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Interferon therapy-induced reduction in PD-1 + CD8 + and CD160 + CD8 + T cells is associated with functional cure in hepatitis B

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Abstract

Background and aims HBsAg seroclearance is a key indicator of functional cure in chronic HBV. We evaluated the efficacy of pegylated interferon-α (Peg-IFN-α) in HBeAg-negative patients and its association with T-cell subsets.

Methods We retrospectively analyzed 618 patients, 34 of whom underwent longitudinal immune profiling.

Results Among 618 patients, a total of 214 cases (34.6%) achieved HBsAg clearance, namely 214 cases in group R and 404 cases in group NR. The R group was younger, had longer treatment, and had lower baseline HBsAg levels compared to the NR group. In 34 HBeAg-negative patients with chronic HBV infection, we studied the distribution of CD4 + and CD8 + T cells at different stages of differentiation. After 24 weeks of interferon therapy, responders exhibited increased CD4 + TCM and stable CD8 + TCM levels, along with decreased CD8 + TEMRA and Th2 cells. In contrast, non-responders showed reductions in TCM, elevated TEMRA, and Th2 cells. Responders exhibited a marked decline in PD-1 + CD8 + and CD160 + CD8 + T cells (at 0.97 and 0.95 times the baseline), whereas non-responders showed relative increases (at 1.74 and 1.55 times the baseline).

Conclusion Peg-IFN-α may promote HBsAg clearance in selected HBeAg-negative patients with low antigen burden and partially reversible T-cell exhaustion.

Keywords Chronic HBV infection, HBsAg seroclearance, T cell immunity, T cell subsets, Inhibitory receptors

Introduction

Chronic hepatitis B virus (HBV) infection remains a major global health burden. Nucleos(t)ide analogs (NUCs), the most widely used therapy, provide durable viral suppression and reduce the risk of hepatocellular carcinoma, but relapse often occurs after treatment discontinuation [1]. In contrast, HBsAg seroclearance is considered the optimal marker of functional cure [2]. Recent evidence suggests that pegylated interferon-α (Peg-IFN-α) can enhance HBsAg clearance in carefully selected patients with low baseline antigen levels. For example, a 2024 cohort study reported a 52.1% clearance rate after 24 weeks of Peg-IFN-α in patients with

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ultra-low HBsAg [3], while immunoprofiling studies have highlighted Peg-IFN-α–induced immune activation [4].

Chronic HBV infection is characterized by a decrease in the frequency and function of HBV-specific CD4 and CD8 T cells [5]. During chronic infection, T cells exhibit reduced effector function and upregulation of inhibitory receptors such as programmed cell death-1(PD-1), CD160, cytotoxic T-lymphocyte antigen-4 (CTLA-4), and 2B4, limiting their ability to clear infection (Supplementary Table 5) [6]. However, depleted T cells are not completely inert, and this depletion is thought to be reversible, providing a clinical opportunity to enhance immunity using immunotherapy [7]. Therefore, restoration of T cell function may be a useful therapeutic strategy for HBV control [8]. Importantly, T-cell dysfunction is not irreversible, offering opportunities for immunomodulatory therapy. Peg-IFN-α, beyond its antiviral activity, exerts pleiotropic effects on T-cell differentiation and exhaustion, as supported by recent single-cell analyses [9–11]. However, the dynamics of T-cell subsets, especially those expressing inhibitory receptors, in patients with chronic HBV infection treated with Peg-IFN-α-based therapy during treatment and the relationship with functional cure are unknown.

Despite these insights, the dynamics of T-cell subsets—particularly those expressing inhibitory receptors—during Peg-IFN-α therapy and their relationship with functional cure remain incompletely understood. Therefore, this study aimed to investigate how Peg-IFN-α modulates T-cell phenotypes in HBeAg-negative patients and to identify immune correlates of HBsAg seroclearance.

Materials and methods

Collection of clinical data

Study population

Patients with HBeAg-negative chronic HBV infection who were retrospectively selected for this study had attended Beijing You'an Hospital of Capital Medical University between January 2008 and February 2023. A total of 618 HBeAg-negative CHB patients were retrospectively included, representing all eligible patients treated with Peg-IFN-α. The availability of eligible cases determined the sample size. The subgroup of 34 patients for longitudinal immune-phenotyping was selected based on the availability of serial PBMC samples at baseline, week 12, and week 24. To ensure that this subgroup was representative, we compared baseline demographic and clinical characteristics with those of the full 618-patient cohort (Supplementary Table 1). No significant differences were identified.

Inclusion criteria: (1) HBsAg-positive for at least 6 months; (2) HBsAg < 1000 IU/ml, HBeAg-negative, HBV DNA < 2000 IU/ml; (3) no previous Peg-IFN-α treatment. HBV genotype testing was not routinely performed during the study period; thus, genotype information was unavailable for the present analysis. Exclusion criteria: (1) patients with comorbid hepatitis A virus (HAV), hepatitis C (HCV), human immunodeficiency virus (HIV), or other viral infections; (2) patients with comorbid other chronic liver diseases, such as autoimmune liver disease and alcoholic liver disease; (3) patients with cirrhosis or HCC; (4) patients suffering from diseases that are contraindicated for the application of Peg-IFN-α, including hyperthyroidism, depression, severe cardiovascular and cerebrovascular diseases. The overall study design is illustrated in Fig. 1.

Study follow-up and outcomes

This was a retrospective cohort study in which enrolled patients were treated with Peg-IFN-α, and all were followed up every 12 weeks during treatment.

Achievement of a virologic response after treatment was defined as two consecutive measurements of HBV DNA < 10 IU/mL at three-month intervals. Achievement of a functional cure (i.e., HBsAg seroclearance) was described as a post-treatment test of HBsAg < 0.05 IU/mL with or without HBsAb positivity, which was determined by a repeat test within three months with an HBsAg still < 0.05 IU/mL.

HBsAg seroclearance was defined as undetectable HBsAg confirmed by at least two consecutive measurements, including one at least 24 weeks after the end of Peg-IFN-α therapy. Patients were grouped according to whether they achieved HBsAg seroclearance after treatment and were categorized into two groups: HBsAg response (R) and non-response (NR).

Experimental methods

Specimen collection

Based on an established Peg-IFN-α treatment cohort of HBeAg-negative chronic HBV infection (HBsAg < 1000 IU/mL, HBeAg-negative, and HBV DNA < 2000 IU/mL), to reduce immunological confounding, 34 sex- and age-matched patients were selected and divided into two groups based on HBsAg clearance or non-clearance, with 22 HBsAg-responsive patients (group R) and 12 non-responsive patients (group NR). These individuals were drawn from the larger Peg-IFN-α–treated cohort based on the availability of paired PBMC samples at 0, 12, and 24 weeks. All enrolled subjects signed the informed consent, and this study was reviewed by the Ethics Committee of Beijing You'an Hospital.

