Original article

Reduced Toll-like receptor 9 expression on peripheral CD14+ monocytes of chronic hepatitis B patients and its restoration by effective therapy

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Background: Chronic hepatitis B (CHB) patients display Toll-like receptor 9 (TLR9)-dependent defective immune responses. We aimed to study TLR9 expression on CHB patients and its alteration during therapy.

Methods: We compared TLR9 expression on fresh peripheral CD14+ monocytes from a cohort of 97 CHB patients and 35 HBsAg-negative, anti-HCV-negative controls, during pegylated interferon or entecavir therapy. TLR9 expression on liver tissue was also investigated.

Results: Compared with controls, peripheral CD14+ monocytes of CHB patients displayed reduced expression of TLR9 mean fluorescence intensity (MFI; 9.90 ±3.64 versus 7.95 ±3.61; *P*=0.007) independent of age, gender and alanine aminotransferase (ALT; -2.09, 95% CI -3.568, -0.613; *P*=0.006). Furthermore, age, gender, ALT, HBeAg status, quantitative HBsAg (qHBsAg) or HBV DNA did not predict the TLR9 expression (*P*=0.863). Hepatic TLR9 messenger RNA (mRNA) was significantly reduced in 54 patients compared with 3 controls (0.45 ±0.32 versus 1-fold). Using response-guided therapy by qHBsAg levels and pretreatment TLR9 MFI as a reference, TLR9 MFI restored to a mean of 1.7- to 2.7-fold in pegylated interferon responders and reduced to a mean of 0.6- to 0.7-fold in non-responders starting from treatment week 12. Among 10 entecavir-treated patients, TLR9 MFI gradually restored to a mean of 1.2- to 2.1-fold starting from treatment week 48.

Conclusions: CHB patients display reduced TLR9 expression on peripheral CD14+ monocytes, which is independent of host and viral markers, and on liver tissue. Responders to pegylated interferon and those under entecavir demonstrate restoration of TLR9 expression. On-treatment TLR9 expression on peripheral monocytes might predict response to pegylated interferon therapy.

Introduction

HBV is a DNA virus that infects about 2 billion people in the world, and 350 million of them become chronic carriers of the virus [1]. The infection can cause acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma [1,2]. The pathogenesis of HBV infection involves interactions between virus and host immune responses [3]. In recent years, our understanding of innate immunity is much improved due to the discovery of pathogen-associated pattern recognition receptors, including the Toll-like receptor (TLR).

TLR9 is known to recognize CpG DNA, and human TLR9 has a significant homology with TLR3,

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7 and 8, which also recognize viral nucleic acids [4]. In transgenic mice models, TLR3, TLR4, TLR5, TLR7 and TLR9 ligand binding inhibit HBV replication in an α/β interferon (IFN)-dependent manner [5]. HBV might escape host immune control by inhibition of TLRs. We have shown the reduction in TLR3 expression on peripheral CD14+ monocytes and liver tissue of chronic hepatitis B (CHB) patients, which can be restored in IFN responders and by entecavir (ETV) therapy [6]. CHB patients have deficient plasmacytoid dendritic cells (pDCs) with reduced TLR9 expression [7]. In addition, HBsAg inhibits TLR9-mediated IFN-α production in pDCs [8]. Furthermore, HBsAg inhibits TLR9-mediated IFN-α production on peripheral monocytes from healthy controls [9]. Thus, CHB patients display TLR9-mediated impaired IFN-α production and defective immune responses. TLR9 signalling demonstrates therapeutic potential by inducing massive expansion of cytotoxic T-lymphocytes in mice liver leading to eradication of chronic HBV infection [10]. However, TLR9 expression on peripheral CD14+ monocytes of CHB patients and its alteration during therapy remain unclear. We aimed to investigate TLR9 expression on peripheral CD14+ monocytes of CHB patients and its confirmatory expression on liver tissue, as well as its alterations during pegylated (PEG)-IFN or nucleoside analogues therapy.

