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Original Article

Predictive value of Th17 and Treg cells at baseline for HBsAg loss in chronic hepatitis B patients with low HBsAg quantification treated with pegylated interferon and nucleos(t)ide analogue☆

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ABSTRACT

Background and aims: The primary goal of chronic hepatitis B (CHB) treatment is to reduce hepatitis B surface antigen (HBsAg). T helper 17 (Th17) and regulatory T (Treg) cells are essential for the development of CHB. However, how Th17 and Treg cells contribute to HBsAg loss is still unknown. Therefore, this study aimed to search for the predictive value of Th17 and Treg cells for HBsAg loss in CHB patients with low HBsAg quantification.

Methods: The study included 99 hepatitis B e antigen (HBeAg)-negative CHB patients who had completed a year of nucleos(t)ide analogue (NA) monotherapy and had received both NA and pegylated interferon (PEG-IFN) treatment for less than 96 weeks (96 wk). In the cured group, 48 patients lost HBsAg within 48 wk, while 51 patients did not (uncured group). Blood samples and clinical data were collected for research.

Results: During PEG-IFN and NA combination therapy, the proportion of Th17 cells in the cured group increased significantly, while the proportion of Treg cells in the uncured group increased. From 0 to 24 wk, the proportion of Th17 cells in the cured group was significantly higher than in the uncured group, while the opposite was true for Treg cells. Patients with alanine aminotransferase (ALT) $\geq$ 2.5 upper limit of normal (ULN) at 12 wk had a higher proportion of Th17 cells and a lower proportion of Treg cells than those with ALT <2.5 ULN at 12 wk. Additionally, the proportion of Th17 cells is inversely associated with the level of HBsAg, whereas the level of Treg cells is positively related to HBsAg quantification. The clinical cure index, including age, HBsAg quantification, and the proportions of Th17 and Treg cells, had a higher area under the curve (0.957) for predicting HBsAg loss when compared to the proportions of Th17 and Treg cells and HBsAg quantification alone.

Conclusions: Combined with quantification of HBsAg, the proportions of Th17 cells and Treg cells at baseline can be used as good predictors of HBsAg loss in patients with low HBsAg quantification treated with NA and PEG-IFN.

1. Introduction

Chronic hepatitis B (CHB) is a worldwide disease caused by the hepatitis B virus (HBV) infection that kills approximately 687,000 people each year.[1,2] Long-term and long-lasting immune responses play an essential role in the progression of the disease. The

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excessive immune response would result in hepatic injury and liver failure, whereas insufficient response could result in persistent HBV infection.[3] Pegylated interferon (PEG-IFN) therapy represents a promising therapeutic alternative to the prolonged use of nucleos(t)ide analogues (NAs) in CHB.[4]

Combining PEG-IFN and NA therapy may improve the rate of hepatitis B surface antigen (HBsAg) loss, a condition known as clinical cure (also called functional cure).[5] CHB patients with HBsAg <1000 IU/mL and an HBsAg decline of more than 0.5 log10 IU/mL in 12 weeks (wk) achieved an optimal rate of clinical cure.[4,6,7] Loss of HBsAg (<0.05 IU/mL) improves long-term outcomes and is the ideal therapeutic goal recommended by current guidelines.[8–10] According to previous research, combining PEG-IFN and NA therapy can cure approximately 30% of patients with low HBsAg levels.[11–14] PEG-IFN has been widely used in the last decade due to its dual capabilities of antiviral activity and immunomodulation.[8] Therefore, we wondered whether immune-related factors are associated with HBsAg loss in different patients. In previous research, that serum transforming growth factor-beta (TGF-β) level could be used as an early indicator of clinical cure in CHB patients treated with PEG-IFN.[15] Uncertainty persists regarding the functional distinction and early prognostic significance of $CD4^+$ T cells between patients who can experience clinical cure and patients who cannot.

$CD4^+$ T cells are an important element of adaptive immune responses and protect the host from a wide variety of pathogens. In chronic HBV infection, $CD4^+$ T cells are linked to liver damage and viral clearance.[16] $CD4^+CD25^+$ regulatory T (Treg) cells play an important role in maintaining immune balance and can suppress the HBV-specific immune response,[17–19] leading to the persistence of HBV infection.[20,21] TGF-β-producing regulatory T helper 3 (Th3) cells and type 1 regulatory T (Tr1) cells, which secrete interleukin-10 (IL-10), are the two main classes of $CD4^+$ Treg cells.[22,23]

Previous research has shown that the balance of Th17 cells (which primarily secrete IL-17 and IL-21) and Treg cells (which primarily secrete TGF-β and IL-10) is associated with the prognosis of infectious diseases and autoimmune disorders.[24,25] Previously, we found that patients with acute-on-chronic liver failure in the recovery phase had an imbalance of Th17 to Treg cells, which could be used as a prognostic marker to predict disease progression.[26] Furthermore, previous research showed that patients with advanced-stage HBV-related liver fibrosis had an increase in hepatic Th17 cells and Treg cells.[27] However, the imbalance of Th17/Treg cells was not clear during HBsAg loss.

In this study, Th17 cells were defined as $IL\text{-}17^+CD4^+$ T cells and $IL\text{-}21^+CD4^+$ T cells. $CD25^+CD4^+$ T cells, $TGF\text{-}\beta^+CD4^+$ T cells (Th3 cells), and $IL\text{-}10^+CD4^+$ T cells (Tr1 cells) were classified as Treg cells. Because Th17 and Treg cells are involved in the progression of CHB disease,[25] we want to dig deeper into the expression differences of Th17 and Treg cells in different populations and their relationship with HBsAg loss.

2. Patients and methods

2.1. Ethical approval

This study was approved by the Medical Ethics Committee of The Third Affiliated Hospital of Sun Yat-sen University (zssy [2018] 02-218-02). Written informed consent was obtained from each participant. The study protocol adhered to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by The Third Affiliated Hospital of Sun Yat-sen University's Independent Central Ethics Committee. All experiments were carried out following the approved guidelines and regulations.

