subset co-expresses high levels of TIM-3 and LAG-3, as is characterized by low IFN- γ and TNF production, indicating that the expression of PD-1 on CD8⁺ T cells arises as a result of the HCC microenvironment (119). A previous



study has shown that the upregulation of Lnc-TIM-3, which specifically binds to TIM-3, can result in CD8⁺ T cell exhaustion in HCC (120). During chronic liver diseases, CD8⁺ T cells with upregulated TIM-3 expression contribute to CD8⁺ T cell exhaustion. The membrane-bound TIM-3 can be cleaved from the cell membrane and yield serum soluble TIM-3, which is associated with liver dysfunction in patients with HCC (121).

Professional or conventional APCs, which can negatively affect T cell function, also play important roles in the regulation of the immune response. Recently, myeloid (m)DCs were found to be functionally impaired in patients with HCC (122), while PD-1 expression on mDCs contributed to the inhibition of CD8⁺ T cell function (123). Kupffer cells also mediate the suppression of CD8⁺ T cells in human HCC, via the B7-H1/PD-1 axis, whereby tumor-associated IL-10 production contributes to the increased B7-H1 expression on Kupffer cells (124).

An important subset of innate immune cells, dysfunctional NK cells are also associated with tumor development (125) and are implicated in the development of HCC. For example, the high expression of NKG2A on NK cells contributes to NK cell exhaustion, which correlates with a poor prognosis for patients with HCC (126). Similarly to NKG2A⁺ NK cells, the HCC microenvironment harbors high numbers of functionally exhausted CD96⁺ NK cells and a

few functionally active CD160⁺ NK cells in patients with HCC (127, 128).

Liver-infiltrating CD11b⁺CD27⁺NK cells represent another dysfunctional subset, closely associated with HCC progression (129). In line with the above findings, dysfunctional DCs, Kupffer cells, and NK cells are associated with T cell dysfunction in the HCC microenvironment. Further study is required to delineate the molecular mechanisms involved in the induction of T cell dysfunction, since the heterogeneity of various innate immune cell phenotypes and functions have been well-described.

STRATEGIES FOR REVERSING T CELL DYSFUNCTION IN LIVER DISEASE

In the liver, T cell-mediated immune tolerance is associated with chronic liver disease. Therefore, reversing immunotolerance is thought to be an effective strategy for restoring effective T cell function, and several approaches have been proposed. For example, novel T cell-based vaccines counteract T cell anergy and restore normal CD8⁺ T cell function, contributing to therapeutic immunity in chronic infection (130). A promising report showed that human redirected T cells with HBV-specific TCR can induce antiviral effects in HBV-infected human liver chimeric

mice (131). Furthermore, TCR-redirected T cells exhibited the potential for functional degranulation and reduced HBsAg levels in a patient with HBV-related HCC (132).

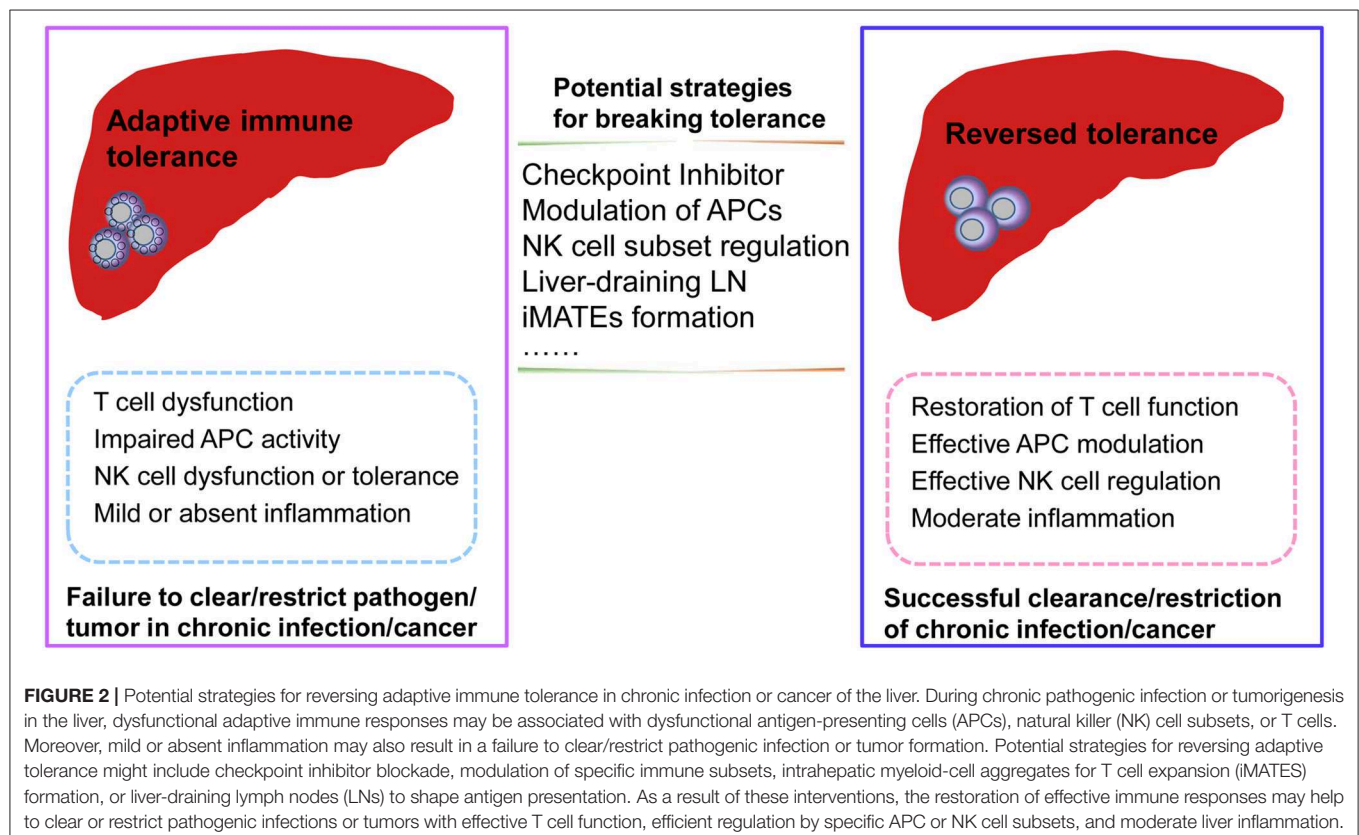
Interestingly, clinical evidence supports the theory that leukemia recipients with HBV infection undergoing bone marrow transplantation can be cured of functional HBV after bone marrow transfer from naturally HBV-immune or actively immunized donors (133, 134). Using IL-12-based vaccination to counteract liver-induced immunotolerance is also an effective strategy for eliciting robust HBV-specific T cell immunity in an HBV-carrier mouse model (135). Moreover, the blockade of inhibitory signaling pathways to reinvigorate exhausted T cell immune responses is thought to be a promising therapeutic strategy, with the blockade of PD-1 signaling proving the most effective to date in the context of HBV infection (136). Notably, IL-12, as the third signal cytokine, enhances the ability of PD-1 signaling blockade to promote the recovery of functional HBV-specific CD8⁺T cells in patients with chronic HBV (137). The addition of CTLA-4 blocking antibodies can partially lead to the rescue of the effective HBV-specific CD8⁺T cell response in patients with persistent HBV infection (138).

The year 2013 marked a major breakthrough for cancer immunotherapy (139). Among effective cancer immunotherapies, blockage of the checkpoint inhibitors, CTLA-4 and PD-1, has shown the most promise, with many HCC patients increasingly benefiting from more treatment

options and combinatorial immune checkpoint inhibitor blockade (140). For instance, blocking NKG2A potentiates tumor-infiltrating CD8⁺ T cell immunity but not NK cells (141). However, this immunosuppressive strategy is hindered by some immunological obstacles, thus resulting in only a minority of tumor patients achieving durable immune responses. Moreover, under certain conditions, CD8⁺ T cell exhaustion may occur in the absence of PD-1 upregulation (142). Therefore, other viable strategies for reversing T cell dysfunction are required to supplement immunotherapy in the context of liver disease.

Several types of parenchymal and nonparenchymal cells also exhibit immunomodulatory functions through their association with T cells in the liver. Therefore, the targeting of immune regulation between APCs or innate immune cells and dysfunctional T cells is expected to have a positive effect on the treatment of liver disease. Evidence suggests that the impairment of DC function is associated with exhausted T cell responses and that CD40-mediated mDC activation rescues intrahepatic anti-HBV CD8⁺ T cells from PD-1-mediated exhaustion (143).

In patients with HCC, both the peripheral and blood DCs co-express PD-1, while the intratumoral transfer of PD-1-deficient DCs elicits tumor-specific CD8⁺ T cell immune responses and restricts tumor growth (123). In chronic HBV infection, HBV-induced monocytes educate NK cells to produce IL-10 via the PDL1/PD-1 pathway, which then contributes to autologous CD4⁺ and CD8⁺ T cell inhibition (144). This suggests that



NK cells could be targeted for CHB therapy. Furthermore, the blockade of the checkpoint receptor, TIGIT, promotes NK cell-based tumor-specific T cell immunity, further highlighting the contribution of NK cells to the restoration of tumor-specific CD8⁺ T cell immune responses (145). In particular, the blockade of the inhibitory receptor NKG2A increases NK cell effector function and the associated anti-viral and anti-tumor immunity in chronic liver diseases, such as CHB infection and HCC (126, 146).

Interestingly, the same anti-NKG2A blocking mAb was recently reported to enhance anti-tumor immune responses by unleashing both NK and T cell effector functions (147). In patients with CHC, TRAF1^{low}HCV-specific CD8⁺ T cell function is restored through IL-7 plus 4-1BBL and PD-1 blockade treatment, indicating a promising immunotherapy for patients with CHC (148).

CONCLUDING REMARKS

The liver has developed various mechanisms for the induction and maintenance of immune tolerance. Hepatic immunotolerance is associated with the presence of dysfunctional T cells, and the processes of clonal deletion, anergy and exhaustion, dysfunctional regulatory cells, and altered liver inflammatory processes. During chronic liver disease, this tolerogenic state prevents the mounting of an effective adaptive immune cell response against pathogens or tumor cells.

In addition to the immunotherapeutic strategies employed to overcome tolerance in liver disease, several approaches have been developed to reverse T cell dysfunction. For instance, Knolle and colleagues found that even in chronic viral infection, antigen-activated intrahepatic CD8⁺ T cell proliferation was induced by intrahepatic myeloid-cell aggregates for T cell expansion (iMATEs) without causing liver immune pathology via the TLR pathway (149). Although it has not been determined whether

iMATEs have a similar structure to that of tertiary lymphoid tissue in local presentation and priming of CD8⁺ T cells, such findings may provide a new way to break T cell tolerance and induce effective anti-pathogen immune responses.

Furthermore, as secondary lymphoid organs, the liver-draining LNs help to shape immune responses in the liver and may play a role in reversing T cell dysfunction by modulating antigen presentation. Moreover, liver-resident NK cell subsets also inhibit T cell function via the PD-L1/PD-1 pathway, while the blockade of PD-L1 abrogates the suppression of T cell function (59). Recent advances in the field of innate immune cell biology, focusing on specific innate immune cell subsets and their different phenotypes and functions, will likely further clarify the regulatory mechanisms and molecular regulators needed to break liver-mediated immune tolerance and reverse adaptive immune cell dysfunction in liver disease. The questions of where and how hepatic immune subsets interact to generate dysfunctional T cells in the context of hepatic immunotolerance remain to be addressed. In summary, additional research is required to identify the innate immune subsets that are involved in inducing T cell dysfunction, the site of their interaction with T cells to render them dysfunctional, and the specific molecular mechanisms that are involved in this complex process (Figure 2).

AUTHOR CONTRIBUTIONS

ZT devised this manuscript. MZ wrote this manuscript. ZT and MZ revised the manuscript.

FUNDING

This work was supported by the Ministry of Science and Technology of China (2018YFA0507403), the Chinese Academy of Sciences (XDB29030201), and the National Natural Science Foundation of China (Grant Nos. 81788101, 81821001, 91542000, and 81771685).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Advances in Targeting the Innate and Adaptive Immune Systems to Cure Chronic Hepatitis B Virus Infection

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OPEN ACCESS

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Specialty section:

This article was submitted to
Viral Immunology,
a section of the journal
Frontiers in Immunology

Received: 23 August 2019

Accepted: 23 December 2019

Published: 07 February 2020

Citation:

Meng Z, Chen Y and Lu M (2020)
Advances in Targeting the Innate and
Adaptive Immune Systems to Cure
Chronic Hepatitis B Virus Infection.
Front. Immunol. 10:3127.
doi: 10.3389/fimmu.2019.03127

“Functional cure” is being pursued as the ultimate endpoint of antiviral treatment in chronic hepatitis B (CHB), which is characterized by loss of HBsAg whether or not anti-HBs antibodies are present. “Functional cure” can be achieved in <10% of CHB patients with currently available therapeutic agents. The dysfunction of specific immune responses to hepatitis B virus (HBV) is considered the major cause of persistent HBV infection. Thus, modulating the host immune system to strengthen specific cellular immune reactions might help eliminate HBV. Strategies are needed to restore/enhance innate immunity and induce HBV-specific adaptive immune responses in a coordinated way. Immune and resident cells express pattern recognition receptors like TLRs and RIG I/MDA5, which play important roles in the induction of innate immunity through sensing of pathogen-associated molecular patterns (PAMPs) and bridging to adaptive immunity for pathogen-specific immune control. TLR/RIG I agonists activate innate immune responses and suppress HBV replication *in vitro* and *in vivo*, and are being investigated in clinical trials. On the other hand, HBV-specific immune responses could be induced by therapeutic vaccines, including protein (HBsAg/preS and HBcAg), DNA, and viral vector-based vaccines. More than 50 clinical trials have been performed to assess therapeutic vaccines in CHB treatment, some of which display potential effects. Most recently, using genetic editing technology to generate CAR-T or TCR-T, HBV-specific T cells have been produced to efficiently clear HBV. This review summarizes the progress in basic and clinical research investigating immunomodulatory strategies for curing chronic HBV infection, and critically discusses the rather disappointing results of current clinical trials and future strategies.

Keywords: immunotherapy, hepatitis B virus, innate immunity, therapeutic vaccination, T cell therapy

INTRODUCTION

The rate of newly acquired hepatitis B virus (HBV) infection is well-controlled by prophylaxis with conventional HBsAg vaccines; however, the vast reservoir of nearly 300 million chronic HBV-infected individuals worldwide still represents a serious threat to humans, leading to up to about 900,000 deaths every year (1–3). Persistent HBV infection could result in liver cirrhosis and/or failure, and liver cancer, accounting for most end-stage liver diseases (4, 5).

Currently, PEGylated interferon- α (PEG-IFN- α) and nucleos(t)ide analogs (NUCs) are available antiviral drugs for the effective treatment of chronic HBV infection (5–8). NUCs, with daily oral administration, are widely welcomed by chronic hepatitis B (CHB) patients, and suitable for

individuals with liver cirrhosis, liver failure, and pregnancy, due to their excellent safety profiles. Although NUCs can control HBV replication profoundly, and reduce the HBV associated end-stage liver disease and liver cancer, hepatitis B surface antigen (HBsAg) clearance, suppression, and/or seroconversion seldom occur in patients administered monotherapy with NUCs. The discontinuation of NUC treatment might result in liver flare or failure. Thus, an uncertain or even lifelong period of NUC treatment may be needed for most patients with chronic HBV infection. Alternatively, PEG-IFN- α treatment can lead to HB e-antigen (HBeAg) clearance and HBsAg seroconversion in 10–30% of cases within a definite duration of therapy (6, 9, 10). Beside direct antiviral effects, the immunomodulatory property of IFN- α may ultimately induce an immune control of HBV. Meanwhile, poor tolerability with frequent severe undesirable effects and the requirement for subcutaneous administration limit PEG-IFN- α application.

Recently, “functional cure,” which is characterized by loss of HBsAg whether or not anti-HBs antibodies are detected, is becoming an accessible ideal endpoint of antiviral treatment in CHB (3, 11). “Functional cure” represents continued suppression of the activity of covalently closed circular DNA (cccDNA) in the patient liver without the serum markers of viral replication. The remaining cccDNA may be reactivated once the immune system is deeply damaged, leading to the recurrence of hepatitis B. Thus, cccDNA eradication is being pursued intensively as an ultimate therapeutic goal. It is believed that host immune control of HBV infection implies complete elimination or functional inactivation of HBV cccDNA though the underlying molecular mechanisms are not fully understood (12). Based on this assumption, enhancing host immunity to HBV is rationally an attractive approach to cure chronic HBV-infected patients.

In this review, we summarized the available information about strategies for enhancing host innate and adaptive immunity for controlling HBV infection. The relevant basic research resulting from preclinical studies is reviewed in sections enhancing innate immunity to establish an antiviral state: results from preclinical studies and induction of HBV-specific immune responses. The available results of clinical trials are presented in sections current clinical trials based on immunotherapy and IFN- α -based immunotherapy plays an important role in HBV “cure” in individuals with functional intrinsic immune responses. A critical consideration of the results of current clinical trials and discussion about the future strategies are included in section conclusions and future perspectives.

IMMUNE PATHOGENESIS OF PERSISTENT HBV INFECTION

The molecular mechanisms accounting for HBV persistence are not fully elucidated. It is generally accepted that dysfunctional immune responses play an essential role in persistent HBV infection as well as liver inflammation, when comparing the characteristics of immune responses in acute hepatitis B and CHB (3, 13–16). Immune responses during CHB are characterized by (1) dysfunctions and exhaustion of HBV-specific

CD4⁺ and CD8⁺ T cells (2, 17–19) decreased numbers and dysfunction of DCs and NKs/NKTs (3, 13, 20–23) up-regulated/enhanced expression of regulatory factors, including the immune checkpoint proteins PD-1, CTLA-4, and T cell immunoglobulin domain and mucin domain-3 (Tim-3) (24–26); and (4) impaired innate immune response, especially toll-like receptor (TLR) downregulation and dysfunction (27–32).

To maintain homeostasis, the hepatic immune system preferentially induces tolerance to antigens flushed from the portal vein. In CHB, the suppressive mechanisms in the liver regulate and inhibit T cell functions. It has been confirmed that intrahepatic inflammatory reactions induce multiple suppressive pathways *in situ* in the liver, leading to T cell function suppression (25). Enzymes such as arginase (33) and IDO (34) are released by damaged hepatocytes and cause depletion of amino acids, which are important in maintaining T cell functions (35). Arginine depletion leads to reduction of CD3 ζ levels in T cells, subsequently causing TCR-pathway dysfunction (36). Intrahepatic inflammation recruits regulatory T cells (37–41), B cells, and myeloid-derived suppressor cells (42–44), and activate stellate cells, leading to IL-10 and TGF- β production (25). The suppressive events in the liver are vital for protection from severe damage primed by inflammation, while further impairing the functionality of HBV-specific T cells.

In general, high HBV DNA, HBsAg, and HBeAg levels contribute to maintain HBV-specific immune tolerance in chronically HBV-infected individuals. Reduction of both circulating and intrahepatic HBV virions and proteins is a prerequisite for (re-)establishing efficient HBV-specific T-cell responses (45–48). The first evidence that HBV clearance can be achieved by adoptive transfer of bone marrow from anti-HBs-positive donors (49) provides a certain way to cure HBV infection through immune modulation. Liver transplantation may also transfer immune cells from vaccinated donors to recipients, and partially control reinfection of the liver (50). An increasing number of studies have been carried out to explore therapeutic strategies including those involving small molecules to boost HBV immunity in patients, aiming to a functional cure for HBV infection (51–53).

THERAPEUTIC STRATEGIES FOR CHB

Based on the knowledge about the immune pathogenesis of chronic HBV infection, a number of innovative strategies may be applied to enhance HBV-specific immune responses in patients (Figure 1). On one hand, oral, intranasal, or subcutaneous application of agonists of pathogen recognition receptors (PRRs), including TLRs, retinoic acid-inducible gene 1 (RIG-I), and stimulator of interferon genes (STING), activates host immune cells and hepatocytes/non-parenchymal liver cells, leading to the production of IFN/expression of interferon-stimulated genes (ISGs) and proinflammatory cytokines, which jointly mount an antiviral state (Figure 2). On the other hand, HBV-specific CTLs can be induced by therapeutic vaccines, boosted through checkpoint blockade, or renewed by adoptive transfer of *in vitro* activated T/NKT cells or genetically edited HBV-specific T cells such as chimeric antigen receptor T (CAR-T) or T cell receptor (TCR)-T cells (Figure 3). These strategies have been explored in

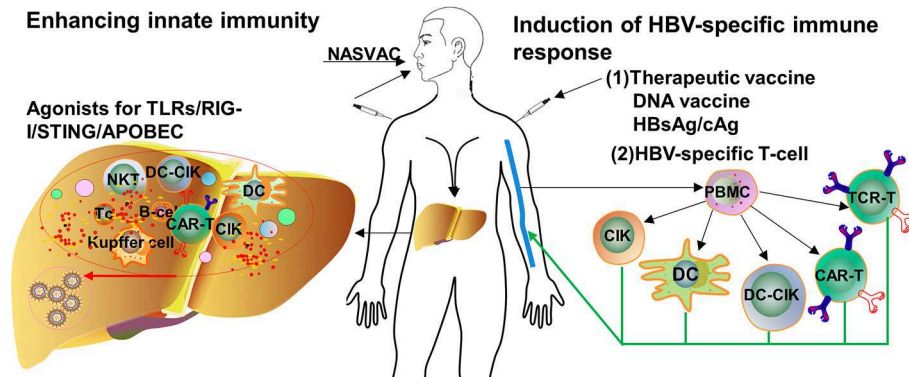


FIGURE 1 | Approaches for the treatment of chronic HBV infection. Available knowledge about HBV immune control and immunopathogenesis; a number of immunomodulatory strategies have been tested to enhance innate and adaptive immunity in preclinical models and clinical trials. TLR, toll-like receptor; RIG-I, retinoic acid-inducible gene 1; STING, stimulator of interferon genes; APOBEC, apolipoprotein B mRNA-editing enzyme catalytic subunit; PBMC, peripheral blood mononuclear cell; DC, dendritic cell; CIK, cytokine-induced killer; CAR-T, chimeric antigen receptor T-cell; TCR, T cell receptor. Dots in various colors indicate different cytokines.

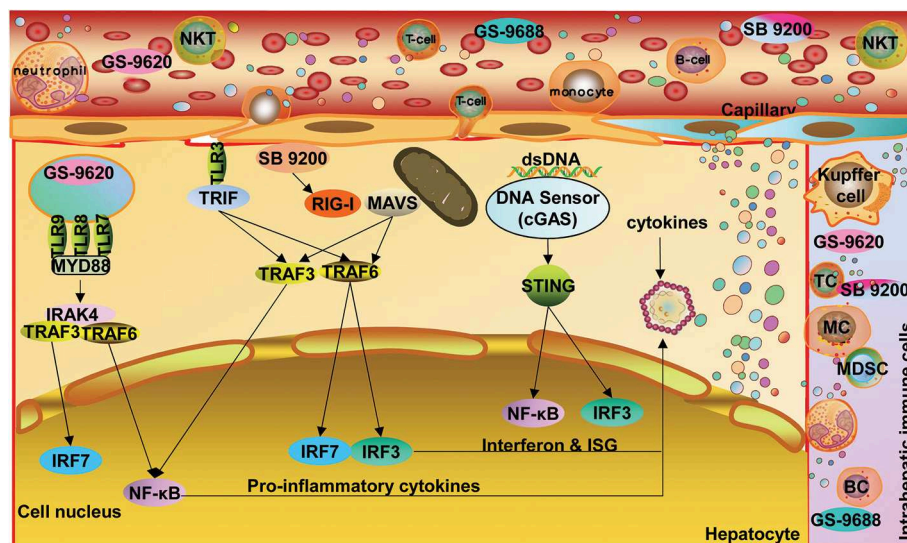


FIGURE 2 | Options for enhancing innate immunity and establish an antiviral state. Oral, intranasal, or subcutaneous application of agonists of PARS, including TLRs, RIG-I, and STING, activates host immune cells and hepatic parenchymal and non-parenchymal cells, leading to the production of IFN and proinflammatory cytokines as well as ISG expression. TLR, toll-like receptor; RIG-I, retinoic acid-inducible gene 1; STING, stimulator of interferon genes; NF- κ B, nuclear factor kappa-B; ISG, interferon-stimulated gene; cGAS, cyclic GMP-AMP synthetase. Dots in various colors indicate different cytokines. STING expression in hepatocytes remains controversial.

the past years. Though their potential usefulness is partly proven, many obstacles hindering the clinical use of these approaches are still to be overcome in the future.

ENHANCING INNATE IMMUNITY TO ESTABLISH AN ANTIVIRAL STATE: RESULTS FROM PRECLINICAL STUDIES

TLR Ligands

TLRs play an important role in the innate immune response through sensing viral and bacterial PAMPs and bridging to adaptive immunity. TLRs are widely expressed in

immune cells, hepatocytes, and non-parenchymal liver cells (NPCs), which contribute to immune control of HBV (32, 51, 52, 54, 55).