Isolation and freezing of PBMC

Peripheral venous blood was collected into 5 mL tubes containing anticoagulant EDTA, and the blood samples were diluted with PBS buffer and centrifuged in a density gradient to isolate the PBMC. After centrifugation, the

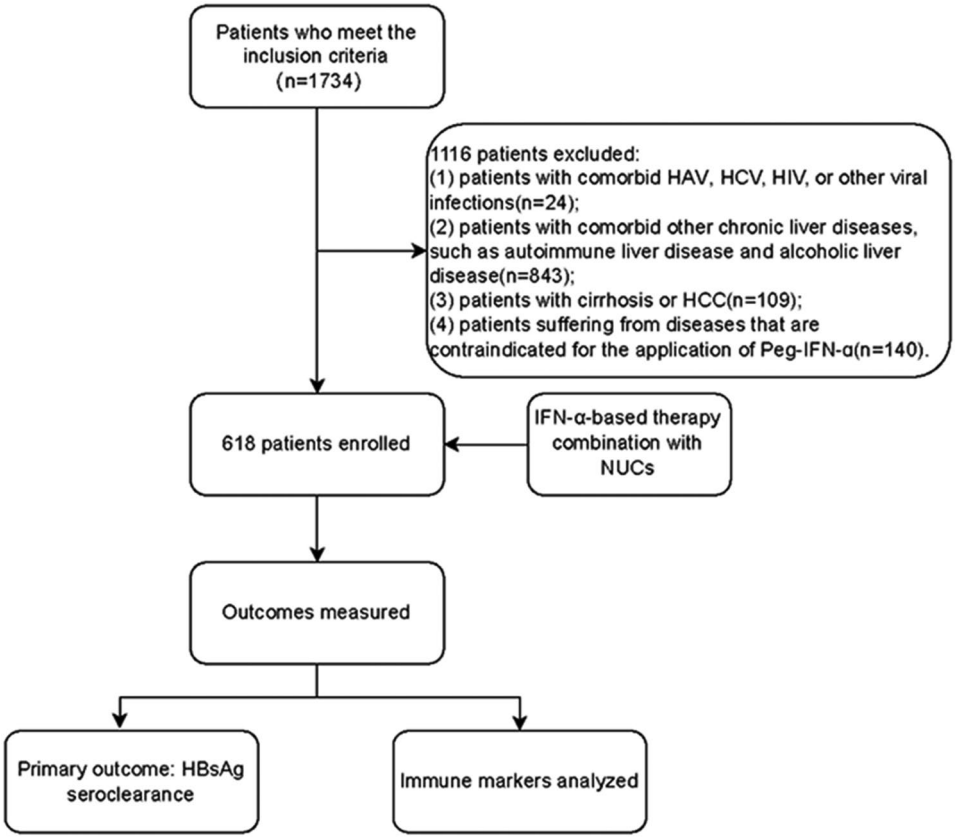

Fig. 1 Study design flowchart

cells were resuspended by adding cell cryopreservation solution (inactivated fetal bovine serum), and then the resuspension solution was dispensed into multiple cryostat tubes for spare parts. A gradient freezing method was applied to freeze the specimen, i.e., the cryobox was first placed in the refrigerator at -4℃ for 30 min, then removed and placed in the refrigerator at -20℃ for 2 h for freezing, and then the cryobox was placed in the refrigerator at -80℃ for freezing, and then after 24 h of freezing in the refrigerator at -80℃, the cells in the cryobox were again transferred to the refrigerator at -130℃ for medium-term preservation or put into the liquid nitrogen for long-term preservation.

Flow cytometry and cell sorting

A subset of 34 patients from the treatment cohort was selected for immunological analysis based on PBMC availability at baseline, week 12, and week 24. The resuscitated cells were transferred into centrifuge tubes containing culture medium (37 °C RPMI-1640 medium, 8 ml), and the cells were resuspended in PBS buffer. The cells were analyzed using anti-FVS510-BV510-A, anti-CD3-Alexa Fluor 700, anti-CD4-FITC, anti-CD8-APC-H7, anti-PD-1-BV650, anti-CTLA-4-PE, anti-2B4-APC, anti-CD160-PE-Cy7, anti-CCR7- BV711, anti-CD45RA-PE-CF594, anti-CXCR5-APC, anti-CD196-BV421, and anti-CD183-PE-Cy7 specific antibodies to stain the cells. The cells were then washed with PBS buffer and fixed with 1% paraformaldehyde before being analyzed using a BD FACS Canto II flow cytometer and analyzing the flow cytometry data using FlowJo software (10.8.1, Tree Star Inc., Ashland, OR, USA).

CD3 + CD4 + cells are CD4 + T cells, and CD3 + CD8 + cells are CD8 + T cells. According to the different stages of differentiation, they are divided into the following four cell populations: CD45RA + CCR7 + cells are initial T cells (naïve T cells); CD45RA-CCR7 + cells are Central Memory T cells (TCM); CD45RA-CCR7- cells are Effector Memory T cells (TEM); and CD45RA + CCR7- cells are Terminally Differentiated Effector Memory T Cells Re-expressing CD45RA (TEMRA).

CD3 + CD4 + CXCR5 + were helper T cells (Th).CD3 + CD4 + CXCR5 + CD183 + CD196- cells were Th1 cells, CD3 + CD4 + CXCR5 + CD183-CD196- cells were Th2 cells, and CD3 + CD4 + CXCR5 + CD183-CD196 + cells were Th17 cells.

CD4 T cells expressing PD-1, CTLA-4, CD160, and 2B4 were denoted as PD-1 + CD4+, CTLA-4 + CD4+,

CD160 + CD4+, and 2B4 + CD4 + T cells, respectively; CD8 T cells expressing PD-1, CTLA-4, CD160, and 2B4 were denoted as PD-1 + CD8+, CTLA- 4 + CD8+, CD160 + CD8+, 2B4 + CD8 + T cells.

Measurement of HBV serum markers

By applying Elecsys MODULAR ANALYTICS E-170 (Roche Diagnostics GmbH, Germany), the lower limit of quantitative detection of HBsAg was 0.05 IU/mL, HBsAb > 10 IU/mL was considered as positive, HBeAg < 1 cut-off index (COI) was considered as negative, and HBeAb > 1 COI was considered as negative. The lower limit of detection of HBV DNA was 10 IU/mL by using the cobas® AmpliPrep/cobas® Taqman fully automated nucleic acid isolation and purification and PCR analysis system (Roche Diagnostics GmbH, Germany).