2.2. Study design and patients

This study is a non-randomized retrospective study. Our study included patients with CHB who were treated at The Third Affiliated Hospital of Sun Yat-sen University from 2018 to 2019. All the patients were seen at the outpatient clinic regularly, with 12 wk intervals between visits. Blood samples at baseline (0 wk), 12 wk, and 24 wk of all patients were collected for research. The baseline point was established when the patient began PEG-IFN combined with NA therapy.

A total of 255 consecutive CHB patients without cirrhosis were included. These patients had been taking NAs (mostly entecavir or tenofovir) for at least a year and had quantification of HBsAg (qHBsAg) less than 1500 IU/mL, hepatitis B e antigen (HBeAg) seroconversion, and HBV DNA <20 IU/mL. For less than 96 wk, they received PEG-IFN 180 μg/wk, in addition to NA therapy (Clinical Trials.gov ID: NCT04035837). After excluding patients with incomplete clinical data and missing blood specimens, 99 patients were finally included in this study. The median duration of PEG-IFN therapy was 48 wk for 48 patients who had HBsAg loss (HBsAg <0.05 IU/mL) and 48 wk for the other 51 patients who did not. Exclusion criteria for similar patients have been described in our previous study.[14,15]

2.3. Data acquisition

Demographic data, including date of birth and sex, hepatic and renal biochemical indices, and hematological and virological parameters (qHBsAg, HBeAg status, and quantitative HBV DNA) were collected from the 99 patients. HBV genotyping was not possible because all participants were on long-term antiviral therapy and HBV DNA was undetectable during screening. Following that, serial hepatic and renal parameters, as well as HBV viral markers, were monitored until the final check-up. A blood sample was retained for a minimum of 24 wk with each check-up.

2.4. HBV serological markers

Elecsys HBsAg II Quant reagent kit (Roche Diagnostics, Indianapolis, IN, USA; lower limit of quantification (LLOQ): 0.05 IU/mL) was used to measure HBsAg. Serum HBV DNA was tested using the Roche COBAS AmpliPrep/COBAS TaqMan HBV Test version 2.0 (Roche Diagnostics; LLOQ: 20 IU/mL).

A Hitachi 7600 automatic analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan) was used to test liver function, and the upper limit of normal (ULN) of the alanine aminotransferase (ALT) level was set to 35 U/L.

2.5. Cell pretreatment steps

Ficoll-Hypaque density gradient centrifugation was used to isolate peripheral blood mononuclear cells (PBMCs) from blood samples, which were then stored in liquid nitrogen. Sequential PBMC samples (0–24 wk) were collected for research. After resuspending the cells, they were treated with eBioscience™ Cell Stimulation Cocktail (phorbol 12-myristate 13-acetate and ionomycin in ethanol, Thermo Fisher Scientific, Cat# 00-4970-03) and eBioscience™ Protein Transport Inhibitor Cocktail (brefeldin A and monensin in ethanol, Thermo Fisher Scientific, Cat# 00-4980-03) for 6 h. The cells were incubated for 20 min at room temperature with a Zombie Violet™ Fixable Viability Kit (Biolegend, Cat# 423113), followed by staining with appropriate surface antibodies.

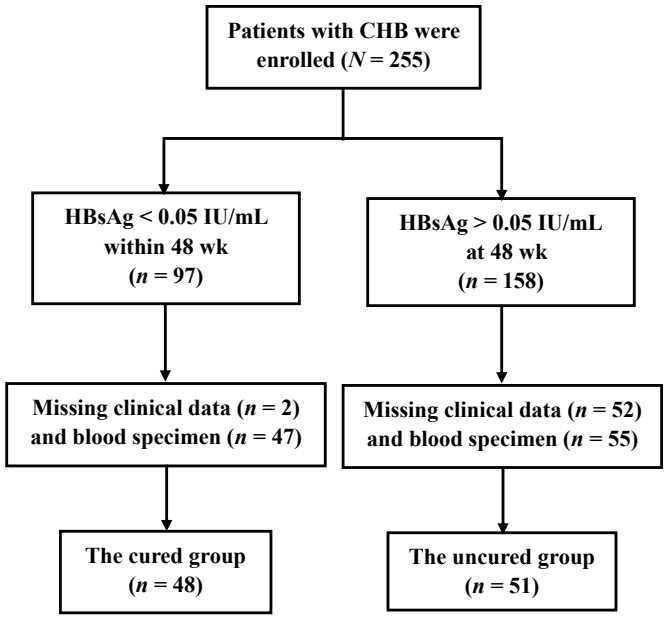

Fig. 1. Flow chart of the patient enrollment. Abbreviations: CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; wk, weeks.

2.6. Cell staining and flow cytometry

Following cell pretreatment, samples were stained with the following Biolegend antibodies (listed in Supplementary Table 1): CD3-AlexaFluor700, CD4-FITC, CD8a-PerCP/Cyanine5.5, and CD25-Brilliant Violet 605. Intracellular staining was done using an intracellular fix/perm buffer (eBioscience™ Intracellular Fixation & Permeabilization Buffer set, Thermo Fisher Scientific, Cat# 88-8824-00), and samples were stained for at least 1 h with TGF-β1-APC, IL-10-PE, IL-17A-PE, and IL-21-APC. Representative flow cytometry data and gating strategy are shown in Supplementary Fig. 1. We found that $CD4^+CD25^+$ T cells have higher forkhead box P3 (foxp3) expression and lower CD127 expression than $CD4^+$ T cells, classifying them as Treg cells (Supplementary Fig. 2). All data were acquired on the BD FACSCanto II and analyzed using FlowJo version 10 software. To ensure the accuracy of the data, PBMCs with poor cell status (less than 50% of viable cells in lymphocytes) were rejected after cryopreservation.