Isogawa and collaborators firstly demonstrated that single application of ligands specific to TLRs-3, 4, 5, 7, and 9 trigger non-cytopathic, IFN-dependent suppression of hepatic HBV replication in HBV transgenic mouse models within 24 h (56). These interesting results ignited hope in treating chronic HBV infection by activating TLR-dependent signaling pathways. Thereafter, various TLR ligands have been tested in cell and animal models with HBV replication, as reviewed previously (52, 57, 58). Direct application of TLR ligands can potently inhibit HBV replication in primary hepatocytes

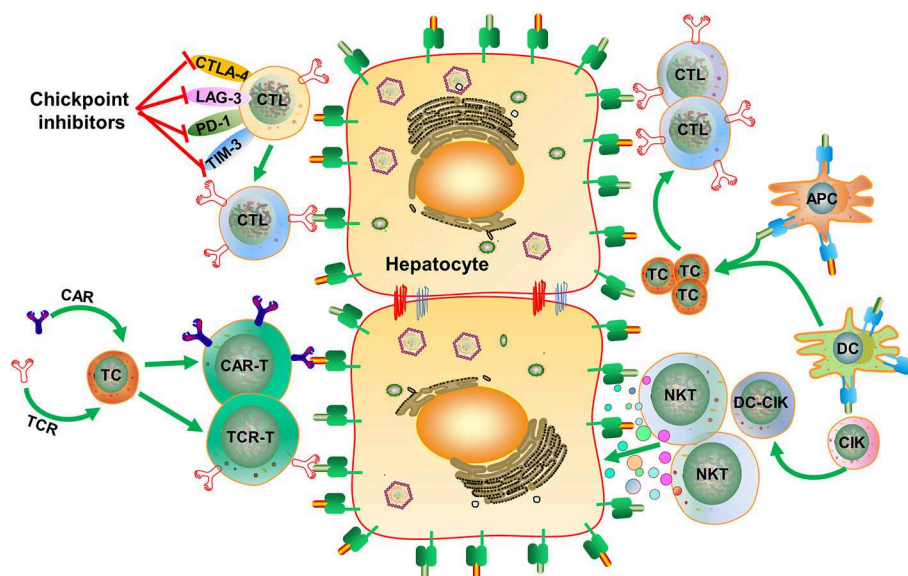


FIGURE 3 | Strategies for inducing HBV-specific immune responses and regaining immunological control of HBV infection. HBV-specific CD8⁺ T cell responses can be induced by therapeutic vaccines, boosted through checkpoint blockade, or renewed by adoptive transfer of *in vitro* activated T/NKT cells or genetically edited HBV-specific CAR-T or TCR-T cells. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; LAG-3, lymphocyte activation gene-3; PD-1, programmed death-1; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; APC, antigen-presenting cell; DC, dendritic cell; CIK, cytokine-induced killer; CAR-T, chimeric antigen receptor T-cell; TCR, T cell receptor. Dots in various colors indicate different cytokines; Hepatitis B surface antigen/epitopes; Hepatitis B core antigen/epitopes; Major Histocompatibility Complex class I molecule; Major Histocompatibility Complex class II molecule.

and hepatoma cells through IFN-dependent and -independent pathways. TLR-2 and -4 activation triggers IFN-independent pathways and leads to a robust inhibition of hepadnaviral replication by various intracellular pathways in hepatoma cells and woodchuck hepatocytes harboring woodchuck hepatitis virus (WHV) (57). On the other hand, stimulating NPCs (KCs and LSECs) and DCs with TLR ligands could induce a panel of antiviral mediators (e.g., type I IFN), which inhibit HBV replication *in vitro* (59, 60). Thus, TLR stimulation may not only activate resident parenchymal cells, NPCs, and infiltrated immune cells in the liver but also recruit circulating immune cells to establish an antiviral state (Figure 2). Recently, TLR stimulation has also been found to directly promote T cell functionality via metabolic regulation, adding to its capacity for immunomodulation (61–65). Meanwhile, systemic inflammatory responses after TLR stimulation should be taken into account.

In woodchucks chronically infected with WHV, weekly subcutaneous injection of CpG ODN for 16 weeks induces IFN synthesis with transient and weak viral inhibition (55). When combined with entecavir, potent inhibition of WHV was evidenced by early viral responses and a significant decrease in serum woodchuck hepatitis surface antigen (WHsAg). However, WHsAg seroconversion was not attained (55). Further data analysis suggested that CpG ODN application enhances viral suppression by antiviral treatment.

GS-9620, a TLR7 ligand, has shown great therapeutic potential in woodchuck and chimpanzee models (54, 57). In chronically WHV-infected woodchucks, GS-9620 monotherapy for 4–8

weeks led to continued, significant decrease of serum WHV DNA and sustained WHsAg loss after cessation of treatment in 13/15 animals. Moreover, 7 of the 13 animals developed an antibody response against WHV surface antigen (66). In chronically HBV-infected chimpanzees, reduced HBV viral load and serum HBsAg level were recorded but without HBsAg loss (67). A recent study showed that GS-9620 could induce multiple HBV suppressive factors in human PBMCs, leading to prolonged type I IFN-associated HBV suppression in primary human hepatocytes (PHH) and HepaRG cells, as well as enhanced biosynthesis of immunoproteasome subunits and display of an immunodominant viral peptide in PHH with HBV-infection. The latter may promote T cell recognition and activation in the host and viral control, though GS-9620 itself could not reduce cccDNA levels in hepatocyte culture systems (68).

RIG-I Activator

SB9200, an activator of RIG-I and nucleotide-binding oligomerization domain-containing protein 2 (NOD2), can induce prolonged IFN- α/β and ISG activation in blood/liver in WHV-infected woodchucks, leading to a 3.7 log₁₀ decrease of serum WHV DNA and an 1.6 log₁₀ decline in serum WHsAg when orally dosed with 30 mg/kg for 12 weeks (69). Interestingly, SB 9200 treatment sequentially followed by ETV administration induces a much more potent suppression of WHV, with a 6.4 log₁₀ decrease in serum WHV DNA load and a 3.3 log₁₀ decline in WHsAg level, delaying the recurrence of viral replication. Thus, SB 9200-induced host responses potentiate the antiviral efficacy of NUCs (70).

STING Activator

An alternative host factor, cyclic GMP-AMP synthetase (cGAS), was reported to be involved in HBV recognition. Indeed, cGAS can recognize HBV DNA and activate its adaptor protein—STING, leading to ISG56 expression and resulting in the suppression of viral assembly (71). Activation of the cGAS-STING pathway by dsDNA or cGAMP markedly inhibits HBV replication in cell and mouse models (72). An agonist of mouse STING, 5,6-dimethylxanthenone-4-acetic acid (DMXAA) significantly induces the expression of ISGs and reduces hepatic HBV DNA production in hydrodynamic HBV mouse models. DMXAA induces a type I IFN-dominated cytokine response, in contrast to TLR agonists which predominantly trigger inflammatory cytokine and chemokine responses (73). A recent study reported that human hepatocytes do not express STING (74). Nevertheless, treatment of HBV-infected hepatoma cells in culture with cGAMP or DMXAA leads to a significant inhibition of HBV replication, evidenced by concentration-dependent reductions in intracellular HBV mRNA, core-associated DNA, and secreted HBsAg, yet without apparent alteration in the amount of cccDNA (75). A splice isoform of MITA/STING, referred to as MITA-related protein (MRP), specifically blocks MITA-associated IFN activation while still inducing the NF- κ B pathway. MRP overexpression significantly inhibits HBV replication by activating the NF- κ B pathway in a hydrodynamic injection mouse model. MITA/STING deficiency (MITA/STING^{-/-}) enhances HBV replication in mice. Moreover, HBV-specific humoral and CD8⁺ T cell responses are reduced in MITA/STING-deficient animals, indicating an important role for MITA/STING in anti-HBV immunity in innate and adaptive responses (76). Altogether, STING might be a potential target for CHB immunotherapy.

APOBEC-Mediated Deamination

APOBEC-3 enzymes are involved in host innate immunity against HBV. It was firstly reported in 2005 that human APOBEC3 enzymes are able to extensively edit HBV DNA strands via cytidine deamination (77). In HBV-harboring HepAD38 and HepG2.2.15 cells, cytidine deaminases found endogenously could edit 10–25% of the HBV rcDNA genome within the viral capsid (78). Meanwhile, Hsp90 enhances APOBEC-3-mediated DNA deamination activity in HBV (79). Recent reports also indicated that APOBEC-3 enzymes may mediate the antiviral activity of type I IFN and lymphotoxin by cytidine deamination, leading to cccDNA degradation (80).

Preclinical studies revealed that triggering of innate immunity leads to the production of antiviral and inflammatory mediators, with viral suppression to various extents. However, activation of innate immunity alone presumably does not control HBV infection, unless the adaptive branch of host immunity subsequently comes into play. Among the tested agonists, only the TLR7 ligand GS-9620 has been tested in clinical trials (see below). Other candidate drugs are not yet ready for clinical testing. It will be useful to characterize such candidates not only for activating innate immunity but also for their ability to bridge to adaptive immunity, given that the specific immune response to HBV is critical for effective HBV control.

INDUCTION OF HBV-SPECIFIC IMMUNE RESPONSES

Protein/Polypeptide Vaccines

HBsAg/preS Vaccine

Conventional HBsAg vaccines failed to achieve a significant therapeutic effect in either preclinical animal models or patients with CHB. This failure was attributed to HBsAg-specific immune tolerance. Using IL-12 as an adjuvant, Zeng et al. showed that HBsAg immunization efficiently reverses systemic tolerance toward HBV proteins, with enhanced HBV-specific CD8⁺/CD4⁺ T cell responses and reduced CD4⁺Foxp3⁺ Treg cell frequency in HBV-harboring mice (81). The majority of animals administered IL-12-based vaccine acquired HBsAg seronegativity, and hepatitis B core antigen (HBcAg) became undetectable in hepatocytes. Meanwhile, preS1-polypeptide has been shown in HBV carrier mice to induce robust immune responses. Anti-preS1 antibody could clear HBV virions and even lead to HBsAg/HBsAb seroconversion through sequential administration of preS1 and HBsAg vaccines (82).

HBcAg Vaccine

Markedly elevated frequencies of HBcAg-specific CTLs have been found in CHB cases capable of controlling HBV replication in comparison with those who did not (83). Thus, HBcAg-based vaccines are considered a promising candidate for CHB treatment. In a pilot study, a peptide-based vaccine containing HBcAg amino acids 18–27 in combination with a Th epitope initiated a low-level CTL activity in CHB patients but failed to clear HBV (84). A synthetic HBcAg vaccine, originally designed to reduce the risk of liver tumors by Inovio Pharmaceuticals Inc., was also reported to be highly potent. However, only very limited information about this vaccine candidate is available.

HBsAg/HBcAg Compound Vaccines

Therapeutic vaccines comprising HBsAg and HBcAg and the CpG adjuvant have been shown to elicit strong HBsAg/HBcAg-specific humoral responses and balanced Th1/Th2 responses to HBsAg as well as Th1-type responses to HBcAg in wild-type C57BL/6 mice and HBV transgenic animals. Enhanced HBsAg/HBcAg-specific cellular immune responses lead to significantly reduced serum HBsAg levels without liver injury in HBV transgenic mice (85). A particulate vaccine composed of HBsAg, HBcAg, and the adjuvant ISCOMATRIXTM could induce multi-specific and multi-functional T cells in HBV-Tg mice, especially HBc-specific CD8⁺ T cells with elevated IFN- γ , TNF- α , and IL-2 production. Anti-HBsAg titers reached >10,000 IU/L in 7/8 animals after 4 vaccinations. However, titers of circulating HBV DNA decreased in vaccinated HBV-Tg mice after two and four vaccinations although statistical significance was not reached. HBcAg-positive hepatocytes were also dramatically decreased without obvious liver damage (86).

Anti-HBsAg Antibody

Antibody-mediated immunotherapy has been assessed in several preclinical and clinical studies but failed to achieve long-lasting HBV suppression. Zhang et al. developed a new monoclonal

antibody (mAb) against HBsAg (mAb E6F6) with remarkable effects in the treatment of persistent HBV replication in several mouse models (87). Indeed, a single dose of E6F6 markedly reduced HBsAg and HBV DNA amounts by over 3 logs for many weeks in HBV-transgenic animals. E6F6 could not only potently prevent primary HBV infection but also reduce secondary spread of HBV from infected hepatocytes in the human-liver-chimeric mouse model. After E6F6-based immunotherapy, anti-HBV T-cell response was restored in mice with persistent HBV replication established by hydrodynamic injection. Fcγ receptor-dependent phagocytosis is considered to play the most critical role in E6F6-associated viral immune clearance, independently on ADCC and CDC (88).

DNA Vaccines

DNA vaccines encoding HBsAg and HBcAg induce both humoral and cellular immunity against both HBV antigens, constituting a promising approach for the control of HBV infection (89–93). Upon intramuscular or intradermal injection, *in situ* expressed HBsAg and HBcAg in transfected cells such as myocytes and APCs are processed and presented to host immune cells, resulting in specific B and T cell activation (94, 95). Encouraging results were obtained in pre-clinical studies in the mouse and woodchuck models assessing diverse technologies to improve the efficacy of DNA vaccines, including (i) integration of immunostimulatory cytokines (96); (ii) combination with NUCs (97, 98); (iii) prime-boost immunization regimens (98, 99); (iv) electroporation delivery of DNA vaccines (93, 100); and (v) combination with checkpoint inhibition (93, 100).

In a recent study, Chuai et al. vaccinated rhesus macaques using a complex procedure. Four animals received three doses of HBV DNA vaccines encoding HBsAg, PreS1, and HBcAg for priming, followed by two boosts with recombinant vaccinia viral vectors encoding HBsAg, PreS1, and HBcAg, with a final boost using fusion protein including HBsAg and PreS1. Anti-PreS1 antibodies were induced quickly upon initial priming with DNA vaccination, followed by anti-HBsAg and anti-HBcAg antibodies. Upon boosting with recombinant vaccinia, both humoral and cellular immune responses to HBsAg, PreS1, and HBcAg were markedly induced, with HBcAg-specific CTL response being the most robust and durable. Further boosting with the fusion protein maintained the immune responses to all three HBV antigens until week 98 after the first vaccination. These results suggested that incorporation of PreS1 and HBcAg may improve the effects of therapeutic vaccines (101).

Vaccines Based on Viral Vectors

As mentioned in the previous section, Kosinska et al. tested therapeutic vaccines based on adenoviral vectors in the mouse and woodchuck models and obtained very promising results (98, 99). Moshkani et al. reported a vesicular stomatitis virus (VSV)-based vaccine platform (102). Using a highly attenuated VSV strain expressing MHBs by either intranasal or intramuscular application, they successfully induced MHB-specific CD8⁺ T cell and humoral responses in naive mice capable of preventing HBV replication after challenge by adeno-associated virus harboring HBV (AAV-HBV). In mice with persistent HBV replication, the

VSV-MHB system could also induce significant multi-specific T cell responses, leading to decreased serum and hepatic HBV antigen and DNA levels and transient elevation of serum alanine aminotransferase activity. These data provide evidence for the potential utility of vaccine platforms based on viral vectors as alternative therapeutic vaccines against CHB.

The design and effectiveness of HBV vaccines have been improved over the years. Clearly, HBV-specific T cells have been stimulated by these vaccine candidates in different experimental settings with variable levels of HBV suppression. The most effective vaccines are those based on viral vectors, which show superiority over other types in terms of T cell induction. Nevertheless, other types such as DNA vaccines could be applied repeatedly without limitation and used in combination with viral vectors. Currently, preclinical and clinical studies using the available vaccine candidates have been rather unfruitful, leaving the major question as to whether the currently available HBV vaccines are potent enough for immunotherapeutic approaches. The hurdles to be overcome likely lie in the recruitment of activated immune cells into the liver and the maintenance and amplification of primed HBV-specific immunity within the liver. Thus, combinations of antiviral treatment and additional immunomodulatory drugs including TLR ligands and checkpoint inhibitors may be necessary to achieve effective, long-lasting T cell immunity.

HBV-Specific T-Cell Therapy

Recently, useful technologies to induce or generate antigen-specific T cells were developed. This is a fast-growing research field with great potential for the treatment of chronic viral infections. HBV-specific T cells could be induced by DC vaccines, boosted by checkpoint blockade, or renewed by adoptive transfer of lab-produced HBV-specific T cells like CAR-T and TCR-T cells (103).

DC Vaccines

As the most powerful professional antigen-presenting cells, DCs play vital roles in bridging the innate immunity and the adaptive immunity. Meanwhile, myeloid DCs (mDCs) were found to regulate the functional differentiation of HBV-specific CD8⁺ T cells in immune transfer experiments (104). While PD-1 was identified to mediate functional exhaustion of HBV-specific CD8⁺ T cells, T cell functions could be restored by CD40 stimulation of mDCs. This has been validated from bench to bedside in CHB treatment using HBsAg/HBcAg-pulsed DCs. In HBV transgenic mouse models, HBsAg-pulsed DCs could induce HBsAg-specific immune responses. Interestingly, HBcAg-pulsed DCs induce both HBsAg- and HBcAg-specific T cell responses, leading to loss of HBsAg with anti-HBsAg seroconversion (105, 106).

CIK/DC-CIK

Cytokine-induced killer (CIK) cells, produced *ex vivo* via treatment of PBMCs or cord blood mononuclear cells with IFN-γ, anti-CD3 antibody, IL-1, and IL-2, are featured as cells with a mixed T- and NK cell-like phenotype (CD3⁺CD56⁺). CIK cells target infected and cancer cells in both MHC-restricted

and MHC-unrestricted manners, inducing rapid and unbiased immune reactions. Therefore, CIK cells attract attention as a potential therapeutic tool in malignancies and viral infections (107, 108).

DC-CIK refers to the co-culture of CIK cells with DCs or sequential adoptive transfusion of autologous DCs and CIKs, engaging the crosstalk between DCs and CIKs. DCs, especially antigen pulsed ones, can stimulate NK cells and initiate antigen-specific T- and B-cell responses (109). Increasing evidence shows that combination of DCs can reduce the frequencies of regulatory T cells in CIK cell cultures and increase the rate of CD3⁺CD56⁺ cells (110).

Immune Checkpoint Inhibitors

Blockade of immune checkpoints such as PD-1/PD-L1 signaling may relieve the negative regulation of specific T cells or even revive exhausted T cells. Application of antibodies targeting PD-L1 in woodchucks with WHV infection, combined with ETV administration and DNA immunization, successfully enhanced virus-specific T cells, resulting in continued inhibition of viral replication, production of anti-WHsAg antibodies, and complete viral clearance in certain woodchucks (100). A recent *ex vivo* study showed that HBV-specific CD4⁺ T cells isolated from individuals with HBeAg-negative HBV infection could be activated by OX40 stimulation combined with PD-L1 blockade, with remarkably increased IFN- γ and IL-21 production *in vitro*. Functional boost of HBV-specific CD4⁺ T cells via both treatment with OX40 and PD-1 pathway blockade might be useful in curing CHB (111).

Checkpoint inhibitors alone have shown only limited efficacy in chronic HCV patients (112). Virus-specific CD8⁺ T cells could be restored through PD-1 blockade in latent HBV carriers (113). Similarly, blockade of other checkpoint molecules such as Tim-3 and CTLA-4 can also restore virus-specific CD8⁺ T-cell responses in CHB patients (114, 115).

Genetically Edited T Cells (CAR/TCR-T)

Spontaneous HBsAg clearance is observed in more than 90% of adults with acute HBV infection accompanied by strong intrinsic or adaptive immune responses (116, 117). Patients with CHB lack effective T cell responses for viral clearance due to various immune tolerance mechanisms. Current T-cell-based therapy uses different types of engineered T cells, which express predefined antiviral characteristics. The basic principle of this process is the use of a new well-functioning T-cell library to replace or enhance the low or depleted energy T-cell library of the host and target the virus-specific immunodominant epitopes (118, 119). Therefore, this strategy may lead to immunological control of HBV infection in patients and CHB cure in long term. However, there are several points that need to be addressed for establishing its effectiveness and safety for CHB cure. First, strong T-cell-mediated killing may lead to severe liver damage and acute liver failure; secondly, it is necessary to demonstrate that engineered T cells indeed have improved functionalities and are able to remain functional under immune tolerizing conditions in the liver.

CAR-T cells can be engineered to identify antigens in an MHC non-dependent manner, providing a wider range of targets compared with natural T-cells. CAR-T cells show highly effective anti-tumor activities in a variety of tumors, including CD19⁺ acute leukemia, and may be developed into a safe and effective tumor treatment strategy (120). Bohne et al. firstly generated CAR-T cells targeting HBsAg. CAR-T cells directed against the “a” determinant of HBsAg and aa37-43 in the preS1 protein were able to recognize HBsAg-positive primary human hepatocytes and HepG2.2.15 cells, and specifically eliminate HBV-infected target cells (118).

A couple of studies have assessed the *in vivo* effects of CAR-T/TCR-T cells directed to HBV in mouse models (121–124). Kah et al. showed that HBV-TCR-T cells could lyse cultured HBV-harboring hepatoma cells, and viral loads were reduced within 12 days of treatment with three injections of HBV-TCR-T cells in HBV-infected human liver chimeric mice (122). Three reports about HBsAg-CAR-T cells are available. Krebs et al. showed that CD8⁺ T cells expressing HBsAg-specific CARs recognize different HBV subtypes and could be expanded in immunocompetent HBV transgenic mouse models, resulting in efficient control of HBV replication with only transient liver damage (121). Kruse et al. tested HBsAg-CAR T cells in HBV-infected human liver chimeric mice. As a result, an average of 4.7-fold reduction of serum HBsAg, 3.0-fold decrease in viral load, and 70% reduction of HBcAg-positive hepatocytes were found 36 days after adoptive transfer of HBsAg-CAR T cells (123). Meanwhile, human plasma albumin levels were unaltered, suggesting non-cytopathic viral clearance. Festag et al. engineered fully human, second-generation CAR T cells targeting HBsAg and tested them in an AAV-HBV mouse model with specific tolerance to human HBsAg-CAR. In this system, long-lasting antiviral effects were demonstrated with 2 log₁₀ decrease of HBsAg and 60% reduction of HBV-DNA for up to 110 days upon adoptive transfer. However, HBsAg-CAR T cells failed to completely clear HBV in the animals (124). Recently, HBV-specific T cells produced by lymphocytes using HBV-T cell receptor mRNA could reduce the viral load of HepG2.2.15 cells by 50% with no overt liver toxicity (125). Whether HBV-specific T cells could indeed suppress HBV efficiently while not directly killing hepatocytes requires further investigation. The approach with HBV-specific CAR/TCR-T cells represents a technology with great therapeutic potential.

Though DC-CIK based therapy has been applied in patients in China, its systematic analysis has not been performed. It is important to follow up patients administered DC-CIK treatment and determine the actual usefulness of this approach. The recent progress in T-cell-based therapies for tumor treatment is encouraging and provides therapeutic guidance for major chronic viral infections with HIV and HBV. Unlike in tumor treatment, the safety issue in antiviral therapy is significantly stricter and any risk of uncontrolled overshooting immune responses in patients is not acceptable. Similarly, the application of immune checkpoint inhibitors faces similar challenges and needs to balance the enhancement of host immune responses and the control of the risk of undesired immunopathology.

CURRENT CLINICAL TRIALS BASED ON IMMUNOTHERAPY

Many immunotherapeutic approaches have been tested in diverse animal models; however, only few of these attempts reached the phase of clinical trials (**Table 1**). It should be pointed out that combination therapy with potent antivirals and therapeutic DNA and viral-vector-based vaccines was partially successfully tested in the woodchuck model, with complete viral control and induced anti-surface antibody. Yet, similar approaches failed in chronically HBV-infected patients.

GS-4774

GS-4774 is a recombinant yeast-based vaccine that contains HBV-specific antigens such as HBx protein and large HBsAg (126). Its safety, tolerability, and immunogenicity have been verified in normal healthy individuals. However, GS-4774 showed no clinical benefit in virally suppressed individuals with CHB in a Phase II study. There was no significant decrease in mean HBsAg levels, with ≥ 0.5 log₁₀ IU/ml reductions in HBsAg in only three patients administered a high GS-4774 dose of 40 YU, and no case of HBsAg clearance. Five HBeAg-positive individuals administered GS-4774 had HBeAg loss, with none recorded among control patients (127).

Recently, the results of an open-label, multicenter, randomized study (<http://clinicaltrials.gov> no: NCT02174276) of CHB patients using tenofovir disoproxil fumarate (TDF) alone or combined with GS-4774 were published (128). Significantly increased IFN- γ , TNF, and IL2 production was evidenced in HBV-specific CD8⁺ T cells from individuals administered GS-4774 and TDF at weeks 24 and 48, but not in those under TDF monotherapy. Increased T-cell functions were correlated with reduced numbers of regulatory T cells. Again, GS-4774 treatment resulted in no reduction of HBsAg levels in patients. Thus, GS-4774 is able to stimulate host CD8⁺ T cell responses but not sufficient to control HBV.

NASVAC

The nasal vaccine candidate (NASVAC) is composed of HBsAg and HBcAg. In Phase I experiments, nasal spray with NASVAC was shown to induce anti-HBcAg antibodies in all subjects 30 days after administration of 3 doses. Seventy-five percent of the tested subjects developed anti-HBsAg antibodies at the latest time point of 90 days upon the start of vaccination (139). NASVAC was also well-tolerated after intramuscular application, with all the 14 enrolled patients developing HBV-specific lymphoproliferative responses. A total of 80 CHB patients were administered NASVAC intranasally or subcutaneously in a Phase III, randomized, controlled clinical trial. Compared with Peg-IFN treatment, NASVAC therapy resulted in a similar proportion of patients with viral load under the detection limit at the end of treatment (59.0 vs. 62.5%, $p > 0.05$). A higher percentage (57.7% vs. 35.0%) of patients had sustained HBV load under the detection limit at 24 weeks of follow-up (130). However, these results need to be verified in future clinical trials.

YIC

The HBsAg-hepatitis B immunoglobulin (HBIG) complex (YIC) has been tested in HBeAg-positive CHB patients in a Phase II clinical trial with six doses. Compared with the control group administered alum only, the patients vaccinated with YIC showed improved HBeAg seroconversion (9% in the control group vs. 21.8% in the YIC group), enhanced anti-HB production, and decreased viral load (140, 141). However, a Phase III clinical trial with 12 doses failed to show satisfying results (142). Indeed, the trial exploring the immunological mechanisms of YIC (clinical registration number: ChiCTR-TRC-11003189) showed that CHB patients immunized with YIC in combination with adefovir treatment exhibit increased CD4⁺ and CD8⁺ T cell responses. A significant increase in IFN- γ production and reduced expression of inhibitory factors including IL-10, TGF- β , and Foxp3 were detected in CD4⁺ T cells from individuals immunized with YIC (143).

