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Immune drivers of HBsAg loss in HBeAg-negative CHB patients after stopping nucleotide analog and administration of Peg-IFN

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Abstract

Background: The stoppage of nucleoside analog (NA) can lead to immune flare and loss of HBsAg in a proportion of HBeAg-negative chronic hepatitis B (CHB) patients. HBsAg loss could be improved by instituting Peg-Interferon therapy in those who show an immune flare after the stoppage of NA. We investigated the immune drivers of HBsAg loss in NA-treated HBeAg-negative CHB patients after stopping NAs and administration of Peg-IFN- α 2b therapy.

Methods: Fifty-five NA-treated eAg-ve, HBV DNA not detected CHB patients were subjected to stopping NA therapy. Twenty-two (40%) patients relapsed (REL-CHBV) within 6 months (HBV DNA \geq 2000 IU/mL, ALT \geq 2XULN) and were started on Peg-IFN- α 2b (1.5 mcg/kg) for 48 weeks (PEG-CHBV). Cytokine levels, immune responses, and T-cell functionality were assessed.

Results: Only 22 (40%) of 55 patients clinically relapsed, of which 6 (27%) cleared HBsAg. None of the 33 (60%) nonrelapsers cleared HBsAg. REL-CHBV patients had significantly increased IL-6 ($p=0.035$), IFN- γ ($p=0.049$), Th1/17 ($p=0.005$), CD4 effector memory (EM) ($p=0.01$), Tfh1/17 ($p=0.005$), and mature B cells ($p=0.04$) compared with CHBV. Six months after Peg-IFN therapy, immune resetting with a significant increase in CXCL10 ($p=0.042$), CD8 ($p=0.01$), CD19 ($p=0.001$), and mature B cells ($p=0.001$) was observed. HBV-specific T-cell functionality showed increased Tfh-secreting IFN- γ ($p=0.001$), IL-21 ($p=0.001$), and TNF- α ($p=0.005$) in relapsers and IFN- γ -secreting CD4 T cell ($p=0.03$) in PEG-CHBV.

Abbreviations: ALT, alanine transaminase; AST, aspartate aminotransferase; ATM B, atypical memory B cells; CHB, chronic hepatitis B; CHBV, chronic HBV-infected patients on antiviral treatments; CM, central memory; EM, effector memory; HC, healthy control; MFI, median fluorescent intensity; NAs, nucleotide analogs; N-CHBV, naive nontreated chronic HBV-infected patients; PBMC, peripheral blood mononuclear cells; PEG-CHBV, Peg-IFN-treated CHBV; Peg-IFN α 2b, pegylated interferon alpha 2b; REL-CHBV, relapsers in CHBV; TEMRA, terminally differentiated effector memory cells re-expressing CD45RA; TLC, total leukocyte count; UMAP, uniform manifold approximation and projection.

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Conclusions: Stopping NA therapy induces flare in about 40% of HBeAg-negative patients. Peg-IFN therapy given to such patients causes immune restoration with HBsAg loss in one fourth of them.

INTRODUCTION

HBV infection is a major global health burden, and around 350 million patients are chronically infected, leading to end-stage liver diseases, such as cirrhosis and HCC.^[1,2]

All guidelines of major liver associations have considered HBsAg seroclearance as a functional cure for chronic hepatitis B (CHB) and the ideal end point of HBV treatment. Unfortunately, clinical studies have shown that HBsAg seroclearance during long-term nucleotide analogs (NAs) therapy is a rare event, especially in HBeAg-negative CHB patients. However, in this setting, there is an agreement that NA stopping is the best and safest rule for HBsAg loss.^[3,4] The decision to discontinue NA therapy for HBeAg-negative CHB adults requires careful consideration of risks and benefits for health outcomes.^[4,5]