Methods

Subjects

In this prospective cohort study, we consecutively enrolled 97 CHB patients and 35 HBsAg-negative, anti-HCV-negative individuals as controls from June 2009 to August 2012 at the Cathay General Hospital Medical Center (Taipei, Taiwan). CHB was defined as the presence of HBsAg for ≥6 months before enrolment. Patients who were treated with nucleoside/nucleotide analogues or (PEG)-IFN within 1 year, were infected by HCV, hepatitis D or HIV, were receiving immunomodulators, corticosteroids or chemotherapies, or who had autoimmune antibodies, thyroid diseases, metabolic liver diseases, cirrhosis, hepatocellular carcinomas, alcohol abuse, or had undergone transplantations were all excluded. The institutional review board of the Cathay General Hospital approved the study and written informed consent was obtained from each participant.

Patients received either PEG-IFN or ETV treatment based on AASLD guidelines [11]. Responders to PEG-IFN therapy were defined as >10% of decline in quantitative HBsAg (qHBsAg) at treatment week 12 [12]. Non-responders were defined as no decline in qHBsAg and <2 log decline in serum HBV DNA levels at treatment week 12 [12,13].

TLR9 expressions on peripheral CD14+ monocytes of non-HBV non-HCV controls at enrolment and CHB patients before and during PEG-IFN or ETV therapy

Fresh whole blood was drawn from controls at enrolment and CHB patients before and during therapy. Cell surface staining was performed using CD14-FITC, TLR9-PE (eBioscience, San Diego, CA, USA). Rat IgG2a isotype control was used for TLR9 (eBioscience). A total of 50,000 viable cells were collected on a FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA, USA). We gated monocytes based on their scatter profile by picking up the lymphocyte tail and CD14+ cells. We obtained the mean fluorescence intensity (MFI) and percentage of each sample gated on both the scatter profile and CD14+ cells. A total of 50,000 CD14+ monocytes were used for each sample reading. Data was analysed using WinMDI software (Becton Dickinson & Co., Franklin Lakes, NJ, USA).

TLR9 expressions on liver tissue of CHB patients and controls

Liver biopsy specimens from 54 CHB patients and 3 non-HBV non-HCV controls were freshly frozen or immersed into optimal cutting temperature (OCT) compound (Ames Company, Elkhart, IN, USA) and kept at -80°C until use.

RNA extraction and real-time reverse transcriptase PCR

Total RNA was isolated using the RNeasy Kit (Qiagen Inc., Valencia, CA, USA). For reverse transcription, 1 μg of total RNA were transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Real-time reverse transcriptase (RT)-PCR was performed in a DNA Engine Opticon 2 (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). TLR9 primers were as follows: forward primer 5′-CCC ACC TGT CAC TCA AGT ACA-3′; reverse primer 5′-GTG GCT GAA GGT ATC GGG ATG-3′. GAPDH primers were as follows: forward primer 5′-GTC CAC TGG CGT GTT CAC CA-3′; reverse primer 5′-GTG GCA GTG ATG GCA TGG AC-3′. The amplification mixture (20 μl) contained 25 ng of sample RNA (5 μl), 2× Master Mix (10 μl), 5 μM forward and reverse primers (2 μl), and 3 μl ddH20 (Kapa Biosystems, Inc., Woburn, MA, USA). Thermal cycling parameters were as follows: one cycle of 3 min at 95°C for enzyme activation, 40 cycles at 95°C for 10 s and at 58°C for 30 s for the denaturing, annealing/extending phases of the PCR reaction, respectively. Each assay included approximately 25 ng of sample total RNA in duplicate. We used GAPDH messenger RNA (mRNA) as an internal control. The relative amount of tissue TLR9 mRNA, standardized against the amount of GAPDH mRNA, was expressed as

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$-\Delta C_T$=-[$C_{T(TLR9)}$-$C_{T(GAPDH)}$]. The ratio of the number of TLR9 mRNA copies to the number of GAPDH mRNA copies was then calculated as $2^{-\Delta CT}$×K, where K is a constant.

Laboratory assays

Serum qHBsAg, HBeAg and anti-HCV were tested by Architect i2000 SR (Abbott Laboratories, Abbott Park, IL, USA). Quantification of serum HBV DNA was assayed by Abbott m2000 sp (Abbott Laboratories). HBV genotyping was determined by melting curve analysis [14].