HepTcell

HepTcell is a mixture of nine synthetic peptides comprising HBV-specific T-cell epitopes. In a Phase I trial performed in the United Kingdom and South Korea, HepTcell was tested in HBeAg-negative CHB cases treated with entecavir or TDF. Three monthly injections of HepTcell were well tolerated and induced cellular immune responses against HBV antigens in patients (131). A Phase II trial is anticipated to start in 2020 to evaluate the immune responses of HepTcell with more injections in an expanded patient cohort with chronic HBV infection.

DNA Vaccines

At the moment, a number of clinical studies on DNA-based HBV vaccines are still ongoing (**Table 1**). In an early French Phase I/II clinic trials, therapeutic DNA vaccines have been confirmed as safe in CHB patients (132, 133). However, satisfying results have not been obtained even in combination with NUCs, with only transient/weak T cell responses, increase in NK cells, and no sustained virological responses. In HBV carriers receiving lamivudine treatment, a DNA vaccine containing the majority of HBV genes in addition to IL-12 DNA could induce HBV-specific IFN- γ secreting T-cells, maintained for 40 weeks or more upon therapy and correlating with virological responses (144). A non-replicative adenoviral vector harboring HBsAg, HBcAg, and Polymerase (TG1050) was shown to induce strong HBV multi-specific and prolonged T-cell responses in the mouse model (145). A Phase Ib study was performed in CHB patients and demonstrated the safety and immunogenicity of TG1050, supporting future testing in combination with antivirals (146).

The Anti-PD-1 Antibody Nivolumab

In a Phase Ib study (ACTRN12615001133527), the anti-PD-1 antibody nivolumab was tested at a single dose in HBeAg-negative CHB cases with viral suppression, either alone or in combination with GS-4774. Reduced HBsAg levels were detected in all 22 individuals administered 0.3 mg/kg nivolumab alone or with GS-4774 at week 12. Interestingly, 2/10 patients in the GS-4774 + nivolumab group and 1/12 of the nivolumab monotherapy group had serum HBsAg reductions ≥ 0.5 log₁₀

TABLE 1 | Current clinical trials based on immunotherapy.

Therapeutics	Vaccine/agonist	Company/Organization	Status	References
GS4774	X, large-S	Gilead Sciences	I-IIIs	(126–128)
NASVAC	HBsAg+HBcAg	Clinical Research Organization, Dhaka, Bangladesh	III	(129, 130)
INO-1800	Plasmids encoding HBsAg + HBcAg	Inovio	I	No data available
HepTcell	T cell epitopes	Altimmune	I	(131)
DNA vaccine	preS2 +S	Institut National de la Santé Et de la Recherche Médicale, France French National Agency for Research on AIDS and Viral Hepatitis	I/II	(132, 133)
DNA vaccine	JNJ-64300535	Janssen Sciences Ireland UC	I	No data available
DNA vaccine-HB-110	S+preS+Core+Pol.	Genexine, Inc.	I	No data available
DNA vaccine	UN	PowderMed	I	No data available
DNA vaccine	HBs	Genexine, Inc.	NA	No data available
DNA vaccine	INO-1800+INO-9112	Inovio Pharmaceuticals	I	No data available
TG1050	ADV-S+C+Pol.	Transgene	I	No data available
ChAd155-hli-HBV	UN	GlaxoSmithKline	I	No data available
HPDC-T cells	HB-Vac	Sun Yat-Sen University	I/II	No data available
AIC649	Inactivated parapox virus	AiCuris	I	(134)
GS-9620	TLR-7 agonists	Gilead Sciences	II	(135, 136)
RO7020531, RG7795(ANA773), RG7854	TLR-7 agonists	Roche	I	No Results Posted
JNJ-64794964	TLR-7 agonists	Janssen	I	(137)
GS-9688	TLR-8 agonists	Gilead Sciences	II	No data available
SB 9200	RIG-I agonists	Spring Bank Pharmaceuticals	I	No data available
GS-9992	RIG-I agonists	Gilead Sciences	II	No data available
$\gamma\delta$ T Cells	–	Jinan University Guangzhou	I	No data available
Nivolumab	Anti-PD-1	Gilead Sciences	Ib	(138)

IU/ml at 24 weeks. A single individual with significantly decreased HBsAg levels in the nivolumab arm showed HBsAg loss at week 16, and anti-HBsAg responses at 10 weeks upon trial completion with anti-HB titers surpassing 500 IU/L 12 months after treatment (138).

Oral TLR-7/8 Agonists

There are currently several oral TLR-7/8 agonists in clinical trials, including GS-9620, RO7020531, RG7795 (ANA773), RG7854, JNJ-4964 (AL-034/TQ-A3334), and GS-9688 (Table 1). GS-9620 represents an effective, selective, and orally active TLR7 agonist. Its safety has been confirmed in treatment-naïve or currently treated individuals with chronic HBV infection. No marked circulatory IFN- α increase and associated symptoms were observed, although ≥ 2 -fold ISG15 increase was evidenced in serum samples from individuals administered 2- or 4-mg GS-9620 (136, 147). Twelve-week GS-9620 administration in CHB patients with HBV well suppressed by NUCs resulted in increased T- and NK-cell responses and decreased NK cell-mediated T cell inhibition, while no significant decrease in serum HBsAg levels was achieved. The beneficial effect of GS-9620 in strengthening HBV-specific immune responses needs to be validated with a longer assessment period or combination with IFN therapy (148). In another Phase II study, GS-9620 treatment of naïve CHB

patients, even after combination with tenofovir (TDF), did not significantly decrease HBsAg levels (135). Another TLR 7 agonist, RO7020531, is being assessed in a Phase I study.

AIC649

AIC649 is a patented inactivated parapox virus (iPPVO) and a novel biological immunomodulator. AIC649 could induce natural, self-limiting immune responses and boost immune responses to unrelated pathogens. A preclinical study in the woodchuck model demonstrated that AIC649 administration leads to a significant decrease in WHsAg even after cessation of treatment (149). Continuous WHsAg suppression as well as anti-WHsAg antibodies and cell-mediated immune responses were measured in combination with ETV (Poster AASLD 2017-10-24), indicating a potential for treatment of chronic HBV infection.

DC/DC-CIK

Clinical examination indicated that HBsAg-pulsed DCs induce anti-HBsAg antibody and HBsAg-specific cellular immunity in 2/5 and 1/5 CHB patients, respectively, without obvious liver damage. Anti-HB antibodies were detectable 1 month after administration of HBsAg-pulsed DCs and increased

progressively for 5 months in one patient (105). HBsAg-pulsed DC vaccines could accelerate HBsAg clearance as well as HBsAg/HBsAb seroconversion in patients with low HBsAg levels (Meng et al. unpublished data). Autologous DC-vaccines could efficiently inhibit HBV replication, decrease the viral load, clear HBeAg, and induce HBeAg/anti-HBe seroconversion, even leading to loss of HBsAg (150). However, randomized controlled studies are required for further validation of DC vaccines for treatment of CHB.

In a clinical study, over 2 log 10 fold decrease in HBV load was detected in 21/33 (63.6%) of CHB patients administered HBsAg activated autologous DC-CIK (151). CIK without HBsAg pulse could also reduce serum HBV load and promote HBeAg clearance in patients with high ALT levels. At 36 weeks of follow-up, HBeAg negativity and HBeAg seroconversion were found in 33.3 and 9.5% of CHB patients who received CIKs, respectively (152).

Yet, clinical trials based on immunotherapy did not show satisfactory results. The trials with DNA vaccines and YIC failed to deliver positive results, reducing the hope placed in immunotherapy. However, the rather negative results in these studies are not surprising as therapeutic immunomodulation is not simply the induction of adaptive immunity but needs to tackle negative immune regulation established during chronic viral infections. It is important to test these approaches in cohorts of patients under antiviral treatment and suppressed HBV replication. In such patients, the host immune system may recover to some extent from HBV-mediated impairment and respond more robustly to immune stimulation. There are patients with sustained low HBV loads, which may hint to naturally enhanced immune control of HBV infection. It would be useful to select such patients for future clinical trials. There are still a great number of options and combinations of these approaches to be considered, along with new innovations from future research.

IFN- α -BASED IMMUNOTHERAPY PLAYS AN IMPORTANT ROLE IN HBV “CURE” IN INDIVIDUALS WITH FUNCTIONAL INTRINSIC IMMUNE RESPONSES

PEG-IFN- α possesses antiviral and immunomodulatory effects, and remains the most effective drug for the treatment of CHB patients. In the treatment of naïve CHB patients, Peg-IFN- α administration for 48 weeks achieves superior efficacy over lamivudine, as reflected by HBeAg seroconversion, HBV DNA clearance, and HBsAg seroconversion. Although only 4% of HBsAg loss was reported at 6 months off-therapy, this rate reached 11% after 4 years of follow-up (153, 154).

PEG-IFN- α has been demonstrated to enhance HBsAg loss, especially in patients administered NUCs with HBsAg titers of <1,000 IU/ml. HBsAg clearance rates at 48 weeks were 9% (2 out of 22) and 15% (4 out of 26), in switch-to and add-on therapy CHB patients, respectively (155). A systematic review revealed that CHB patients treated with NUCs for at least 48 weeks are more likely to achieve

HBsAg loss (11%), using a PEG-IFN- α -based combination treatment (10). HBsAg loss occurred significantly often in selected CHB patients in a new SWITCH study with initial administration of NUCs and switch to PEG-IFN- α -2a (155). Another study demonstrated the effectiveness of PEG-IFN- α -2a for the treatment of inactive HBsAg carriers, resulting in high rates of HBsAg depletion and seroconversion (9). PEG-IFN- α treatment showed enhanced HBsAg seroconversion rate in CHB cases with low HBsAg and HBV DNA levels. Therefore, more clinical trials (e.g., NCT02745704, NCT02893124, and NCT02838810) are currently on the way to identify the optimal usage of Peg-IFN- α treatment in CHB patients with low HBsAg levels. Peg-IFN- α may contribute significantly to the cure of HBV infection if diversely integrated in multi-drug regimens, e.g., with lower dosage or intermittent application to avoid severe adverse effects.

Peg-IFN-lambda (Peg-IFN- λ), a type-III IFN, has been attributed dual immunomodulatory effects on both innate and adaptive immune responses in chronic HBV infection. IFN- λ shares similar ISG induction pathways as IFN- α , and Peg-IFN- λ exerts antiviral effects similar to those of Peg-IFN- α . Interestingly, Peg-IFN- λ showed substantially improved tolerability than Peg-IFN- α , since IFN- λ binds to type III interferon receptors, which are restricted to cells of epithelial origin, including hepatocytes (156, 157). Thus, the clinical application of Peg-IFN- λ may benefit CHB patients.

CONCLUSIONS AND FUTURE PERSPECTIVES

Chronic HBV infection is considered a result of HBV-specific immune tolerance. Based on this concept, breaking immune tolerance and restoring HBV-specific immune responses may ultimately lead to HBV control and clearance in patients. It becomes possible to induce HBV-specific immune responses in patients, yet not with the desired results of HBV control. Immunotherapeutic approaches are also hampered by the risk of overshooting immune responses in CHB patients and causing uncontrolled liver damage. Thus, combinations of potent antiviral treatment and carefully adjusted immune modulation may achieve a “cure” of CHB, without severe liver damage and disease progression. Nevertheless, HBV-specific CAR-T/TCR-T cells in combination with checkpoint inhibitors may be a potential strategy for HBV control.

In the past years, an essential role for HBV-specific T cell responses in viral control has been emphasized. Though functional T cell response is required for HBV control, it is definitely not sufficient for a successful immunotherapeutic approach. Given that the various therapeutic vaccines tested so far were highly effective in priming specific T and B cell responses to HBV antigens, they generally do not achieve significant and long-lasting viral suppression in animal models and patients. These rather disappointing results may have diverse reasons but the potential conceptual problem should not be ignored. Transfer of large numbers of activated CD8⁺ T cells to HBsAg in HBV Tg mice only led to transient suppression of HBV replication

(158). Transfer of splenocytes from HBsAg-vaccinated mice to HBsAg Tg mice resulted in sustained production of anti-HBsAg antibodies and HBsAg clearance in the peripheral blood of recipient mice (159). However, HBsAg-specific CD8⁺ T cells became undetectable, while HBsAg production in the liver continued in recipients. Importantly, no inflammation and T cell infiltration in the liver of Tg mice were observed. Further, HBV-specific T cells could be detected in mice with persistent HBV replication after hydrodynamic injection, but did not enter the liver unless an intrahepatic immune activation was triggered by TLR3 stimulation (160). A recent report also showed that HBV-specific T cells are detectable in the peripheral blood of young patients in the immune tolerant phase (103, 161). HBV-specific T cells were found to possess the ability to proliferate and produce cytokines (162). Specific CD8⁺ T cells in all mentioned cases apparently could not cause chronic liver inflammation, consistent with the findings reported by other immune transfer studies (163). Therefore, the antiviral effects of HBV-specific T cells may require appropriate conditions in the liver. TLR agonists may be useful for the promotion of T cell functions in the liver (160, 164, 165), by recruiting various immune cells into the liver to form tertiary lymphoid structures (166–168). These aspects have been discussed in recent reviews and need to be investigated in future studies (32, 52, 58, 92).

Beside T cell-mediated antiviral effects, other mechanisms for HBV control need to be considered in future approaches. The roles of other immune cell types are not yet well studied in the context of HBV immunity. NK cells may contribute significantly to HBV control during acute and chronic infection (22, 169, 170), playing an important role for successful IFN- α therapy (171, 172). At the moment, modulation of the NK cell activity to control HBV infection has only been tested in few studies and needs more attention (173). Recently, the function of HBV-specific B cells in HBV infection has been characterized by using fluorescently labeled HBV proteins, showing impairment in chronically infected patients (174–176). Modulation of B cell function and antibody production may represent another option for immunotherapy (87, 177). A number of host genetic determinants have been identified to contribute to HBV control and pathogenesis (178). Many of these determinants play a role in immune control of HBV infection. However, there are other factors such as UBE2L3 gene that regulate HBV replication by controlling cccDNA stability and yet unknown processes (179). By comparing hepatic gene expression profiles in patients

from different phases of the natural course of chronic HBV infection, a number of differently expressed host genes were found to contribute to HBV control in inactive carriers with low HBV loads (180). This result is somewhat relevant as no immune-related gene was actually found to be active in such patients, indicating that immune mechanisms may not be effective in patients with low HBV replication and gene expression. This is rational as antigen-specific T cells may target infected cells only with sufficient levels of antigen production and would ignore hepatocytes with HBV production below the recognition threshold. Apparently, there are diverse non-immune host restrictions for HBV replication that are important for achieving long-term, non-cytotoxic HBV control.

While we appreciate the great relevance of host immune responses for HBV control, successful therapy of chronic HBV infection may require combinations of antiviral treatment with NUCs, activation of intrahepatic innate immunity, stimulation of specific T cell responses, and finally switching on of non-immune mechanisms for sustained HBV control without undesired side effects. Recent findings pointed out the central role of cccDNA and/or integrated HBV DNA in hepatocytes for “functional cure” of chronic HBV infection. It is yet unknown whether HBsAg seroconversion/clearance alone would protect against HCC with cccDNA or integrated HBV DNA still present in hepatocytes. Likely, immunotherapeutic approaches may clear at least a great part of infected hepatocytes with cccDNA and/or integrated HBV DNA if they express HBV proteins, thereby reducing the risk of HCC development. These aspects deserve further investigation.

AUTHOR CONTRIBUTIONS

ZM searched the literature, performed review design, and wrote the manuscript. YC designed the figures and contributed to manuscript preparation. ML contributed to review conception and manuscript revision.

FUNDING

This study was partially funded by the Foundation for Innovative Research Groups of Natural Science Foundation of Hubei Province of China (2018CFA031), the National Key R&D Program of China (2017YFC0908104), and National Science and Technology Major Project (Grant Nos. 2018ZX10302206 and 2018ZX10723203).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Pathogenetic Mechanisms of T Cell Dysfunction in Chronic HBV Infection and Related Therapeutic Approaches

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OPEN ACCESS

Edited by:

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The Catholic University of Korea,
South Korea

Reviewed by:

Claudia Feriotti,
Queen's University Belfast,
United Kingdom
Kyong-Mi Chang,
University of Pennsylvania,
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equally to this work

Specialty section:

This article was submitted to
Viral Immunology,
a section of the journal
Frontiers in Immunology

Received: 12 November 2019

Accepted: 14 April 2020

Published: 12 May 2020

Citation:

Fisicaro P, Barili V, Rossi M, Montali I, Vecchi A, Acerbi G, Laccabue D, Zecca A, Penna A, Missale G, Ferrari C and Boni C (2020) Pathogenetic Mechanisms of T Cell Dysfunction in Chronic HBV Infection and Related Therapeutic Approaches. *Front. Immunol.* 11:849. doi: 10.3389/fimmu.2020.00849

A great effort of research has been devoted in the last few years to developing new anti-HBV therapies of finite duration that also provide effective sustained control of virus replication and antigen production. Among the potential therapeutic strategies, immune-modulation represents a promising option to cure HBV infection and the adaptive immune response is a rational target for novel therapeutic interventions, in consideration of the key role played by T cells in the control of virus infections. HBV-specific T cells are severely dysfunctional in chronic HBV infection as a result of several inhibitory mechanisms which are simultaneously active within the chronically inflamed liver. Indeed, the liver is a tolerogenic organ harboring different non-parenchymal cell populations which can serve as antigen presenting cells (APC) but are poorly efficient in effector T cell priming, with propensity to induce T cell tolerance rather than T cell activation, because of a poor expression of co-stimulatory molecules, up-regulation of the co-inhibitory ligands PD-L1 and PD-L2 upon IFN stimulation, and production of immune regulatory cytokines, such as IL10 and TGF- β . They include resident dendritic cells (DCs), comprising myeloid and plasmacytoid DCs, liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), hepatic stellate cells (HSCs) as well as the hepatocytes themselves. Additional regulatory mechanisms which contribute to T cell attrition in the chronically infected liver are the high levels of soluble mediators, such as arginase, indoleamine 2,3-dioxygenase (IDO) and suppressive cytokines, the up-regulation of inhibitory checkpoint receptor/ligand pairs, the expansion of regulatory cells, such as CD4+FOXP3+ Treg cells, myeloid-derived suppressor cells and NK cells. This review will deal with the interactions between immune cells and liver environment discussing the different mechanisms which contribute to T cell dysfunction in chronic hepatitis B, some of which are specifically activated in HBV infection and others which are instead common to chronic inflammatory liver diseases in general. Therapeutic interventions targeting dysregulated pathways and cellular functions will be also delineated.

Keywords: chronic HBV infection, T cell exhaustion, immune-therapy, liver environment, immunoregulatory mechanisms

INTRODUCTION

Chronic HBV infections remain a major public health problem worldwide (1). Currently, there are no curative treatments and available therapies are effective in inhibiting HBV replication, but are of limited efficacy on cccDNA and HBsAg concentrations, thereby requiring long-lasting administrations to avoid the risk of HBV reactivation at withdrawal (2–4). In the search for more effective therapies, possible candidates are compounds with direct anti-viral or immune modulatory activity. The latter strategy is supported by the evidence of dysfunctional innate and adaptive immune responses in chronic active hepatitis B (CHB), which contribute to HBV persistence (5, 6). Recent studies unveiled a number of altered regulatory mechanisms which are key for the impairment of anti-viral immune responses in chronic infections, involving different cellular populations of the immune system, suppressive soluble mediators, up-regulation of co-inhibitory molecules (7, 8). Most of these inhibitory mechanisms take place within the liver which is a tolerogenic organ that must prevent excessive immune responses against pathogens and antigens derived from the gut, to protect the host against severe immune-mediated damage (8). The tolerogenic properties of the liver are further enhanced by chronic inflammation which can trigger several regulatory mechanisms that make T cell exhaustion in HBV infection particularly severe, allowing HBV to acquire survival advantage over the immune system and to persist in the infected host.

This review will elucidate the relationship between adaptive anti-viral immune responses and the different virus- and host-related mechanisms particularly active within the chronically inflamed liver which favor HBV persistence. We will focus on: (i) T cell dysfunction, including up-regulation of co-inhibitory signaling pathways, metabolic alterations, apoptotic cell death and phenotypic/functional heterogeneity of HBV-specific T cells; (ii) the effect of the persistent exposure of immune cells to high antigen loads; (iii) the features of the liver environment, comprising tolerogenic antigen presenting cells (APC), suppressive soluble factors, local induction of suppressive regulatory cells; (iv) potential immune-therapeutic strategies based on functional T cell reconstitution.

T CELL EXHAUSTION IN HEPATITIS B

During chronic HBV infections virus-specific T-cells appear deeply exhausted (5). Both CD8 and CD4 T cells, up-regulate co-inhibitory receptors which can inhibit the T cell function upon cross linking of their corresponding ligands (9–16). Overexpression of such co-inhibitory molecules was originally described in exhausted T cells from LCMV-chronically infected mice (17, 18), although up-regulation of inhibitory receptors is known to play a physiological role in the contraction of effector acute phase responses to avoid excessive immune pathology and autoimmune disorders. Indeed, during acute, self-limited HBV infection activated functional effector T cells display high PD-1 levels, as expression of activation, which tend to decrease during the recovery phase (9, 19–22) (**Figure 1**). However, in the setting

of virus persistence, chronic antigen stimulation leads to the sustained expression of inhibitory receptors in association with T cell dysfunction (23). Liver-infiltrating HBV-specific T cells show maximal up-regulation of PD-1 and to a lesser extent of other co-inhibitory receptors, such as 2B4, LAG3, and CD160 (10, 11, 24) (**Figure 1**). On the other hand, overexpression of several checkpoint ligands, such as PD-L1 and galectin-9, has been observed on circulating and intrahepatic antigen-presenting cells and on liver resident Kupffer cells (KCs), respectively (14, 25, 26). Mechanistically, PD-1 engagement causes the de-phosphorylation of the costimulatory receptor CD28 and of other TCR-associated components, thereby leading to the attenuation of the corresponding signaling and to up-regulation of inhibitory genes (27–30). Moreover, PD-1 and CTLA-4 signalings also intervene in T cell metabolism, by inhibiting glycolysis (31).

Exhausted HBV-specific T cells have also been depicted as more prone to apoptosis, mediated by the up-regulation of the death receptor TRAIL-2 and the pro-apoptotic mediator BIM (32–34). They harbor dysfunctional mitochondria with an abnormally elevated ROS content, have a poor capacity to use oxidative phosphorylation but are instead strictly dependent on glycolysis to meet cell energy demands (35, 36) (**Figure 1**). The relevance of these metabolic defects to T cell exhaustion is confirmed by the effect of mitochondria-targeted anti-oxidant compounds in the reconstitution of the anti-viral T cell function *in vitro* (36).

As described for CD8 T cells in cancer and in other viral infections, also HBV-specific CD8 T cells from chronically infected patients are not a functionally homogeneous population of exhausted cells, because distinct T cell subsets with different degrees of dysfunction have been identified (37, 38). Moreover, different levels of exhaustion have been reported for T cell subsets of different HBV antigen specificity. Higher expression of exhaustion markers, associated with a lower expansion capacity has been reported for polymerase-specific compared to core-specific CD8 cells from chronic HBV patients with low viral load (39, 40). Such heterogeneity has been associated to variable levels of sensitivity to functional restoration treatments in other models of T cell exhaustion (41–46). Thus, analysis of CD8 T cell heterogeneity in individual chronic patients is worth being investigated as a possible tool to identify those patient populations that are more likely to respond to immune therapeutic interventions.