Both the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver do not recommend treatment discontinuation in patients with cirrhosis owing to the potential for decompensation and death, although data are limited.^[6] However, the Asian Pacific Association for the Study of Liver recommends that stopping NA therapy may be considered in patients with cirrhosis, with a careful off-therapy monitoring plan. Further, both the Asian Pacific Association for the Study of Liver and the European Association for the Study of the Liver guidelines suggested stopping rule for NAs in HBeAg-negative CHB after treatment for at least 2 years with undetectable HBV DNA documented on 3 separate occasions or 6 months apart (Asian Pacific Association for the Study of Liver);^[7,8] or who have achieved the long-term (≥ 3 y) virological suppression (European Association for the Study of the Liver) but with further close post-NA monitoring should be guaranteed.^[9]

After stopping NAs treatment, a virologic relapse is nearly universal, even after prolonged periods of HBV DNA suppression.^[10] The principal aim of stopping NA is to induce a durable remission of HBV infection, with the ideal aim of a functional cure with HBsAg loss. Although stopping NA showed an alteration in the immune component and reduction in HBsAg levels but did not support the anti-HBs production or functional cure.^[3,11,12]

However, HBeAg-negative CHB patients develop virological and clinical relapses after NAs withdrawal.^[13]

But because of the emergence of HBV DNA and no complete loss of HBsAg, there is a debate about the indications of restarting NAs or immune modulators (interferons) therapy. It is believed that clinical relapse could be beneficial in terms of driving the immune-mediated elimination of the infected hepatocytes to achieve immune control.^[5,12] Further, the discontinuation of NAs therapy in HBeAg-negative CHB patients alters the cytokine milieu suggesting that changes in the immune environment occur after virological relapse.^[14]

Although the efficacy of Peg-interferon alfa as an initial therapy in HBeAg-negative CHB is proven, it is not established whether, after NAs stop, patients restarting on Peg-interferon alfa will show complete HBsAg loss or not and what percentage and which subset of immune cells directly play a role in HBsAg loss.^[4,15,16]

Therefore, this study aimed to assess the immune predictors of relapse in HBeAg-negative CHB patients after stopping NAs and to assess the effect of Peg-IFN alpha 2b treatment as immune modulators in patients who develop clinical relapse. To address these questions, we performed high-dimensional immune phenotyping and HBV-specific T cells functionality in eAg-negative CHBV at the time of stopping NA (CHBV), at the time of relapse (REL-CHBV), and post-treatment with Peg-IFN (PEG-CHBV).

METHODS

Patient cohort

This study was conducted on the patients who were recruited in the clinical trial treatment at the Institute of Liver and Biliary Sciences (ILBS), New Delhi, from March 2017 to 2019 and registered at Clinicaltrials.gov.in (identifier number: NCT03123653).

The clinical trial was approved by the ethics and review committee of the Institute of Liver and Biliary Sciences (ILBS) (No-IEC/2017/48/MA04). To analyze the immune components after NA stopping and Peg-IFN in patients recruited in a clinical trial, a separate protocol was submitted to the ethics and review committee of the Institute of Liver and Biliary Sciences (ILBS), which was approved after a month with No-IEC/2017/49/NA02. Written informed consent was taken from all the participants, and the work was done in accordance with the Declaration of Helsinki and Istanbul (details of patient

cohort elaborated in Supplemental information, <http://links.lww.com/HC9/A226>).

Blood sampling

Peripheral blood (10–12 mL) was collected in EDTA tubes. Whole blood (100–120 μ L) was used for immunophenotyping, and the rest of the blood was spun down to collect plasma, which was stored at -80° C for various biochemical, serological tests, and cytokine bead array. The plasma-free blood sample was used for peripheral blood mononuclear cells (PBMC) isolation and T-cell functionality assays, as depicted in the experimental design (Supplemental Figure 2, <http://links.lww.com/HC9/A226>).

Serological assays

Serological markers for HBV infection, including HBsAg (#6C36-29), anti-HBs (#7C18-29), anti-HBc (#8L44-25), HBeAg (#6C32-27), and anti-HBe (#6C34-25), were done as per the manufacturer's protocol and analyzed by fully automated ARCHITECT i2000SR immunoassay analyzer (Abbott Laboratories, Abbott Park, IL). The quantification of HBsAg levels was done with a detection range of 0.05–250 IU/mL. If the HBsAg level was > 250 IU/mL, the samples were diluted from 1:100 to 1:1000 to obtain a reading within the range of the calibration curve. A concentration higher than 0.05 IU/mL was considered HBsAg positive.