Statistical analysis

Categorical variables were expressed using percentages, and comparisons of the 2 ratios were handled using the Crosstabs and Fisher's exact tests. Continuous variables were presented as medians (interquartile range) and compared between groups using the Mann-Whitney U test and the Kruskal-Wallis test. Given the exploratory aim and the relatively small size of the immunophenotyping subgroup, no formal multiple testing correction (e.g., Bonferroni or FDR) was applied to avoid inflation of type II error. Instead, interpretation focused on consistent patterns across immune subsets and time points. For clinical variables, multiple imputation is performed on those with missing values less than 20%, Interpolate 5 times, return 5 different interpolated datasets, perform up to 50 interpolation iterations, and extract the first dataset after interpolation for final analysis, while those with missing values exceeding 20% have been excluded. For the immunophenotyping subgroup, patients were included only if PBMC samples were available at all three time points (weeks 0, 12, and 24), thus minimizing missingness. Statistical analyses were done in IBM SPSS Statistics 27, and statistically significant differences are indicated as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Results

Clinical characteristics and therapeutic effects

Patient clinical characteristics

A total of 618 HBeAg-negative chronic HBV-infected patients who visited Beijing You'an Hospital of Capital Medical University between January 2008 and February 2023 were included in this study. The median age was 40(33,48) years, 72.8% were male, the median duration of treatment with Peg-IFN-α was 53(36,82) weeks, and the median follow-up was 86(51,199) weeks. 139 patients received nucleoside analogs for at least 3 months before interferon therapy.

618 patients were treated with interferon, HBsAg response (R) occurred in 214 patients, and non-response (NR) occurred in 404 patients (Table 1). The median time to HBsAg seroclearance in the response group ($n = 214$) was 33 weeks (range: 21–75 weeks) from treatment initiation. The majority of patients ($n = 183$, 85.5%) achieved HBsAg seroclearance during treatment, while the remainder did so during follow-up. The response group was significantly younger, 39(31,46) vs. 42(35,50) years old, $p < 0.001^{***}$, and had a longer duration of Peg-IFN-α treatment, 63(43,88) vs. 49(34,82) weeks, $p < 0.001^{***}$. Patients in the response group had significantly lower baseline HBsAg and HBV DNA levels than those in the non-response group. The P values were < 0.001 and 0.048.

Table 1 Baseline clinical characteristics

	Total($n = 618$)	R ($n = 214$)	NR ($n = 404$)	*P*
Age	40(33,48)	39(31,46)	42(35,50)	< 0.001***
Sex (Male)	72.8%	72.9%	72.8%	0.526
Previously treated with NUCs (%)	22.5%	19.6%	24%	0.127
Duration of Peg-IFN-α treatment (weeks)	53(35.6,82.4)	63(43,88)	49(34,82)	< 0.001***
HBsAg (Log_{10} IU/mL)	1.96(1.06,2.50)	1.42(-0.02,2.02)	2.2(1.5,2.7)	< 0.001***
HBsAb Positivity (%)	10.0%	12.6%	8.7%	0.080
HBV DNA (Log_{10} IU/mL)	0(0,2.35)	0(0,2.11)	1.10(0,2.43)	0.048*
Virologic Response Rate (%)	51.4%	55.4%	49.4%	0.121
ALT (U/L)	27.2(18.7,44.1)	27.3(18.2,47.5)	25.4(18.0,39.4)	0.139
AST (U/L)	26(21,34.9)	25.6(21,31.3)	24.7(20,33.4)	0.480
TBIL (μmol/L)	15.4(12,20.4)	15(11.4,20.6)	16(12.5,21.3)	0.009**
ALB (g/L)	46.5(44.6,48.2)	46.5(44.8,48.5)	46.4(44.5,47.9)	0.183
GLO (g/L)	28(25.7,30.3)	28.1(25.9,30.5)	28.1(25.7,30.2)	0.376
γ-GT (U/L)	22.6(15.8,36.8)	22.4(15,67.5)	21(15,36.7)	0.662
WBC (*10^9/L)	5.01(3.9,6.4)	5.2(4.1,6.5)	5.3(4.1,6.6)	0.391
ANC (*10^9/L)	2.78(1.95,3.74)	2.9(2.04,3.81)	2.93(2.12,3.9)	0.248
PLT (*10^9/L)	189(143,233)	193(160.5,243)	195(156,238)	0.399

No significant differences were observed between these two groups at baseline in terms of sex ratio, HBV DNA virologic response rate, ALT, AST, ALB, GLO, γ-GT, WBC, ANC, and platelet counts.

Changes in HBsAg, HBsAb, HBV DNA, and ALT during treatment

The changes in viral response indicators during interferon therapy for both groups are presented in Table 2. In the response group, HBsAg levels consistently decreased throughout treatment until a serologic response to HBsAg was achieved. However, the changes in HBsAg levels in the non-response group were not significant (Fig. 2A). The response group consistently demonstrated higher HBV DNA response rates compared to the non-response group, particularly at weeks 36 and 48 (Fig. 2B), with P values of 0.003** and 0.031*, respectively. ALT levels increased in both groups at 12 weeks of treatment, with a greater increase in the response group, and decreased to baseline levels in both groups at 48 weeks (Fig. 2C). During the treatment period, the incidence of HBsAb was significantly higher in the response group compared to the non-response group. Among responders, HBsAb typically appeared after or concurrently with HBsAg seroclearance (Fig. 2D), indicating successful seroconversion. A few patients exhibited detectable HBsAb at baseline while still HBsAg-positive, possibly reflecting early immune reactivation. These patients generally had low baseline HBsAg levels.

Changes in the number of T-cell subsets and subpopulations expressing inhibitory receptors during interferon therapy

Changes in T-Cell subset frequencies during interferon therapy

We have identified and studied the distribution of CD4 + and CD8 + T cells in different stages of differentiation in 34 HBeAg-negative patients with chronic HBV infection. 13 patients who received NUCs prior to Peg-IFN-α therapy, and the remaining patients were treated with Peg-IFN-α-based therapy combination with NUCs concurrently. Patients were grouped based on their response to treatment: 22 responded, and 12 did not. Importantly, there were no significant differences in the

Table 2 Comparison of changes in HBsAg, HBsAb, HBV DNA, and ALT between the response and non-response groups during interferon therapy