Inhibitory checkpoint blockade has been widely studied as a strategy for immune reconstitution in chronic HBV infection. Many *in vitro* studies showed that PD-1/PD-L1 blockade, alone or in combination with the manipulations of other pathways, can induce variable levels of improvement of both T and B cell responses, because high PD-1 levels have also been detected in dysfunctional HBV-specific B cells from chronic HBV patients (9–12, 14, 15, 24, 47–50). A reduction of the pro-apoptotic Bim molecule expression and an increase in cytokine-producing CD8 T cells have been observed upon CTLA-4 blockade (15) and manipulation of the 2B4 and Tim-3 pathways as well (14, 16). All different checkpoint modulation approaches, however, are not free of toxicities or immune-related adverse events, as reported in cancer patients (51–54). Moreover, additional limitations to

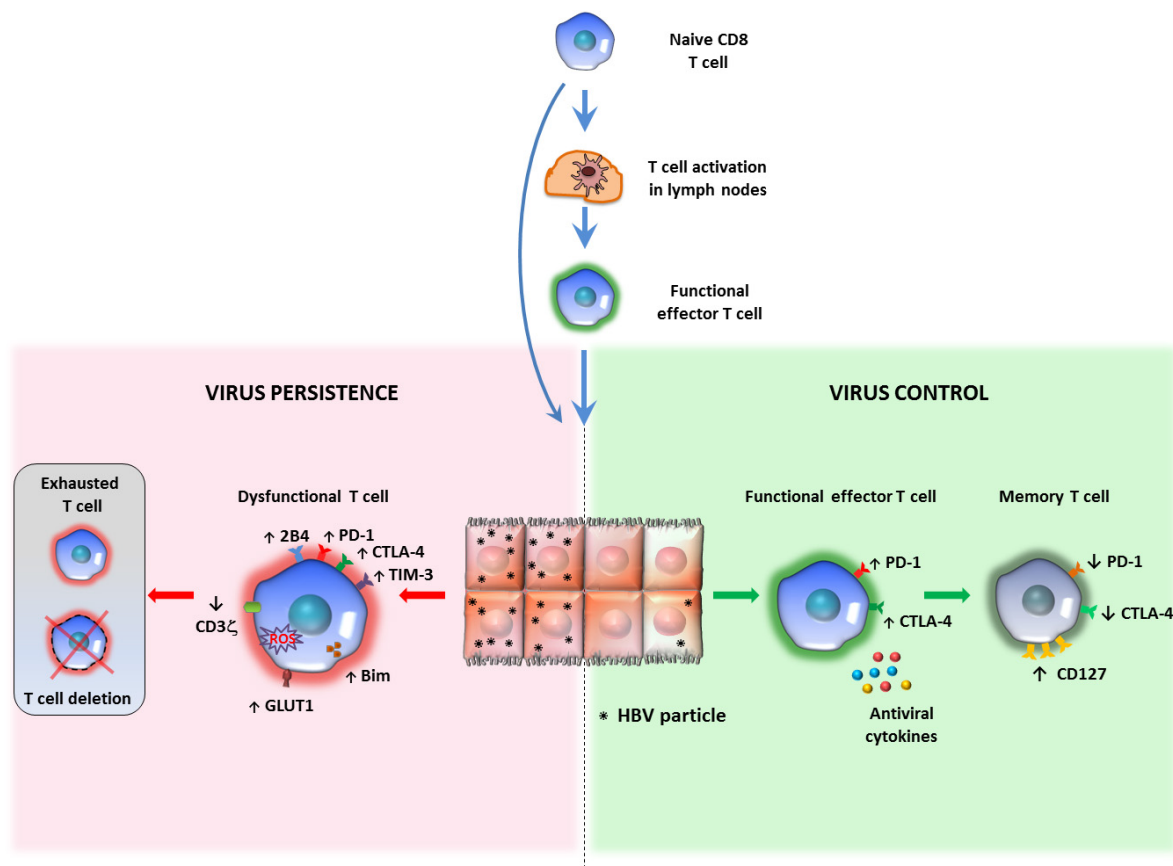


FIGURE 1 | Relationship between antigen persistence and anti-viral T cell responses in the liver. Priming of naïve CD8 T cells can occur in lymph nodes (LNs) or within the liver. In the lymph nodes naïve CD8 T cells differentiate into functional effectors; after migration to the liver, if the majority of hepatocytes are infected and express high antigen levels, virus-specific CD8 T cells undergo functional impairment or physical deletion. Some specific features of exhausted HBV-specific CD8 T cells are the over-expression of multiple inhibitory receptors and pro-apoptotic molecules, CD3 ζ chain down-regulation and various metabolic alterations (*left panel*). If only a minor proportion of liver cells express limited amounts of viral antigens, virus specific CD8 cells can maintain efficient anti-viral activity and can promote virus control and antigen clearance (*right panel*). This model of T cell activation derives from studies performed in mouse models of HBV infection, but no definitive evidence is available in human infection to confirm that induction of functionally efficient effector CD8 T cells is driven by the amount of antigen (number of infected hepatocytes and amount of antigen expressed by individual liver cells) and that decline of antigen can allow restoration of functionally efficient HBV-specific CD8 T cell responses.

the *in vivo* use of checkpoint inhibitors is the wide heterogeneity of T cell responses reported *in vitro* to this treatment (11), and the lack of simple predictors to identify with some level of accuracy those patients who could benefit from PD-1 blockade either alone or in association with other costimulatory (for example, CD137 or OX40 stimulation) (10, 48), or co-inhibitory (CTLA-4 or TIM-3) (14, 15), pathway manipulation. The *in vitro* study of HBV-specific T cell functionality cannot be widely used to predict response to therapeutic immune modulation *in vivo* because of its complexity and the need of a better standardization of functional assays. A much simpler possibility, which is being explored in different laboratories, consists in the use of phenotypic panels, including exhaustion and memory molecules, to study total, unfractionated T cells, in view of data indicating that exhaustion can partially affect also the overall CD4 and CD8 T cell populations. In this regard, the downregulation of CD3 ζ and CD28 has been associated to functional defects

in total non-antigen-specific CD8 T cells *in vitro* and *in vivo* in CHB patients with high viral load (55). Besides, a more recent investigation confirmed the presence of both HBV-specific and global T cell dysfunction mediated by multiple regulatory mechanisms, including overexpression of PD-1 and CTLA-4 by CD4 T cells (47). Moreover, additional studies have highlighted also the unconventional $\gamma\delta$ T cell role in HBV pathogenesis both in acutely infected chimpanzees (56) and in acute and chronic patients (57). Despite some controversial findings (58–61), a recent report described innate-like phenotypes based on the expression of Tbet/Eomes, and reduced PD-1, in association with a functional alteration in circulating V δ 2 + $\gamma\delta$ T cells during ALT flares in CHB patients (57).

In spite of the multifaceted nature of the immune dysfunction, an improvement of the virus-specific immunity has been detected in nucleoside analog (NUC)-treated chronically infected woodchucks upon the association of PD-L1 blockade with

therapeutic DNA vaccination, leading to suppression of viral replication and anti-WHs antibody seroconversion in two out of three animals (62). In another study, a durable control of viremia and antigenemia was induced in 2 of 11 WHV-infected woodchucks when anti-PD-L1 was associated to NUC treatment (63).

In a recent study in human HBV infection a single dose anti-PD-1 (Nivolumab) was administered to NUC treated, virally suppressed HBeAg negative chronic HBV patients who were compared to NUC treated patients who received anti-PD-1 plus therapeutic vaccination. Reduction of HBsAg titers was detected only in a limited proportion of patients who received NUC combined with anti-PD-1, with a total and persistent HBsAg loss in only one of them. Remarkably, no severe adverse events were reported, but, disappointingly, no additional effect was observed on serum HBsAg concentrations as well as on strength and quality of anti-viral T cell responses in the cohort of patients treated also with therapeutic vaccination (64). Low dose and short time administration of anti-PD-1 in this study don't allow, however, to draw definitive conclusions about possible future applications of this potential treatment.

In summary, *in vitro* data support the concept that only a proportion of patients should partially benefit from check-point blockade because only a limited percentage of patients respond *in vitro* and very rarely functional restoration involves simultaneously all important anti-viral cell-mediated immunological parameters. Moreover, in view of the multi-factorial nature of T cell exhaustion pathogenesis in HBV infection, the possibility to reconstitute T cell functionality with a single intervention selectively focused on immune check-points seems to be highly unlikely.

This conclusion is further reinforced by the recent finding that PD-1 blockade doesn't allow complete correction of the specific epigenetic profiles which are associated with CD8 T cell exhaustion (65, 66). This finding gives theoretical supports to the concept that inhibition of the PD-1 pathway cannot allow persistent and complete correction of T cell dysfunction in chronic viral infections. Based on our present understanding of HBV-specific T cell exhaustion, PD-1/PD-L1 blockade should be likely seen as a possible adjuvant therapy to be used only in a selected group of HBV infected patients to improve T cell function and antigen responsiveness before the use of more specific HBV antigen-based therapies, such as vaccination with an appropriate antigenic composition. Moreover, the lack of an epigenetic effect provides the rationale for combining checkpoint blockade with epigenetic drugs, a therapeutic strategy already tested in the setting of anti-tumor immunotherapy.

PERSISTENT T CELL EXPOSURE TO HIGH VIRAL ANTIGEN CONCENTRATIONS

The amount of antigen expressed by liver cells is believed to influence the fate of effector CD8 cells but available data do not allow to draw definitive conclusions on this issue.

In vitro studies have addressed the interplay between infected hepatocytes and anti-viral T cells, showing the strengthening of the T cell function as antigen expression increases, suggesting that high viral antigen production is needed for efficient T-cell activation within the liver (67). On the same line, an antigen dose-dependent anti-viral T cell function increment has been observed also in an infectious HCV-hepatoma cell co-culture model, in which a cognate epitope expression threshold has been investigated, indicating a peptide concentration range for effector T cell activation (68). Also the effector:target ratio has been shown to modulate the HCV-specific T cell function in an HCV replicon system, with non-cytolytic mechanisms prevailing at lower ratio values (69). However, as *in vivo* high antigen loads are associated to co-inhibitory receptor/ligand overexpression, when PD-L1 expressing hepatoma cells were used, recapitulating more closely the liver environment, the T cell cytolytic activity resulted significantly inhibited (68). Indeed, experiments performed in animal models, evidenced a more complex T cell regulation in the liver environment.

By exploiting a recombinant adeno-associated viral vector system (rAAV8 transduction) to obtain selective antigen expression in mouse hepatocytes *in vivo*, a threshold of antigen in the liver was identified by the Bertolino's group as a crucial factor tuning T cell differentiation. In this model, persistent cytotoxic T lymphocyte (CTL) function was maintained only when less than 25% of hepatocytes were transduced, indicating that low frequencies of antigen-expressing hepatocytes can elicit a functional CD8 T cell response (70, 71). Conversely, when antigen was expressed by a high percentage of hepatocytes, virus-specific CD8 T cells became less responsive and over time underwent T-cell exhaustion and deletion. The phenotypic analysis of intrahepatic T cells isolated from mice treated with high doses of rAAV revealed that T cell exhaustion was associated with high levels of the inhibitory PD-1 and Tim-3 receptors as well as with a deficiency in cytotoxic activity and anti-viral cytokine production (71). Therefore, these data suggest that the level of antigen expression and the proportion of infected hepatocytes represent key factors in driving the development of adaptive T cell responses in chronic viral hepatitis.

These results are consistent with previous findings in a transgenic mouse model where intrahepatic antigen presentation triggered negative regulatory signals leading to a dysfunctional differentiation of naïve CD8⁺ T lymphocytes. Indeed, naïve HBV-specific CD8 T cells adoptively transferred into transgenic mice with high intrahepatic HBV antigen expression displayed proliferative responses but lacked the capacity to differentiate into functional effector T cells. The mechanism of CD8 T cell dysfunction involved PD-1 signaling and could be rescued by CD40-dependent mDC activation (72, 73).

More recently, intrahepatic T cell differentiation was studied in a HBV transgenic mouse system in which T cell priming was restricted to the liver. In this model, naïve CD8⁺ TCR transgenic T cells specific for HBV core were injected into major urinary protein (MUP)-core transgenic mice which exclusively expressed a non-secretable version of the HBV core protein in 100% of hepatocytes. Naïve T cells primed in the presence

of high levels of HBV core antigen expressed proliferative function but failed to differentiate into functionally competent effector cells. Remarkably, even when CD8 T cell priming occurred within a liver environment where HBV core antigen expression, induced by injection with a low dose of a hepatotropic adeno-associated viral vector (AAV) encoding the HBV core protein, was limited to less than 5% of hepatocytes, effector differentiation was not supported despite much lower levels of antigen expression (74). These different T cell differentiation fates observed in different experimental models may likely be related not only to variable thresholds of antigen expression within the liver in different infection/transfection models, but may also depend upon TCR affinity which may restrict effector priming to high affinity TCR/MHC interactions. The evidence that a substantial reduction of hepatic antigen expression by more than 15-fold in individual hepatocytes was still insufficient to induce a functionally efficient effector CD8 T cell differentiation, force some caution in predicting what antigen decline induced by therapy can actually do on the T cell function in chronic hepatitis patients. Future experiments in chronic patients treated with anti-viral drugs able to diminish the antigen load are mandatory to address this fundamental issue because no evidence is available at present in natural HBV infection of whether decline of antigen can actually cause functional T cell improvement, and, if so, which magnitude of antigen decline is required for induction of a biological effect. This is particularly puzzling in a clinical setting where multiple factors simultaneously contribute to T cell exhaustion and where individual patients harbor a widely heterogeneous HBV-specific CD8 T cell population comprising cells with different levels of functional impairment and different degrees of affinity for target cell recognition.

In another HBV-transgenic (tg) mouse model of therapeutic vaccination, based on a heterologous vaccination strategy with initial protein priming followed by recombinant Modified Vaccinia Ankara virus (MVA) vector-boost, serum HBV antigen levels have been reported to influence the immunological responsiveness to therapeutic vaccination. Indeed, vaccine-induced HBV-specific CD8⁺ T cell responses inversely correlated with antigenemia levels before vaccination. In mice with high antigen levels both HBsAg/MVA-S and HBcAg/MVA-core immunization failed to induce envelope and core-specific CD8 T cell responses in the spleen and in the liver, while vaccination in mice with low or intermediate antigen levels allowed expansion of anti-viral T cell responses and control of infection (75). Interestingly, HBV-specific CD8 T-cells were induced efficiently by therapeutic vaccination after RNAi-mediated suppression of hepatic antigen expression in highly antigenemic mice which were non-responsive to antigen stimulation before anti-viral therapy (76). Although very promising, translation of this therapeutic approach to the human natural infection may be not so easy because antigenemia in chronic patients is generally much higher and of longer duration than in this mouse model and because exposure to high antigen loads is only one of the multiple mechanisms of T cell inhibition which are simultaneously in place after decades of infection in chronic hepatitis patients.

In the setting of natural human HBV infection, a hierarchy of T cell functional efficiency was described in different conditions of HBV control in relation to serum HBsAg concentration (77). For example, maximal T cell functional efficiency was observed in the resolution phase of an acute self-limited hepatitis, associated with complete control of infection and lack of HBsAg in the serum, followed by intermediate levels of T cell functionality in chronic inactive carriers with partial control of infection and low levels of HBsAg, and finally maximal impairment of T cell responses in chronic active hepatitis patients with high levels of viremia and antigenemia (77).

Although level of antigen and duration of T cell exposure to antigenic epitopes certainly affect T cell functionality and responsiveness to exogenous stimulation, we must consider, however, that the level of functional T cell efficiency is the final result of the interplay between T cells and a wide range of inhibitory mechanisms (Figures 1, 2). Thus, the presence of a high antigen load in chronically infected hosts certainly represents an important obstacle for curative immunotherapeutic approaches but we must be aware that decline of antigen alone is unlikely to be sufficient for successful recovery of protective immune responses because multiple mechanisms contribute simultaneously to T cell exhaustion in chronic HBV infection (78).

THE LIVER ENVIRONMENT

A number of inhibitory mechanisms are active within the liver making the intrahepatic environment highly tolerogenic. The unique composition of the hepatic cell population, the expression of inhibitory checkpoint ligands and the presence of soluble regulatory mediators are some of the factors which contribute to the tolerogenic nature of the liver, which is maximal in the presence of chronic inflammation, irrespective of the etiology. Therefore, most of the mechanisms which will be described in this section are common to different chronic inflammatory liver diseases and are not specifically expressed only in chronic HBV infection (Figure 2).

Tolerogenic APC

Liver APC are not functionally mature as their counterparts in other organs (79). Hepatic dendritic cells (HDCs) and liver resident macrophages, known as KCs, which represent conventional APC, but also non-professional, unconventional APC, such as liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), as well as the hepatocytes themselves, contribute to immune tolerance, because they differ significantly from cells present in the circulation or in secondary lymphoid organs (80).

Hepatic dendritic cells show an immature phenotype, with a lower expression of MHC and costimulatory molecules (i.e., CD40, CD86, and CD80), poor endocytotic capacity and low IL-12 production, compared to their peripheral counterparts (81, 82). As reported for HDCs, low level expression of costimulatory and MHC class I and II molecules at the steady state makes also KCs, LSECs, and HSCs poorly efficient in

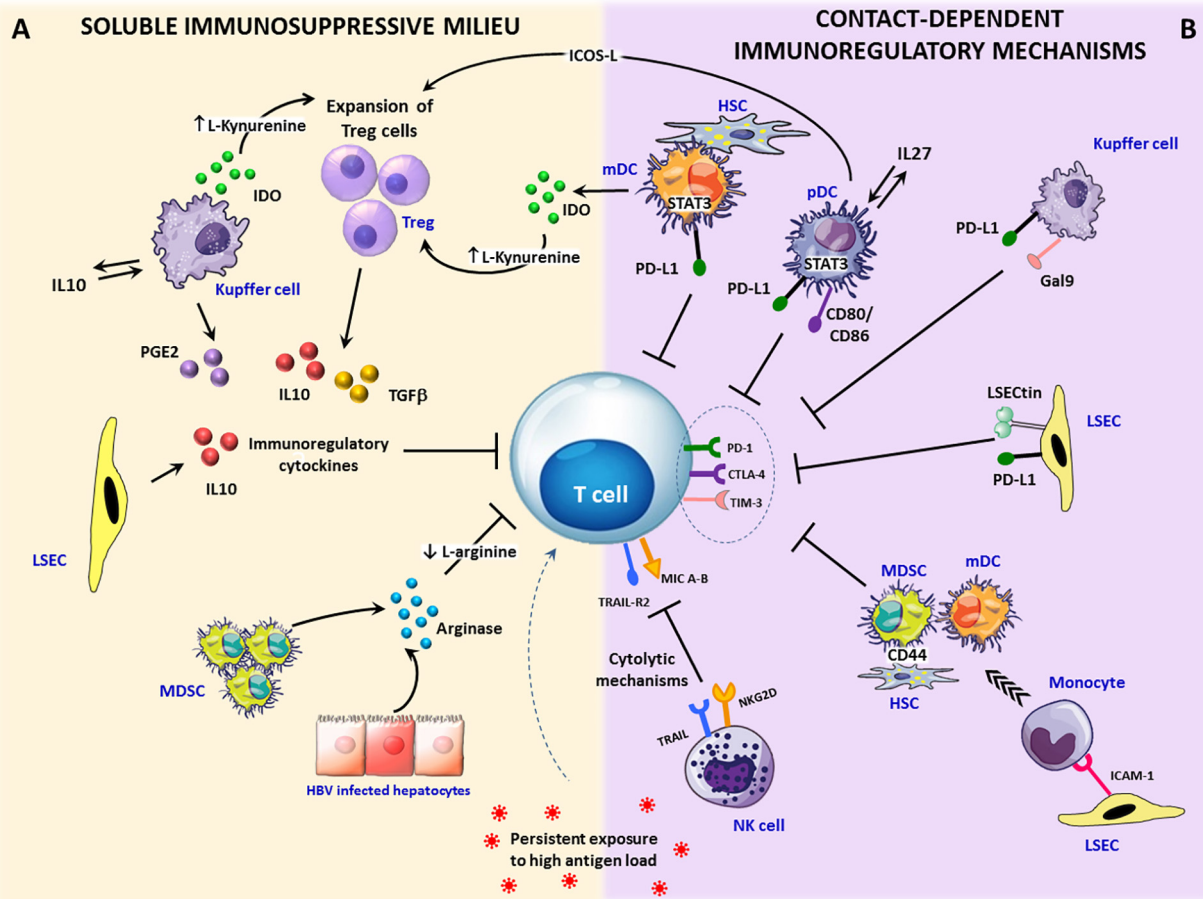


FIGURE 2 | Immunosuppressive circuits in the liver. Within the infected liver, persistent expression of viral antigens in concert with different immunoregulatory pathways drive T cell differentiation toward exhaustion. Upon inflammation, IFN- γ stimulation activates immunosuppressive mechanisms that can be mediated by soluble factors or require cell to cell contact. **(A)** Soluble factors: immunosuppressive cytokines, such as TGF- β and IL-10 can be produced by expanded regulatory T cells (Treg) as well as by stellate cells (HSCs), dendritic cells (DCs) and Kupffer cells (KCs). In addition, a number of liver infiltrating cells, such as monocytes, macrophages and dendritic cells, can release the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO), which causes either tryptophan depletion with consequent dampening of T cell proliferation and function or the generation of toxic catabolites, such as kynurenines, which can cause T cell apoptosis and CD4 differentiation in Treg cells. Another key soluble mediator is represented by arginase, released by damaged hepatocytes and myeloid-derived suppressor cells (MDSC), inducing arginine depletion. Lack of arginine determines CD3 ζ downregulation and suppression of T cells proliferation. Moreover, the enzyme cyclooxygenase-2 produced by activated KCs participates in the synthesis of the immunosuppressive prostaglandin E2 (PGE-2). **(B)** Contact-dependent mechanisms: antigen-specific CD8 T cells can be killed by NK cells through NKG2D- and TRAIL-dependent lysis. Over-expression of inhibitory ligands (e.g., PD-L1, Galectin-9) on liver endothelial sinusoidal cells (LSECs), dendritic cells, KCs, and stellate cells facilitates the triggering of T cell inhibitory pathways. In addition, inflammatory monocytes recruited into the liver through ICAM-1 expression on LSECs can differentiate into myeloid-derived suppressor cells by a CD44-dependent mechanism driven by activated stellate cells. Finally, Treg cell expansion and IL-10 production can be caused by pDCs and mDCs via the ICOS/ICOSL-mediated interaction or IDO secretion.

T cell stimulation (83–88). They can also induce CD8 T cell apoptosis by Fas-FasL interaction, as described for KCs following reactive-oxygen species (ROS) production induced by FasL up-regulation on their surface (80, 89), and for HSCs through induction of intracellular signaling pathways triggered by PD-L1 and B7-H4 crosslinking (90–93). Hepatocyte priming of CD8 T cells generally results either in clonal T cell deletion by BIM-mediated apoptosis (90, 94), or in dysfunctional CD8 T cells without effector functions (74), as already discussed in detail in the previous section. HSCs and LSECs can also inhibit CD8 T cell priming/activation through a TRAIL and ICAM-dependent

mechanism or through LSECtin-CD44 interaction, respectively (95). Moreover, antigen presentation by all these cells is often associated to the secretion of immune-regulatory mediators, such as IL-10, TGF- β , prostaglandin E2 (PGE2), and to the up-regulation of PD-L1 (87, 96–100).

High co-inhibitory molecule expression by HDCs can be induced by the nucleotide-binding oligomerization domain 2 (NOD2) signaling, significantly more expressed in hepatic plasmacytoid (pDCs) than in conventional or myeloid (mDCs) dendritic cells, which is also responsible for their low IFN- α secretion (101). The CD141 $^{+}$ subset of mDCs, which produces

IL-12 and plays a central role in antigen cross-presentation (102), have also been reported in healthy human livers to express high levels of the tolerogenic molecules immunoglobulin-like transcript 3 (ILT3) and ILT4 (103), which can inhibit T cell activation through tyrosine-based inhibitory motifs (ITIMs) contained in their cytoplasmic tails (104, 105).

In chronic HBV patients hepatic and peripheral dendritic cells have been reported by several studies to be functionally impaired, with a possible inhibitory effect of viral antigens on their function (106–112). However, this concept is still widely debated (113) because other studies did not observe any significant alterations of the DC function (114, 115).

Among the possible DC defects in chronic hepatitis B, a reduced production of the immunoregulatory cytokine osteopontin (OPN) has been described and suggested to represent a potential cause of TH1 response impairment (116). Impaired DCs maturation and function in chronically HBV-infected patients has also been associated with an altered expression of innate sensors, such as TLRs (117–120).

Although most studies on DCs in chronic HBV infection have been performed on peripheral cells, a recent report shows alterations in frequency and basal activation status of DC subsets from both blood and liver of chronic HBV patients compared to healthy controls. In addition, also a defective up-regulation of maturation markers upon TLRs triggering was reported in this study, but only circulating DCs displayed a significant impairment of cytokine production in response to TLR agonist stimulation (118). The poor effect on the NK cell cytolytic function by TLR9 ligand stimulation of HDCs was related to reduced OX40L expression as well as to high plasma IP-10 and HBV antigen levels (121). Mechanistically, HBV particle internalization can block pDCs IFN α production by inhibiting TLR9 signaling, through down-regulation of TLR9 transcription (122), which can also be indirectly caused by TNF α and IL-10 production induced by HBsAg on monocytes (123).

Also KCs have a role in the induction of tolerance to HBV. This is supported by the observation in a mouse model of HBV infection that earlier HBV clearance in older mice was accompanied by a severe reduction in KC numbers, concomitant with a sharp induction of TNF α -producing Ly6C⁺ monocytes, followed by the proliferation of IFN γ +TNF α + CD8 T cells. Instead, younger mice, which failed to clear the virus, maintained high frequencies of IL-10-secreting KCs (124).

Functional skewing of the T cell response toward a TH2 profile can also be supported by macrophages with a predominant M2-like phenotype, known to be potent immune suppressor cells (125), detected at high frequencies in a humanized mouse model of persistent HBV infection (126). The evidence that KCs and liver infiltrating macrophages show up-regulation of the CD86 co-stimulatory molecule, which is known to drive T cell differentiation toward a functional TH2 profile (127), detected by immunohistochemistry staining of liver biopsies from chronic HBV patients, further support this finding (128). A direct interaction between HBsAg and KCs with HBsAg uptake, was documented both *in vitro* and *in vivo* isolated KCs from CHB patients (129). *In vitro* exposure to HBV antigens preferably induced TGF- β , rather than pro-inflammatory cytokine secretion

by primary rat KCs (130); moreover, HBV antigen interaction with TLR2 on KCs caused T cell inhibition through IL-10 secretion (131, 132) and TLR2 knockout or KC depletion resulted in enhanced HBV elimination and improved CD8⁺ T cell responses in mice (131). This inhibitory effect mediated by HBV proteins was further documented by more recent studies showing that HBV protein uptake by intrahepatic macrophages from CHB patients can favor anti-inflammatory over pro-inflammatory functions and can ultimately promote hepatocyte infection (133).

Despite these tolerogenic features, the liver environment must ensure a fine tuning of divergent functions to guarantee tolerance to antigens introduced through the gut, but also to initiate efficient immune responses needed for pathogen control. For example, in case of liver inflammation KCs secrete pro-inflammatory cytokines and can mediate full CD8 T cell differentiation (83, 134), while the propensity of hepatic DCs toward immunogenicity or tolerance has been associated to their lipid content and metabolism. Indeed, high lipid content can make HDCs able to secrete high levels of pro-inflammatory cytokines and to efficiently activate T or NK cells, while low lipid content is associated with tolerance induction (135).