Statistical analyses

Continuous variables were evaluated by Student's *t*-test. Categorical variables were expressed as frequencies with proportions and compared using Pearson's χ^2 test. All of the tests were two-tailed and a *P*-value of <0.05 was considered statistically significant. Linear regression analysis was applied for predictors of TLR9 expression on peripheral CD14+ monocytes using SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA). Male gender was categorized as '1' and female as '0'. CHB patients were categorized as '1' and controls as '0' in the regression analysis.

Results

TLR9 expression on peripheral CD14+ monocytes and liver tissue of CHB patients versus controls

Baseline characteristics of 97 consecutively enrolled CHB patients and 35 non-HBV non-HCV controls are shown in Table 1. Among the CHB patients, there were more males and alanine aminotransferase (ALT) levels were higher than controls. MFI of TLR9 expression on peripheral CD14+ monocytes of CHB patients was significantly lower than that of controls (7.95 ±3.61 versus 9.90 ±3.64; *P*=0.007; Figure 1A). Linear regression analysis on predictors of TLR9 MFI on peripheral CD14+ monocytes is shown in Table 2. Compared with controls, peripheral CD14+ monocytes of CHB patients had lower expression of TLR9 MFI independent of age, gender and serum ALT level (-2.09, 95% CI -3.568, -0.613; *P*=0.006; Table 2). Age, gender, HBeAg status, serum ALT, qHBsAg or serum HBV DNA level were not predictive of TLR9 MFI on peripheral CD14+ monocytes of CHB patients (*P*=0.863; Table 2). In addition, hepatic TLR9 mRNA expression was lower in 54 CHB patients than 3 controls (0.45- ±0.32-fold versus 1-fold; Figure 1B).

TLR9 expression on peripheral CD14+ monocytes of CHB patients during PEG-IFN or ETV therapy

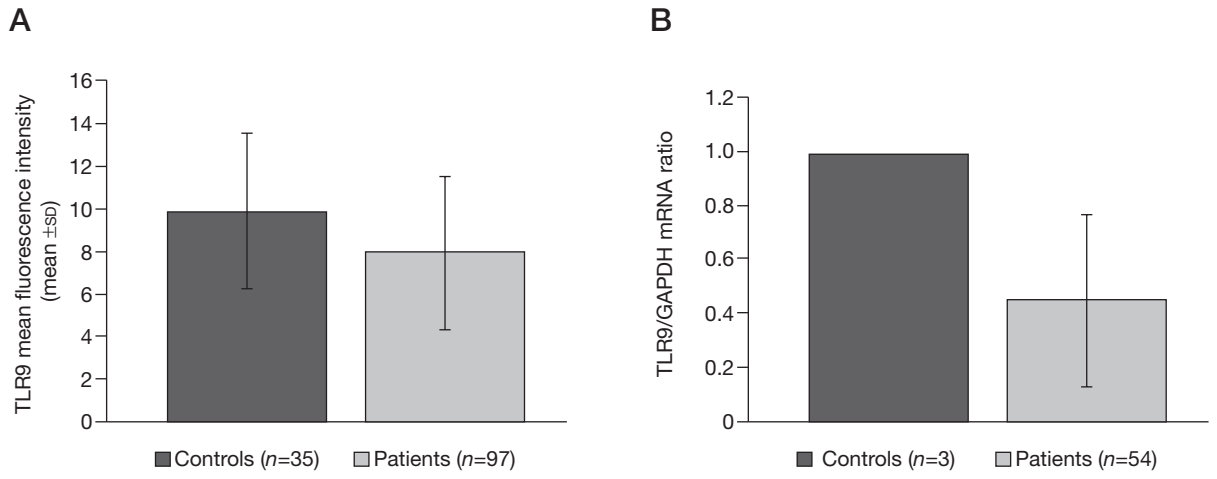
There were three responders (male/female *n*=3/0, mean [range] age 43 years [37–47], HBeAg-negative 100%, log HBV DNA 5.6 ±2.0 IU/ml and HBV genotype B 66.7%) and five non-responders (male/female *n*=5/0, mean [range] age 36 years [31–45], HBeAg-negative 40%, log HBV DNA 6.4 ±1.5 IU/ml and HBV genotype B 100%) to PEG-IFN therapy. Using pretreatment TLR9 MFI as a reference, fold changes of TLR9 MFI during PEG-IFN therapy are shown in Figures 2 and 3. Among responders to PEG-IFN therapy, TLR9 MFI restored to a mean of 1.7- to 2.7-fold starting from treatment week 12 (Figure 3) and restored to a mean (range) of 1.7-fold (1.3–2.3) at 18 to 34 weeks post-treatment (Figure 2A). Among non-responders, TLR9 MFI reduced to a mean (range) of 0.57-fold (0.50–0.64) in three patients with available data at treatment week 12 (Figure 3) and reduced to a mean (range) of 0.67-fold (0.45–0.84) at treatment week 24 (Figure 2B).