	R (*n* = 214)	NR (*n* = 404)	*P*
HBsAg (Log10 IU/mL)			
Baseline	1.43(0.22,2.18)	2.25(1.59,2.68)	< 0.001***
12 W	0.59(-0.59,1.65)	2.00(0.96,2.50)	< 0.001***
24 W	-0.70(-1.30,0.65)	1.71(0.40,2.31)	< 0.001***
36 W	-1.30(-1.30, -0.20)	1.42(0.21,2.26)	< 0.001***
48 W	-1.30(-1.30, -0.51)	1.46(0.22,2.25)	< 0.001***
Delta Value of HBsAg Compared with Baseline (Log_{10} IU/mL)			
12 W	0.47(0.04,1.10)	0.13(-0.01,0.45)	< 0.001***
24 W	1.23(0.38,2.51)	0.37(0.08,0.87)	< 0.001***
36 W	1.86(0.81,3.02)	0.44(0.14,2.14)	< 0.001***
48 W	2.09(0.97,3.05)	0.50(0.14,1.14)	< 0.001***
Virologic Response Rate (%)			
Baseline	55.4% (92/166)	49.4% (163/330)	0.121
12 W	76.5% (127/168)	71.9% (241/335)	0.223
24 W	81.1% (133/164)	79.3% (256/323)	0.362
36 W	92% (138/150)	81.7% (214/262)	0.003**
48 W	91.5% (118/129)	83.9% (183/218)	0.031*
Incidence of HBsAb Positivity (%)			
Baseline	12.6% (27/214)	8.7% (35/404)	0.080
12 W	20.4% (43/211)	8.4% (34/404)	< 0.001***
24 W	35% (75/214)	10.6% (42/397)	< 0.001***
36 W	48.8% (98/201)	14.7% (49/333)	< 0.001***
48 W	58.3% (109/187)	15.1% (41/271)	< 0.001***
ALT(U/L)			
Baseline	30.0(19.1,50.0)	26.7(18.3,40.9)	0.139
12 W	47.8(31,77.0)	40.2(24.1,67.2)	0.047*
24 W	43.8(27.4,71.0)	36.2(23.0,59.0)	0.004**
36 W	37.0(24.2,60.0)	32.0(29.0,53.8)	0.025*
48 W	31.0(22.0,47.3)	28.8(20.0,44.0)	0.171

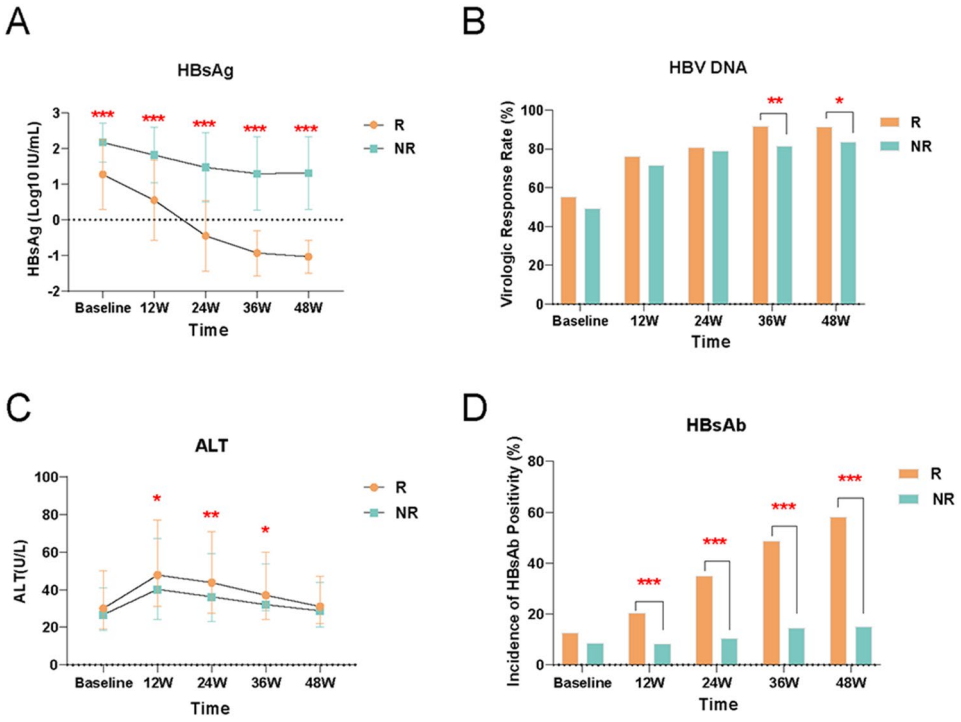

Fig. 2 (**A**) Changes in HBsAg quantification; (**B**) Virologic response rate; (**C**) ALT quantification; (**D**) HBsAb incidence during interferon therapy in the HBsAg-response and non-response groups. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

baseline clinical characteristics between the two groups (Supplementary Table 2). To provide hematological context for T cell phenotypic changes, we evaluated baseline and dynamic changes in total lymphocyte counts and neutrophil-to-lymphocyte ratio (NLR) in the 34 patients (Supplementary Table 3), and there were no significant differences were observed between responders and non-responders.

The frequency of CD4 + T cells and CD8 + T cell subsets did not show significant differences between the response and non-response groups (Fig. 3.B&C), corresponding absolute frequencies are provided in Supplementary Table 4. However, at 24 weeks of interferon treatment, the percentage of CD4 + central memory T (TCM) cells in the responder group increased to 1.34 times the baseline level, whereas in the non-responder group it decreased to 0.85 times the baseline (Fig. 3D). The difference in changes between the two groups was statistically significant ($P = 0.044$*). A similar trend was observed in CD8 + TCM cells: their frequency remained relatively stable in the responder group after 24 weeks, while it declined to 0.61 times the baseline level in the non-responder group (Fig. 3E, $P = 0.022$*). In contrast, CD8 + TEMRA cells showed an opposite pattern, decreasing to 0.92 times the baseline in the responder group but increasing to 1.10 times the baseline in the non-responder group at week 24 (Fig. 3E, $P = 0.033$*).

Changes in Th cell frequency during interferon therapy

Helper T cells are critical for the maturation, differentiation, and survival of B cells and are closely related to antibody production. To understand the dynamic changes of Th cells during interferon treatment regarding HBsAg response, we further examined Th cells. The results showed that the frequency of Th1 cells in the response group was consistently higher than that in the non-response group at baseline and during treatment (Fig. 4B). Still, this difference did not reach statistical significance. At 12 weeks of interferon treatment, the percentage of Th2 cells increased in both groups compared to baseline; however, the increase was less pronounced in the responder group, reaching 1.01 times the baseline level, compared to 1.13 times in the non-responder group ($P = 0.030$*). By week 24, the frequency of Th2 cells had decreased slightly in the responder group to 0.94 times the baseline, while it remained elevated in the non-responder group at 1.13 times the baseline (Fig. 4C, $P = 0.020$*).