2.7. Statistical analysis

The SPSS version 25.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis, and MedCalc version 19.0 (MedCalc Software bvba, Ostend, Belgium) was used for receiver operating characteristic (ROC) curve analysis. Because continuous variables do not have

Table 1
Demographic and laboratory characteristics of the seventy matched CHB patients.

Variable	Time	Total (n = 70)	Cured group (n = 33)	Uncured group (n = 37)	P-value
Demographic characteristics					
Age (years)	0 wk	44.0 (36.0–48.0)	42.0 (32.0–47.0)	46.0 (40.5–48.0)	0.110
Male, n (%)	0 wk	65 (92.9)	29 (87.9)	36 (97.3)	0.180
NAs, n (%)	0 wk				0.250
ETV		36 (51.4)	15 (45.5)	21 (56.8)	
TDF		32 (45.7)	18 (54.5)	14 (37.8)	
TAF		2 (2.9)	0	2 (5.4)	
PEG-IFN (wk)		48.0 (44.0–60.0)	48.0 (36.0–58.0)	48.0 (48.0–64.0)	0.120
Laboratory characteristics					
qHBsAg, log10 IU/mL	0 wk	2.71 (2.36–2.89)	2.61 (2.16–2.83)	2.78 (2.50–2.90)	0.060
	12 wk	2.35 (1.93–2.69)	1.92 (0.41–2.23)	2.65 (2.39–2.80)	< 0.001
	24 wk	1.85 (−0.95–2.46)	−0.98 (−2.0–(−0.11))	2.44 (2.09–2.68)	< 0.001
AST, U/L	0 wk	23.0 (20.0–26.3)	23.0 (21.0–27.0)	22.0 (19.0–25.5)	0.080
	12 wk	50.0 (37.0–70.3)	66.0 (44.5–76.5)	43.0 (30.0–54.0)	< 0.001
	24 wk	48.0 (39.0–63.5)	58.0 (45.0–84.0)	40.0 (34.0–51.0)	< 0.001
ALT, U/L	0 wk	24.0 (18.8–31.3)	26.0 (18.0–35.5)	23.0 (19.5–29.0)	0.260
	12 wk	61.0 (41.0–84.0)	78.0 (48.0–129.0)	52.0 (35.0–67.5)	0.006
	24 wk	53.5 (37.0–79.3)	66.0 (49.0–96.5)	44.0 (31.0–64.0)	0.001
FBS, μmol/L	0 wk	5.4 (5.0–5.9)	5.2 (4.9–5.5)	5.6 (5.2–6.2)	0.006
	12 wk	5.3 (5.0–5.8)	5.3 (5.0–5.6)	5.7 (5.1–6.2)	0.030
	24 wk	5.4 (5.1–5.8)	5.3 (5.1–5.6)	5.5 (5.2–6.2)	0.140
$IL\text{-}21^+CD4^+$ T cells (%)	0 wk	2.70 (1.57–4.40)	4.20 (2.91–5.62)	2.01 (0.93–2.74)	< 0.001
	12 wk	3.05 (1.39–4.62)	4.62 (3.86–5.93)	1.91 (0.94–2.91)	< 0.001
	24 wk	2.98 (1.37–5.31)	5.30 (4.00–6.79)	1.48 (0.62–2.62)	< 0.001
$IL\text{-}17^+CD4^+$ T cells (%)	0 wk	1.70 (1.09–2.97)	2.19 (1.47–2.99)	1.21 (0.81–2.97)	0.030
	12 wk	1.93 (0.99–3.43)	2.90 (1.85–3.84)	1.14 (0.62–2.68)	< 0.001
	24 wk	1.77 (0.86–3.19)	2.83 (1.64–3.49)	0.95 (0.50–2.04)	0.001
$TGF\text{-}\beta^+CD4^+$ T cells (%)	0 wk	0.42 (0.26–0.66)	0.22 (0.31–0.52)	0.58 (0.36–1.25)	< 0.001
	12 wk	0.49 (0.30–0.83)	0.36 (0.25–0.51)	0.68 (0.47–1.36)	< 0.001
	24 wk	0.46 (0.26–0.79)	0.32 (0.20–0.59)	1.05 (0.49–1.51)	0.002
$IL\text{-}10^+CD4^+$ T cells (%)	0 wk	0.46 (0.31–0.69)	0.40 (0.30–0.51)	0.52 (0.32–1.30)	0.020
	12 wk	0.58 (0.35–0.92)	0.39 (0.25–0.61)	0.76 (0.52–1.21)	< 0.001
	24 wk	0.48 (0.29–0.73)	0.32 (0.26–0.57)	0.71 (0.50–1.31)	0.001
$CD25^+CD4^+$ T cells (%)	0 wk	2.30 (0.81–3.08)	0.93 (0.56–2.64)	2.54 (1.17–3.56)	0.008
	12 wk	2.83 (0.95–4.34)	1.15 (0.55–3.05)	3.21 (2.23–4.40)	0.007
	24 wk	2.39 (0.75–3.71)	1.51 (0.59–3.21)	2.97 (1.57–4.15)	0.020

Data were expressed as n (%) or median (interquartile range).
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHB, chronic hepatitis B; ETV, entecavir; FBS, fasting blood sugar; IL, interleukin; NAs, nucleos(t)ide analogues; PEG-IFN, pegylated interferon; qHBsAg, quantification of hepatitis B surface antigen; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate; TGF-β, transforming growth factor-beta; wk, week(s).

a normal distribution, they are expressed as the median (interquartile range). Qualitative data are presented as numbers (percentage). While the data did not conform to a normal distribution, the Mann–Whitney *U* test was used to compare the differences between different groups. Comparisons between two categorical variables were performed using the chi-square test. The correlation between immune cell levels and clinical indices was investigated using linear regression analysis. To analyze data from the same group at different time points, the Wilcoxon matched-pairs signed rank sum test was used. The association of parameters was analyzed using multivariable logistic regression. The area under the curve (AUC) was used to assess the predictive value of immune cells and HBsAg levels. A *P*-value of <0.05 (two-tailed) was considered statistically significant.