Table 1. Baseline characteristics of chronic hepatitis B patients and healthy controls

Characteristic	CHB patients	Controls	*P*-value
Participants	97	35	–
Male	69 (71)	15 (43)	0.004[a]
Age, years	42.71 ±11.34	41.06 ±8.22	0.431[b]
ALT, U/l	118.25 ±344.73	19.97 ±8.61	0.095[b]
HBeAg positivity	36 (37)	NA	–
Log_{10} qHBsAg, IU/ml[c]	2.90 ±1.07	NA	–
Log_{10} HBV DNA, IU/ml[d]	5.42 ±1.85	NA	–
HBV genotype[e]			
B	53	NA	–
C	19	NA	–
B+C	1	NA	–
Undetermined	2	NA	–

Values are expressed as mean ±SD or *n* (%). [a]Pearson's χ^2. [b]Student's *t*-test. Available number of participants: [c]*n*=97, [d]*n*=86, [e]*n*=75. ALT, alanine aminotransferase; CHB, chronic hepatitis B; NA, not applicable; qHBsAg, quantitative HBsAg.

Figure 1. TLR9 expressions on peripheral CD14+ monocytes and in liver cells of non-HBV non-HCV controls and chronic hepatitis B patients



(A) Toll-like receptor 9 (TLR9) mean fluorescence intensity on peripheral CD14+ monocytes between non-HBV non-HCV controls (n=35) and CHB patients (n=97; 9.90 ±3.64 versus 7.95 ±3.61; P=0.007). **(B)** TLR9 messenger RNA (mRNA) level in liver tissue between non-HBV non-HCV controls (n=3) and CHB patients (n=54; relative quantification 1-fold versus 0.45- ±0.32-fold). Error bars are ±SD.

Table 2. Predictors for mean fluorescence intensity of Toll-like receptor 9 expression on peripheral CD14+ monocytes by simple and multiple regression analysis

Variable	Coefficient	95% CI	*P*-value
CHB patients and controls by simple regression analysis			
CHB patients versus controls	-1.951	-3.362, -0.540	0.007
Age	-0.040	-0.100, 0.020	0.191
Gender	-0.219	-1.550, 1.112	0.746
ALT	0.001	-0.001, 0.003	0.335
CHB patients and controls by multiple regression analysis[a]			
(Constant)	10.942	8.143, 13.740	<0.001
CHB patients versus controls	-2.09	-3.568, -0.613	0.006
Age	-0.028	-0.088, 0.032	0.359
Gender	0.209	-1.140, 1.558	0.760
ALT	0.001	0.000, 0.003	0.243
CHB patients by simple regression analysis			
Log_{10} quantitative HBsAg	0.277	-0.408, 0.962	0.425
Age	-0.028	-0.093, 0.036	0.386
Gender	0.182	-1.432, 1.795	0.824
ALT	0.001	0.000, 0.004	0.163
HBeAg	0.002	-0.003, 0.007	0.396
Log_{10} HBV DNA	0.081	-0.354, 0.517	0.711
CHB patients by multiple regression analysis[b]			
(Constant)	8.789	3.534, 14.043	0.001
Log_{10} quantitative HBsAg	0.177	-0.872, 1.226	0.738
Age	-0.023	-0.104, 0.058	0.571
Gender	0.126	-1.741, 1.993	0.894
ALT	0.001	-0.001, 0.003	0.331
HBeAg	0.001	-0.006, 0.008	0.737
Log_{10} HBV DNA	-0.109	-0.736, 0.518	0.730

[a]P=0.04. [b]P=0.863. ALT, alanine aminotransferase; CHB, chronic hepatitis B.

