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ORIGINAL ARTICLE

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A genetic variant in the CD40 gene is related to HBV infection in the Chinese Han population

Wennan Wu[1,2,3,4,5,6] | **Siyi Xu**[1,2,3,4,5,6] | **Yongbin Zeng**[1,2,3,4,5,6] | **Luoli Yu**[1,2,3,4,5,6] | **Tianbin Chen**[1,2,3,4] | **Hongyan Shang**[1,2,3,4,5,6] | **Can Liu**[1,2,3,4,5,6] | **Bin Yang**[1,2,3,4,5,6] | **Qishui Ou**[1,2,3,4,5]

[1]Department of Laboratory Medicine, Gene Diagnosis Research Center, The First Affiliated Hospital, Fujian Medical University, Fuzhou, China

[2]Department of Laboratory Medicine, National Regional Medical Center, Binhai Campus of the First Affiliated Hospital, Fujian Medical University, Fuzhou, China.

[3]Fujian Key Laboratory of Laboratory Medicine, The First Affiliated Hospital, Fujian Medical University, Fuzhou, China

[4]Fujian Key Laboratory of Laboratory Medicine, the First Affiliated Hospital, Fujian Medical University, Fuzhou, China

[5]Gene Diagnosis Research Center, Fujian Medical University, Fuzhou, China

[6]Fujian Clinical Research Center for Clinical Immunology Laboratory Test, the First Affiliated Hospital, Fujian Medical University, Fuzhou, China

Correspondence
Qishui Ou, Department of Laboratory Medicine, The First Affiliated Hospital of Fujian Medical University, Fuzhou 350004, China.
Email: ouqishui@fjmu.edu.cn

Abstract

Background: CD40 is an important immune costimulatory molecule that has recently been found to be associated with chronic hepatitis B. This study aims to explore the association between CD40 polymorphisms and HBV infection, as well as to investigate the impact of different rs1883832 genotypes on CD40 expression and its effect on the progression of chronic HBV infection.

Methods: We genotyped rs1883832 in 3433 individuals using MassARRAY, and quantified the CD40 expression, including CD40 mRNA, sCD40, and mCD40. The CD40 and HBV infection indicators were assessed to investigate the potential function of rs1883832 in suppressing HBV replication in HepG2.2.15 and HepAD38, CD40L in cytotoxic t lymphocytes (CTLs) and interferon-γ, TNF-α, granzyme B, and perforin were measured to elucidate the mechanism by which CD40 inhibits HBV replication.

Results: Our study revealed that the frequencies of CC genotype and C allele of rs1883832 were significantly higher in immune recovery compared to chronic hepatitis B. Individuals with CC genotype exhibited significantly elevated CD40 in serum and B cells compared to TT genotypes in chronic hepatitis B. Additionally, CD40 is capable of inhibiting HBV replication and transcription in hepatocytes by means of interaction with CD40L. A significant negative correlation was found between HBV DNA, HBeAg, and mCD40. Conversely, the expressions of ALT and mCD40 showed a positive correlation, which aligns with the trend of CD40L.

Conclusions: rs1883832 C allele may have a protective role in HBV immune recovery. This protective effect could potentially be attributed to the regulation of CD40 expression. The activation of the anti-HBV immune

Abbreviations: CHB, chronic hepatitis B; eQTL, expression quantitative trait loci; IFN, interferon; SNPs, single-nucleotide polymorphisms.

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response, which occurs through binding CD40L on CTL, can suppress HBV DNA replication and potentially facilitate immune recovery in HBV infection.

INTRODUCTION

HBV infection is a widespread infectious disease affecting over 350 million individuals globally, leading to varied clinical outcomes such as spontaneous resolution, acute and chronic hepatitis, hepatic cirrhosis, and HCC.[1] Despite long-term HBV infection, the immune mechanisms governing the diverse outcomes of HBV infection remain unclear.[2] Nevertheless, accumulating evidence confirms the pivotal role of cellular immunity, particularly $CD4^+$ T helper type cells, in the progression of HBV infection. These cells may provide assistance to cytotoxic T cells and generate cytokines, thus influencing the course of HBV infection.[3]

CD40 is a crucial immune costimulatory molecule that interacts with CD40L and provides a second signal for the activation of T cells, facilitating the interaction of T, B, and antigen-presenting cells (APC) cells and upregulating the expression of costimulatory molecules, thereby playing a pivotal role in humoral immunity and cellular immunity.[4] Inhibition of CD40 signaling has been shown to impair immune responses.[5,6] Multiple studies have demonstrated that serum CD40 (sCD40) levels are significantly elevated in patients with HBV infection in comparison to healthy individuals.[7–9] Additionally, in patients with chronic hepatitis B (CHB), sCD40 levels are positively correlated with serum alanine aminotransferase and aspartate aminotransferase levels, implying that sCD40 is linked to the severity of liver inflammation. Recent research has identified single-nucleotide polymorphisms (SNPs) in CD40's Kozak sequence that may affect the start of transcription, and studies have also suggested that SNPs in CD40 are closely linked to autoimmune diseases.[7,10,11] SNPs in CD40 have the potential to alter CD40's function, thereby affecting protein expression or activity, and may play a potential role in HBV infection.

However, the current understanding of the relationship between CD40 gene polymorphisms and the clinical outcomes of HBV infection is still incomplete. Although several related reports exist, studies on the functionality of CD40 SNPs in HBV infection are still required. To address this question, we conducted a genetic association study in a Chinese Han population to investigate the influences of CD40 SNP on HBV infection progression. Additionally, functional studies were carried out to investigate the impact of the variants on CD40 expression and HBV infection.

METHODS

Subjects

In this study, we collected whole blood samples and clinical data from 1766 unrelated patients diagnosed with CHB, as well as 531 immune recoveries and 1136 healthy controls. The diagnosis of CHB and recoveries was based on the Asian-Pacific clinical practice guidelines on the management of hepatitis B, with exclusion criteria including infection with other hepatitis viruses (HAV, HCV, and HEV), HIV infection, autoimmune disorders, and other non-HBV diseases, were excluded. This study was approved by the ethics committee of First Affiliated Hospital, Fujian Medical University. Written informed consent was obtained from all subjects.