3. Results

3.1. Differences in clinical characteristics between the cured and the uncured group

Fig. 1 depicts the patient enrollment process. The clinical and laboratory characteristics of all the ninety-nine patients are shown in Supplementary Table 2. We matched seventy patients (33 cured and 37 uncured patients) with no significant difference in age, gender, transaminase levels, or viral indicators at baseline to make the data of the two groups of patients (HBsAg loss at 48 wk or not) comparable. The clinical and laboratory characteristics of the matched seventy patients are given in Table 1. Table 1 shows that the levels of transaminases and fasting blood sugar (FBS), as well as the proportions of Th17 and Treg cells, differed between the cured and uncured groups from 0 to 24 wk.

3.2. Differences in the proportions of Th17 and Treg cells between the cured and uncured group

Within 24 wk, the proportions of $CD4^+$ T cells and $CD8^+$ T cells in the same group did not change significantly, and there was no difference in the two types of cells between the cured and uncured patients (Fig. 2A). However, we discovered that the proportions of Th17 cells ($IL\text{-}17^+CD4^+$ T cells) in the cured patients (Fig. 2B) and Treg cells ($CD25^+CD4^+$ T cells and $TGF\text{-}\beta^+CD4^+$ T cells) in the uncured patients (Fig. 2C) fluctuated significantly within 24 wk of PEG-IFN and NA treatment. In all patients with CHB treated with PEG-IFN and NA, the proportion of Th17 and Treg cells has a transient increase. The difference is that the proportion of Th17 cells in the cured group increased when compared to the uncured group. In contrast, compared with the cured group, the proportion of Treg cells in the uncured group increased. Even though the data only partially demonstrated statistical significance in all cell subsets, the proportions of different cell subsets exhibited comparable patterns. Importantly, the proportions of Th17 and Treg cells differed significantly between cured and uncured patients at baseline, 12 wk, and 24 wk (Fig. 2D–F). Typical graphs of flow cytometry analysis data for cured and uncured patients are presented in Fig. 3.

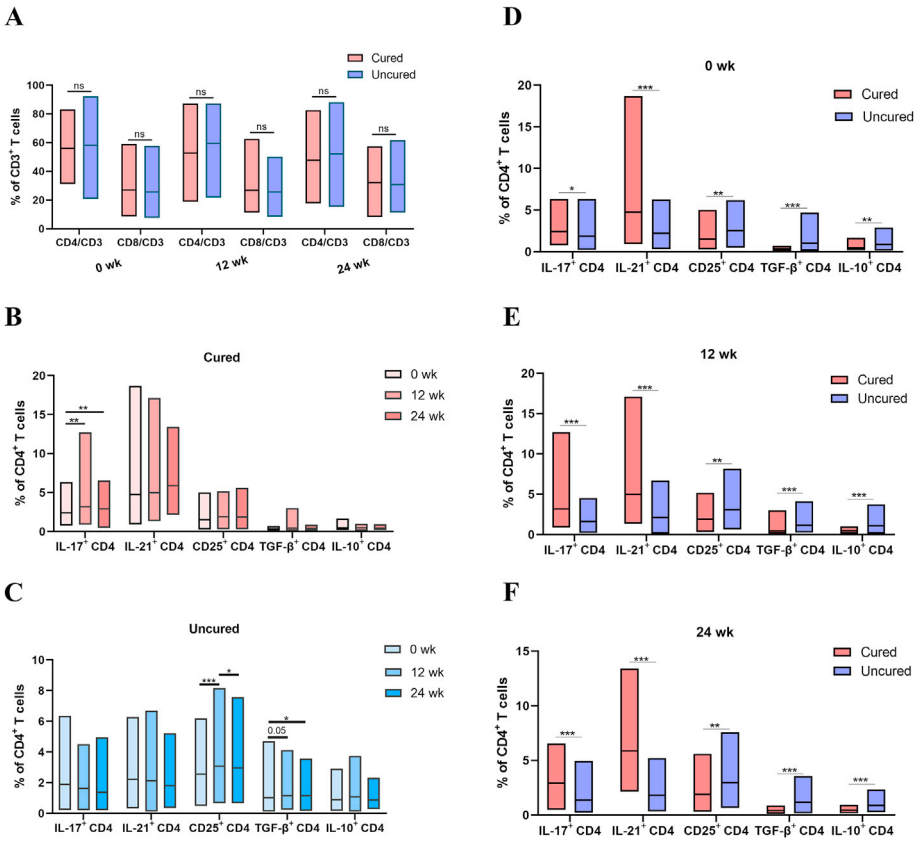

Fig. 2. Dynamic fluctuations of Th17 and Treg cells at baseline (0 wk), week 12 (12 wk), and week 24 (24 wk) in the cured and uncured groups and the differences of proportions of Th17 and Treg cells from 0 to 24 wk between the two groups. (A) Within 24 wk, the proportions of $CD4^+$ and $CD8^+$ T cells did not change significantly in the same group, and there were no significant differences between cured and uncured patients. **(B)** The proportions of Th17 cells ($IL\text{-}17^+CD4^+$ T cells) showed significant fluctuation within 24 wk in the cured patients. **(C)** In the uncured group, the proportions of Treg cells ($CD25^+CD4^+$ T cells and $TGF\text{-}\beta^+CD4^+$ T cells) changed significantly in 24 wk. Proportions of Th17 and Treg cells showed a substantial difference between the cured and uncured patients at **(D)** baseline, **(E)** 12 wk, and **(F)** 24 wk. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, not significant. Abbreviations: IL, interleukin; TGF-β, transforming growth factor-beta; Th17, T helper 17; Treg, regulatory T.