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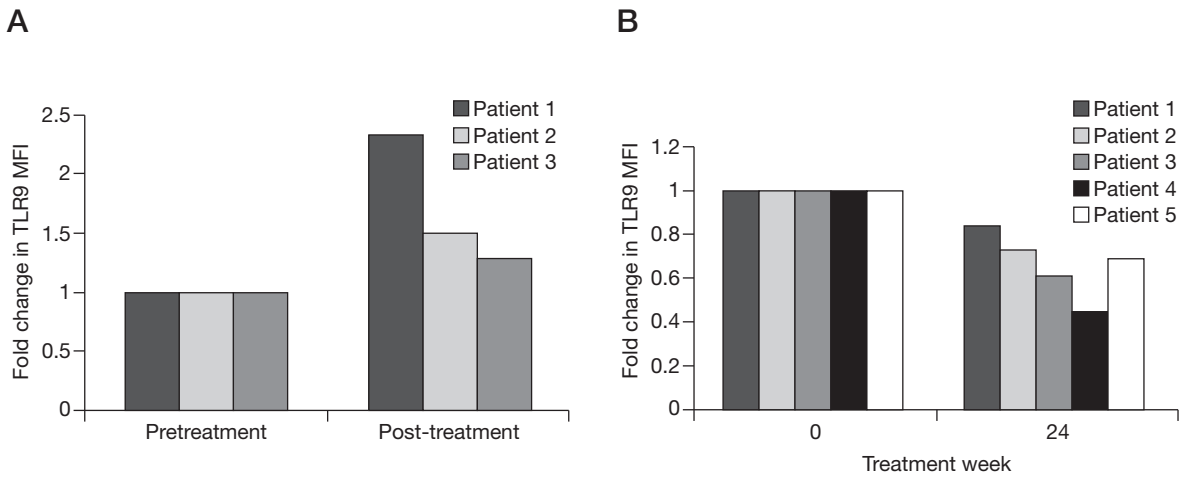
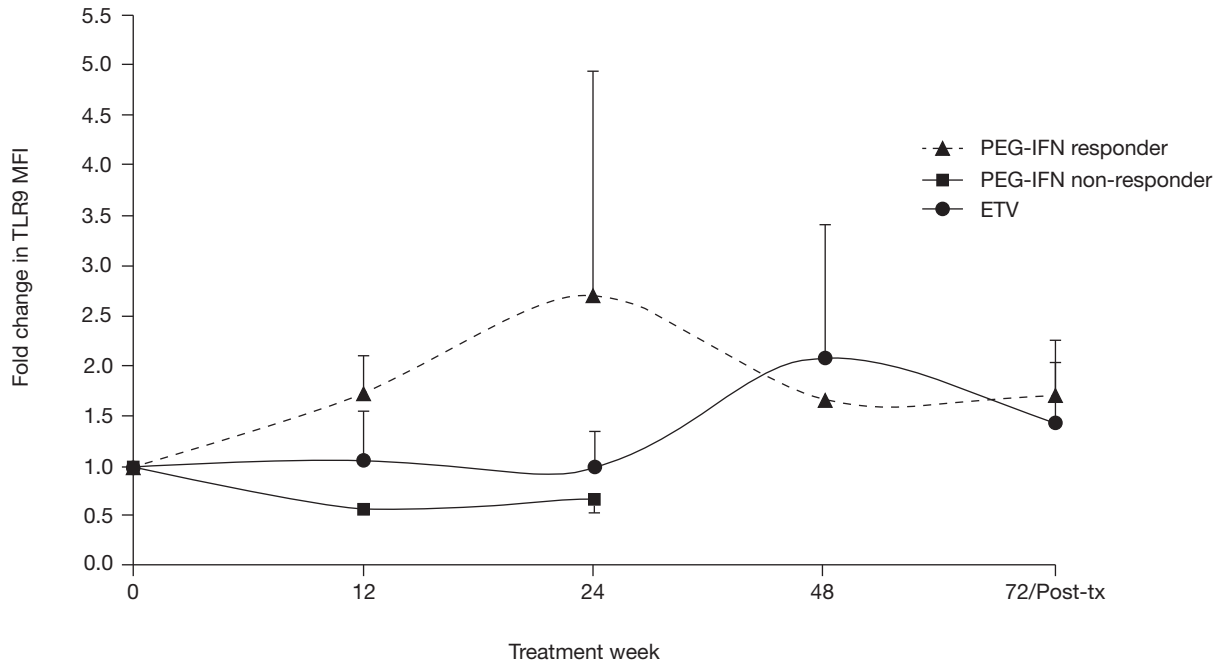
Figure 2. Fold change of TLR9 expression on peripheral CD14+ monocytes of chronic hepatitis B patients under PEG-IFN therapy