Genotyping

Genomic DNA was extracted from whole blood by standard procedures using TIANamp Genomic DNA kits (Tiangen Biotech, China). Genotyping was conducted using the MassARRAY system (Sequenom, San Diego, CA). All of the primers were designed using the MassARRAY Assay Design 3.1 software (Sequenom) (Table 1). DNA samples were amplified by multiplex PCR reactions and then the PCR products were used for locus-specific single-base extension reactions. The resulting products were desalted and subsequently transferred to a 384-well SpectroCHIP bioarrary (Sequenom) using a MassARRAY Nanodispenser RS1000 (Sequenom). Allele detection was performed

TABLE 1 The primer information used for the amplification of CD40 polymorphism loci

SNP	Primer
rs1883832	1st-PCRP: ACGTTGGATGGGCAAAAACAACTCACAGCG
	2nd-PCRP: ACGTTGGATGTGGTCCTGCCGCCTGGTCT
	UEP_SEQ:GAGGCAGACGAACCAT
	EXT1_SEQ:GAGGCAGACGAACCATA
	EXT2_SEQ:GAGGCAGACGAACCATG

Note: 1st-PCRP, forward-PCR primer, 2nd-PCRP, reverse-PCR primer; EXT1_SEQ; extension sequence 1; EXT2_SEQ: extension sequence 2; SNP, single-nucleotide polymorphisms; UEP_SEQ; single-nucleotide extension primer.

using MALDI-TOF mass spectrometry and analyzed by the MassARRAY Typer 4.0 software (Sequenom). We conducted a search for expression quantitative trait loci (eQTL) evidence of positive loci using the public GTEx Portal V6p database (http://www.gtexportal.org/). The GTEx project has generated comprehensive transcriptome data in different human tissues, providing valuable insights into the regulatory impact of genetic variation.

Clinical chemistry analysis

Serum HBsAg and HBeAg were detected using the ARCHITECT i2000SR automatic biochemical immunoassay system (Abbott Laboratories, USA). Aspartate transaminase (AST) and alanine aminotransferase (ALT) were determined using an automated biochemical technique (Siemens Healthcare Diagnostics, USA). Serum HBV DNA levels were measured on the ABI 7500 Real-Time PCR System (Life Technologies, USA), which has a detection limit of 20 IU/mL.

sCD40 and mCD40 level measurement

Forty patients with treatment-naive CHB and 40 immune recovery patients were recruited in our study. Plasma samples were isolated immediately and stored at −80°C until required for an analysis by ELISA (R&D, USA). The levels of sCD40 were measured by ELISA methods.

Murine anti-human monoclonal antibodies anti-CD19-FITC, anti-CD16-APC, anti-CD14-PE-Cy7, and anti-CD40-PE (Cell Signaling Technology, USA) were used to determine CD40 expression on B cells and monocyte. Peripheral blood mononuclear cells (PBMCs) of patients with CHB and immune recovery patients were operated follow the instructions of manufacturer's recommended (Absin, China).

CD40L and intracellular molecules expression measurement

Murine anti-human monoclonal antibodies anti-CD3-FITC, anti-CD8-PE-Cy7, and anti-CD40L-APC (Cell Signaling Technology, USA) were used to determine CD40L expression on CTL. PBMCs from patients with CHB and immune recovery patients were operated follow the instructions of manufacturer's recommended (Absin, China). For intracellular cytokines detection, after surface antigen staining, cells were fixed, permeabilized, and stained with anti-interferon (IFN)-γ, anti-TNF-α, anti-perforin, and anti-granzyme B antibodies (Cell Signaling Technology, USA). Navios flow cytometer (Beckman Coulter, USA) was used to analyze the stained cells.

qPCR analysis

Purified RNA was reversely transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Vazyme, China). Real-time quantitative polymease chain reaction (qPCR) analysis was performed with ABI 7500 Real-Time PCR System (Life Technologies, USA) using TB Green Premix Ex Taq (Vazyme, China). The thermal profile was as follows: pre-denaturation at 95°C for 30 s), 40 cycles of denaturation at 95°C for 5 seconds, 62°C for 30 seconds followed by melt curve analysis. The primers used in this study are shown in Table 1.

Cell culture

HepG2.2.15 and HepAD38 cell lines were obtained from the Cell Bank of Type Culture Collection (Chinese Academy of Sciences, Shanghai, China). Cells were maintained in DMEM (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) at 37°C in a 5% CO_2 incubator. Control lentivirus negative control [NC], lentivirus-CD40-CC (CC), and lentivirus-CD40-TT (TT) were transferred to HepG2 and HepAD38 cells, respectively. Puromycin was used to select stable clones. All viruses were purchased from GeneChem (Shanghai, China). The infection efficiency was confirmed by qPCR. The genotypes of CD40 were carried out by the Sanger sequence.

Statistical analysis

The Hardy-Weinberg equilibrium of genotype was evaluated by using the Chi-squared test obtained from the healthy subjects. Logistics regression models were used to compare the genotypes and allele frequencies between the groups and to calculate OR with 95% CI with the adjustments of the sex, age, serum HBsAg, HBeAg, HBeAb and ALT, and HBV DNA levels. sCD40 and mCD40 were expressed with average and compared using the Student *t* test. All statistical analyses were performed using the SPSS20.0 software (SPSS Inc.). All tests were two-sided, and a *p*-value <0.05 was considered statistically significant.

RESULTS

Characteristics of the study subjects

The age of individuals enrolled in our study was between 35 and 50 years old. The 792 of 1766 (44.8%) patients with CHB were female, and 974 of 1766 (55.2%) were male with a mean ± SD age of 38.60 ± 12.30 years. The sex and age showed no difference among the groups ($p > 0.05$, Table 2). Additionally, the distributions of rs1883832 genotypes in our study populations,

TABLE 2 Demographic and clinical characteristics of the participants

	Total	HC	IR	CHB	*p*
Case	3433	1136	531	1766	—
Sex					
Male	1916 (55.8)	631 (55.5)	311 (58.6)	974 (55.2)	> 0.05
Female	1517 (44.2)	505 (44.5)	220 (41.4)	792 (44.8)	—
Age	41.21 ± 11.8	40.73 ± 12.4	41.53 ± 12.0	38.60 ± 12.3	0.096
HBV infection indicators					
HBsAg (log_{10} IU/mL)	—	—	—	3.39 ± 0.9	—
HBeAg	—	0.34 ± 0.1	0.37 ± 0.2	314.77 ± 483.0	0.218
HBV DNA (log_{10} IU/mL)	—	—	—	4.89 ± 2.0	—
ALT (U/mL)	—	22.24 ± 5.2	31.76 ± 9.1	207.04 ± 305.3	**< 0.05**
AST (U/mL)	—	20.26 ± 5.00	27.11 ± 5.8	127.13 ± 219.1	**< 0.05**

Measurement data were expressed as median ± SD.
Enumeration data were presented as number (%) for every group.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate transaminase; CHB, chronic hepatitis B; HC, healthy controls; H-W, hardy-weinberg; IR, immune recovery.

including patients with CHB, HBV immune recovery subjects, and healthy controls, were in Hardy-Weinberg equilibrium ($p > 0.05$, Table 3), suggesting the representativeness of the populations in our study.