Overall, many pieces of evidence indicate a preferential anti-inflammatory response induction by antigen-presenting cells in chronic HBV infection, suggesting the possibility of a direct innate immunity inhibition by the virus itself (119), which has also been reported to be poorly sensed by innate immunity sensors, behaving as a stealth virus (136). Interference of viral proteins with innate signaling pathways (119) represents another theoretical reason why reduction of antigenemia may be relevant in the perspective of therapeutic strategies for CHB based on functional immune reconstitution. In addition to novel direct anti-viral agents acting at different levels of the HBV life cycle (see below in the “Perspectives for novel therapeutic strategies” section), stimulation of pathogen recognition receptors, such as TLRs and Retinoic acid-Inducible Gene I (RIG)-like receptors (137) represents a possible strategy, currently under evaluation, to increase the efficiency of anti-viral immune activation.

Dendritic cells manipulation has also been proposed. Indeed, administration of HBV peptide-loaded DCs was reported to elicit functional HBV-specific CD8 cells and to reduce the viral load in Hepato-HuPBL mice (138) and a targeted antigen delivery to DCs was proven to efficiently induce local immunity to hepatotropic viruses in mice (139). Interestingly, in the context of cancer immunotherapy, PD-L1 silenced DCs were used for a DC vaccine that induced potent T cell responses (140). Finally, IL-2 administration could recover dysfunctional HBV-specific CD8 cells originated by an inefficient hepatocyte priming in HBV transgenic mice (74).

Soluble Factors

As a result of its peculiar cell population, the liver microenvironment is enriched in soluble factors with immunosuppressive function (Figure 2A). Not only the immunoregulatory cytokines IL-10 and TGF- β are preferentially secreted over other pro-inflammatory cytokines by several types of hepatic cells (i.e., dendritic cells, macrophages/monocytes, LSECs) (141), but also an abundance of other regulatory

mediators has been detected within the liver. Indeed, constitutive expression by different liver infiltrating cells (e.g., dendritic cells, regulatory myeloid cells) (141, 142), of tryptophan-2,3-deoxygenase (TDO), or Indoleamine 2,3-dioxygenase (IDO) which are tryptophan-degrading enzymes, can lead to tryptophan depletion and the formation of toxic metabolites, such as kynurenines (143, 144). These factors have been shown to constrain T and NK cell proliferation (145) and to induce T cell apoptosis (143, 144).

The shortage of another essential amino acid, i.e., L-arginine, causing T cell arrest in the G0/G1 phase and CD3 ζ chain down-regulation, is due to an excess of arginase derived from damaged hepatocytes (146) and other liver-infiltrating cells (147). In HBV-infected patients the latter mechanism was demonstrated both in acutely and in chronically infected subjects and could be corrected *in vitro* by T cell transfection of CD3 ζ or replenishment of the amino acid arginine required for its expression (19, 55).

Interestingly, a granulocytic subset of myeloid-derived suppressor cells (gMDSC), which release arginase I upon degranulation, was demonstrated to be expanded in CHB patients with high HBV replication levels without liver necro-inflammation, highlighting the capacity of these cells to contain tissue damage by limiting arginine supply to T cells (148).

Local Induction of Regulatory Cells

In addition to HSCs, KCs, and macrophages, the liver is enriched in populations of regulatory cells, such as myeloid derived suppressor cells (MDSCs) and regulatory T cells (Tregs), that can promote tolerance by producing immunosuppressive cytokines (IL-10 and TGF- β) and by expressing high levels of co-inhibitory ligands, such as PD-L1 and Galectin-9 (**Figures 2A,B**). Importantly, the direct contact between circulating T cells and resident regulatory cells is favored by the slow blood flow in liver sinusoids and by the unique liver architecture, which is characterized by the presence of sinusoidal wall fenestrations which facilitate cell to cell contact (85).

Myeloid Derived Suppressor Cells

Myeloid cells play a key regulatory role within the liver contributing to the outcome of immune responses, thanks to their functional plasticity. In fact, they can differentiate from monocytes into macrophages, monocyte-derived dendritic cells or myeloid suppressor cells. During liver inflammation, inflammatory monocytes can be recruited into the liver as a result of ICAM-1 (CD54) expression on LSECs (141). They can subsequently differentiate into myeloid-derived suppressor cells by a CD44-dependent mechanism driven by activated stellate cells (141), as shown by the possibility to prevent acquisition of the suppressive phenotype by blocking CD44-mediated interaction between monocytes and HSCs (149). During prolonged hepatic inflammation, MDSCs inhibit immune responses through different mechanisms, including IL10 and TGF- β secretion, as well as production of arginase 1 and reactive oxygen species (ROS) (150). Expansion and suppressive function of MDSCs was reported to be modulated by cysteine-rich protein 61 (CCN1), a multifunctional protein highly expressed in impaired cholangiocytes and hepatocytes. This has been

described in primary biliary cholangitis (PBC) but it may likely represent a general mechanism of MDSC modulation shared by chronic liver inflammations of different etiology (151).

As already mentioned, the immunosuppressive function of MDSCs was described in chronic HBV infection where gMDSC could dampen HBV-specific T cell responses in a arginase-dependent manner, documenting the capacity of expanded arginase-expressing gMDSCs to regulate liver immunopathology (148). A recent study in chronically HBV infected patients with high levels of HBsAg revealed the capacity of MDSCs to promote immune dysfunction through the induction of regulatory T cells, primarily via TGF- β and IL-10 dependent signaling pathways. Interestingly, a year tenofovir treatment did not result in the immune restoration of the regulatory MDSC and Treg populations (152). In addition, circulating MDSCs were significantly expanded in patients with HBV-related acute-on-chronic liver failure (ACLF) and closely associated with disease progression and severity. In this setting, CD3 ζ chain expression was decreased in T cells and negatively correlated with MDSC frequency (153).

Monocyte differentiation within the liver can also be driven by TLR-9 signaling toward the acquisition of anti-viral protective properties through the formation of inflammatory monocyte aggregates, called iMATEs, where virus-specific CD8 cells can expand upon OX40- and CD28-mediated signaling initiated by inflammatory dendritic cells of monocyte origin (154). This unique iMATEs structure, which, however, seems to be generated selectively in the mouse liver, can contribute to chronic virus infection control. iMATE formation induced by systemic TLR-9 treatment has also been reported to induce effector CD8 T cell expansion and control of tumor growth in a murine hepatoma model (155).

Such functional plasticity of MDSCs represents a unique challenge for future therapeutic interventions in the setting of chronic liver disease (156). Indeed, all these results suggest that a therapeutic regimen that either blocks MDSC suppressive functions and Treg amplification or supports iMATES formation facilitating CD8 T cell proliferation might represent a potential strategy to cure HBV-related liver disease. However, further studies of this unique regulatory cell population are needed to better characterize its real therapeutic potential.

Treg Expansion

During viral infection, regulatory T cells are recruited into the inflamed liver and compete with effector CD8 T cells for IL-2, limiting the amplification of virus-specific T cell responses (8). Both mDCs and pDCs have been described to promote Treg proliferation through a mechanism mediated by STAT3 signaling (102, 157–159). Specifically, the expansion of Treg cells, which can inhibit T cell responses either by direct cell-cell contact or by secretion of suppressive cytokines, can be caused by plasmacytoid dendritic cells (pDCs) through an IL27-based circuit which can lead to PD-L1 expression and subsequent Treg proliferation (158, 160). pDCs further foster T cell tolerance by stimulating IL-10 producing Tregs via an ICOS/ICOSL-mediated interaction (102, 159). In parallel, mDCs can also promote Treg expansion and T cell apoptosis as a result of the cross-talk with HSCs, leading

to PD-L1 up-regulation and IDO induction, and subsequent generation of immunosuppressive kynurenine compounds (161–163). Recent findings highlighted a novel OX40L + DC subset able to selectively expand Tregs, which plays an essential role in Treg homeostasis maintenance under inflammatory conditions; indeed, co-culture of DCs from GM-CSF treated mice and CD4⁺ T cells induced an increase in Treg proliferation in an OX40L dependent manner (164). Treg cell expansion can be also stimulated by LSECs and IFN- γ activated stellate cells in a PD-L1 independent manner (141, 161). LSECs are the major liver cells responsible for TGF- β dependent hepatic FoxP3⁺ Treg induction, thanks to their unique capacity to secrete TGF- β and to bind exogenous LAP/TGF- β to their membrane through the anchor molecule GARP. By this mechanism, LSEC-induced Tregs have been reported to become functional suppressor cells in a mouse model of autoimmune encephalomyelitis (165). Beyond this tolerogenic role, LSECs have been recognized as a population that can efficiently stimulate naïve CD8 T cells to differentiate into a liver-primed memory T cell population providing protection and contributing to clearance of viral infections (8, 166).

Also HSCs can function as tolerogenic regulators in the liver, by enhancing TGF- β -dependent Treg generation and inhibiting TGF- β dependent Th17 differentiation, via a retinoic acid (RA)-mediated mechanism (167). HSCs have been associated with the production of TGF- β and all-*trans* retinoic acid (ATRA), both important for Treg differentiation (168). In particular, generation of FoxP3⁺ Tregs have been reported to occur in the presence of DCs and low concentrations of TGF- β in an ATRA-dependent manner, since blocking experiments of RA-RA receptor interaction inhibited HSC-induced FoxP3 expression (169). Furthermore, a recent report indicates that HSCs from HBV patients with advanced liver fibrosis play an important role in modulating the intrahepatic Treg population via a PGE2/EP2 and EP4 pathway (170).

T Cell Killing by NK Cells

NK cells are highly enriched within the liver where they exert a key role in anti-viral control but can also exert a regulatory effect with possible inhibition of adaptive immune responses (32, 34) (**Figure 2B**). Initial studies performed in LCMV-infected mice demonstrated that T cells are susceptible to NK-cell-mediated killing (171, 172). The elimination of activated T lymphocytes by NK cells can be mediated by NKG2D- and TRAIL-dependent mechanisms, as demonstrated *in vitro* (34, 173–175) and in LCMV infected mice *in vivo* (172). Moreover, regulation of T-cell responses by a direct perforin-dependent NK-cell-mediated elimination of CD4 T cells leading to the loss of help for CD8 T cells was observed in the same murine model of chronic viral infection (171).

In this negative modulation of T cell responses by NK cell killing, the NK cell NCR1 (NKP46)-receptor may have an important role and inhibition of NCR1 ligand expression on T cells by type I IFN signaling can protect T cells from NK cell killing allowing T cell evasion from NCR1 mediated NK cell attack (176).

In chronic HBV patients NK cells are more pathogenic than protective because defective in cytokine production but efficiently able to express cytolytic function (32, 34). Moreover, apoptosis of HBV-specific CD8 T cells up-regulating the death-inducing receptor TRAIL-R2 can be caused by TRAIL-positive NK cells in chronically infected HBV patients (34). By these mechanisms NK cells can deeply impair T cell responses, protecting the host from fatal immunopathology during viral infections but also diminishing the anti-viral activity of effector T cells, thereby contributing to the exhausted T cell phenotype of chronic infections. Thus, therapeutic strategies aimed at promoting the protective over the pathogenic and inhibitory effects of NK cells may represent potential options to treat chronic HBV infection.

PERSPECTIVES FOR NOVEL THERAPEUTIC STRATEGIES

Immune reconstitution of functionally efficient T cell responses can be crucial for the cure of chronic HBV infection. An efficient strategy to improve the HBV-specific T cell function should probably target multiple mechanisms, because the protective anti-viral T cell response is affected by multiple factors simultaneously during chronic infections. Decline of antigen can allow correction of a single inhibitory mechanism and is expected for this reason to be insufficient alone for successful immune restoration. It thus represents only a part of the overall functional reconstitution strategy which should be put in place to cure HBV infection. Instead of currently available NUCs, which are poorly effective in diminishing HBV antigen concentrations, new and more efficient antivirals should be employed in the future, once clinically available, to reduce more rapidly and more efficiently the antigen load and the number of infected hepatocytes. They include RNA interference (RNAi) molecules, which can directly target HBV mRNAs, allowing reduction in HBsAg serum titers (177), HBV capsid assembly inhibitors and nucleic acid polymers (NAPs), as HBsAg release inhibitors (178–180). Since decline of antigen alone is expected to be insufficient, additional mechanisms should be targeted to further improve T cell responsiveness. Immune checkpoint blockade represents a possibility, but also metabolic and epigenetic modulations are very promising. In addition, recent studies performed in a transgenic mouse model of neonatal HBV infection, which closely recapitulates the immunological events occurring in the early immune tolerant phase of chronic HBV infection, indicate that IL-2, rather than checkpoint blockade, can improve defective T cell responses (74). As predictable, IL-2 treatment induced functional improvement only of HBV-specific CD8 T cells from immune-tolerant, but not immune-active chronic HBV patients.

In the perspective of a therapeutic correction of T cell exhaustion, a crucial limitation is represented by the incomplete information we have on level and quality of T cell functional reconstitution which is needed to achieve control of chronic HBV infection.

In order to address this issue, the optimal comparator to define level and quality of the T cell response to be restored for control of infection would be represented by patients able to

resolve an acute infection spontaneously. However, one major limitation to the definition of the immune parameters in these subjects is represented by the heterogeneity of T cell reactivities in relation to the time elapsed from the initial exposure to the virus, which is generally undefined. Certainly the study of adult-onset infections with known duration evolving either toward resolution or chronicization would be crucial for the identification of T cell features associated to virus control. Nonetheless, the paucity of clinically overt acute HBV infections nowadays and the much greater rarity of those chronically evolving make such a study hardly feasible. In addition, difficult recruitment of a significant control patient cohort is further compounded by technical curbs due to patients' HLA heterogeneity, as most of the studied T cell epitope specificities are mainly limited to the HLA-A*02 restriction, which is only prevalent in the Caucasian population.

Even more important, still unknown, is whether the maximal improvement of T cell responses that a given chronic patient can reach after long-term exposure to HBV and its antigens is sufficient for complete and durable HBV cure. In the lack of definitive data ruling out the possibility that T cell functional defects derived from decades of T cell/virus interplay are only partially and insufficiently reversible by therapeutic T cell correction strategies, an alternative possibility to overcome T cell exhaustion is represented by the *in vitro* generation and expansion of functionally efficient, genetically engineered HBV-specific T cells for adoptive transfer to chronically infected patients. To this purpose, autologous T cells of HBV-unrelated specificity have been engineered to express HBV-specific TCR able to recognize HLA/peptide complexes in a HLA restricted manner (TCR-redirected T cells) or chimeric antigen receptors (CAR) composed of synthetic antibody fragments, combined with costimulatory domains, such as the CD28 and the CD3 zeta molecules, that allow the receptor to recognize viral antigens on infected cells in a HLA independent way, without the need of antigen processing (181–184). Thus, an important advantage of CAR T cells over TCR-redirected T cells is that CAR T cells can be used in all patients, irrespective of their HLA profile, while TCR-redirected T cells can only be used in patients with the appropriate HLA haplotype and can recognize only individual epitopes generated by intracellular antigen processing.

Potential problems related to these T cell transfer approaches are the possible risk of severe liver damage and the possible inhibitory effect on transferred effector T cells of the tolerogenic liver environment. To reduce cell lifespan and limit the risk of uncontrolled proliferation with progressive liver damage, a transient expression of the modified TCR has been developed by mRNA electroporation (185). Moreover, combination of checkpoint blockade and CAR T cell therapy (186), as well as shRNA knockdown of PD-1 in TCR-redirected T cells (187) have been used to prevent immunosuppressive mechanisms mediated by inhibitory co-receptors (188). In addition, CAR T cells have recently been further engineered by over-expressing the canonical AP-1 transcription factor c-Jun to render them resistant to exhaustion. These cells showed different chromatin accessibility, enhanced expansion and functional capacity, and improved anti-tumor activity in five different tumor mouse models *in vivo* (189).

In summary, T cell modulation remains a promising therapeutic strategy to cure chronic HBV infection, but the proof of concept that it can actually work in this clinical context still remains to be provided. A number of different elements contribute to the complexity of its practical application: first of all, the still partial knowledge of the immunological correlates of protection in HBV infection. The functional efficiency of HBV-specific T cell responses is certainly essential for a final and persistent control of infection, but we don't know whether a selective improvement of the T cell function can be sufficient to cure infection. This uncertainty raises the issue of which other effectors of the immune system should be modulated in combination with a functional T cell reconstitution, in consideration of the evidence that also intracellular innate responses and NK cells are defective in chronic HBV infection (32, 190). This opens in turn a series of additional questions. In particular, the number of different therapeutic interventions to be used simultaneously or sequentially in order to restore a functional immune system, considering that T cell dysfunction *per se* is multifactorial and may require for its correction the coordinated application of different therapeutic approaches. This is particularly relevant in consideration of the fact that available therapies are easy to take and almost totally free of side effects and that chronic HBV patients under therapy feel absolutely well (191). This implies that new therapies should be easy to take, free of important side effects, and highly effective against the virus in a short time. In light of these considerations, all different strategies exposing the patient to the risk of a strong stimulation of T cells of HBV-unrelated specificity (i.e., epigenetic therapies, checkpoint inhibition) or of extensive and severe liver damage (i.e., therapeutic vaccines) or the combination of different approaches may be ethically problematic. This concern may also apply to the adoptive T cell transfer of genetically engineered T cells which is also technically very complex for a wide application in the clinical practice.

FINAL REMARKS

A number of specific drugs able to intervene on the different steps of the HBV life cycle and on different mechanisms involved in the pathogenesis of T cell exhaustion are now available and under clinical evaluation. In a first step of therapy, new direct anti-viral drugs acting on HBV replication, antigen production and liver inflammation may be needed to make immune therapies more effective, because their efficacy in improving the T cell function is believed to be affected by the negative regulatory mechanisms triggered by the exposure to high antigen quantities and by the environment of the chronically inflamed liver. This initial step of anti-viral therapy may be also essential to diminish the risk of severe liver pathology which may follow a strong activation of anti-viral effector T cells induced when the percentage of infected liver cells is elevated. Assuming that decline of antigen and control of liver inflammation may be insufficient for optimal restoration of anti-viral T cell functions, the possible choice for a second step of therapy to further overcome T cell exhaustion is in principle between checkpoint blockade, metabolic correction

and epigenetic modulation strategies. On one hand, correction of mitochondrial and proteasomal defects in chronic HBV infection by the use of mitochondrial targeted anti-oxidants and polyphenolic compounds seems to be comparably or even more efficient than PD-1/PD-L1 blockade in functional T cell restoration *in vitro* with very limited effects on T cells of HBV-unrelated specificity (192). On the other hand, epigenetic modulators are expected to be highly effective on T cell responses given the massive transcriptional down-regulation detected in exhausted HBV-specific CD8 cells (36), but their use *in vivo* may be precluded by their potentially severe side effects.

Once T cell exhaustion is maximally corrected and responsiveness to antigen stimulation is optimally reacquired, effector T cell responses will likely need to be boosted in a third step of therapy by vaccines containing not only HBsAg and HBcAg but also polymerase, in consideration of its high immunogenicity and the severe level of exhaustion of pol-specific CD8 T cells reported in chronic HBV infection (39, 40).

Finally, an important goal for future research will be also to develop reliable predictors of response to immune therapies to be used in individual patients. Indeed, it is becoming increasingly clear that chronic patients are a heterogeneous population with variable levels of T cell functionality, which can probably confer propensity to respond more or less efficiently to immune modulation (37, 38). Identification of cell-mediated immunologic profiles predictive of response to immune modulation will be pivotal for the success of immune therapies because we can

predict that only a proportion of chronic HBV patients will take advantage from immune modulation.

Thus, the possible scenario that recent research results allow to depict for a near future would be a therapy for chronic hepatitis B with new direct anti-viral compounds started after an immunological characterization of individual treated patients to predict their likelihood of responsiveness to immune therapies aimed at functional T cell reconstitution. Only patients predicted to be immunologically responsive will receive this second step of therapy based on immune modulation (i.e., metabolic or checkpoint modulators) and therapeutic vaccination, either sequentially or simultaneously.

AUTHOR CONTRIBUTIONS

PF, CB, and VB: design and writing of the manuscript. MR, GA, and DL: contribution to figure drawing. IM, AV, and AZ: contribution to the selection of the references to quote. AP and GM: discussion of the concepts to be introduced in the text. CF: critical revision of text and figures.

FUNDING

This work was supported by a grant from Regione Emilia-Romagna, Italy (Programma di Ricerca Regione-Università 2010–2012; PRUa1RI-2012-006), and by a grant from the Italian Ministry of Health (Ricerca Finalizzata RF 2013-02359333).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Diverse Virus and Host-Dependent Mechanisms Influence the Systemic and Intrahepatic Immune Responses in the Woodchuck Model of Hepatitis B

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OPEN ACCESS

Edited by:

Allan Randrup Thomsen,
University of Copenhagen, Denmark

Reviewed by:

Robert Thimme,
University of Freiburg, Germany
Jianzhong Zhu,
Yangzhou University, China

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Specialty section:

This article was submitted to
Viral Immunology,
a section of the journal
Frontiers in Immunology

Received: 19 July 2019

Accepted: 14 April 2020

Published: 27 May 2020

Citation:

Michalak TI (2020) Diverse Virus and Host-Dependent Mechanisms Influence the Systemic and Intrahepatic Immune Responses in the Woodchuck Model of Hepatitis B. *Front. Immunol.* 11:853. doi: 10.3389/fimmu.2020.00853

Woodchuck infected with woodchuck hepatitis virus (WHV) represents the pathogenically nearest model of hepatitis B and associated hepatocellular carcinoma (HCC). This naturally occurring animal model also is highly valuable for development and preclinical evaluation of new anti-HBV agents and immunotherapies against chronic hepatitis (CH) B and HCC. Studies in this system uncovered a number of molecular and immunological processes which contribute or likely contribute to the immunopathogenesis of liver disease and modulation of the systemic and intrahepatic innate and adaptive immune responses during hepadnaviral infection. Among them, inhibition of presentation of the class I major histocompatibility complex on chronically infected hepatocytes and a role of WHV envelope proteins in this process, as well as augmented hepatocyte cytotoxicity mediated by constitutively expressed components of CD95 (Fas) ligand- and perforin-dependent pathways, capable of eliminating cells brought to contact with hepatocyte surface, including activated T lymphocytes, were uncovered. Other findings pointed to a role of autoimmune response against hepatocyte asialoglycoprotein receptor in augmenting severity of liver damage in hepadnaviral CH. It was also documented that WHV in the first few hours activates intrahepatic innate immunity that transiently decreases hepatic virus load. However, this activation is not translated in a timely manner to induction of virus-specific T cell response which appears to be hindered by defective activation of antigen presenting cells and presentation of viral epitopes to T cells. The early WHV infection also induces generalized polyclonal activation of T cells that precedes emergence of virus-specific T lymphocyte reactivity. The combination of these mechanisms hinder recognition of virus allowing its dissemination in the initial, asymptomatic stages of infection before adaptive cellular response became apparent. This review will highlight a range of diverse mechanisms uncovered in the woodchuck model which affect effectiveness of the anti-viral systemic and intrahepatic immune responses, and modify liver disease outcomes. Further exploration of these and other

mechanisms, either already discovered or yet unknown, and their interactions should bring more comprehensive understanding of HBV pathogenesis and help to identify novel targets for therapeutic and preventive interventions. The woodchuck model is uniquely positioned to further contribute to these advances.

Keywords: woodchuck model of hepatitis B, virus-hepatocyte interaction, major histocompatibility complex presentation, asialoglycoprotein receptor, hepatocyte as cytotoxic immune effector, intrahepatic innate and adaptive immune responses, pre-acute infection, toll-like receptors

INTRODUCTION

Woodchuck hepatitis virus (WHV) was discovered in wild-caught woodchucks housed at the Philadelphia Zoological Garden (Pennsylvania, USA) where chronic hepatitis (CH) and hepatocellular carcinoma (HCC) were observed at high rates (1, 2). Studies that followed demonstrated that the virus is highly compatible to HBV considering ultrastructure, genome organization and size, nucleotide sequence (~65% homology), and in replication strategy. The number and functions of viral proteins and the range of organs targeted, namely the liver and the immune system, were also alike (3–7). WHV and HBV also share similar profiles of endurance of viral antigens in circulation and liver, and liver disease displays the stepwise progression where acute hepatitis (AH) spontaneously resolves and is followed by persistent occult infection or advances to CH and HCC (2, 5, 8–13). The profiles of WHV-specific humoral and cell-mediated, as well as innate immune responses closely model those in human hepatitis B. Resolution of AH coincides with vigorous, polyclonal T cell response, intrahepatic upregulation of interferon (IFN)- γ and IFN- α , and appearance of otherwise protective antibodies against WHV envelope (surface) antigen (WHsAg). In contrast, CH is characterized by weak or absent T cell reactivity toward virus, T cell exhaustion, and a decreased hepatic expression of interferons (14–19). Importantly, both infections comparably respond to antiviral and to the majority of immunomodulatory approaches tested so far, and the pharmacokinetic and drug toxicity are congruent (12, 20–22). For these reasons, naturally or experimentally infected eastern North American woodchucks (*Marmota monax*) have been recognized as the pathogenically and immunologically relevant model of human HBV infection and HBV-induced liver diseases, and as the preferable system for assessing potential future therapeutics. Nevertheless, there are certain differences between HBV and WHV and liver diseases caused. On the molecular level, there are discrepancies in the activities of individual enhancers and promoters (23) and differences in glycosylation of the virus envelope proteins (24). Considering liver pathology, the leading variances are that CH does not progress to cirrhosis and that HCC advances at much higher rate (80–90%) in chronically infected woodchucks than in CH patients who acquired infection in adulthood in whose HCC rate is considerably lower (~5%) (8, 10, 11).

This review summarizes studies according to their focus but not based on the time line when the data were reported. Also, the review does not cover the entire scope of contributions made by the woodchuck model, particularly not those where the model

was applied for evaluation of antiviral and immunomodulatory therapies since such summaries were recently published by others (12, 25). The purpose of this review was to bring together the range of the mechanisms uncovered, on one hand, to illustrate their vast diversity and, on the other, to encourage broader exploration of this highly valuable model to advance our knowledge beyond the scope explored so far. This should expedite discovery of new therapeutic and preventive strategies against both virus and diseases which it causes.