There were three responders and five non-responders to pegylated interferon (PEG-IFN) therapy. **(A)** Among responders, using pretreatment Toll-like receptor 9 (TLR9) mean fluorescence intensity (MFI) as a reference, TLR9 MFI restored to a mean (range) of 1.7-fold (1.3–2.3) at 18 to 34 weeks post-treatment. **(B)** Among non-responders, TLR9 MFI reduced to a mean (range) of 0.67-fold (0.45–0.84) at treatment week 24.

Figure 3. Fold change of TLR9 expression on peripheral CD14+ monocytes of CHB patients under PEG-IFN or ETV therapy



Among pegylated interferon (PEG-IFN) responders, using pretreatment Toll-like receptor 9 (TLR9) mean fluorescence intensity (MFI) as a reference, TLR9 MFI restored to a mean of 1.7- to 2.7-fold starting from treatment week 12. Among PEG-IFN non-responders, TLR9 MFI reduced to a mean (range) of 0.57-fold (0.50–0.64) in three patients with available data at treatment week 12. A total of 10 patients were treated with entecavir (ETV); all had undetectable serum HBV DNA starting from treatment week 12. Using pretreatment TLR9 MFI as reference, TLR9 MFI gradually restored to a mean (range) of 1.2–2.1-fold in 8 of 10 (80%) patients at treatment weeks 48 to 72.

A total of 10 patients were treated with ETV (male/female *n*=8/2, mean (range) age 47 years (22–68), HBeAg-negative 60%, log HBV DNA 5.5 ±2.1 IU/ml and HBV genotype B 50%); all of them had undetectable serum HBV DNA starting from treatment week 12. Using pretreatment TLR9 MFI as reference, TLR9 MFI gradually restored to a mean of 1.2–2.1-fold in 8 of 10 (80%) patients at treatment week 48 to 72 (Figure 3).

Discussion

In this study, we demonstrated that CHB patients had a reduced expression of TLR9 on peripheral $CD14^+$ monocytes, regardless of age, gender, and serum ALT level, as compared with controls. In addition, the restoration of TLR9 expression on peripheral $CD14^+$ monocytes in the responders to PEG-IFN therapy and majority of ETV-treated patients further confirmed our findings. Taken together, our data showed that HBV reduced the expression of TLR9, followed by TLR9 rescue under effective treatment. This finding supports the hypothesis that innate immunity is inhibited during HBV infection, which may contribute to chronicity.

A crucial role of dysfunctional or inhibited innate immune mechanisms in the establishment of CHB has been suggested by previous studies demonstrating the down-regulation of functional TLR2, 4 and 9 expression by HBV [7,8,15–17]. Furthermore, TLR9 ligand activation in CHB patients impairs peripheral natural killer (NK) cells IFN-γ production [18] and cytolytic activity as well as pDCs function [19].