Association between CD40 SNP and HBV infection status

Logistic regression models were used to calculate OR, 95% CI, and *p*-values while adjusting for sex and age. For the rs1883832, genotype TT showed significantly different among their groups ($p < 0.001$, Table 3), and genotype TT was found more in the CHB group compared to controls and immune recovery patients ($p < 0.001$, Table 3), so as in the dominant model, which showed CT+TT genotype was more common to be found in the CHB group ($p < 0.001$, Table 3). Beyond that, the allele frequency of the rs1883832 was also distributed differently among the patients, recoveries, and controls. The frequency of allele T in the CHB group was significantly higher than that in immune recovery patients and healthy controls. ($p < 0.001$, Table 3). The distribution of rs1883832 genotypes and alleles showed no significant difference between immune recovery patients and healthy controls.

CD40 expression increased in patients with CHB

To evaluate the association between CD40 and HBV infection status, 40 patients with treatment-naive CHB and 40 immune recovery patients were recruited to discover CD40 expression. First, we measured CD40 mRNA by real-time PCR, and our results suggested that CD40 mRNA in patients with CHB was significantly higher than that in immune recovery ($p = 0.015$, Figure 1A). ELISA was used to detect soluble CD40 (sCD40) in serum. We found that sCD40 in the CHB group was markedly higher than that in the immune recovery ($p < 0.001$, Figure 1C). In addition, in our study, flow cytometry was performed to evaluate the membrane CD40 (mCD40). mCD40 was reported to express on B cells and myeloid cells, including monocytes, in PBMC. In order to investigate the role of mCD40 in combating HBV infection, we examined its expression on B cells and monocytes. We observed that mCD40 expression on B cells was significantly higher in CHB patients compared to immune recoveries ($p = 0.013$, Figure 1B). However, there was no significant difference in mCD40 expression on monocytes between the CHB and recovery groups ($p = 0.133$, Figure 1D).

Association of CD40 polymorphism and CD40 expression

Next, the eQTL analysis was performed in our study using the public GTEx Portal V6p database (http://www.gtexportal.org/). We found that the correlation between rs1883832 and CD40 expression in whole blood was significantly stronger than that in liver tissue ($p = 1.6 \times 10^{-13}$ and $p = 0.08$, respectively, Supplemental Figure S1A, http://links.lww.com/HC9/A668). And we observed that allele C of rs1883832 significantly upregulated CD40 mRNA levels ($p = 1.56 \times 10^{-13}$, Supplemental, Figure S1B, http://links.lww.com/HC9/A668). To further analyze the function effects of rs1883832 on CD40 expression in patients, the rs1883832 genotypes and CD40 expression were measured in all subjects. For CD40 mRNA, individuals carrying rs1883832 CC genotype had the highest CD40 mRNA among all genotypes in the CHB group, while there is no significant difference among recovery patients ($p = 0.040$ and 0.045, Figure 1E). Beyond that, our results showed CC genotype presented a higher mCD40 on B cells than patients with TT genotype in the CHB and recovery group

TABLE 3 Genotype and allele frequencies of CD40 gene polymorphism (rs1883832) in patients with chronic HBV infection, HBV immune recovery subjects, and healthy controls

	CHB	Recovery	Controls		CHB vs. controls			CHB vs. recovery			Controls vs. recovery	
rs1883832	n = 1766	n = 531	n = 1136	*p*	*p*	OR 95% CI		*p*	OR 95% CI		*p*	OR 95% CI
Genotype, n (%)												
CC	522 (29.6)	201 (38.9)	426 (37.5)	**< 0.001**		1			1			1
CT	906 (51.30)	250 (47.0)	522 (46.0)	—	**< 0.001**	0.706 (0.597–0.835)		**0.003**	0.717 (0.578–0.888)		0.909	1.015 (0.810–1.272)
TT	338 (19.1)	80 (15.1)	188 (16.5)	—	**0.001**	0.682 (0.547–0.849)		**0.001**	0.615 (0.459–0.824)		0.530	0.902 (0.662–1.231)
H-W	0.11	0.97	0.19	—	—	—		—	—		—	—
Dominant	(CT+TT) vs. CC		—	**< 0.001**	**< 0.001**	0.699 (0.597–0.819)		**< 0.001**	0.689 (0.562–0.844)		0.914	0.985 (0.796–1.218)
Recessive	(CT+CC) vs. TT		—	0.084	0.084	0.838 (0.688–1.020)		**0.034**	1.334 (1.023–1.740)		0.475	1.118 (0.841–1.486)
Allele, n (%)												
C	1950 (55.2)	652 (61.4)	1374 (60.5)	**< 0.001**		1			1			1
T	1582 (44.8)	410 (38.6)	898 (39.5)	—	**< 0.001**	0.806 (0.724–0.897)		**< 0.001**	0.775 (0.674–0.892)		0.621	0.962 (0.829–1.117)

Note: Data were presented as number (%) for every group.
The differences in genotype frequencies between any 2 groups were analyzed using logistic regression models.
ORs were calculated and reported within the 95% CI.
p-values (< 0.05) are considered statistically significant and highlighted in bold.
Age and sex were included as covariates.
Abbreviation: CHB, chronic hepatitis B.

($p = 0.040$ and 0.020, Figure 1F). In the CHB group, patients who carried CC genotype also had a higher expression compared to CT genotype, while it not showed in the recovery group ($p = 0.010$, Figure 1F), so as sCD40 ($p = 0.023$ and 0.003, Figure 1G). The expression of CD40 on monocytes in patients and recovery individuals was observed to be slightly elevated in individuals with the CC genotype compared to those with the CT and TT genotypes. However, no statistically significant difference was found between the CHB and immune recovery groups ($p > 0.05$, Figure 1H). These findings support the notion that CD40 expression and its associated polymorphism are implicated in the status of chronic HBV infection. Furthermore, our investigation revealed that individuals carrying the C allele exhibited higher concentrations of CD40 across all groups, suggesting that the rs1883232 polymorphism may exert an influence on CD40 expression.

CD40 rs1883832 genotypes affect HBV infection status in HCC cell lines

To examine whether the CD40 affect the HBV infection status, we transfected CD40 overexpression plasmid (rs1883832 CC genotype and rs1883832 TT genotype) to 2 HCC cell lines (HepG2.2.15 and HepAD38). qPCR was used to quantify the CD40 relative expression in HepG2.2.15 and HepAD38. HBsAg, HBeAg, and HBV DNA were detected at 24 and 48 hours. First, we detected CD40 basic expression in the 2 cell lines, and the results showed that the expression of CD40 mRNA was significantly upregulated in 2 HCC cell lines ($p < 0.05$, Figure 2).