3.3. Differences in the proportions of Th17 and Treg cells between patients with ALT≥2.5 ULN or ALT < 2.5 ULN at 12 wk

Since ALT flares are linked with HBsAg loss in CHB patients.[16,28] As revealed in Table 1, ALT levels were higher (≥2.5 ULN) in cured patients than the uncured patients at 12 wk. Based on the level of transaminase increase (2.5 ULN) by the 12th week, we divided the ninety-nine patients into two groups: those with ALT levels higher than 2.5 ULN, and those with ALT values lower than 2.5 ULN. As a result, at baseline, we discovered that patients with ALT≥2.5 ULN had a higher proportion of Th17 cells and a lower proportion of Treg cells than those with ALT <2.5 ULN (Fig. 4A). Although the difference in Th17 cell populations between the two groups was not statistically significant at 12 and 24 wk, the *P*-value was already close to 0.05 (Fig. 4B and C). These outcomes indicated that the ALT levels at 12 wk could reflect the immune status of patients to some extent. The findings revealed that the proportions of Th17 and Treg cells are critical in an ALT flare and HBsAg loss.

3.4. Correlation of Th17 and Treg cell levels with qHBsAg and ALT levels

Meanwhile, we discovered that the proportions of IL-17$^+$CD4$^+$ T cells and TGF-β$^+$CD4$^+$ T cell subsets were either negatively or positively related to qHBsAg (Fig. 5A and B). Furthermore, as expected, the ALT level was linearly positively correlated with the proportion of IL-17$^+$CD4$^+$ T cells and linearly negatively correlated with TGF-β$^+$CD4$^+$ T cells (Fig. 5C and D). This linear relationship between qHBsAg levels and IL-21$^+$/IL-10$^+$CD4$^+$ T cell subsets was similar (Supplementary Fig. 3). The results indicated that low levels of HBsAg quantification were closely related to the immune status of patients. ALT levels, on the other hand, primarily reflected patients' transient inflammatory states in the liver and were also correlated with immune markers at 12 wk.

3.5. The proportions of Th17 and Treg cells at baseline can predict HBsAg loss in CHB patients treated with NA and PEG-IFN

According to the above findings, the proportions of Th17 and Treg cells in cured and uncured patients differed significantly from the baseline. This phenomenon suggests that the difference in Th17 and Treg cell levels from baseline is directly related to the CHB patient's clinical prognosis during PEG-IFN and NA therapy. Therefore, we speculated that the proportions of Th17 and Treg cells at baseline might predict HBsAg loss in CHB patients. The proportions of IL-17$^+$/IL-21$^+$/CD25$^+$/IL-10$^+$/TGF-β$^+$CD4$^+$ T cells, qHBsAg, and clinical indices at baseline were then analyzed using multivariable logistic regression to predict HBsAg loss. We added

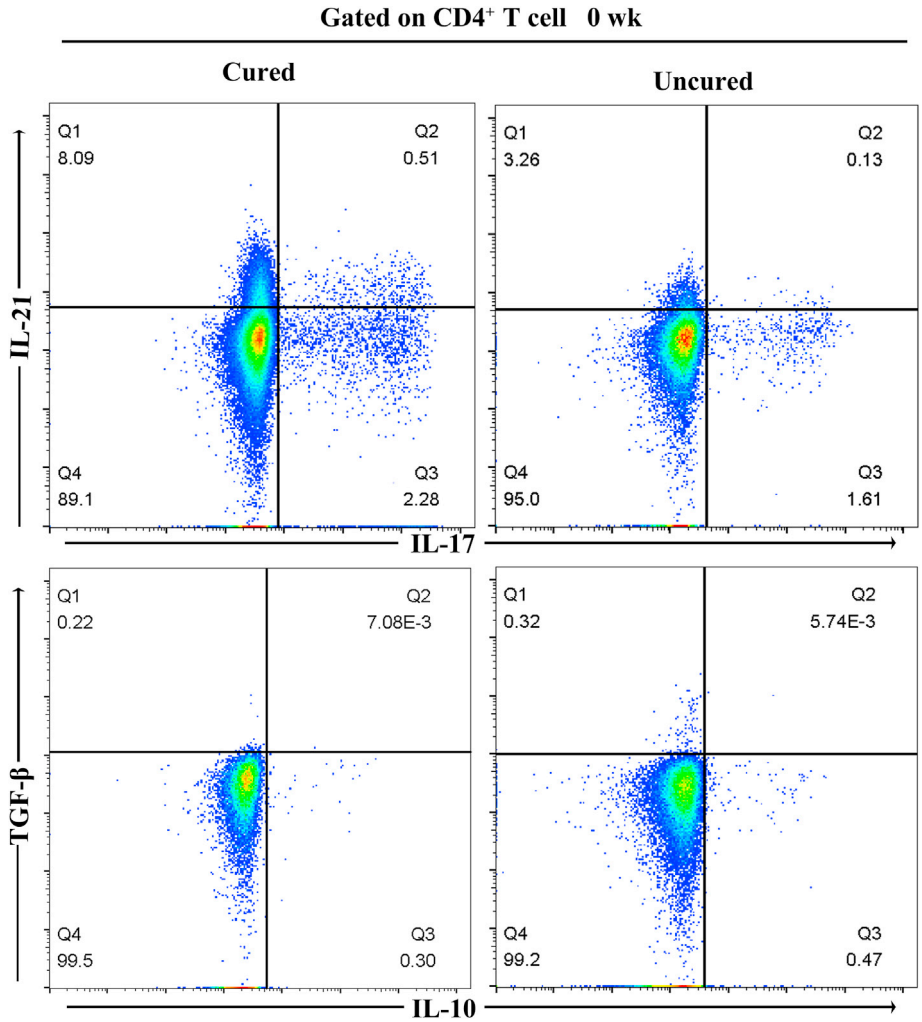

Fig. 3. Typical graphs of flow analysis data of Th17/Treg cells for cured and uncured patients at baseline (0 wk). Abbreviations: IL, interleukin; TGF-β, transforming growth factor-beta; Th17, T helper 17; Treg, regulatory T.

the above indices of ninety-nine patients at baseline to the formula ($\alpha_{in} = 0.05$, $\alpha_{out} = 0.05$).