TYPES OF WHV INFECTION AND STAGES OF WHV-INITIATED LIVER DISEASE

Approximately 250 million people have serum HBV surface antigen (HBsAg)-reactive CH and up to two billion may have occult HBV infection occurring in the absence of detectable HBsAg and clinically apparent hepatic disease (26). Symptomatic infection usually begins as serum HBsAg-positive AH which subsides without treatment in the majority of adults and, therefore, was named as self-limited AH (SLAH). SLAH is followed by asymptomatic low-level persistence of HBV, termed as secondary occult infection (SOI) or seropositive occult infection (OBI) (27, 28). This infection continues in the absence of serum HBsAg but is accompanied by antibodies to HBV core (anti-HBc) antigen and to HBsAg (anti-HBs), and by low levels of circulating HBV DNA, while virus replication is readily detectable in the liver and the immune system. Up to one-tenth of patients with AH advances to CH which is serum HBsAg and anti-HBc reactive disease usually accompanied by high levels of circulating HBV DNA of up to 10^{10} – 10^{11} virus genome copies, also called virus genome equivalents (vge), per mL. CH displays biochemical and histological indicators of liver protracted inflammation and hepatocyte death, robust HBV replication, and it may progress to liver cirrhosis and HCC, which are the main causes of mortality among chronically infected patients.

Woodchucks naturally or experimentally infected with WHV develop AH which is seropositive for WHsAg and antibodies to WHV core (nucleocapsid) antigen (anti-WHc), and is accompanied by biochemical and histological signs of liver injury. Like in HBV-infected humans, AH can advance to CH and HCC or may resolve and lifelong SOI is established (Figure 1A) (5, 13, 29). Resolution of AH coincides with apparent complete clearance of serum WHsAg (see section below), arise of antibodies to WHsAg (anti-WHs), decrease in serum WHV DNA to levels below 100–200 vge/mL, and normalization of liver

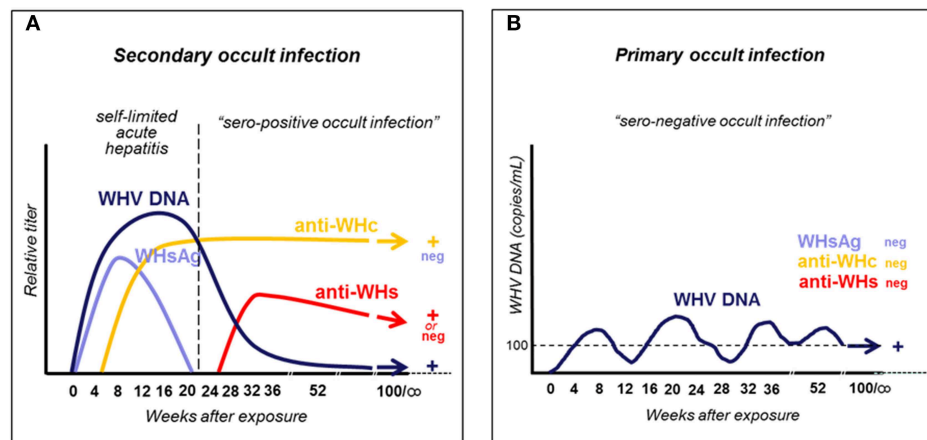


FIGURE 1 | Schematic presentation of serum immunovirological and molecular profiles of occult infections caused by WHV. **(A)** Secondary occult infection (SOI) continuing after resolution of self-limited acute hepatitis caused by a liver pathogenic dose greater or equal to 1×10^3 WHV characterized by persistent asymptomatic infection negative for serum WHsAg but reactive for anti-WHc antibodies, positive or negative for anti-WHs antibodies, and positive for WHV DNA at levels below 100–200 virus copies/mL. **(B)** Primary occult infection (POI) developing after exposure to a liver non-pathogenic dose of $<1 \times 10^3$ WHV virions that persists as a seronegative infection but remains serum WHV DNA reactive at levels usually below 100–200 virus copies/mL.

biochemical function. Notably, like in HBV-infected patients, CH is diagnosed when WHsAg reactivity in plasma or serum persists for longer than 6 months. In addition, animals infected with very low doses of WHV ($<10^3$ virions) develop another form of persistent asymptomatic infection, termed as primary occult infection (POI), that progresses in the absence of WHsAg, anti-WHc and anti-WHs, but WHV DNA is detectable in serum at similar levels as in SOI (Figure 1B) (29, 30). This infection is accompanied by essentially normal liver biochemistry and histology but HCC may develop (31). The human equivalence of this infection is seronegative OBI (28, 32). More details on the natural history and characteristics of SOI and POI are given in sections on pages 3 and 5, respectively, while features of coinciding immunological responses are summarized in section beginning on page 7.

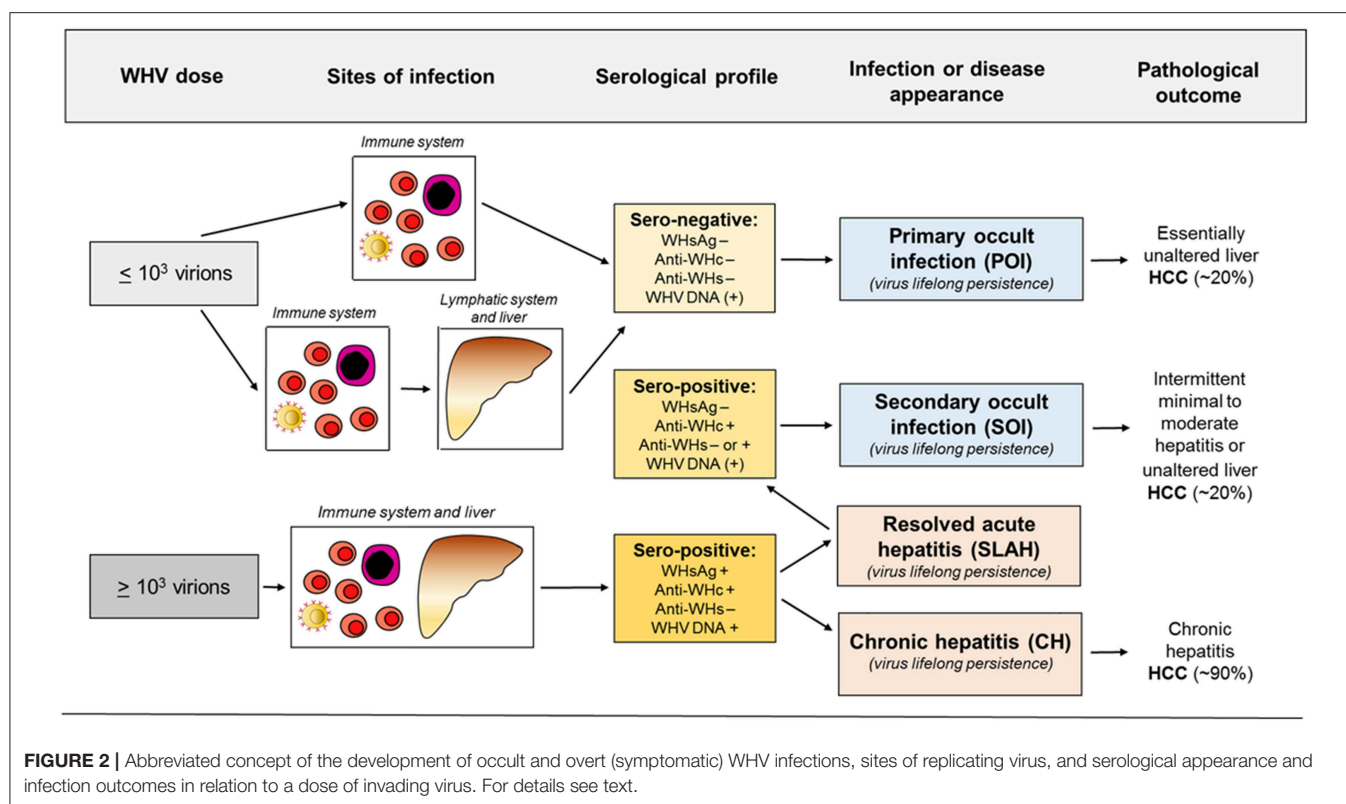
PERSISTENCE OF PATHOGENIC WHV AFTER RESOLUTION OF SYMPTOMATIC INFECTION: SECONDARY OCCULT INFECTION (SOI)

HBV persistence in the absence of serum HBsAg, biochemical indicators of liver injury and clinical symptoms was rarely reported prior to introduction of HBV DNA detection by nucleic acid amplification tests (NAT) (33–36). Application of an increasingly sensitive and controlled NAT made identification of serologically silent HBV infection (i.e., OBI) more frequent and reliable (27, 37–40). With time, OBI became defined as persistence of HBV DNA in the absence of serum HBsAg. It later became apparent that this asymptomatic infection may have severe clinical consequences due to reactivation of hepatitis following immunosuppression, radiation or administration of immunomodulatory and cytotoxic therapies

(41–45). With accumulation of molecular and immunological evidence, including data from the woodchuck model (28–30, 32, 46, 47), it became apparent that there are two distinct forms of OBI (Figures 1, 2). Both are serum HBsAg-negative but one is accompanied by anti-HBc with or without detectable anti-HBs, and another in which the antibodies are absent (13, 32).

One of the earliest studies demonstrated HBV persistence in serum and peripheral blood mononuclear cells (PBMC) collected from seemingly healthy persons after spontaneous resolution of AH type B (27). In these individuals, HBV DNA remained detectable in both serum and PBMC in the presence of anti-HBs and anti-HBc antibodies for up to 70 months after clinical resolution of AH. HBV DNA was detected by nested polymerase chain reaction (PCR) with primers specific for different HBV genes. Specificity of HBV amplicons was verified via nucleic acid hybridization (NAH). The estimated sensitivity of the PCR-NAH assays was <10 vge/mL. It was also found that HBV DNA in plasma co-sedimented with HBsAg reactivity in sucrose gradients and that some particles reactive for HBV DNA displayed buoyant density of intact virions in cesium chloride gradients. It was postulated that traces of infectious HBV persist after termination of SLAH in these otherwise healthy individuals. The existence of asymptomatic HBV infection after resolving AH was confirmed in subsequent studies [e.g., Penna et al. (48), Rehmann et al. (37), Uemoto et al. (38), Yotsuyanagi et al. (49), Yuki et al. (40), Murakami et al. (50)].

The findings from the 1994 study (27) triggered investigations in the woodchuck model which have brought a number of important discoveries. These findings have solidified data on the existence, molecular and immunological characteristics, and pathogenic significance of occult hepadnaviral infection continuing after resolution of a symptomatic disease. Because this form of infection follows clinically and serologically apparent infection, it was designated as SOI (secondary occult



infection) (5, 30) and later also referred to as seropositive-OBI since antibodies to viral antigens remained detectable (**Figures 1A, 2**) (28).

Studies in woodchucks revealed that silent replication of WHV persists for life after recovery from AH and that SOI can culminate in the development of HCC (**Figure 2**) (29). It was also uncovered that both the liver and the immune system are the sites of virus replication. Thus, after resolution of AH, the serum levels of WHV DNA oscillated from <10 to 100 vge/mL, with occasional rises up to 10^3 vge/mL. However, there were periods when serum WHV DNA was negative, but virus genome could be detected in the liver biopsy and/or PBMC acquired at the time of serum collection. WHV DNA quantities in hepatic tissue, PBMC and lymphatic organs, such as spleen, lymph nodes and bone marrow, ranged between 0.02 to 200 vge/ 10^4 liver cells and 0.005 to 0.5 vge/ 10^4 lymphoid cells. WHsAg in serum remained negative during entire SOI, while anti-WHc were consistently detectable during lifespan (**Figure 1A**). Histological examination of serial liver biopsies revealed minimal-to-mild inflammation with periods of essentially normal liver morphology. However, HCC ultimately developed in about one fifth of animals (**Figure 2**). Importantly, WHV carried by animals with SOI induced typical AH in virus-naïve animals which advanced to CH and HCC in some woodchucks (29). These data unambiguously showed that resolution of AH does not reflect complete elimination of virus and did not prevent HCC development, even when anti-WHs antibodies arise. These were unexpected findings considering the prevailing opinion at that time.

The subsequent study verified that detection of anti-WHc alone, in the absence of conventionally detectable serum WHsAg, indicates existence of SOI (51). Notably, electron microscopic examination of pellets from ultracentrifuged anti-WHc-positive sera demonstrated singular spherical and short tubular particles of WHsAg. This indicated that small amounts of the antigen were produced although they were not detectable by otherwise highly sensitive, clinically compatible assay. The results were consistent with other investigations demonstrating that the WHV persisting in animals with the anti-WHc alone remained infectious (29). They also supported the notion that persistence of anti-core alone is a consequence of re-stimulation of the immune system by traces of nucleocapsid protein transcribed during subdued virus replication. Thus, the anti-core alone showed to be an excellent marker of enduring hepadnaviral replication accompanied by quantities of circulating virus DNA which may not be consistently detected due to their fluctuating levels.

TRACES OF WHV INDUCE PRIMARY OCCULT INFECTION (POI) THAT IS PERSISTENT, PRIMARILY LYMPHOTROPIC AND MAY CAUSE HCC IN THE ABSENCE OF HEPATITIS

POI was originally discovered in offspring born to woodchuck mothers which resolved AH and established SOI, including those which developed anti-WHs (46). All of the offspring

demonstrated low levels of circulating WHV DNA and WHV DNA, covalently closed circular DNA (cccDNA) and mRNA in the lymphatic system, whereas hepatic tissue was infected only in some of them. Surprisingly, no markers of WHV infection, including serum WHsAg, anti-WHc or anti-WHs, could be detected after birth (**Figure 1B**). Nonetheless, particles reactive for WHV DNA with biophysical features of intact (enveloped) virions were found in offspring sera. Histological examination of serial liver biopsies revealed normal morphology. Further, sera and supernatants of cultured lymphoid cells from these animals induced typical serologically apparent AH in WHV-naïve animals which verified pathogenic fitness of the persisting virus. Remarkably, the offspring were not protected from challenge with a large, liver pathogenic dose of WHV [see (30)]. This implied that this low level infection observed in offspring did not generate immunity against reinfection with larger doses of the same virus (5, 46). The study was the first to uncover that hepadnaviral infection can be restricted to the immune system arguing that hepadnavirus at low quantities is preferentially lymphotropic (**Figure 2**).

The subsequent study was designed to determine if POI can be established in adult immunocompetent woodchucks and to find WHV dose required to induce POI. For this purpose, adult healthy animals were intravenously (*i.v.*) injected with serial 10-fold dilutions of a well-defined WHV inoculum known to cause serologically evident infection and AH (30). The results of the study not only confirmed the existence of this form of occult infection, but also showed that the infection is triggered by WHV doses lower than or equal to 1×10^3 virions and can be reproducibly established in woodchucks. The same study demonstrated that the same WHV inoculum that caused POI induced at WHV doses larger than 1×10^3 virions classical WHsAg-positive AH that advanced to CH in some animals. Taken together, the data revealed that: (1) the amount of virus is the principal determinant of whether infection is serologically evident (overt) and symptomatic or serologically silent and asymptomatic (occult); (2) virus at quantities $>1 \times 10^3$ virions causes WHV hepatitis, and (3) the immune cells are primary targets and can be the only site of replication when virus invades at doses smaller than 1×10^3 virions (**Figures 1, 2**).

It was further examined whether repeated exposures to trace amounts of WHV normally establishing POI could result in serologically apparent infection and hepatitis. Adult animals were *i.v.* injected with twelve 110-virion doses over two rounds of 6 injections delivered weekly (52). At 14 weeks after the last injection, the animals were challenged with the same WHV inoculum at 1.1×10^6 virions and followed for additional 7.5 months (**Figure 3**). The results showed that the multiple injections of liver non-pathogenic virus did not culminate in serum WHsAg and/or anti-WHc positive infection and hepatitis, even though the overall amount of administered WHV was greater than the threshold of 1×10^3 virions known to trigger hepatitis. Not surprisingly, the animals remained susceptible to challenge with a liver pathogenic dose of WHV and responded by establishing AH (52). Repeated exposures to trace quantities of HBV may occur in different occupational and familial situations and in intravenous drug users. The results of this study suggest

that such multiple exposure will unlikely cause serologically evident infection and hepatitis in individuals not protected by vaccination, but HBV DNA in the absence of HBsAg, anti-HBc and anti-HBs might be detected.

To recognize the life-long consequences of POI, young adult woodchucks were investigated in a prospective study in which POI was established by *i.v.* injection with 10 or 100 DNase digestion-protected virions (31). Initially, WHV infection was constrained to cells of the lymphatic (immune) system in all animals. Serum WHV DNA loads were between <100 – 200 vge/mL during lifespan and $<1 \times 10^3$ vge/ μ g of cellular DNA in PBMC. After 2.5 to 3.3 years post-infection (*p.i.*), WHV infection spread to liver and low levels of WHV DNA, cccDNA and/or virus mRNA became detectable despite that hepatic histology remained normal. Notably, temporal increases in serum WHV load above 1×10^3 vge/mL preceded the involvement of the liver in infection. Among other original findings was that 20% of the animals with POI finally developed HCC. This implied that the virus persisting in POI maintained its pro-oncogenic potency and led to HCC in spite of the lack of hepatitis or other protracted liver injury. This was consistent with the finding that POI was accompanied by WHV DNA integration to hepatic, immune organs and PBMC genomes (31). This study pointed to the pathogenic significance of POI and implied that the development of HCC of unknown etiology in humans should consider this clinically and serologically mute infection as a causative factor (54, 55).

SYSTEMIC AND INTRAHEPATIC IMMUNE RESPONSES IN PRE-ACUTE INFECTION AND ACUTE WHV HEPATITIS

Our step-by-step understanding of the events coinciding with the initiation of HBV infection and hepatitis is hindered by the lack of recognition of processes occurring in the liver and the immune system immediately following invasion with virus and during the early pre-acute and acute periods of infection. Identification of patients with these essentially asymptomatic stages of infection is excitingly difficult and collection of liver samples practically impossible because of the absence of clinically sound indications. However, these early events are likely of primary importance for identification of immunological and other factors determining the development of symptomatic or occult infection, recovery from AH or its progression to CH, and initiation of pro-oncogenic processes which may culminate in HCC (56–58). The woodchuck model is particularly well-suited for this type of studies.

The hepatic kinetics of WHV replication and transcription of the genes encoding cytokines, immune cell markers and cytotoxic effector molecules, as well as the profiles of WHV-specific and generalized (mitogen-induced) T cell responses have been delineated in experimentally infected woodchucks soon after virus administration and in the very early stages of infection. Serial liver biopsies acquired from 1 h *p.i.* up to 36 months *p.i.*, when AH resolved and SOI was established, were analyzed by quantitative real-time PCR assays preceded

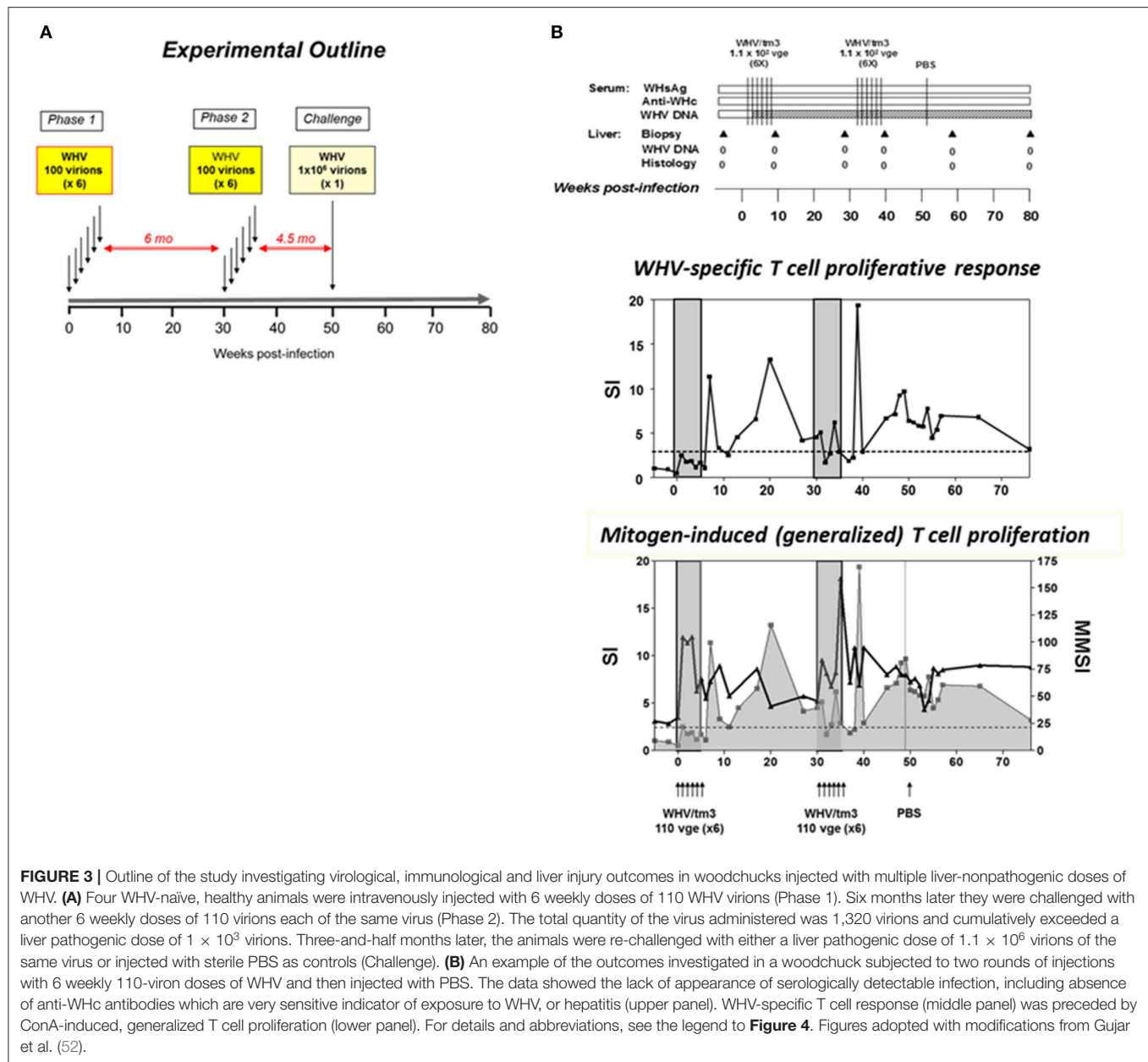


FIGURE 3 | Outline of the study investigating virological, immunological and liver injury outcomes in woodchucks injected with multiple liver-nonpathogenic doses of WHV. **(A)** Four WHV-naïve, healthy animals were intravenously injected with 6 weekly doses of 110 WHV virions (Phase 1). Six months later they were challenged with another 6 weekly doses of 110 virions each of the same virus (Phase 2). The total quantity of the virus administered was 1,320 virions and cumulatively exceeded a liver pathogenic dose of 1×10^3 virions. Three-and-half months later, the animals were re-challenged with either a liver pathogenic dose of 1.1×10^6 virions of the same virus or injected with sterile PBS as controls (Challenge). **(B)** An example of the outcomes investigated in a woodchuck subjected to two rounds of injections with 6 weekly 110-virion doses of WHV and then injected with PBS. The data showed the lack of appearance of serologically detectable infection, including absence of anti-WHc antibodies which are very sensitive indicator of exposure to WHV, or hepatitis (upper panel). WHV-specific T cell response (middle panel) was preceded by ConA-induced, generalized T cell proliferation (lower panel). For details and abbreviations, see the legend to **Figure 4**. Figures adopted with modifications from Gujar et al. (52).

by the reverse transcription step (RT-qPCR) (17). The results uncovered that WHV replication became detectable in the liver in an hour after i.v. injection with virus. This was in contrast to the previous studies in woodchucks and HBV-infected chimpanzees indicating that virus replication remains undetectable in the liver until 3–4 week p.i. (15, 59, 60). However, hepatic replication of these viruses was never before evaluated in the first hour p.i. by highly sensitive PCR-NAH assays. Briefly, it was found that in 3 to 6 h p.i., hepatic transcription of interleukin (IL)-12, which is a key cytokine produced by antigen presenting cells (APC) (61), was significantly (~20-fold) augmented together with an increase (12.5-fold) in IL-8 expression, a cytokine mediating chemotaxis of phagocytic cells. This also coincided by increased expression

of CD1d, a molecule facilitating antigen presentation by APC to natural killer (NK) T cells (62), and CD40 ligand (CD40L), which is implicated in APC activation via CD40-CD40L interaction. In 48 to 72 h, NK and NK T cells became active as implied by significant increases in hepatic expression of CD3, IFN- γ , OAS (2',5'-oligoadenylate synthetase), CD1d and CD40L, and in NKp46 and perforin expression. Most interestingly, WHV replication was significantly inhibited at the same time in the liver, implying that this early innate intrahepatic response was at least partially effective in inhibiting WHV replication (17). Nonetheless, hepatic CD4+ and CD8+ T cells became robustly activated much later at 4 to 5 weeks p.i. when hepatitis became evident. Overall, the findings showed that liver replication of

WHV is initiated and the innate response activated very soon after infection with a liver pathogenic dose of WHV (30). Nevertheless, this transient response was insufficient to right away activate T cells similarly as in other viral infections. In a somewhat similar study in chimpanzees in which liver biopsies were collected from 1 week post-HBV injection, hepatic tissue did not express of genes coinciding with innate immune response when evaluated by microarray (60). The conclusion was that HBV is a stealth virus unable to induce innate immunity in the infected host. However, activation of the innate response may have occurred earlier and already subsided at 1 week, as it was apparent in the WHV infection model. Subsequent studies showed HBV ability to trigger different branches of the innate response, although it remains not well-explained what their effector functions are in hosts naturally susceptible to HBV infection [reviewed in Thomas and Baumert (63)].