Changes in the number of T cells expressing inhibitory receptors during interferon treatment

We examined the frequency of CD4 + and CD8 + T cells expressing PD-1, CTLA-4, 2B4(CD244), and CD160 at baseline, 12 weeks, and 24 weeks of interferon therapy in both groups. Our data showed that at baseline, the frequencies of the three cell populations, 2B4 + CD4+,

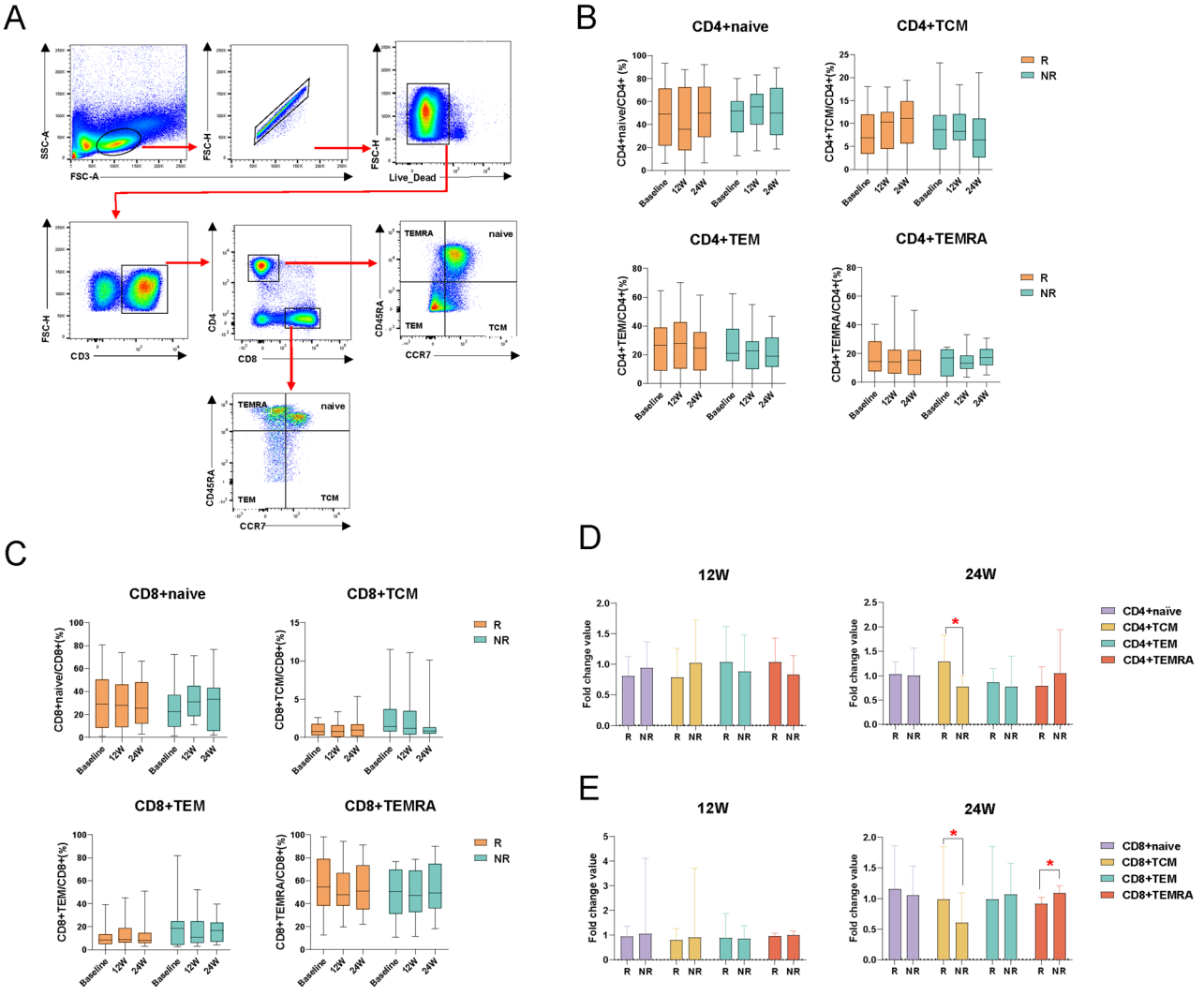

Fig. 3 (**A**) Gated sorting strategies for memory T cell subsets; (**B**) Changes in frequencies of differentially differentiated subpopulations of CD4 + T cells in the response and non-response groups during treatment; (**C**) Changes in frequencies of differentially differentiated subpopulations of CD8 + T cells in the response and non-response groups during treatment; (**D**) Fold change values(12 or 24 weeks/baseline, the value greater than 1 indicates an increase, and a value less than 1 indicates a decrease) relative to baseline were calculated for subpopulations of CD4 + T-cell at 12 and 24 weeks; (**E**) Fold change values(12 or 24 weeks/baseline) relative to baseline were calculated for subpopulations of CD8 + T-cell at 12 and 24 weeks. **P* < 0.05; ***P* < 0.01; ****P* < 0.001

PD-1 + CD8+, and CD160 + CD8 + T, were significantly higher in the response group than in the non-response group (Fig. 5B&C), with P values of 0.007**, 0.031*, and 0.005**, respectively. At week 12 of interferon treatment, the frequencies of PD-1 + CD8 + and CD160 + CD8 + T cells in the responder group were 0.89 and 0.98 times their respective baseline levels, whereas in the non-responder group, they increased significantly to 2.16 and 2.69 times the baseline (Fig. 5D), with P values of 0.022* and 0.007**, respectively. At week 24, the frequencies of PD-1 + CD8 + and CD160 + CD8 + T cells in the responder group were 0.97 and 0.95 times the baseline, while in the non-responder group, they remained elevated at 1.74 and 1.55 times the baseline (Fig. 5E), with corresponding P values of 0.031* and 0.007**.

Discussions

HBsAg seroclearance is currently the best criterion for evaluating antiviral therapy and represents a clinical cure. In addition to biochemical, virological, and histological improvements, people who achieve HBsAg seroclearance have a 20-fold reduced risk of HCC [12, 13]. HBV-specific T-cells play HBV-specific T cells play an important role in disease progression (including viral response, hepatitis, cirrhosis, and liver cancer development) and the response to antiviral therapy [14]. In the current standard of care for chronic HBV infection, Peg-IFN-α-based therapy has shown greater efficacy than NUC monotherapy in achieving HBsAg seroconversion when administered over a comparable treatment duration, and higher HBsAg seroclearance and sustained increases in HBsAg