The findings revealed that the levels of age, qHBsAg, TGF-β$^+$CD4$^+$ T cells (%), and IL-21$^+$CD4$^+$ T cells (%) were all independently associated with HBsAg loss at 48 wk. The following formula was developed: CCI (clinical cure index) = 14.872−0.263 × age (years) - 0.006 × qHBsAg (IU/mL) + 0.762 × IL-21$^+$CD4$^+$ T cells (%) - 9.133 × TGF-β$^+$CD4$^+$ T cells (%). The CCI had a higher AUC (0.957) for predicting HBsAg loss when compared to IL-17$^+$/IL-21$^+$/IL-10$^+$/TGF-β$^+$CD4$^+$ T cells (%) and qHBsAg alone (Fig. 6A and B). The cutoff value > 0.028 of the CCI for predicting HBsAg loss had a Youden index of 0.778, a sensitivity of 82.93%, and a specificity of 94.87%. It is worth noting that the AUC of IL-21$^+$CD4$^+$ T cells (%) at baseline (0.842) was higher than that of HBsAg (AUC = 0.792) (Fig. 6A and B). The above findings suggested that a combination of immune and clinical indicators at baseline could predict clinical cure within 48 wk in CHB patients treated with both PEG-IFN and NA. The patient's immune status, such as the baseline proportions of Th17 and Treg cells, maybe a better predictor of clinical cure after 48 wk of NA and PEG-IFN therapy than HBsAg levels.

4. Discussion

We discovered that the immune status of CHB patients is closely related to clinical prognosis in our study. During PEG-IFN plus NA therapy, proportions of Th17 and Treg cells showed a significant difference between the cured and uncured patients from 0 to 24 wk. Following PEG-IFN and NA combination therapy, the proportion of Th17 cells in the cured group increased significantly, while the proportion of Treg cells in the uncured group increased significantly when compared to the cured group. In CHB patients treated with both PEG-IFN and NA, combining baseline immune and clinical indicators could well predict clinical cure within 48 wk. Previous studies showed that a higher level of Th17/Treg ratio was associated with subsequent HBeAg seroconversion.[29,30] Our findings suggested that HBsAg loss may be associated with the higher proportions of Th17 cells and lower proportions of Treg cells in CHB patients.

The levels of Th17 and Treg cells in CHB patients and the development and severity of the disease have been linked in a sizable body of literature. Compared to healthy individuals, CHB patients have a higher percentage of Treg cells, and a higher Th17 cell count is directly linked to liver injury and the development of liver cirrhosis or liver failure in CHB patients.[3,31–34] On the other hand, NAs inhibiting viral replication results in an increase in Th17 cells and a concomitant decrease in Treg cells.[35,36] Similarly, higher levels of Treg cells result in a poorer response to antiviral therapy, causing HBV infection to persist and the disease to become chronic.[37]

In contrast to the previous study, all participants in this study had low-level HBsAg quantification and had undergone NA treatment followed by PEG-IFN plus NA therapy to achieve a clinical cure. Because the various stages of CHB and the host's immune status are closely linked. Different immune status affects the occurrence, progress, and prognosis of the disease. Therefore, the function of immune cells cannot be defined according to particular disease status. Higher levels of Th17 cells and lower levels of Treg cells are generally associated with HBV clearance, but when HBV cannot be cleared, it can lead to disease progression and poor outcomes.

The proportions of Th17 and Treg cells have pronounced dynamic fluctuations in patients from 0 to 24 wk of PEG-IFN and NA combination treatment, and the changes of Th17 and Treg cells have opposite trends in the cured and uncured groups (Fig. 2B and C). The above results reflect the immunomodulatory role of IFN-alpha (α), and different patients respond differently to IFN-α. Because of IFN-α therapy's potent antiproliferative effect, the proportions of Th17 and Treg cells were temporarily elevated or consistently suppressed in the majority of patients.[38,39] Micco *et al.*,[39] discovered that IFN-α-mediated divergent effects on the innate and adaptive arms of the immune system occur *in vivo*, and the efficacy of PEG-IFN may be limited by its depleting effect on CD8$^+$ T cells; however, PEG-IFN can cumulatively drive the proliferation, activation, and antiviral potential of CD56 (bright) natural killer cells.[39] Similar studies also support our results.[40–43] As a result, for some patients, particularly those in the uncured group, IFN-α treatment did not improve Th17 cell proportions and may