The common characteristic of HBV and WHV infections is the postponement of virus-specific T cell response and the reason behind this was unknown. This is in contrast to infections with other viruses where specific T cells usually appear in about 10 days to 2 weeks p.i. To investigate this issue, woodchucks were inoculated with 1.9×10^{11} WHV virions and challenged with the same virus dose several weeks later (53). As anticipated, the WHV-specific T cell response appeared 5 to 7 weeks p.i., remained elevated during AH, and then subsided but remained measurable during SOI. However, soon after administration of WHV, i.e., in the first 7 days p.i., T cells demonstrated significantly augmented proliferation in response to mitogenic stimuli, i.e., concanavalin A (ConA), pokeweed mitogen (PWM), and phytohemagglutinin (PHA) (Figure 4). Thus, this strong antigen-nonspecific, generalized T cell response occurred before the appearance of WHV-specific T cell reactivity, which was previously unknown consequence of hepadnaviral infection. Analysis of cytokine transcription in weekly PBMC samples confirmed very early activation of T lymphocytes and impairment in transcription of tumor necrosis factor- α (TNF- α) and IFN- γ up to week 6 p.i., thus until AH and WHV-specific T cell response became apparent. The pre-acute phase of WHV infection was also associated with reduced transcription of IFN- α , IL-2, and IL-12, and increased expression of IL-10. This cytokine expression profile was compatible with the profiles observed in the early infections with other viruses and was found to be indicative of defective activation of APC and presentation of viral epitopes to T cells (64, 65).

Interestingly, re-exposure of woodchucks which resolved AH to the same WHV again immediately triggered strong, generalized proliferative T cell response which was followed by the delayed virus-specific T cell reactivity (53). It is commonly acknowledged that re-exposure of immunocompetent hosts to the same biological agent swiftly mounts secondary adaptive response detectable within a week or two after re-challenge [e.g., Flynn et al. (66), Doherty et al. (67), Rocha and Tanchot (68)]. In this context, the results obtained suggested that temporal, generalized, polyclonal activation of T cells delayed adaptive T cell response not only after primary but also after secondary

exposure to WHV. This was the first of this kind finding that was not yet examined in HBV infection.

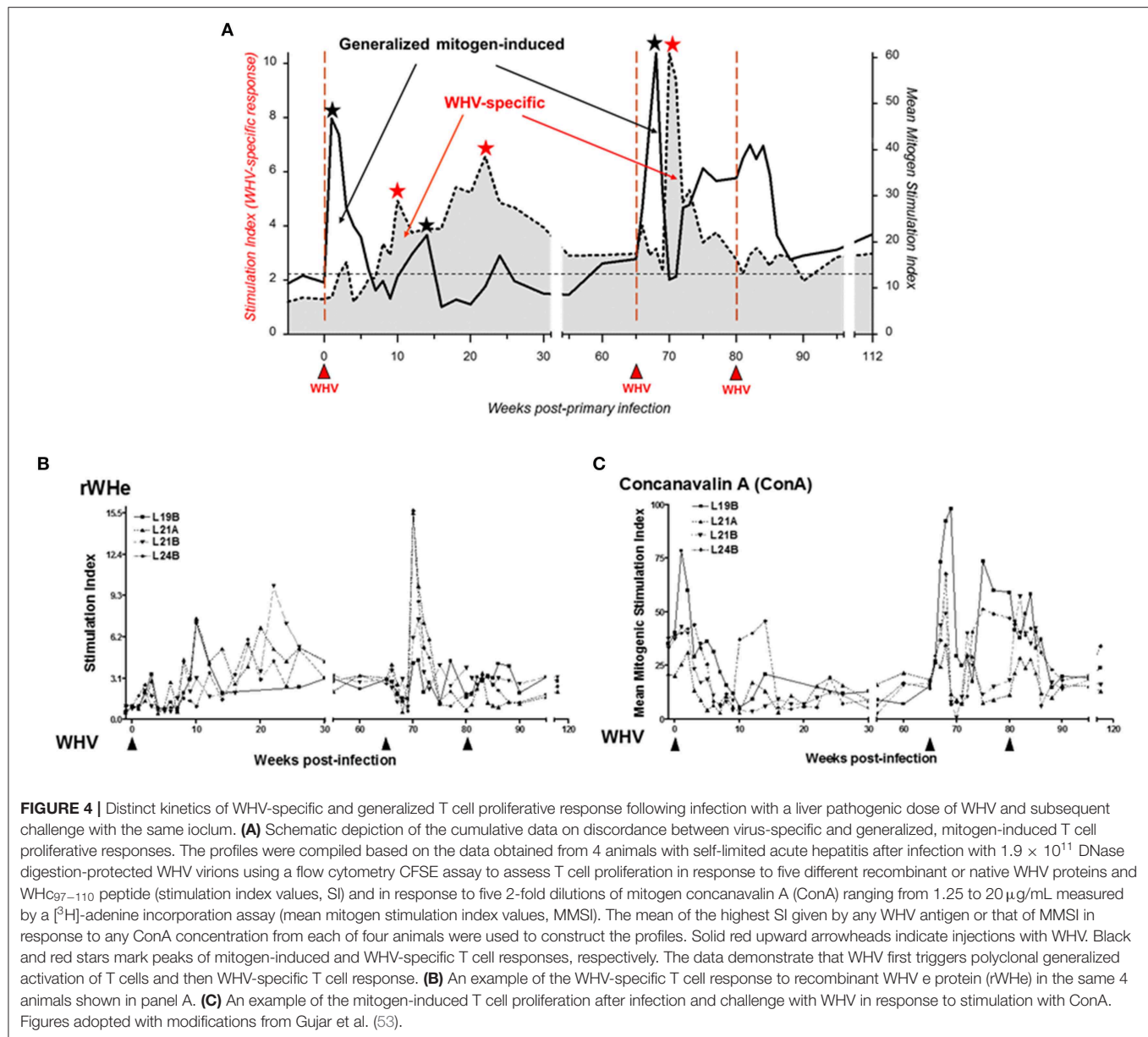
It was noticed that the generalized proliferative capacity of T cells varied depending on the phase of acute WHV infection (53, 69) and was significantly heightened promptly after infection and then dramatically declined before WHV-specific T cell response appeared (Figure 4). Hence, it became of interest to examine what the mechanism of this rapid T cell decline was. For this purpose, a flow cytometric CFSE [5-(and-6)-carboxyfluorescein diacetate succinimidyl ester]-based assay coupled with staining for annexin V-7 in the presence of actinomycin-D (7-AAD) was adopted to simultaneously measure lymphocyte proliferation and apoptotic lymphocyte death (53, 69, 70). The evaluation of lymphocyte behavior after WHV infection leading to SLAH showed that the rapid contraction of the generalized T cell response was associated with the strongly augmented susceptibility of T cells to activation-induced apoptotic death. This indicated that T cells became compromised in the early phase of acute WHV infection and this may directly contribute to the postponement of WHV-specific T cell response.

In summary, both primary and secondary exposures to liver-pathogenic doses of WHV caused generalized activation of T lymphocytes accompanied by the cytokine expression profile suggesting defective activation of APC and priming of virus-specific T cells. These processes occurred in the initial phase of infection and were likely responsible for postponement of WHV-specific T cell response. Consequently, this implied that the hindered initial recognition and elimination of virus permitted its replication and spreading in the very early, asymptomatic phase of infection. Similar findings were reported in the early infection with other viruses, including simian immunodeficiency virus (SIV) which infects T cells [e.g., Wallace et al. (65)]. As previously mentioned, WHV and HBV are lymphotropic viruses which infect cells of the immune system. For more details on WHV lymphotropism see section on page 9.

WHV-SPECIFIC T CELL AND HUMORAL IMMUNE RESPONSES IN CHRONIC SYMPTOMATIC AND PERSISTENT OCCULT INFECTIONS

CH remains the main burden of HBV infection despite significant progress in treatment options. The woodchuck model meaningfully contributed to identification of features of virus-specific T cell responses during symptomatic chronic infection and recognition of ways to modulate them for therapeutic purposes. Due to a very weak or absent activity of these responses, their augmentation either alone or in combination with antiviral agents may offer a prospect of more effective therapy for CH type B.

Identification of immunodominant T cell epitopes within WHV core and envelope proteins, and development of the first generation of assays measuring helper and CTL responses initiated recognition of the anti-WHV cellular immunity in woodchucks (14, 71, 72). These first tests included $2[^3\text{H}]$ -adenine



incorporation T cell proliferation assay and flow cytometric CD107a degranulation test to assess WHV-specific cytotoxic T cells (CTL) response (14, 15, 72). The data showed a close compatibility between woodchucks and humans in adaptive T cells responses both during resolution of AH and CH. Thus, recovery from AH coincided with strong T cell proliferative and CTL reactivity directed to multiple WHV antigenic epitopes which coincided with rapid decline in circulating virus. In contrast, weak or undetectable T cell responses were identified in CH in the presence of usually high loads of circulating WHV (14, 71, 72). With time more sensitive or applying different approaches assays were developed, including a flow cytometric CFSE-based assay capable of evaluation of relative strength and kinetics of WHV-specific T cell proliferation (52, 53, 70) and

flow cytometric assays quantifying WHV-specific CTLs based on defining CD3-positive, CD4-negative (73) and CD4-negative, IFN- γ positive T cell subsets (Mulrooney-Cousin and Michalak, unpublished). The results from these assays remained in good agreement with the findings previously reported.

The weakened or absent virus-specific T cell response in chronic infections was found to be a consequence of T cell exhaustion in which programmed death 1(PD-1)/PD-ligand 1 (PD-L1) interaction plays a dominant role [e.g., Barber et al. (74), Maier et al. (75), Velu et al. (76)]. The restoration of this interaction *in vitro* brought promising results, however experiments with PD-1 blocking anti-PD-L1 antibodies alone *in vivo* were not as much successful (77, 78). Chronically infected woodchucks, like HBV-infected humans, can have

elevated liver PD-L1 expression and increased display of PD-1 on CD8⁺ cytotoxic T cells. Woodchuck PD-1 and PD-L1 and PD-L2 were cloned and characterized, and antibodies against PD-L1 produced (18, 73). Function of WHV-specific CTLs was significantly enhanced *in vivo* in some woodchucks with CH when anti-PD-L1 antibodies were given together with entecavir (ETV), a clinically used anti-HBV nucleoside analog, and DNA vaccination with plasmids expressing WHc and WHs antigens (19). In more recent study, the effect of anti-PD-L1 in combination with ETV was only seen in a minority of chronically infected animals (73). Nonetheless, this approach may represent valuable therapeutic strategy for CH type B after further improvements in consistency and durability of the T cell response.

SOI continuing after recovery from an episode of AH is associated with low levels of T cell response toward WHV antigenic epitopes which is intermittently detectable throughout lifetime (Figure 4). This profile of T cell reactivity during SOI closely resembles the profiles of proliferative and CTL responses against HBV in patients who resolved AH type B (37, 48) who, like woodchucks, continue to carry after SLAH traces of replicating virus for years. It is now acknowledged that the residual transcription of small amounts of viral proteins provides continuous antigenic stimulation that maintains an active antiviral immune response during occult infection. This response sustains persisting virus at levels which may no be longer liver pathogenic; however, this control may fail and reactivation of hepatitis may occur (32, 45).

The features of WHV-specific T cell response were also investigated in POI and after challenge of woodchucks with POI with liver pathogenic or non-pathogenic doses of WHV (79). Similarly as AH, POI was associated with the delayed appearance of WHV-specific T cell proliferative response against multiple virus epitopes (53). This T cell reactivity persisted intermittently at low levels as it was seen in the course of SOI. Like in WHV AH, immediately after inoculation with WHV establishing POI, lymphocytes displayed an augmented capacity to proliferate in response to mitogenic stimuli prior to arise of virus-specific response (79). Interestingly, the profiles of both virus-specific and generalized T cell proliferative responses were again very similar to those observed after infection with liver pathogenic doses (Figures 3, 4). These results well-supported the view that WHV-specific T cell reactivity is an extremely sensitive indicator of exposure to hepadnavirus, even to amounts as low as 10 virions (31). However, there were two major differences between POI and SOI considering immune response. In contrast to SOI, POI was not accompanied by anti-viral antibodies, including anti-WHc which as anti-HBc normally accompany WHV or HBV infection. Another distinctive feature was that POI did not induce protective immune response against WHV, while infection with liver-pathogenic doses leading to SOI invariably did (52, 53, 79). This confirmed a central role of humoral anti-viral immunity in protection against re-exposure to hepadnavirus. Overall, the discrepancy between virus-specific cellular and humoral responses to infection with a low dose of WHV was consistent with the data from other asymptomatic infections, including those with hepatitis C virus, human immunodeficiency virus type

1 or SIV (80–84). In these infections, T cell responses occurred in the absence of serological or pathological signs of infection. Comparable findings were reported for humans exposed to HBV (85).

THE IMMUNE SYSTEM AS SITE OF REPLICATION AND LIFELONG PERSISTENCE OF WHV

It became evident during studies of the woodchuck model that the immune system is the site of productive WHV replication as well as reservoir of persisting virus independent of whether infection is symptomatic or occult (Figure 2). Infectivity of WHV derived from lymphoid cells of infected animals and from *in vitro* infected immune cells have been comprehensively documented and reviewed (5, 9, 29–31, 46, 47, 86–93).

Among others, the method of WHV DNA identification within intact lymphoid cells by employing *in situ* PCR combined with flow cytometric quantification of cells containing WHV amplicons was established to enumerate infected cells (91). The data showed that 3.4 to 20.4% (mean 9.6%) of the circulating lymphoid cells carried WHV DNA in animals with AH and CH, while cells from SOI or POI were positive at lower numbers ranging from 1.1 to 14.6% (mean 4.4%) (91). There was no meaningful difference in numbers of lymphoid cells carrying WHV genome between SOI and POI, which was consistent with comparable loads of WHV DNA in PBMC as measured by PCR-NAH (29, 30, 46, 94, 95).

To investigate whether WHV ability to infect immune cells is a feature of wild-type virus or a result of a particular virus variant predisposed to infect these cells, WHV with a homogenous sequence in both the liver and the immune system was subjected to serial passage in woodchuck hepatocytes and splenocytes (92). It was hypothesized that such repeated passage should enrich a lymphotropic variant if it exists. This repeated passage did not lead to the appearance of cell type-specific WHV variants, has not changed virus cell tropism or its infectivity when administered to WHV-naïve woodchucks. The resulting infection profiles in the animals infected dependent upon virus dose but not on virus cellular origin, and the virus retained its initial DNA sequence.

Although hepatocytes and lymphoid cells are targets of HBV and WHV (96–109), there is very limited knowledge regarding the receptor/s determining these viruses lymphotropism. However, it was demonstrated that there is a protease-activated cell binding site in the large preS1 protein of WHV envelope that facilitates *in vitro* binding to woodchuck hepatocytes and lymphoid cells (110, 111). The core epitope of this site was mapped to amino acid residues 10–13 at the N-terminal sequence of the preS1 protein. Peptides comprising this site sequence interacted with woodchuck hepatocytes and lymphoid cells with characteristics of a specific ligand-receptor interaction. Interestingly, their ability to bind lymphoid cells was about 1000-fold greater than that for hepatocytes. This cell recognition site was found to be protected within the WHV preS1 protein tertiary structure and its activity was not identifiable until the protein was subjected to limited digestion with proteases. The antibodies

directed against the site appeared as the first immunological indicator of infection in animals experimentally infected with WHV, indicating that proteolytic cleavage of this protein also occurred *in vivo* (111). In addition, the involvement of a 330-kD proteoglycan, as well as heparan sulfate and polymannose in the binding of WHV envelope to hepatocyte and splenocyte plasma membranes was described (112).

INTERACTIONS BETWEEN WHV PROTEINS AND HEPATOCYTE SURFACE PLASMA MEMBRANE AND THEIR SIGNIFICANCE

Hepatitis caused by hepadnaviral infection is in principle a result of the immune response directed against viral peptides carrying epitopes which are presented at the hepatocyte surface in the context of major histocompatibility complexes (MHC). These epitopes are recognized by the epitope-specific CD4+ and CD8+ T lymphocytes which perform supportive (helper) and cytotoxic functions directed against infected cells, respectively (113, 114). However, viral proteins are also directly incorporated into hepatocyte plasma (surface) membranes (HPM) as integral proteins or loosely linked with the HPM structure as peripheral plasma membrane proteins (Figure 5). These HPM-associated viral proteins can themselves serve as targets of cytopathic reactions and potentially modify the extent of immune-mediated liver damage.

In the HBV infection known as asymptomatic, healthy chronic carriage of HBsAg and in the equivalent infection in woodchucks, normal or nearly normal liver histology and unaltered hepatic biochemical functions coincide with the vast quantities of circulating HBsAg or WHsAg, respectively (121, 122). This is accompanied by abundant deposits of virus envelope material in the cytoplasm and in the proximity of the hepatocyte surface in the majority, if not in all, hepatocytes. This naturally occurring situation seemingly perfectly exemplify the state of immune tolerance in the presence of excess of foreign immunogen. The above observations triggered a question whether there is a relationship between the status of hepadnavirus protein association with HPM and the form of inflammatory liver injury.

In a series of studies, HPM purified from woodchucks with different immunomorphological forms of WHV hepatitis and HCC were investigated for the nature of WHV proteins' interaction with the HPM lipid bilayer, the amounts and the molecular profiles of the associated WHV proteins, and the ability of exogenous WHsAg to interact with HPM (115–118). Regarding WHV nucleocapsid (also called core or WHc) proteins, their levels and molecular species displayed by infected HPM were not related to form, severity or duration of hepatitis (Figure 5) (118). Notably, two WHc proteins with molecular weights of 22 and 43 kD were identified in HPM from both AH and CH. Quantitation of the WHc content in HPM from AH (mean 1.62 ± 0.96 ng/ μ g HPM protein) and CH (mean 1.39 ± 0.39 ng/ μ g HPM protein) were not statistically different (118). Conversely, quantities of the WHs proteins were meaningfully greater in HPM from CH (mean $16.36 \pm$

2.05 ng/ μ g HPM protein) than those from AH (mean 2.60 ± 1.15 ng/ μ g HPM protein) (Figure 5) (118). Molecular profiles determined by Western blotting showed that the WHV preS1, preS2, and S polypeptides, with or without equivalents in the purified serum WHsAg and WHV virions, were detected in all infected HPM. Detection of the WHs polypeptides other than those comprising circulating particles implied that the proteins unique for intracellular processing and not utilized in assembly of viral particles were inserted into HPM. Further, staining with antibodies against WHsAg which recognized all envelope proteins and with monoclonal antibody against WHV preS2 peptide demonstrated that the preS2 protein was dominant in all infected HPM (118).

Examination of HPM from animals with AH or CH demonstrated that most of WHc proteins resisted extraction with 6M urea that removes peripheral membrane proteins loosely associated with HPM, indicating their strong bond with the HPM lipid bilayer. Treatment of HPM with Triton X-114 (1%), which dissociates membranes into the hydrophilic and the hydrophobic protein fractions, released the majority of WHc polypeptides confirming their nature as integral proteins. There was no difference in the behavior of the HPM-associated WHc proteins from animals with diverse severity of AH or CH (118).

The majority (~85%) of the proteins carrying WHs specificity was very tightly incorporated into HPM and resisted treatments with strong plasma membrane perturbants (115, 118). Extraction with agents solubilizing the lipid bilayer, such as Triton X-114, Triton X-100 (1%) or deoxycholic acid (50 mM), ascertained their behavior as the integral HPM protein, indicating that their insertion was irreversible (115). This suggested that perhaps only lysis or apoptotic death of infected cells could eliminate these proteins. Furthermore, HPM from animals with CH, but not from healthy woodchucks or these with AH, were unable to bind purified exogenous WHsAg (Figure 5) (118). Similar incapability characterized HPM from chronic WHsAg carriers (123), implying that the HPM potential to interact with WHV envelope was exhausted. This was likely due to a large amount of WHs proteins already incorporated into and associated with HPM and occupation of hepatocyte virus receptors by virus envelope material. However, such exhaustion could also be due to formation of WHsAg-anti-WHs immune complexes. Circulating anti-WHs or anti-HBs antibodies are rarely detectable in CH, however such possibility exists and a contribution of HBsAg-anti-HBs immune complexes to hepatocyte injury in CH was postulated (124). From this perspective, HPM eluates from woodchucks with AH contained immunoglobulins displaying anti-WHc and anti-WHV e antigen (anti-WHe) reactivity, although anti-WHs antibodies were not detected (117).

Taken together, the irreversible incorporation of WHV envelope but not core proteins into HPM lipid bilayer was a specific characteristic of CH (Figure 5). The accumulation of WHs proteins in HPM was not related to CH duration, severity of inflammation or progression to HCC. This raised a concept that such a state could be linked with the development and protraction of liver disease perhaps by creating an immune protective barrier at the hepatocyte surface that shelters virally infected cells from immune elimination. Notably, the increased

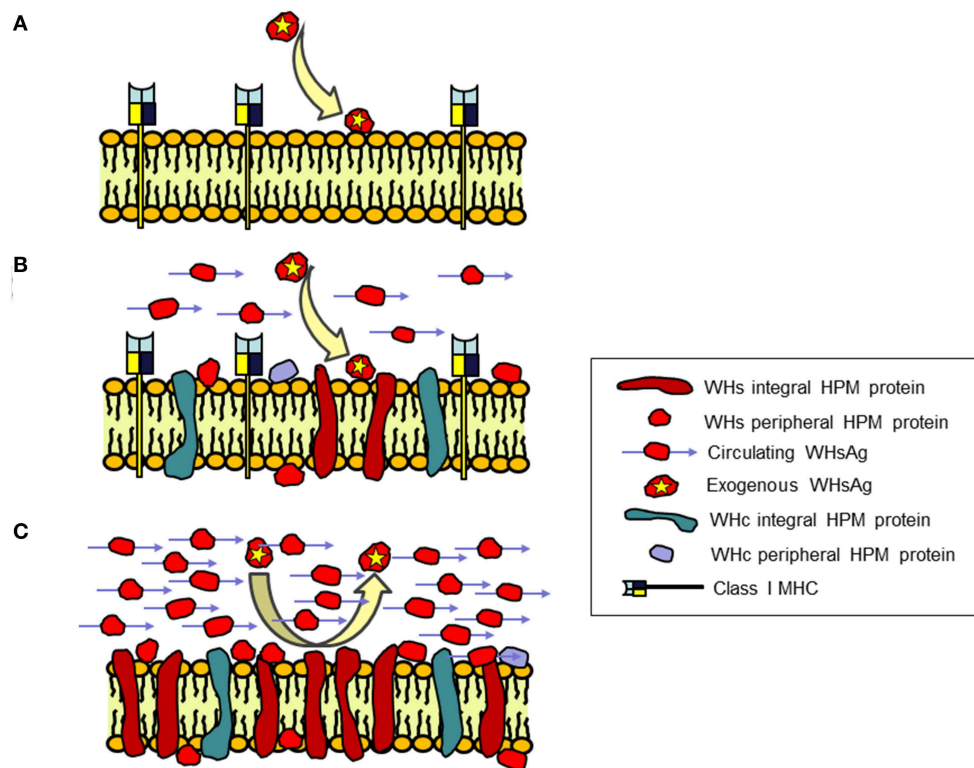


FIGURE 5 | Schematic presentation of the concept of an immune protective barrier at the surface of hepatocytes in chronic WHV infection created by virus envelope (WHs) proteins associated with hepatocyte plasma membranes (HPM) that coincides with depletion of class I MHC on the membranes. HPM from **(A)** Healthy, WHV-negative woodchucks; **(B)** WHV-infected animals with acute hepatitis (AH), and **(C)** WHV-infected animals with chronic hepatitis (CH). CH in contrast to AH is characterized by irreversible incorporation of large (saturating) quantities of WHs proteins into HPM lipid bilayer and presence of WHs reactivity loosely associated with HPM structure behaving as peripheral membrane protein. In addition, CH occurs in the context of large quantities of circulating virus envelope proteins carrying WHs antigenic specificity (WHsAg) and inability of HPM to bind exogenous purified WHsAg in contrast to HPM from healthy woodchucks and animals with AH. WHV core (WHc) proteins are also incorporated as integral and joined with HPM as peripheral membrane proteins, however there are no differences between AH and CH in their HPM quantities and susceptibility to removal by agents dissociating HPM. In addition, HPM from CH are voided of class I MHC presence in contrast to HPM from acutely infected and healthy animals. Together, the concept assumes that WHs proteins incorporate at saturating quantities into hepatocyte surface membranes in combination with large amounts of circulating WHsAg and absence of class I MHC presentation at the membranes form an immune protective barrier that shelters infected hepatocytes from immune elimination. For details see Michalak and Churchill (115), Michalak et al. (116, 117), Michalak and Lin (118), Michalak et al. (119), and Wang and Michalak (120).

display of WHV envelope proteins, particularly pre-S2, was found later to coincide with an inhibition of class I MHC presentation on hepatocytes in CH (119, 120). This occurrence should impact the efficiency of immune elimination of infected hepatocytes by virus-specific CTL (also see section on page 12). Common immunovirological properties of CH and the host immune responses in WHV and HBV infections would suggest that comparable events may happen in CH type B. However, similar studies are not feasible in the HBV-infected individuals mainly due to the lack of liver tissue uncompromised by therapy, comorbidities and/or the-end-of-life events.