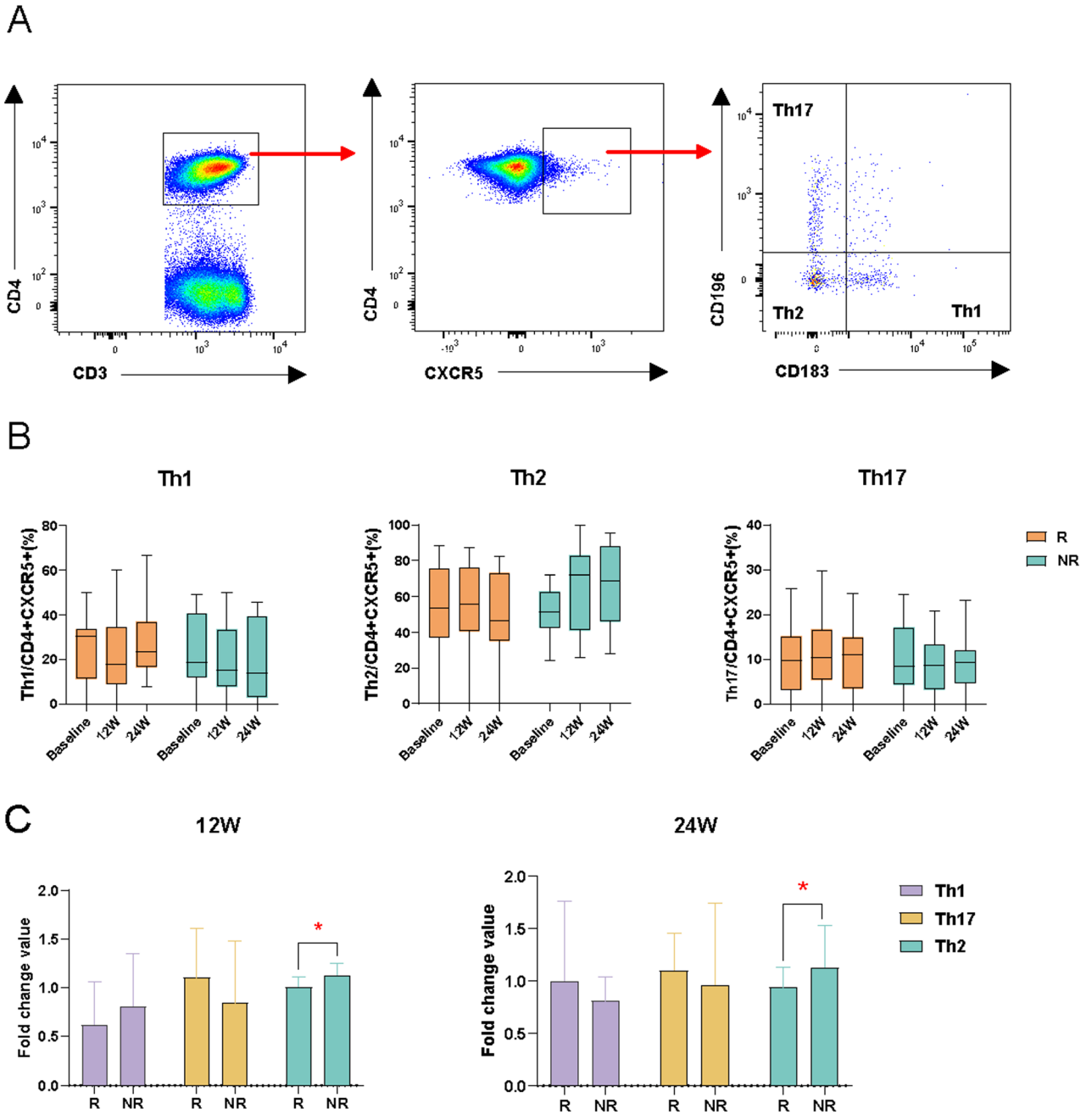

Fig. 4 (**A**) Gated sorting strategies for helper T cells; (**B**) Changes in Th1, Th2, Th17 cell frequencies in the response and non-response groups throughout treatment; (**C**) Fold change values (12 or 24 weeks/baseline) relative to baseline were calculated for Th1, Th2, Th17 cell frequencies in the response and non-response groups at 12 and 24 weeks. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

response can be achieved several years after treatment is stopped [15, 16]. According to EASL 2025 guidelines, patients with HBeAg-negative chronic HBV infection (inactive carriers) typically do not require antiviral therapy [17]. However, Peg-IFN-α-based immunomodulatory therapy has been explored in select patients with low HBsAg levels as a strategy to achieve functional cure [6]. Additionally, in the HBeAg-negative interferon-treated advantaged population, evidence is scarce regarding the correlation between changes in T-cell subsets and inhibitory receptor expression and Peg-IFN-α-induced HBsAg response.

In this study, we found that younger patients, lower baseline HBsAg and HBV DNA levels, and longer duration of Peg-IFN-α treatment were significantly associated with HBsAg response. Patients who achieved HBsAg response had HBsAg levels that continued to decrease over the treatment period, whereas patients who