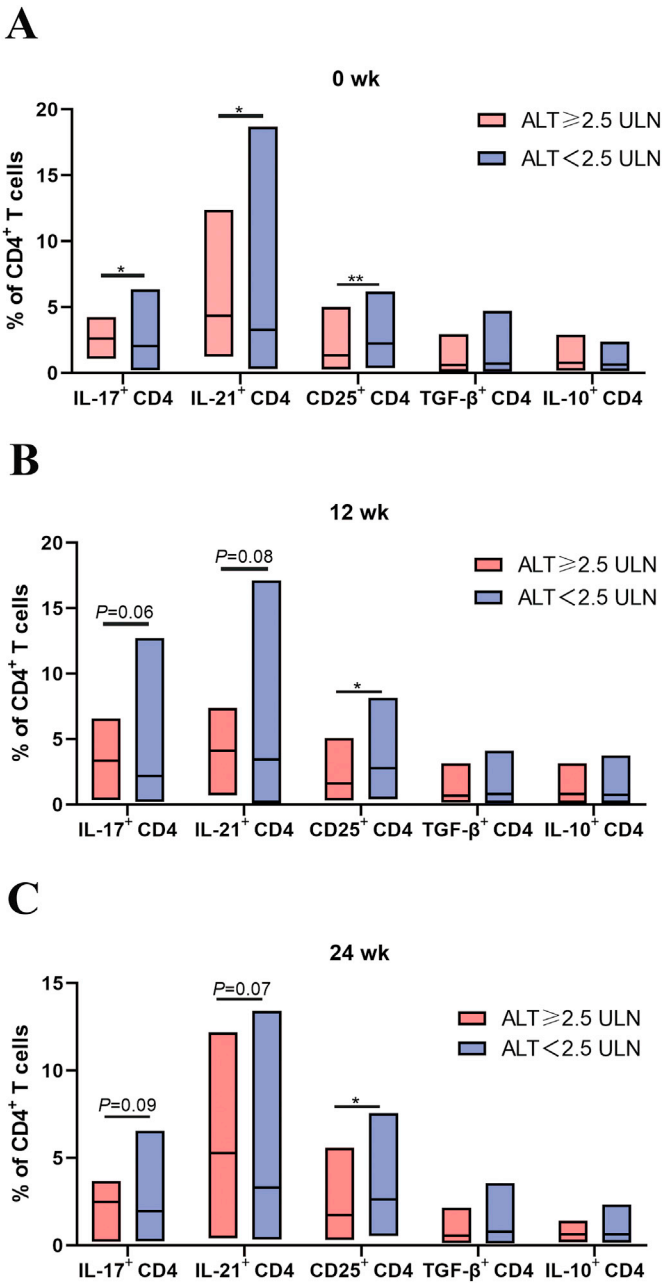

Fig. 4. The differences in proportions of Th17 and Treg cells among patients with ALT ≥2.5 ULN and ALT <2.5 ULN at (A) baseline (0 wk), (B) week 12 (12 wk), and (C) week 24 (24 wk). $^*P < 0.05$, $^{**}P < 0.01$. Abbreviations: ALT, alanine aminotransferase; IL, interleukin; TGF-β, transforming growth factor-beta; Th17, T helper 17; Treg, regulatory T; ULN, upper limit of normal.

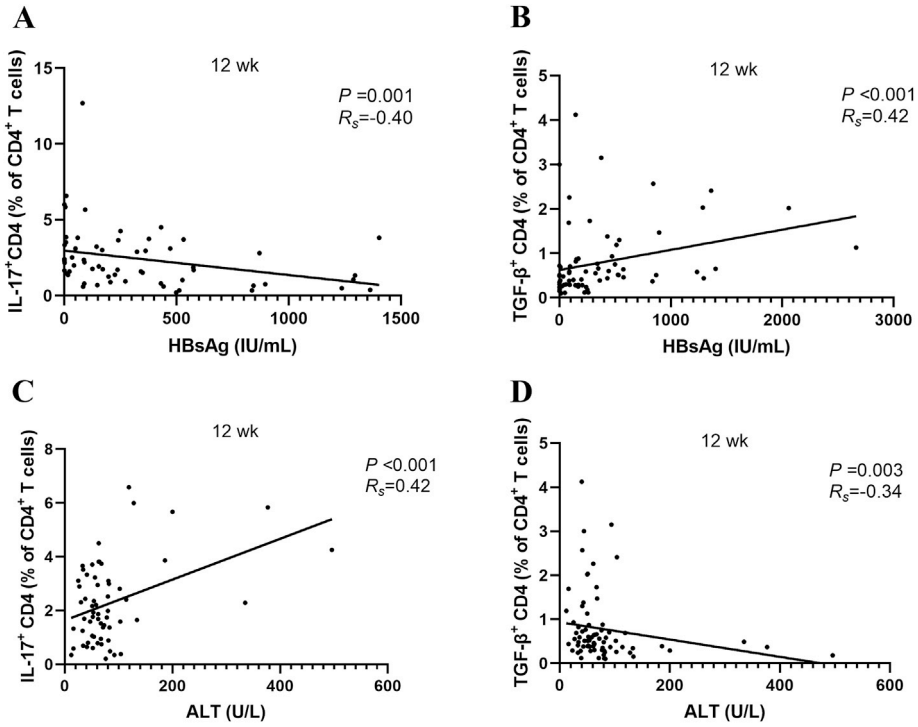

Fig. 5. Proportions of IL-17$^+$CD4$^+$ T cells and TGF-β$^+$CD4$^+$ T cell subsets were linearly correlated with the levels of (A, B) qHBsAg and (C, D) ALT at week 12 (12 wk). Abbreviations: ALT, alanine aminotransferase; IL, interleukin; qHBsAg, quantification of hepatitis B surface antigen; TGF-β, transforming growth factor-beta.