And then, we examined HBsAg, HBeAg, and HBV DNA at 24 and 48 hours, and our results showed HBsAg, HBeAg, and HBV DNA were downregulated in the 2 cell lines (Figure 3). Beyond that, we also focused on the level of HBV infection indicators between CC and TT genotypes. And we found in HepG2.2.15, HBsAg was significantly downregulated at 24 and 48 hours. CC genotype showed a lower HBsAg, HBeAg, and HBV DNA than TT at 24 hours ($p = 0.001$, 0.016, and 0.009, respectively, Figure 3A). At 48 hours, the difference in HBeAg was not obvious, while HBsAg and HBV DNA still showed statistical differences between CC and TT ($p = 0.009$ and 0.025, Figure 3B). In HepAD38, HBV DNA was obviously decreased in CC genotype compared to TT genotype at 48 hours ($p = 0.010$, Figure 3D), and HBeAg was decreased in CC genotype at 24 hours ($p < 0.001$, Figure 3C).

Correlation between HBV infection indicators and CD40 expression

In order to know the relationship between CD40 and the degree of liver inflammation caused by HBV infection,

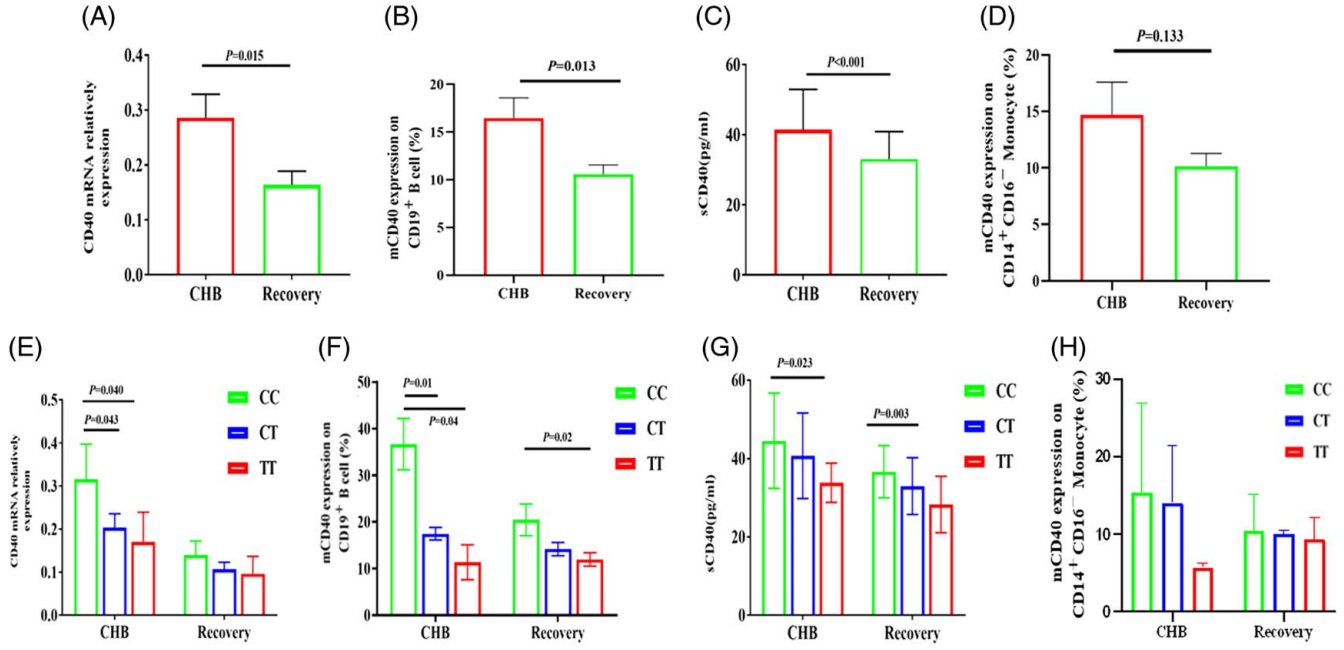

FIGURE 1 Association of CD40 rs1883832 polymorphisms and CD40 expression in CHB and immune recovery patients. CD40 mRNA, soluble CD40 (sCD40), and membrane CD40 (mCD40) were detected in CHB and recovery patients. (A) CD40 mRNA in the CHB group was significantly higher than that in the immune recovery group; (B) mCD40 expressed on B cell in the CHB group was markedly higher than that in the immune recovery; (C) sCD40 in the CHB group showed significantly higher compared to immune recoveries; (D) the expression of mCD40 on monocytes in patients with CHB did not show any significant differences compared to immune recoveries. (E–H) The influence of rs1883832 on CD40 expression (including CD40 mRNA, mCD40 on B cells, sCD40, and mCD40 on monocytes) was analyzed in both CHB patients and immune recovery patients. The most significant difference in CD40 expression was observed in B cells between the CHB and recovery groups. Abbreviation: CHB, chronic hepatitis B.

correlation analysis was carried out in the CHB group, as shown in Figure 4; we analyzed the correlation between the expression of CD40 mRNA and HBV infection indicators in patients with CHB. There were no significant correlations between HBV DNA, HBeAg, ALT, AST, and CD40 mRNA ($p > 0.05$, Figure 4A–D).

Furthermore, we investigated the relationship between HBV infection indicators and the level of sCD40, mCD40 on B cells, and mCD40 on monocyte, respectively. We found a significantly negative correlation between sCD40 and HBV DNA ($p = 0.008$, Figure 4F), while we did not find any statistical correlation between sCD40 and other HBV infection indicators ($p > 0.05$, Figure 4E, G, H). It is noteworthy that the expressions of HBV DNA, HBeAg, and mCD40 on B cells were in a significantly negative correlation ($p = 0.002$ and 0.004, Figure 4I, J). Otherwise, the expressions of ALT and mCD40 on B cells were in a positive correlation ($p = 0.033$, Figure 4K). There was no