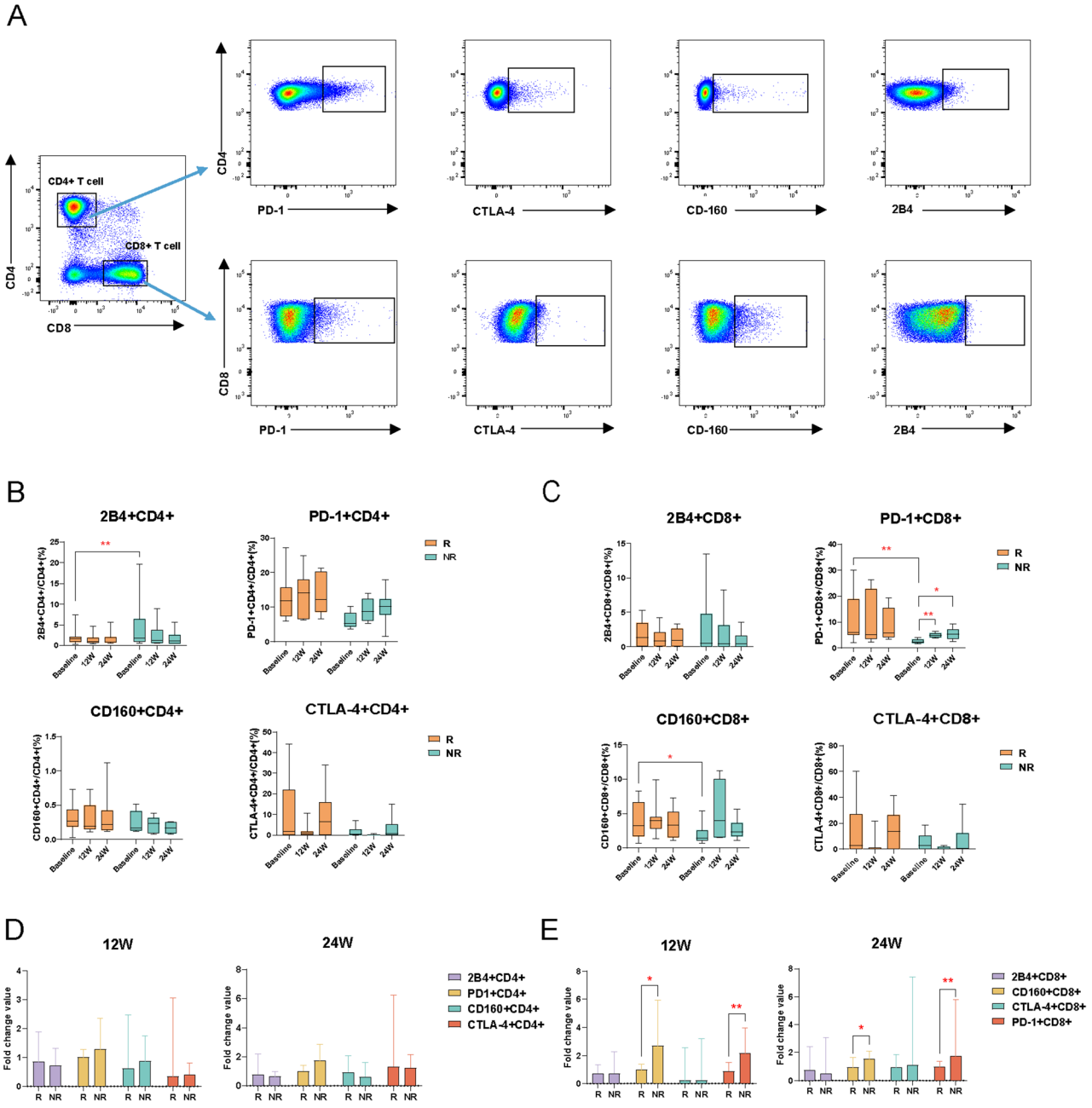

Fig. 5 (**A**) Gated sorting strategies for CD4 + and CD8 + T cells expressing inhibitory receptors; (**B**) Expression of CD4 + T cell surface inhibitory receptors in the response and non-response CD4 + T cell surfaces during treatment; (**C**) Expression of CD8 + T cell surface inhibitory receptors in the response and non-response CD8 + T cell surfaces during treatment; (**D**) Fold change values (12 or 24 weeks/baseline) relative to baseline were calculated for CD4 + T cell surface inhibitory receptors frequencies in the response and non-response groups at 12 and 24 weeks; (**E**) Fold change values (12 or 24 weeks/baseline) relative to baseline were calculated for CD8 + T cell surface inhibitory receptors frequencies in the response and non-response groups at 12 and 24 weeks.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

ultimately failed to achieve HBsAg response did not have significant changes in their HBsAg levels over the treatment period.

Younger patients may have more active immune systems that can respond more effectively to interferon therapy [18]. Supporting this notion, our post hoc analysis showed that patients aged ≤ 35 years had lower baseline CD8 + TEMRA and higher CD8 + naïve T cell frequencies, suggesting a more responsive immune status to interferon therapy (Supplementary Fig. 1). Low baseline HBsAg and HBV DNA levels may imply a lower viral load that is more easily responded to. Several studies have shown that serum HBsAg levels are negatively correlated with HBsAg response and are good predictors of

sustained HBsAg response in patients with chronic HBV infection [19, 20]. The levels of HBsAg in the response group continued to decrease during the treatment period until HBsAg serologic response was achieved. In contrast, there was no significant change in the non-response group. These findings are in line with previous studies and highlight the importance of monitoring HBsAg levels and their changes during treatment to predict HBsAg serological response [21]. Upon cessation of Peg-IFN-α therapy, some patients with HBsAg response achieve HBsAg seroconversion, i.e., production of HBsAb. The most recent studies demonstrate that higher levels of HBsAb are associated with sustained functional cure in HBeAg-negative patients treated with Peg-IFN-α-based therapy, and that patients with an HBsAb > 2 LogIU/L at the end of therapy are at much lower risk of reversal was much lower than that of other patients [22–24]. We observed that the incidence of HBsAb during treatment was significantly associated with HBsAg response. In addition, HBsAb positivity at baseline in a minority of patients suggests partial immune control or early seroconversion despite ongoing HBsAg expression. This group may represent a unique immunological subset with higher potential for HBsAg seroclearance.

During HBV infection, initial T cells are activated by antigens and can differentiate into effector T cells. Some of these effector T cells can further differentiate into antigen-specific memory T cells [25]. However, when the infection becomes chronic, effector T cell function is severely depleted, affecting T cell homeostasis and preventing the formation of antigen-specific memory T cells [26]. Peg-IFN-α-based therapy has been shown to promote the recovery of memory T cells in HBeAg-positive CHB patients [27], and it was observed in cross-sectional studies that CHB patients who achieved spontaneous HBsAg response had significantly higher CD8 + TEM than HBeAg-response individuals [28]. In this study, we analyzed global CD4 + and CD8 + T cell phenotypes ex vivo. Although TEM cells did not change significantly over time, we observed a notable increase in CD4 + TCM cells in responders and a marked decrease in CD8 + TCM cells in non-responders, suggesting that restoration of central memory compartments may be more relevant to functional cure. Furthermore, CD8 + TEMRA cells increased in non-responders and decreased in responders, which may reflect divergent terminal differentiation trajectories under interferon therapy.

Th cells regulate immunity and promote inflammation and antibody production. Th1 cells mainly secrete IFN-γ, which enhances antigen presentation and promotes the bactericidal function of macrophages. Th2 cells secrete IL-4, which promotes B-cell proliferation and IgE production, thereby mediating humoral immunity and suppressing extracellular pathogens [29]. In this study, a negative correlation was observed between the frequencies of Th1 and Th2 cells during the treatment. Patients with unresponsive outcomes showed a higher prevalence of Th2 cells, possibly due to increased levels of Th2 and secretion of IL-4. This can impede IFN-γ production and Th1-type immune responses, leading to sustained HBV replication and immune tolerance, affecting the serological response to HBsAg. The frequency of Th1 cells was consistently higher in the response group compared to the non-response group. This suggests that the cellular immune responses, mediated by anti-inflammatory cytokines such as IFN-γ and IL-2, produced by Th1 cells, play an important role in the response to persistent HBV infections [30].Th17 cells, with a unique gene expression profile that differs from that of Th1 and Th2 cells, mainly secrete IL-17, which is associated with the pathogenesis of various autoimmune diseases such as rheumatoid arthritis, autoimmune encephalomyelitis, and alcoholic liver disease [31]. A negative correlation between Th17 cell frequency and Th1 and a positive correlation with ALT levels were observed in CHB patients, suggesting that Th17 cells may play an important role in immune activation in chronic HBV infection [32]. However, no significant changes in Th17 levels were observed in patients with HBV infection treated with Peg-IFN-α-based therapy.