In addition to the expected immunomodulatory effect exerted by HPM-associated virus envelope proteins, viral proteins displayed at the hepatocyte surface can be targets of immunopathogenic reactions. As mentioned, the eluates of HPM from animals with AH demonstrated anti-WHc and anti-WHe but not anti-WHs reactivity, although all three antigenic

specificities were detectable in HPM (117). Also, while anti-WHc was readily identifiable, anti-WHe could be detected only in eluates from HPM of animals recovering from AH (117). These findings indicated that hepadnaviral proteins associated with HPM can be recognized by specific antibodies and became targets of hepatocytotoxic reactions. This concept was consistent with demonstration that heterologous anti-HBc and anti-HBs, as well as antibodies to hepatocyte asialoglycoprotein receptor (ASGPR) were cytopathic in the presence of active complement when hepatocytes from treatment-naïve patients with CH type B were used as targets (125). The data also indicated that anti-HBc-directed complement-mediated cytotoxicity was augmented against hepatocytes from individuals with chronic active hepatitis over that against hepatocytes from chronic mild hepatitis or inactive cirrhosis due to HBV infection. Overall, the data suggested that both HBV proteins and ASGPR (also see section on page 12), are recognized by circulating antibodies

and that antibody-mediated cytotoxicity may contribute to hepatocyte damage in HBV infection.

INHIBITION OF CLASS I MHC PRESENTATION ON HEPATOCYTES IN CHRONIC WHV HEPATITIS

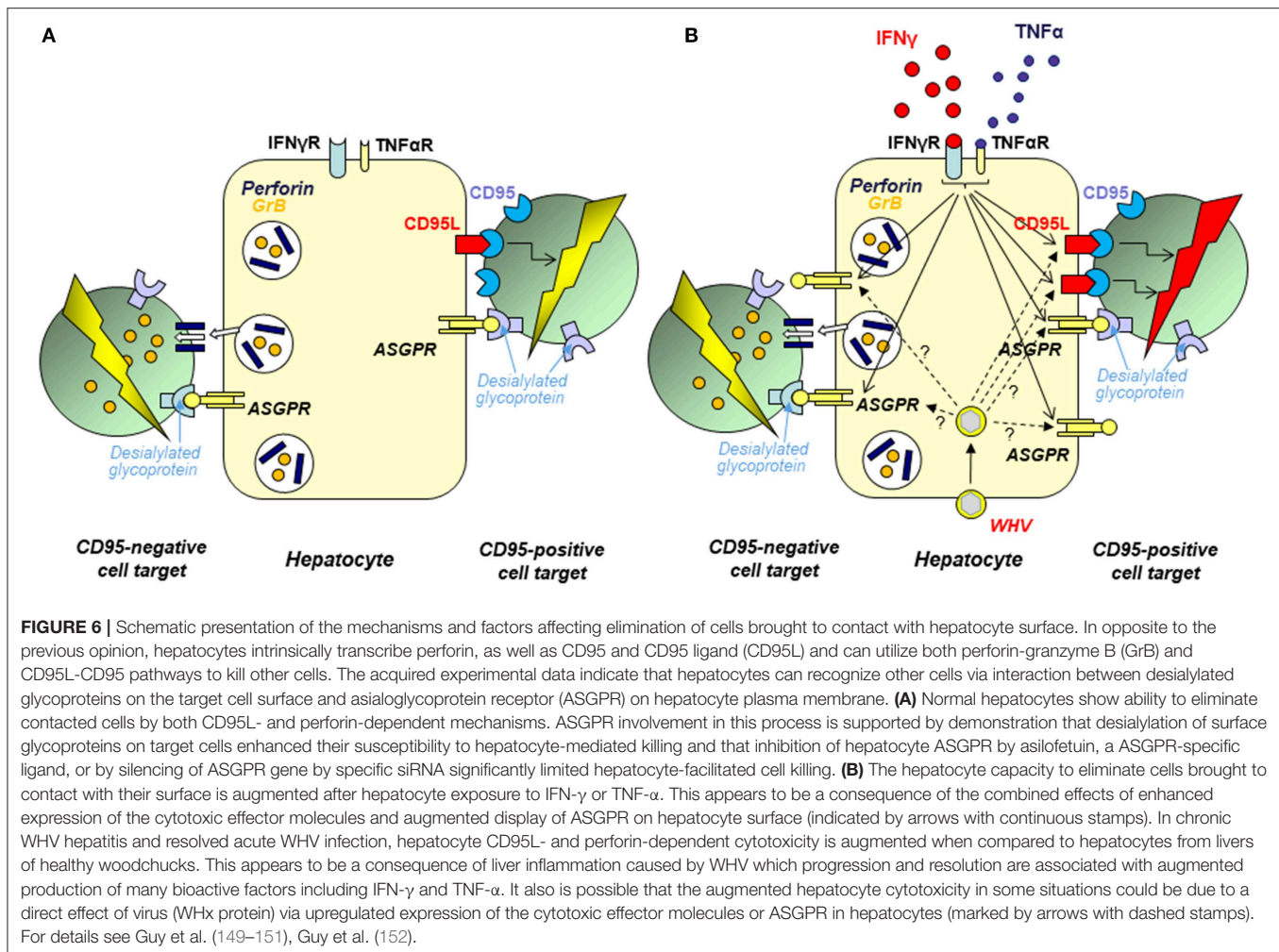
Generation of an antibody against a non-polymorphic epitope of woodchuck class I MHC heavy chain allowed identification of the MHC localization by flow cytometry and immunoblotting methods in woodchucks (126). Presentation of class I MHC was found on normal woodchuck hepatocytes. This was in contrast to the previous assumption based on immunohistochemical staining that normal hepatocytes do not display this complex. Further study of hepatocytes and HPM isolated from animals with WHV hepatitis revealed that AH, but not CH coincided with significantly augmented display of the class I MHC (119). This was associated with the enhanced expression of genes encoding for class I MHC heavy chain, β_2 -microglobulin, transporters associated with antigen processing (TAP1 and TAP2), and IFN- γ . However, despite the similarly increased transcription of these genes in hepatocytes from AH and CH, the class I MHC display was suppressed only on hepatocytes from CH. Furthermore, the level of inhibition was not associated with the histological degree of hepatocellular damage, the severity of inflammation, the hepatic level of IFN- γ expression, and the liver load of WHV. This together implied a profound posttranscriptional inhibition in the class I MHC presentation on hepatocytes in CH and that this represents a common hallmark of CH (119). Interestingly, the class I MHC was also inhibited on splenocytes of chronically infected woodchucks which are also known to support WHV replication (29, 86, 92). Since, the class I MHC is principal to presentation of viral peptides to cytotoxic CD8⁺ T cells, this finding was of an utmost significance to the understanding one of the mechanisms responsible for development and endurance of CH in hepadnaviral infection. Because a similar study has not yet been accomplished in either human or any other animal model of hepadnaviral infection, it is appropriate to emphasize these results.

Subsequent investigations asked which of the WHV proteins could be responsible for the impairment of class I MHC presentation on hepatocytes. To study this issue, WCM-260 hepatocytes isolated from a healthy woodchuck (127, 128) were transfected with the genes encoding individual WHV proteins as well as with the complete WHV genome (120). It was found that the class I MHC display was significantly impaired after transfection with the entire WHV DNA or with genes coding WHV envelope preS2 middle or preS1 large protein, which also contains the preS2 protein. In opposite, transfection of hepatocytes with the WHV X gene alone augmented the class I MHC display, while hepatocytes expressing after transfection WHV major S protein or WHV core alone did not alter the class I presentation on WCM-260 hepatocytes. Interestingly but not surprisingly, hepatocytes treated with woodchuck recombinant IFN- γ reestablished the class I presentation suppressed by the transcription mentioned above (120). Overall, these data implied

that the impaired display of class I MHC on hepatocytes transcribing WHV is a result of posttranscriptional inhibition due to an interference from the virus preS2 protein and that this impairment can be fully reversed by treatment with IFN- γ . In this regard, exposure of primary hepatocytes from WHV-infected woodchucks to woodchuck IFN- γ was also found to upregulate class I MHC transcription (129).

WHV-INDUCED ASIALOGLYCOPROTEIN RECEPTOR AUTOIMMUNITY AND ITS EFFECT ON THE OUTCOME OF HEPADNAVIRAL HEPATITIS

The induction of humoral and cellular autoimmune reactions by viruses is well-documented, while the contribution of virus-induced autoimmunity to the development and maintenance of chronic liver diseases, including viral hepatitis, although postulated for a long time, remained sparsely recognized. Circulating autoantibodies to non-organ and liver-specific antigens frequently accompany CH type B (130, 131). Regarding liver-specific autoantibody responses, autoantibodies to hepatic ASGPR (anti-ASGPR) have been encountered in up to 73% of patients with CH type B (132, 133), but rarely in hepatitis C (<15%) (134). For comparison, approximately 80% of patients with chronic autoimmune hepatitis may carry anti-ASGPR (132, 135). The target for anti-ASGPR is a HPM-associated receptor, a hepatic C-type lectin, which facilitates removal through endocytosis and lysosomal degradation of desialylated glycoproteins carrying galactose-terminal oligosaccharides (136, 137). The structure and biological properties of ASGPR have been well-characterized for human and other species (136–138), including woodchuck (139). The woodchuck ASGPR (WcASGPR) is a hetero-oligomeric complex composed, similarly as human ASGPR, by two polypeptides with molecular masses of 47 and 40 kD (139). Since the expression of ASGPR is essentially restricted to hepatocytes and anti-ASGPR antibodies coincide at a high frequency with HBV infection, it was postulated that HBV may trigger anti-ASGPR which, in turn, could be implicated in liver damage (140). In this context, we have embarked to recognize whether hepadnaviral infection induces ASGPR autoreactivity and what consequences of this response are regarding hepatocyte function and liver pathogenicity in experimental CH. The woodchuck model is certainly well-positioned to answer such questions since a relationship between virus infection and development of autoimmunity can be investigated from the time of virus entry and easily related to the status of autoimmune response before infection. From this perspective, the woodchuck model has been previously used to investigate the impact of hepadnavirus on the induction of non-organ specific autoantibodies (141). It was uncovered that WHV infection undeniably induces a non-organ specific autoimmune response that appears during the incubation period before detection of serum WHsAg and clinical appearance of hepatitis. The presence of smooth muscle autoantibodies (SMA) was most pronounced among the autoantibodies tested, however



their kinetics and levels did not differentiate whether AH resolves or progresses to CH.

The studies on induction of anti-ASGPR in the course of WHV infection and on potential pathogenic roles of this autoimmune response revealed that: (1) Inoculation with WHV triggered anti-ASGPR autoantibodies in healthy woodchucks at a frequency close to 90% (142); (2) Existence of anti-ASGPR reactivity prior to WHV infection was associated with development of CH at significantly greater rate (55.5%) than in woodchucks non-reactive for anti-ASGPR before infection (15.6%) (142); (3) WHV-induced anti-ASGPR antibodies inhibited recognition of asialoglycoprotein by woodchuck hepatocytes and human HepG2 cells suggesting that the induced anti-ASGPR might impair hepatocyte clearance of desialylated proteins (127), and (4) Anti-ASGPR antibodies triggered by WHV were able to induce complement-mediated hepatocytotoxicity implying that they may contribute to the pathogenesis, impact severity, and hinder recovery from liver damage in hepadnaviral hepatitis (127). In another related study, healthy woodchucks which were first immunized with a WcASGPR-closely compatible rabbit ASGPR (RbASGPR) and then infected with WHV advanced at a higher frequency to

CH, while animals with ongoing CH challenged with RbASGPR demonstrated exacerbated histological degree of liver injury when compared to the unchallenged animals with CH (143). In general, the data pointed out that immune response directed to hepatocyte ASGPR has potential to modify the severity and the outcome of hepadnaviral hepatitis and likely liver status in other diseases accompany by this autoreactivity.

HEPATOCTES AS CYTOTOXIC EFFECTORS CAPABLE OF CELL ELIMINATION BY BOTH PERFORIN AND CD95 (FAS) LIGAND-DEPENDENT PATHWAYS

It is now acknowledged that the liver is an immunologically competent organ which plays important roles in maintenance of peripheral immune tolerance and surveillance over the gut originating pathogens. While the contribution of Kupffer cells and sinusoidal endothelial cells to the hepatic immune responses is relatively well-recognized (144–148), involvement of hepatocytes, which number reaches $\sim 8 \times 10^{11}$ and they

comprise ~80% of the adult liver, remained unknown. We uncovered that hepatocytes can function as cytotoxic effectors and constitutively display ability to eliminate cells directly contacted with their surface *via* both perforin-granzyme B (GrB) and CD95 ligand (CD95L)-CD95 (formerly Fas ligand-Fas) death pathways (**Figure 6**) (149, 152). These were unexpected findings since the ability to eliminate cells *in vivo* was previously known only regarding immune cells, such as CTLs, which are endowed with the same cytotoxic mechanisms. Related data showed that hepatocyte cell killing can be differentially modified by cytokines. Thus, while IFN- γ and tumor necrosis factor alpha (TNF- α) upregulate hepatocyte expression and usage of CD95L, the ability of hepatocytes to eliminate cells *via* perforin-GrB was unaltered upon exposure to either cytokine (152) (**Figure 6**). Remarkably, it was also uncovered that hepatocyte cytotoxic potency depends upon interaction of terminally desialylated glycoproteins on target cells with ASGPR on the hepatocyte surface (150). While the most acknowledged role of ASGPR is the removal of desialylated glycoproteins (for more details see section on page 12), it has also been postulated that the receptor facilitates the trapping of activated lymphocytes in the liver. In this regard, it was reported that activated T cells carrying the B220 epitope, a CD45 molecule depleted of sialic acid, accumulate in the livers of CD95-deficient mice (153). Binding of activated T cells by hepatic ASGPR could facilitate the retention of the cells and their removal *via* an apoptotic mechanism involving the death signaling *via* CD95 (153, 154). Based on the data implying that hepatocytes can act as cytotoxic effectors, we hypothesized that they may play a role in removal of activated T cells (150). To test this possibility, we investigated whether hepatocyte surface ASGPR could be directly involved in recognition and removal of activated T cells. The results demonstrated that desialylation of glycoproteins on the surface of T cells augmented their hepatocyte-mediated apoptosis *in vitro*, while inhibition of hepatocyte ASGPR binding by a soluble ligand, such as asialofetuin, or silencing of the ASGPR gene by small interfering RNA (siRNA) significantly reduced hepatocyte-mediated cell killing. The study also revealed that hepatocytes can wipe out affinity-purified, mitogen-activated CD4+ T lymphocytes brought into contact with their surface (150).

It was further shown that both CH and resolved AH are associated with enhanced hepatocyte cytotoxicity that is dependent on an increased activity of both CD95L-CD95 and perforin-granzyme B mediated pathways (**Figure 6**) (151). This was supplemented by the study utilizing intact woodchuck WCM-260 hepatocytes and WCM-260 stably transfected with WHV DNA or singular WHV genes (120). We uncovered that hepatocytes treated with IFN- γ , but not those transfected with the complete WHV genome or individual WHV genes, excluding the WHV X gene, displayed augmented cytotoxicity facilitated by both CD95L and perforin. This implied that increased intrahepatic production of IFN- γ rather than virus replication itself led to increased hepatocyte-mediated cell death. In this context, an increased hepatic transcription of IFN- γ was reported in woodchucks during both CH and SOI continuing after resolution of AH, as well as in POI (16, 53). Interestingly, hepatocytes transfected with the WHV X gene

alone displayed significantly higher levels of CD95L and perforin, and killed cell targets more efficiently (151). This suggested that WHV under certain circumstances may directly heighten hepatocyte cytotoxicity.

The studies summarized above for the first time revealed the cytotoxic phenotype of hepatocytes and showed that they are naturally equipped with the machinery facilitating death of other cells *via* perforin-granzyme B and CD95L-CD95-mediated pathways (**Figure 6**). The data also suggested that the recognition of target cells by hepatocytes involves ASGPR that recognizes desialylated glycoproteins on the surface of cells predestined for elimination. The appearance of these proteins is a natural outcome of the glycoproteins physiological usage, including activation of T lymphocytes (155–158). It was also shown that hepatocytes can directly eliminate activated T cells. This intriguing finding suggests that hepatocytes may actively contribute to local immune regulation and moderation of inflammation. Thus, hepatocytes might be much more than passive targets of immune reactions and may function as immunological effectors. Whether hepatocytes directly contribute to outcome of viral hepatitis *via* immunoregulation and/or contraction of inflammation remains to be established. Nonetheless, examination of the mechanisms underlying liver pathology should consider hepatocytes as potentially immunologically competent participants.

PREDICTORS OF SPONTANEOUS RESOLUTION OF ACUTE INFECTION AND ITS PROGRESSION TO CHRONIC HEPATITIS

Woodchucks infected with WHV display pattern of liver disease and age-dependent rates of development of CH similar to those in human HBV infection. In search for indicators capable of predicting in advance whether hepadnaviral infection will resolve or progress to CH, liver biopsies collected during AH from adult animals which finally either resolved AH or developed CH after administration of the same dose of WHV inoculum were investigated (16). The dynamics of intrahepatic expression of selected cytokines, liver T cell influx, histological severity of hepatitis, and serum and hepatic WHV loads were assessed. The data revealed that recovery from AH was characterized by a significantly greater hepatic expression of IFN- γ and CD3, an indicator of T cell infiltration, an increased transcription of TNF- α , a greater histological severity of liver inflammation, and by lower hepatic loads of WHV than those detected in animals in which AH advanced to CH. For instance, the estimated liver load of WHV during AH was 2.7-fold lower for animals that resolved AH than for those which established CH. The study also revealed that the elevated hepatic transcription of IFN- γ , TNF- α , and CD3 endured for years after resolution of AH. This was in agreement with the findings of residual WHV replication and minimal intermittent liver inflammation continuing normally for life after seemingly complete resolution of AH (29). Evaluation of similar parameters in liver biopsies acquired from healthy woodchucks in which the outcome of WHV AH was known

did not demonstrate such predictive value. Overall, the study documented that the development of CH can be predicted well in advance by analyzing liver tissue in the acute phase of infection.

The above study was completed in the adult animals in which AH either subsided or advanced to CH in the setting of the mature immune system. It is noted that woodchucks infected as adults with liver pathogenic doses of WHV develop CH at the rate not >25–30%. This is in contrast to WHV infection acquired in the neonatal period, which likely due to the immunological immaturity of the host, establishes CH in the majority of neonates. This somewhat resembles a situation in children born to mothers chronically infected with HBV or those exposed to virus very early in life which tend to develop much higher rates of CH than adults if untreated. Despite significant immunological differences between adults and neonates, the parameters typifying the early phase of WHV infection progressing to recovery or CH identified in the adult woodchucks were closely comparable to those reported for neonatal infection (159, 160). Hence, recovery from AH in the neonatal period coincided with greater levels of hepatic IFN- γ and TNF- α transcription, augmented severity of hepatitis, and with lower hepatic loads of WHV. Taken together, despite significantly different frequencies of spontaneous recovery or progression to chronic disease in adult and neonatal animals, the same liver parameters measured in the acute phase of infection predict outcome of hepatitis.

TOLL-LIKE RECEPTOR 1–10 EXPRESSION IN DIFFERENT FORMS OF WHV INFECTION AND STAGES OF EXPERIMENTAL HEPATITIS

Toll-like receptors (TLRs) are important mediators of immune responses which contribute to immune recognition of microbes and to the immunopathogenesis of diseases and cancers in general. There is now a wealth of data on the TLRs' expression, their functions and roles in a variety of diseases. A significant progress has also been made in recognition of TLRs' role in the pathogenesis of hepatitis B and in therapeutic potential of their antagonists in HBV infection (25). The woodchuck model plays a particularly important role in these studies. As an example, a potent agonist of TLR-7 (GS-9620) has been developed and tested in chimpanzees and woodchucks, and its evaluation has advanced to clinical trials. The results indicate that both innate and adaptive immunity contribute to successful antiviral response (161–164). However, the response was not uniformly effective among animals and patients treated. Recently, a new TLR-7 activating compound (APR002), designed to be preferentially delivered to the liver, was investigated in WHV-infected woodchucks in combination with ETV (165). The virological and immunological equivalence of functional HBV cure was achieved in some animals which appeared to be due to immunological modulation rather than augmented antiviral efficacy of the treatment (166). In another recent study, agonist of TLR-8 (GS-9688) has shown ability of sustained inhibition of WHV replication in some chronically infected woodchucks

when administered alone, as evidenced by decline in serum WHsAg and hepatic WHV cccDNA to levels undetectable by the assays employed (22). In this context, a variety of factors among which individual discrepancies in the TLRs' expression and cooperation between TLRs and functionally important downstream molecules, and individual differences in the kinetics of virus infection and in the degree of inflicted inflammation and liver damage could play a role.

Considering the above, comprehensive recognition of the TLR expression profiles in sequential stages of WHV infection and hepatitis was recently completed (167). The assessment of the transcription profiles of woodchuck TLRs 1 to 10 in serial liver biopsy and PBMC samples from the pre-infection and pre-acute periods to AH followed by SLAH and SOI or CH, and from animals with POI was made. This was supplemented by analysis of the TLR transcription profiles in whole livers and in primary hepatocytes isolated from these livers. This last comparison was important to determine how TLRs' expression differs between hepatocytes, which are the principal site of virus replication, and the liver where TLRs' transcription could be influenced by cells of inflammatory infiltrations, sinusoidal lining and by blood cells within hepatic vasculature. Since there are not yet specific antibodies against the majority of woodchuck TLRs and molecules facilitating their downstream effects, quantitative TLRs' gene expression analysis provided the most direct approach to recognize TLR characteristics of different stages of WHV infection and associated liver disease. The study generated an abundance of data among which the most interesting showed that liver biopsies from AH and CH demonstrated significantly enhanced transcription of the majority of TLRs when compared to healthy woodchucks and animals with other stages of infection. Also, in contrast to whole liver tissue, hepatocytes from CH displayed significantly lower transcription or a trend toward suppression of several TLRs when compared to hepatocytes from healthy woodchucks and animals with other forms of infection or hepatitis. This implied that hepatocyte innate immune response is in fact downregulated in the course of CH. In contrast to hepatocytes, upregulated expression of some TLRs characterized PBMC during CH. Overall, the study indicated that: (1) TLRs' expression widely varied between different forms of infection and to a significant degree depended on whether infection was accompanied or not by hepatitis; (2) Analysis of the TLRs' transcription in hepatocytes provided more distinct association with form of infection or stage of hepatitis than analysis of total liver tissue. This was best exemplified in CH and SOI continuing after SLAH, and (3) TLRs' expression profiles in PBMC overall poorly mirrored those in livers and hepatocytes from infected animals. The study also delineated the profiles of TLRs 1–10 expression during sequential stages infection in individual animals from the healthy state to AH/SLAH/SOI or AH/CH and the baseline TLRs' expression in healthy woodchucks.

Potential applications of TLRs in therapy of CH type B which were examined in the woodchuck model were reviewed previously (12, 25, 168). Among others, it was shown that activation of TLR-2-dependent innate immune response was associated with a decreased replication of

WHV in primary woodchuck hepatocytes and HBV in HBV-transcribing HepG2.2.15 cells (169). This coincided with the activation of NF- κ B and cell MAPK/ERK and PI3K/Akt signaling pathways, as well as with production of pro-inflammatory cytokines by woodchuck hepatocytes. WHV infection modulated TLR-2 expression on PBMC and this reversely correlated with WHV DNA levels in woodchucks with AH and in animals with CH treated with ETV. In related study, TLR-2 agonists transiently enhanced production of IL-6 and TNF- α , increased CD8 $^{+}$ T cell response, and augmented clearance of HBV in the hydrodynamic injection model of HBV infection (170).

CONCLUDING REMARKS

The woodchuck model of hepatitis B has significantly contributed to the recognition of the natural history of hepadnaviral infection and the biological and pathogenic properties of hepadnaviruses, and to identification and characterization of occult HBV infection and its pathological outcomes. Reproducible experimental systems to investigate primary occult infection persisting indefinitely as a serologically and clinically silent but molecularly evident infection and secondary occult infection that continues in the presence virus-specific antibodies after resolution of a symptomatic infection were established. The woodchuck model significantly contributed to the identification of the pivotal, although not yet fully appreciated, role of the immune system as a reservoir of biologically competent hepadnavirus, even when the liver is seemingly not involved. The model assisted in the discovery of an extensive range of molecular and immunological mechanisms underlying development of liver disease of which some should be further explored, but many others await discovery. The uniqueness of the model relies on the close virological and pathogenic similarity to a human disease situation and on the unaltered environment in which interactions between virus and host occur. As such, the woodchuck-WHV

infection should remain the standard for the discovery and validation of new pathogenic mechanisms and for preclinical drug testing.

AUTHOR CONTRIBUTIONS

TM conceived, designed, and wrote the manuscript.

FUNDING

The studies from the author's laboratory summarized in this review were supported by operating grants MA-9256, MT-11262, MT-14818, RO-15174, MOP-14818, and PJT-153001 from the Canadian Institutes of Health Research (CIHR), formerly the Medical Research Council of Canada, Ottawa, Canada awarded to TM. TM was a recipient of the Senior (Tier 1) Canada Research Chair in Viral Hepatitis/Immunology sponsored by the Canada Research Chair Program and funds from the CIHR, the Canada Foundation for Innovation, and Memorial University, St. John's, NL, Canada.

ACKNOWLEDGMENTS

The author thanks graduate and undergraduate students, postdoctoral research fellows and staff members from the Molecular Virology and Hepatology Research Group and the Woodchuck Viral Hepatitis Research Facility at the Faculty of Medicine, Memorial University of Newfoundland for their contributions summarized in this publication. He in particular wishes to thank Patricia M. Mulrooney-Cousins, Norma D. Churchill, Colleen L. Trelegan, Carla S. Coffin, Shashi A. Gujar, Clifford S. Guy, Jinguo Wang, Paul D. Hodgson, Tram N. Q. Pham, Jingyu Diao, Yan-Yee Lew, Charles J. Ming, Ingrid U. Pardoe, Annie Y. Chen, Ranjit Chauhan, John B. Williams, Christopher P. Corkum, Danielle P. Ings, and Jacques R.B. De Sousa.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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