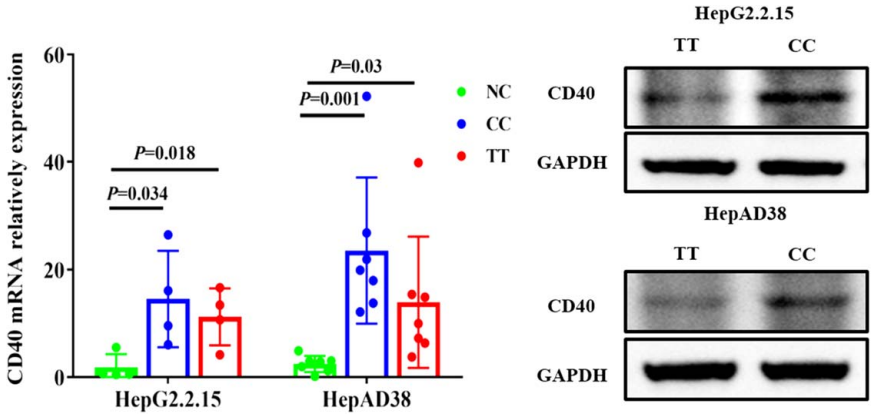

FIGURE 2 CD40 expression in HepG2.2.15 and HepAD38 after transfected overexpression plasmid (rs1883832 CC genotype and rs1883832 TT genotype). After transfected CD40 overexpression plasmid (rs1883832 CC genotype and rs1883832 TT genotype) to 2 HCC cell lines (HepG2.2.15 and HepAD38), the expression of CD40 mRNA was significantly upregulated in 2 HCC cell lines. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NC, negative control.

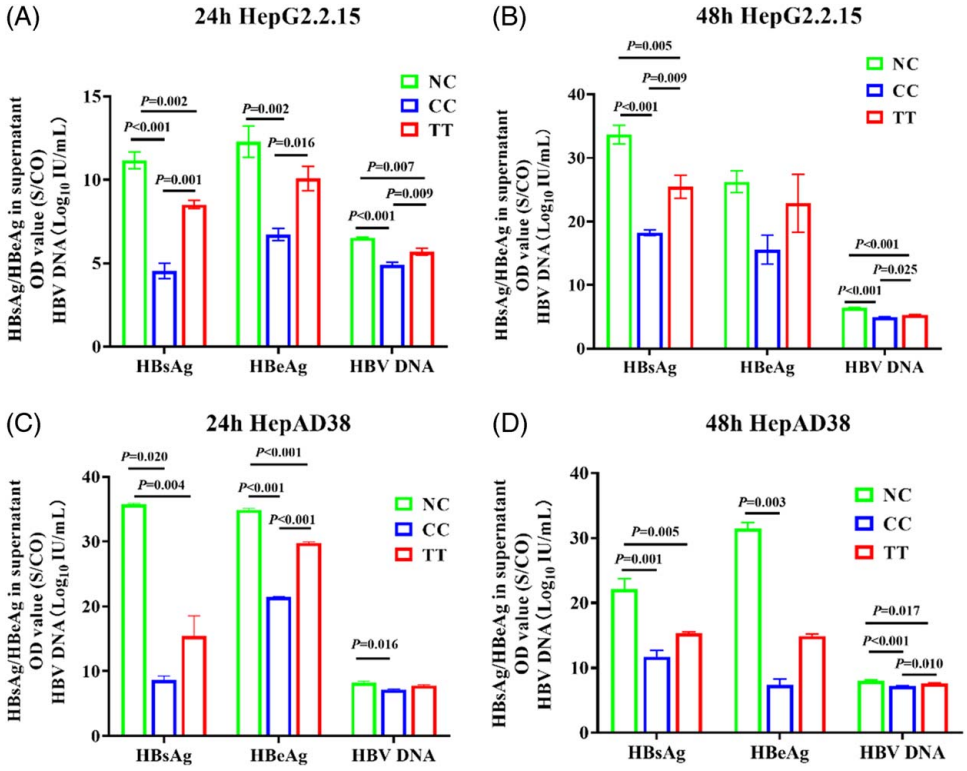

FIGURE 3 Overexpress CD40 rs1883832 CC and TT in HepG2.2.15 and HepAD38 significantly decreased the level of HBV infection indicators, including HBsAg, HBeAg, and HBV DNA. (A, B) The secretion of HBsAg, HBeAg, and HBV DNA at 24 and 48 hours in HepG2.2.15, CC genotype showed a lower HBsAg, HBeAg, and HBV DNA than TT at 24 hours. At 48 hours, the difference in HBeAg was not obvious, while HBsAg and HBV DNA still showed statistical differences between CC and TT; (C, D) Examination of HBsAg, HBeAg, and HBV DNA at 24 and 48 hours in HepAD38, HBV DNA was obviously decreased in CC genotype compared to TT genotype at 48 hours. The secretion of HBeAg was decreased in cells carried rs1883832 CC genotype at 24 hours. Abbreviations: NC, negative control; OD, optical density; S/CO, sample optical density/cutoff.

significant correlation between AST and mCD40 on B cells ($p > 0.05$, Figure 4L). Besides, no significant correlations were found between HBeAg, HBV DNA, ALT, AST, and mCD40 expressed on monocytes ($p > 0.05$, Figure 4M–P).

Combination of CD40 and CD40L promotes CTL activation and inhibits HBV DNA replication

The cellular immune response of the host plays a crucial role in the chronic progression of HBV infection. Specifically, CTLs are integral to the host's cellular immune response following HBV infection. To further investigate the potential mechanism of CD40 inhibiting HBV replication, CD40L in CTL and the intracellular cytokines was detected in our study. First, we compared the CD40L levels of CHB and recovery patients. Our results indicate that the expression of CD40L is significantly higher in the CHB group than in the immune recovery group ($p < 0.001$, Figure 5A), and that this expression is consistent with CD40. Beyond that, the concentration of IFN-γ, TNF-α, perforin, and granzyme B in CHB patients was significantly higher than in the immune recovery group ($p < 0.05$, Figure 5B–E). Further investigation into the correlation between CD40L expression levels and cytokine expression levels revealed a positive correlation with IFN-γ, TNF-α, perforin, and granzyme B ($p < 0.05$, Figure 5F). Therefore, we posit that CD40 binding with CD40L promotes CTL activation, cytokine secretion, and ultimately inhibits HBV DNA replication in patients with CHB.

Correlation between HBV infection indicators and CD40L expression

The correlation of HBV infection indicators and CD40L was also performed in our study. We observed a positive correlation between CD40L expression and ALT levels, indicating that higher levels of CD40L expression are associated with increased ALT ($p = 0.043$, Figure 6C). Conversely, a negative correlation was observed between CD40L expression and HBV DNA and HBeAg, suggesting that higher CD40L expression is linked to lower levels of HBV DNA and HBeAg ($p < 0.05$, Figure 6A, B). However, we did not find a significant correlation between CD40L expression and AST levels ($p = 0.159$, Figure 6D).