In HBV infection, the expression of inhibitory receptors such as PD-1, CTLA-4, 2B4, and LAG-3 on the surface of functionally depleted CD4 + and CD8 + T cells significantly increases as the infection becomes chronic, and the antigenic and viral loads increase [33]. Peg-IFN-α treatment downregulates the expression of inhibitory receptors on T cells to restore their immune response function [27]. This improvement is likely to apply to both multispecific and multifunctional T cells and is most evident in patients with greater decreases in serum HBsAg levels and lower basal HBcrAg values [34]. In this study, interestingly, we observed higher baseline PD-1 + CD8 + T-cell frequencies in responders compared to non-responders, which appears counterintuitive given that PD-1 is typically a marker of T cell exhaustion. However, recent evidence suggests that PD-1 expression does not always indicate irreversible dysfunction [35]. In patients with low viral antigen load, PD-1 + T cells may retain proliferative and cytotoxic potential and represent a state of reversible exhaustion that can be restored with appropriate therapy [36, 37]. In the context of interferon therapy, it is possible that the higher baseline PD-1 + CD8 + frequencies in responders reflect an immune subset with restorable function, which can be activated by Peg-IFN-α treatment. Nevertheless, this finding should be interpreted with caution and viewed as exploratory. Further mechanistic studies are needed to confirm the role of PD-1 + T cells in response to

Peg-IFN-α therapy and to better understand the dynamics of T cell exhaustion and reactivation in chronic HBV infection. However, at 24 weeks, the frequencies of PD-1 + CD8 + and CD160 + CD8 + T cells remained relatively stable in the responder group (close to baseline), whereas in the non-responder group, both markers increased markedly compared to baseline, suggesting a continued immune exhaustion phenotype in patients failing to clear HBsAg. PD-1 and CD160 are well-known inhibitory receptors that play significant roles in the regulation of T-cell responses during chronic viral infections. However, the immune checkpoint landscape is more complex, and other inhibitory receptors such as TIM-3 and LAG-3 are also involved in immune regulation. For instance, TIM-3 is upregulated in chronic infections and can contribute to T-cell exhaustion and impaired immune responses [38]. Similarly, LAG-3, another critical immune checkpoint receptor, has been implicated in T-cell dysfunction in chronic viral infections [7]. Future work should explore the combined effects of these multiple immune checkpoints to provide a more comprehensive understanding of T-cell exhaustion and reactivation during interferon therapy.

Although our analysis was not limited to HBV-specific T cells, the phenotypic shifts observed in total CD4 + and CD8 + T cell populations are consistent with known immunological alterations in chronic viral infections [39]. Prior studies have shown that systemic expression of PD-1 and other exhaustion markers is elevated in HBV-infected individuals compared to healthy controls, reflecting chronic antigen exposure and immune dysregulation [37, 40]. Similar patterns have been reported in chronic HCV [41] and HIV infections [42]. Thus, the observed changes in T-cell subsets may represent both HBV-specific responses and broader immune activation. While not antigen-specific, it can provide meaningful insights into immune restoration dynamics in the context of antiviral therapy.

In addition, the data from this study showed that the number of some T-cell subsets and the expression of inhibitory receptors showed inconsistent fluctuations between the first 12 weeks and the second 12 weeks of interferon therapy during the first 24 weeks of treatment. A recent study suggested that such changes may be related to fluctuations in ALT levels caused by Peg-IFN-α treatment [43].

Strengths and limitations of this study: strengths: a large and complete cohort of Peg-IFN-α-treated patients, and focused on changes in T-cell subsets and phenotypes during Peg-IFN-α treatment. Limitations: (1) The study was retrospective and may have a selection bias (2). HBV genotype is a well-established predictor of interferon response. Unfortunately, due to the retrospective design and limited genotyping data, we were unable to include genotype in our analysis (3). It did not consider changes in T-cell immunity in HBeAg-positive patients, limiting a comprehensive exploration of Peg-IFN-α treatment effects on T-cell immunity (4). We only studied T-cell subpopulations and inhibitory receptor phenotypes, and did not analyze T-cells that secrete inflammatory cytokines, such as IFN-γ and IL-2. This is crucial for T-cell function during Peg-IFN-α treatment. Frequency, which is not known about the changes in T cell function in Peg-IFN-α treatment (5). This study did not include the direct measurement of HBV-specific T cells. While we assessed the frequency and phenotype of total CD4 + and CD8 + T cells, virus-specific responses were not identified, limiting the interpretation of antiviral immune restoration (6). The relatively small size of the immunophenotyping subgroup ($n = 34$) limits statistical power and may restrict the robustness of subgroup findings. Although baseline characteristics did not differ significantly from the overall cohort, results should be interpreted with caution. These factors limit the generalizability of our findings and mean that the associations observed should be interpreted cautiously as hypothesis-generating. Prospective, genotype-stratified studies with standardized treatment histories are needed to validate these results.

In conclusion, our findings suggest that Peg-IFN-α may still have a role in carefully selected HBeAg-negative patients with low baseline HBsAg levels. In patients with low antigen burden and immunological profiles consistent with reversible T-cell exhaustion, Peg-IFN-α may provide an additional pathway toward HBsAg clearance. Importantly, the immune biomarkers we observed (e.g., reduction of PD-1 + CD8 + and CD160 + CD8 + T cells, expansion of CD4 + TCM). These biomarkers should be regarded as exploratory indicators, may provide valuable insights into patient stratification and inform the development of biomarkers for guiding therapeutic decisions in chronic HBV infection.

Abbreviations

HBV	Hepatitis B virus
NUC	Nucleoside (t) analogs
HCC	Hepatocellular carcinoma
Peg-IFN-α	Pegylated interferon alpha
CHB	Chronic HBV infection
HAV	Hepatitis A virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
PBMC	Peripheral blood mononuclear cells
COI	Cut-off index
TCM	Central Memory T cells
TEM	Effector Memory T cells
TEMRA	Terminally Differentiated Effector Memory T Cells Re-expressing CD45RA
Th	Helper T cells
PD-1	Programmed cell death-1
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DPT	Duration of Peg-IFN-α treatment
TBIL	Total bilirubin
ALB	Albumin

GLO	Globulin
γ-GT	γ-glutamyl transpeptidase
WBC	White blood cell
ANC	Absolute neutrophil count
PLT	Platelet
NLR	Neutrophil-to-lymphocyte ratio

Supplementary Information

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Supplementary Material 1

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Author contributions
Daqiong Zhou (Conceptualization; Writing – original draft), Jiangyu Liu (Data curation and analysis), Jianru Jia (Formal analysis; Project administration), Hong Li (Methodology; Resources), Ling Qin (Software; Visualization), Zichen Zhang (Investigation), Zhenhuan Cao (Conceptualization; Writing – review & editing).

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Data availability
The data used to support the findings are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate
All patients provided written informed consent at the time of enrollment, which included permission for storage and future research use of PBMC samples. The protocol and consent form for the study were approved by the research ethics committee of the Beijing You'an Hospital, Capital Medical University, China ([2016]16), and conform to the principles of CFDA/GCP and Declaration of Helsinki.

Consent for publication
All authors were informed and consented to the publication of the study.

Competing interests
The authors declare no competing interests.

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