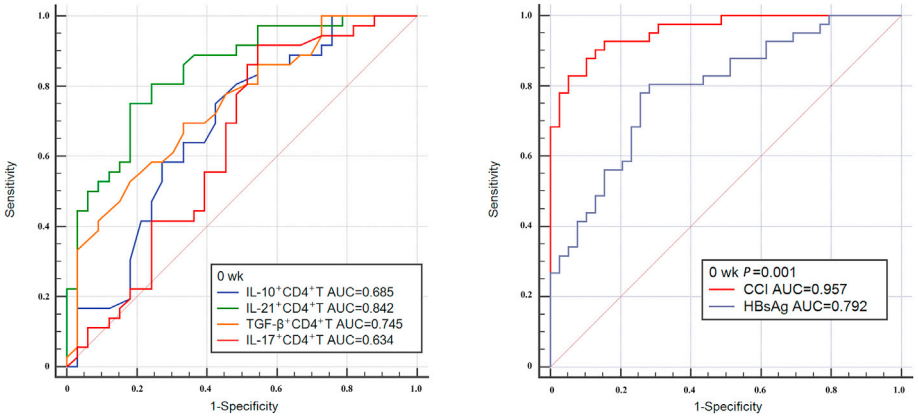

Fig. 6. The predictive value of the proportions of Th17 and Treg cells, qHBsAg, and CCI at baseline (0 wk) for HBsAg loss. (A) The receiver operator characteristic curve of proportions of IL-17$^+$/IL-21$^+$/IL-10$^+$/TGF-β$^+$CD4$^+$ T cells at baseline in predicting HBsAg loss. **(B)** Baseline CCI values were significantly better than qHBsAg levels in predicting clinical cure within 48 weeks. Abbreviations: AUC, area under the curve; CCI, clinical cure index; IL, interleukin; qHBsAg, quantification of hepatitis B surface antigen; TGF-β, transforming growth factor-beta; Th17, T helper 17; Treg, regulatory T.

inhibit Th17 cell function instead. Thus we think IFN-α therapy may not be appropriate for patients in such an immune state.

Furthermore, we found that the immune status of cured and uncured patients was significantly different at baseline. Important differences in the proportions of Th17 and Treg cells between the two groups remained at 12 and 24 wk. The findings suggested that a patient's immune status at baseline was the most important factor in determining whether or not they could achieve a clinical cure. We hypothesized that the patient's baseline immune status would affect his or her response to IFN-α, as well as directly contribute to the patient's subsequent HBsAg clearance. Because NA antiviral therapy can restore the immune response to HBV,[44–46] long-term antiviral treatment results in long-lasting viral suppression. When qHBsAg drops to a specific level, the function of CD4$^+$ T cells can be restored to a certain extent. Currently, a low HBsAg level is the best predictor of clinical cure,[4] however, we believe that recovery of antiviral immune cells after NA therapy is closely linked to clinical cure. HBsAg levels indirectly reflected this phenomenon in the process. However, because the immune effect is a slow and long-lasting process, IFN-α may induce a long-lasting therapeutic response in a subset of patients.[6] As a result, we will extend the study to see if this pair of immune cells is linked to the ongoing maintenance or recurrence of HBsAg loss.

According to our data, Th17 cells were negatively correlated with qHBsAg but positively correlated with ALT level while Tregs did the opposite. From another angle, after PEG-IFN treatment, the

proportions of Th17 and Treg cells in many patients change dynamically, because different patients respond differently to IFN-α. Given that there was already a significant difference in the baseline proportions of Th17 and Treg cells between the two groups. In contrast, the qHBsAg levels in the two groups of patients are more or less declining, with significant fluctuations in ALT levels, particularly in the cured patients. As a result, the results indicated that the Th17 and Treg proportions could not fully reflect the dynamic changes in qHBsAg and ALT levels. Nonetheless, our findings suggest that the proportions of Th17 and Treg cells are important in an ALT flare and HBsAg loss during PEG-IFN and NA therapy.

To date, there is no better predictor of clinical cure than qHBsAg itself, and more studies are needed to confirm the predictive value of the immunologic and virological biomarkers for HBsAg loss. We used baseline data to predict HBsAg loss based on the difference in overall proportions of Th17 and Treg cells between the two groups of patients. The immune index of our data at baseline could indeed be used as a good predictor of clinical cure and was better than qHBsAg itself. García-López *et al*.,[7] recently discovered that in CHB patients with low qHBsAg, the presence of functional HBV-specific T cells at baseline is associated with a successful outcome after treatment withdrawal and that HBV-specific T cell responses do not increase during HBsAg loss.[7] Previous research has reported that some immune-related indicators can predict HBeAg seroconversion and response to IFN-α.[47–49] Good early predictors (for clinical cure) can assist patients in maximizing benefits and stopping IFN-α therapy in time to minimize adverse effects. Our findings suggest that baseline levels of Th17 and Treg cells, in addition to viral markers, can be used to predict the outcome of CHB patients treated with PEG-IFN and NA. We can best predict the outcome of INF-α and NA treatment for CHB patients with low levels of qHBsAg by combining immune cells and virus indicators.

A limitation of our study was lack of the HBV genotype, which could not be obtained at enrollment. HBV DNA was undetectable. In addition, our sample size was still limited. Our findings require external validation, and we hope to expand the sample size for future research to back up our current findings. Meanwhile, this study's data is restricted to peripheral blood, and there is no available information on intrahepatic pathology. Because the effects of IFN-α may be long-lasting, we will continue to follow up with current patients to provide more solid evidence for our recent findings.

5. Conclusions

In summary, baseline Th17 and Treg cell levels were closely associated with HBsAg loss in HBeAg-negative patients with low qHBsAg treated with PEG-IFN combined with NA. Our finding suggested that a patient's immune status at baseline was the most important factor in determining whether or not they could achieve a clinical cure. Combined with qHBsAg, the baseline proportions of Th17 and Treg cells could better predict HBsAg loss. The findings of this study can help clinicians make medication decisions, screen out patients who have a poor response early on, and help patients avoid unnecessary financial burdens and adverse reactions.

Authors' contributions

L.-L. Wu and X.-Y. Li contributed equally to this study. L.-L. Wu, X.-Y. Li, Y.-H. Huang, and Z.-L. Gao designed the study. L.-L. Wu, X.-Y. Li, H.-Deng, G.-L. Zhang, and Q.-Y. Zhao contributed to the draft. L.-L. Wu, X.-Y. Li, D.-Y. Xie, B.-L. Lin, and Z.-S. Mo collected the data. L.-L. Wu, X.-Y. Li, and G.-L. Zhang analyzed and interpreted the data. K. Deng, Q.-Y. Zhao, D.-Y. Xie, B.-L. Lin, Y.-H. Huang, and Z.-L. Gao critically revised the manuscript. All authors read and approved the final version of this manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.livres.2023.04.002.

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