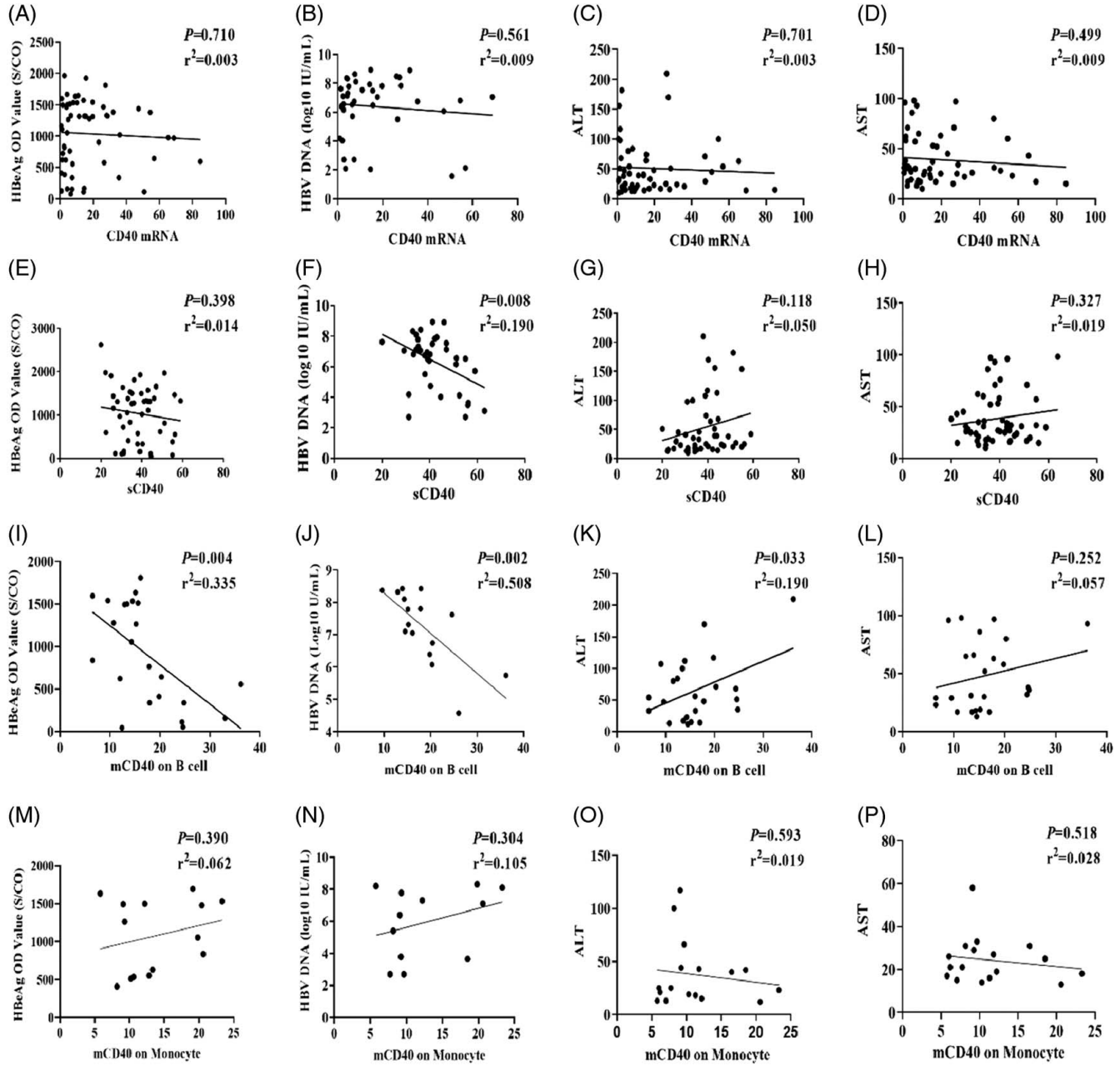

FIGURE 4 Correlation between HBV infection indicators and CD40 expression in patients with CHB. The correlation analysis revealed no significant associations between HBeAg, HBV DNA, ALT, AST, and CD40 mRNA (A–D). Conversely, a statistically significant negative correlation was observed between sCD40 and HBV DNA, while no statistical correlation was found between sCD40 and other HBV infection indicators (E–H). Furthermore, a significantly negative correlation was observed between the expressions of HBeAg, HBV DNA, and mCD40 on B cells, whereas a positive correlation was observed between ALT and B cell mCD40 (I–L). On the other hand, no significant correlations were found between HBeAg, HBV DNA, ALT, AST, and mCD40 expressed on monocytes (M–P). Abbreviations: ALT, alanine aminotransferase; AST, aspartate transaminase; OD, optical density; S/CO, sample optical density/cutoff.

DISCUSSION

CHB infection involves complex interactions between inflammation, immune response, and viral variation, in which the host's cellular immune response is a critical component.[12,13] Recent studies have demonstrated that blocking CD40 signaling can impair the immune response,[14] while CD40 activation is essential for restoring HBV-specific $CD8^+$ T cells with suppressed function.[15,16] Notably, the CD40 rs1883832 polymorphism has been linked to asymptomatic carriers. Studies have demonstrated an association between the CD40 rs1883832 polymorphism and asymptomatic carrier status following HBV infection.[7,17] In this study, our objective was to elucidate the relationship between the CD40 rs1883832 polymorphism and susceptibility to HBV infection within a substantial cohort of the Chinese Han population. To address this objective, we