The down-regulation of TLR9 was supported by reduced hepatic TLR9 mRNA level in our study. Wu *et al*. [20] provided evidence that HBV suppresses TLR-induced antiviral activity in the liver. Persistent HBV infection may also be due to the absence of antiviral cytokines producing cells in the liver [21]. TLR9 signalling induces myeloid cell aggregates that enable massive expansion of cytotoxic T-lymphocytes in the liver leading to eradication of chronic HBV infection in mice [10]. Nanoparticle-encapsulating HBV-CpG induces therapeutic immunity against HBV carrier mice [22]. Further studies are needed to explore and characterize the therapeutic potential of TLR9 stimulation in CHB patients.

The interaction between TLR9 and HBV has been explored. HBsAg preferentially binds to TLR9-triggered pDC, whereas HBV directly interferes with pDC function and monocyte-pDC interaction [17]. HBsAg inhibits IFN-α production in pDC that is mediated specifically by TLR9, but not TLR7 [8], through tumour necrosis factor-α and interleukin-10 induction in monocytes [9].

We also showed the reduction in TLR3 expression on peripheral $CD14^+$ monocytes and liver tissue of CHB patients, which can be restored effectively in IFN responders and by ETV therapy [6]. Synergistic effect of multiple TLRs has been reported in herpes simplex virus [23,24] and murine cytomegalovirus infection [25]. TLRs cooperate with other pattern recognition receptors (PRRs) in sensing of modified vaccinia virus Ankara [26]. Further studies are needed to explore the synergistic effect among TLRs or other PRRs in chronic HBV infection.

In our study, PEG-IFN responders displayed more rapid and striking restoration of TLR9 expression compared with those successfully treated with ETV. This may be due to the known immune-modulatory properties of IFN. ETV-treated patients with undetectable HBV DNA had a gradual restoration of TLR9 expression, suggesting the restoration of TLR9 expression may occur after viral suppression in this special setting. By contrast, PEG-IFN non-responders exhibited an immediate decline in TLR9 MFI after initiation of therapy. CHB patients are known to have deficient pDCs [7], and HBV interferes with pDC function and monocyte-pDC interaction [17]. IFN non-responders may fail to activate and restore pDCs and monocyte-pDC interaction, which immediately reduces TLR9 expression.

Chen *et al*. [16] showed comparable TLR9 mRNA of peripheral blood mononuclear cells (PBMCs) between CHB patients and healthy donors, and Wang *et al*. [27] reported up-regulation of TLR9 mRNA of PBMCs in active CHB patients as compared with controls. These two studies are limited by a small sample size. Furthermore, Wang *et al*. [27] and Zhou *et al*. [28] showed the correlation of TLR9 expression with serum HBV DNA and other variables. However, apart from serum HBV DNA, other variables might also affect TLR9 expression, such as age, gender, serum ALT levels, HBeAg status and serum qHBsAg, which were not adjusted or stratified by regression analysis in the latter studies as in our study.

There were several limitations in this study. First, the enrolled number of patients receiving treatment was relatively small, however, we disclosed the differential expression of TLR9 among responders in contrast to non-responders in this limited number of patients. There were also challenges to timely performance of TLR9 staining on fresh peripheral monocytes of patients under pretreatment and several on-treatment time points, which limited the enrolled number of patients. Second, most patients refused post-treatment liver biopsy; however, the TLR9 expression on peripheral $CD14^+$ monocytes may represent its expression on the liver as we shown in the pretreatment study. Furthermore, it reflects the real world situation where peripheral monocytes are more accessible than liver tissue, thus are more clinically useful for monitoring treatment response.

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In summary, CHB patients display reduced TLR9 expression on peripheral $CD14^+$ monocytes, independent of age, gender, serum ALT level, HBeAg status, qHBsAg or HBV DNA, and on liver tissue. Responders to PEG-IFN therapy and those under ETV therapy demonstrate substantial restoration of TLR9 expression. On-treatment TLR9 expression on peripheral $CD14^+$ monocytes might predict the response to PEG-IFN therapy.

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Disclosure statement

The authors declare no competing interests.

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