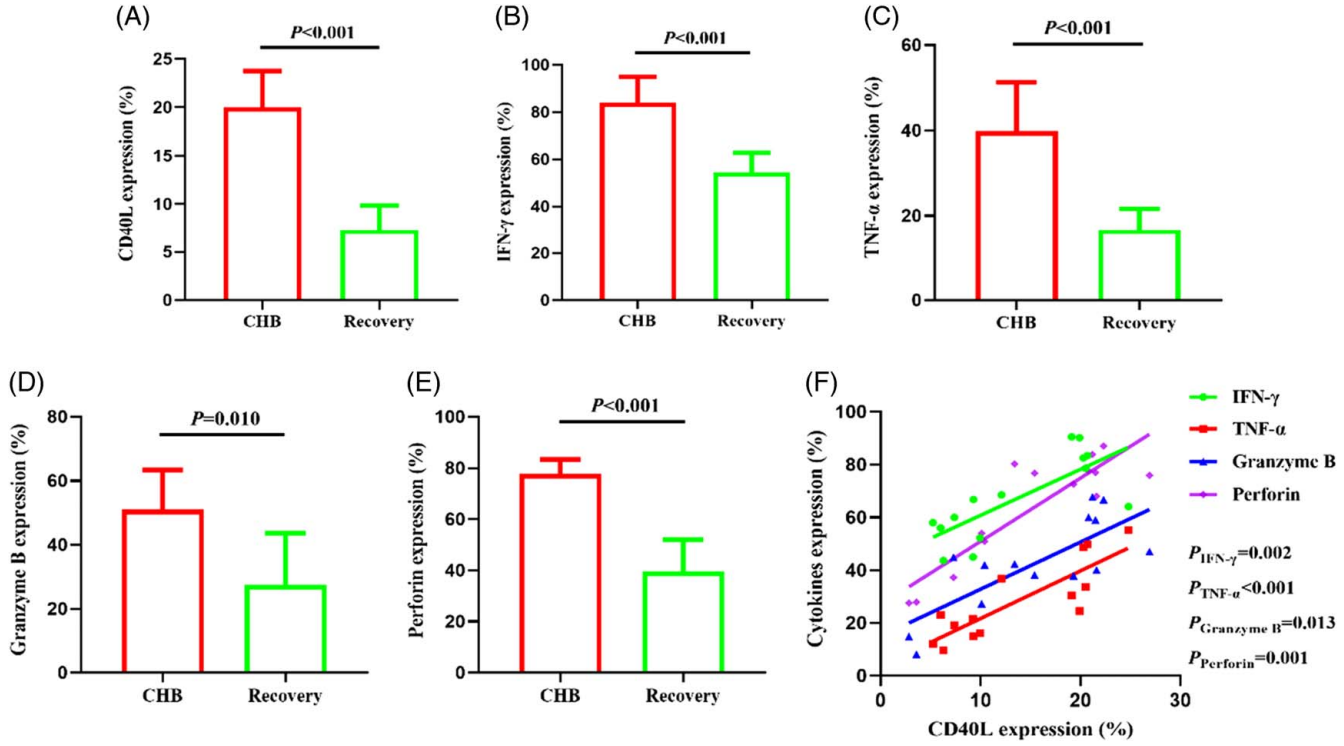

FIGURE 5 CD40L expression is positively correlated with cytokines released by CTL in CHB patients. (A) The expression of CD40L in the CHB group was significantly higher than that in the immune recovery group; (B-E) The intracellular cytokine in the CHB group was markedly higher than that in the immune recovery; (F) The expressions of intracellular cytokine were in a significantly positive correlation with CD40L in all patients. Abbreviations: CHB, chronic hepatitis B; IFN, interferon.

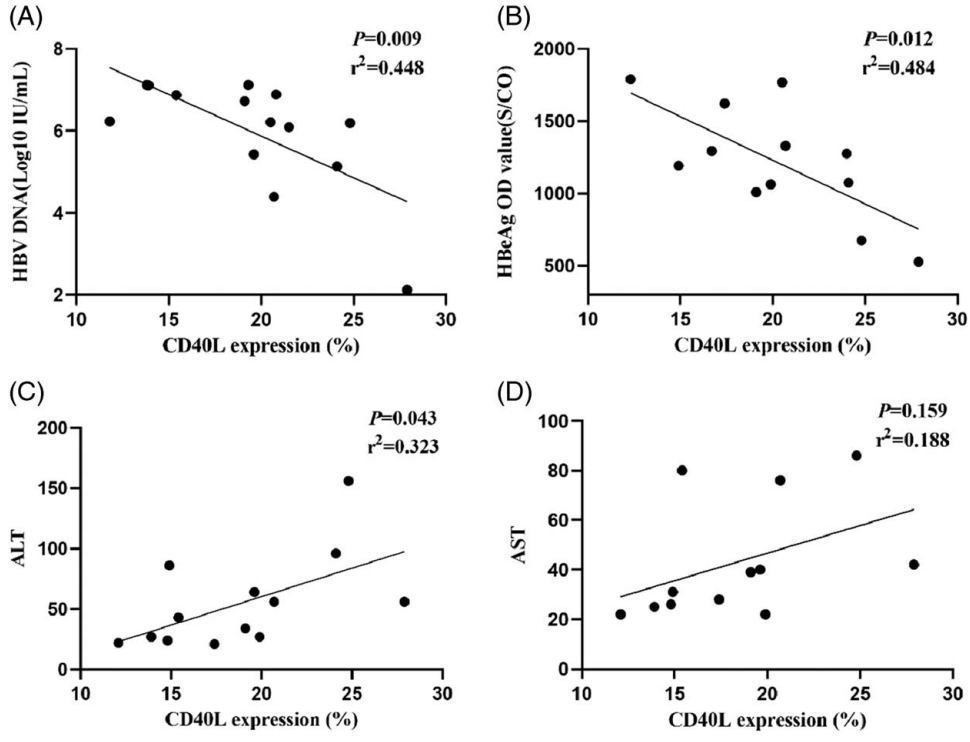

FIGURE 6 Correlation between HBV infection indicators and CD40L expression in patients with CHB. HBV DNA, HBeAg, and CD40L were in a significantly negative correlation, and the ALT and CD40L were in a positive correlation, which is consistent with mCD40. Abbreviations: ALT, alanine aminotransferase; AST, aspartate transaminase; OD, optical density; S/CO, sample optical density/cutoff.

conducted a comprehensive investigation employing cytological experiments, flow cytometry, and real-time fluorescence quantitative PCR to assess the potential functional implications of the rs1883832 polymorphism. Notably, our findings revealed that the presence of the rs1883832 C allele could potentially confer a protective effect, facilitating immune recovery among individuals affected by HBV infection. Moreover, individuals with the rs1883832 CC genotype exhibited augmented CD40 expression and a reduced HBV infection indicator within their serum. Furthermore, our study brings attention to the pivotal role of CD40 and CD40L in promoting CTL activation and inhibiting HBV DNA replication, which occurs through the release of perforin, granzyme B, IFN-γ, and TNF-α. Additionally, we emphasize the crucial involvement of CD40 in peripheral blood B cells during the immune response against HBV infection. Collectively, these findings underscore the potential significance of the rs1883832 genotype in fostering the recovery process in individuals affected by hepatitis B infection.

According to reports, rs1883832 polymorphism has been linked to increased susceptibility to systemic lupus erythematosus sepsis and acute coronary syndrome in the Chinese population.[18–20] In our study, a large number of Chinese Han population were recruited to investigate CD40 rs1883832 and HBV infection status. Statistically different genotypic and allelic frequencies were observed between CHB and recovery, CHB and control. The frequency of rs1883832 TT genotype was detected more frequently in the CHB group compared to immune recoveries and healthy controls, so as in the codominant model. In the recessive model, CT+CC genotype was detected more in recovery patients compared to patients with CHB. Beyond that, rs1883832 T allele has been identified as a potential risk factor for developing chronic HBV infection in the present study. Conversely, the rs1883832 C allele may play a protective role for individuals in response to HBV infection.

Research has shown that the SNP found in the Kozak sequence has the ability to impact the rate at which a gene is translated.[21] Our results indicated rs1883832 TT was a risk genotype to develop CHB, and we also verified the significantly higher expression of CD40 mRNA, mCD40, and sCD40 in patients with CHB compared to immune recoveries; it is consistent with Mao et al,[8] which suggested sCD40 concentrations increased with a rise in the severity of liver necroinflammation and fibrosis. In addition, Zhou et al[7] found that rs1883832 polymorphism can lead to changes in CD40 translation on human B cells, and they suggested that in both the asymptomatic HBV carriers and healthy controls, individuals with the TT genotype had significantly lower CD40 expression compared to those with CC and CT genotypes. Our findings demonstrated partial concurrence with Zhou et al,[7] as we observed a noteworthy elevation in CD40 expression (including CD40 mRNA, mCD40 on B cells, and sCD40) among individuals harboring the rs1883832 CC genotype in the CHB group, in contrast to those with the TT genotype. However, we did not detect any significant disparity in CD40 expression on myeloid cells within PBMCs between the groups. Based on these observations, we propose that CD40 expressed on peripheral blood B cells assumes a crucial role in orchestrating the immune response against HBV infection. Prior studies have reported that aberrant CD40 expression can trigger excessive production of proinflammatory cytokines, thereby augmenting the risk of CHB.[22,23] However, these results were not seen in the recovery group. Our findings indicate that patients with the TT genotype are at a higher risk of liver damage following HBV infection, as evidenced by their chronic HBV infection status. These results suggest that individuals with the TT genotype may have weaker immunity against viral infections, leading to an ineffective antiviral immune response that fails to eliminate HBV. Additionally, the lower intensity of specific CTL response to HBV may also contribute to this susceptibility. Furthermore, it was observed that CC genotype patients exhibited a higher expression of CD40 compared to those with the TT genotype, which is consistent with the results of eQTLs. The eQTLs showed rs1883832 and CD40 expression in whole blood was significantly stronger than that in liver tissue, which suggested that CD40, particularly on lymphocytes' surface in peripheral blood, holds greater significance for the immune response following HBV infection. This increase in CD40 expression can potentially promote the interaction between CD40 and CD40L, leading to the activation of T cells and an auxiliary killing effect of $CD40^+$T cells on CTL. Consequently, this can enhance the function of $CD8^+$ T cells and ultimately result in a state of immune recovery.

Emerging studies have demonstrated that the activation of CD40 signaling is crucial in initiating a potent virus-specific $CD8^+$ T cell response, which is necessary for the elimination of the virus.[15,22,24] In our study, we observed that overexpression of CD40 in HepG2.2.15 and HepAD38 cell lines resulted in a reduction of HBsAg, HBeAg, and HBV DNA levels, with a more pronounced effect at the 24-hour time point. Our findings also suggest that the CC genotype has a greater inhibitory effect on viral replication than the TT genotype, which aligns with the results from our gene polymorphism study on a large sample. The CC genotype appears to act as a protective genotype, favoring virus replication suppression and promoting transition to an immune recovery state. These results demonstrate the potential therapeutic benefits of CD40 as a target for the treatment of HBV infection. Consistent with the known functions of CD40 in inhibiting HBV replication and enhancing inflammation,

the expression of sCD40 and mCD40 showed a significant negative correlation with the levels of HBV DNA in the plasma, while mCD40 was positively associated with ALT in patients with CHB. These results collectively suggest that the deficiency of CD40 may be biologically plausible for the heightened susceptibility to chronic HBV infection.

Moreover, the CTL plays a pivotal role in exerting antiviral effects against HBV infection through the release of perforin and granzyme B, as well as the secretion of IFN-γ and TNF-α, thereby inhibiting HBV DNA replication.[25] In our investigation, we observed a decline in HBV DNA and HBeAg levels among patients exhibiting elevated CD40 and CD40L expression, accompanied by increased ALT levels. Furthermore, a positive correlation was identified between the levels of perforin, granzyme B, IFN-γ, and TNF-α. These findings suggest that the interaction between CD40 and CD40L enhances the antiviral efficacy of CTLs, consequently leading to heightened liver inflammation. Additionally, it impedes HBV DNA replication and facilitates immune recovery subsequent to HBV infection. It is noteworthy that the aforementioned findings primarily pertain to B cells within PBMCs, as opposed to monocytes, thereby aligning with the findings of Jiang et al.[26] In their study, Jiang and colleagues demonstrated a marked elevation of CD40 expression in $CD19^+$ B cells compared to $CD14^+$ monocytes. Furthermore, they noted that the CC/CT genotype of rs1883832 exhibited heightened CD40 expression specifically in $CD19^+$ B cells, while such an association was not observed in $CD14^+$ monocytes. Moreover, current studies indicate that CD40 is also prominently expressed in myeloid cells, including myeloid-derived suppressor cells, which are activated neutrophils and monocytes exhibiting potent immunosuppressive activity. However, the study conducted by Pal et al[27] demonstrated the minimal impact of myeloid-derived suppressor cell–derived CD40 on the cytokine response of HBV-specific CTLs, aligning with our research findings. Thus, we postulate that CD40 expressed on peripheral blood B cells assumes a critical role in mounting an immune response against HBV infection.

Our study possesses several noteworthy limitations that warrant acknowledgment. First, it is crucial to recognize that the immune recovery of patients with HBV may also be influenced by additional genetic polymorphisms within the host. Second, it is important to note that the outcomes of HBV infection can be influenced by environmental factors and the diverse genotypes of HBV. Lastly, in order to comprehend the precise functionality of key SNP linkage disequilibrium loci, further investigation is required to unravel the intricate relationship between individual polymorphic loci and specific diseases. Considering the intricate interplay between genetic and environmental factors in determining the clinical status of HBV infection, it is imperative to account for these variables in future research.

In conclusion, our study suggests that the CD40 rs1883832 CC genotype continues to be a potential protective factor for HBV immune recovery and highlights that CD40 and CD40L promote CTL activation and inhibit HBV DNA replication by means of release of perforin, granzyme B, IFN-γ, and TNF-α, and the role of CD40 on B cells in the immune response to HBV infection is crucial. These results may provide insights into the pathogenesis of HBV infection and facilitate the development of innovative therapeutic interventions.

AUTHOR CONTRIBUTIONS

Qishui Ou planned the study. Wennan Wu wrote and submitted the paper. Siyi Xu, Yongbin Zeng, and Luoli Yu conducted the relevant experiments. Hongyan Shang and Tianbin Chen conducted the survey and specimen collection. Can Liu and Bin Yang were responsible for the statistical work.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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