HBV DNA quantification

DNA quantitation was done with 500 μ L patient plasma using COBAS AmpliPrep/COBAS TaqMan HBV Test, version 2.0 (Roche Diagnostics, Rotkreuz, Switzerland, #04894570190), as per the manufacturer's protocol. The results were expressed as IU/mL, and the lower limit of detection for the assay was 6 IU/mL.

Genotyping

HBV DNA was isolated from 200 μ L of plasma using QIAamp MinElute Virus Spin Kit (Qiagen, MD, #57704), and HBV genotyping was performed using HBV genotyping real-time PCR kit (Athenese-Dx, Chennai, India # 8002-25). TaqMan primer and probes (FAM) were used against all 8 genotypes (A–H). The assay was performed on LightCycler 480-II (Roche Diagnostics GmbH, Germany).

Isolation of PBMCs

After plasma removal, the PBMCs were isolated from the blood and stored in liquid nitrogen. In brief, the

blood was diluted with 3 times 1X PBS. Diluted blood was carefully overlaid over 15 mL Ficoll hypaque (Himedia, HiSep LSM 1077, #LS001-500) in 50 mL centrifuge tubes, which was then centrifuged (Eppendorf 5810R, A-4-62, max 4000 rpm) at 400 g without brake with acceleration at 4 for 30 minutes. The buffy coat was collected and washed with 1X PBS at 400 g for 10 minutes. The cells were counted using hemocytometer and used as per the necessity of experiments.

(Details of immune phenotyping, T-cell functional assays, checkpoint inhibitor assays, cytokine bead array, flow cytometry data analysis, and statistical analysis are elaborated in Supplemental Material and Methods, <http://links.lww.com/HC9/A226>).

RESULTS

Patients characteristics

CHBV patients considered for NA stop therapy ($n = 55$) and treatment naïve CHBV patients (N-CHBV) ($n = 19$) were closely matched for sex, age, platelets, leukocytes, and albumin (Supplemental Table 1, <http://links.lww.com/HC9/A226>). N-CHBV patients showed a significant elevation in ALT and HBV DNA compared with treated CHBV (<http://links.lww.com/HC9/A226>). A longitudinal study was conducted with CHBV ($n = 55$), on stopping NA, 22 patients relapsed (REL-CHBV) (Supplemental Figure 1, <http://links.lww.com/HC9/A226>), with a significant increase in ALT, AST, and HBV titer (Table 1). These 22 patients were started on Peg-IFN for 48 weeks and followed up till 72 weeks, but out of 22 patients, we had complete follow-up till 72 weeks in only 13 patients. Nine patients (40%) did not complete the treatment, and the reason for withdrawal was listed (Supplemental Table 4, <http://links.lww.com/HC9/A226>). In 13 PEG-CHBV, 6 patients showed loss of HBsAg, and the other 7 patients persisted with HBsAg at 72 weeks (Table 1).

We have observed differences in the magnitude of HBV DNA and HBsAg in some of the patients who were unable to clear HBsAg (Figure 1A) and who cleared HBsAg (Figure 1B). It has also been reported that during relapse, the difference in the magnitude of HBV DNA upsurge modulates HBV-specific immune response.^[5] Therefore, we have chronologically assessed plasma cytokines and immune cell responses in CHBV patients at the time of NA stopping (CHBV), at relapse (REL-CHBV), and after PEG-IFN treatment at 72 weeks (PEG-CHBV) (Supplemental Figure 2, <http://links.lww.com/HC9/A226>).

Difference in relapsers and nonrelapsers

We have compared CHBV patients after stopping NA, those who relapsed (REL-CHBV) within 6 months, and

TABLE 1 Clinical characteristics of CHBV patients at the time of relapse and after peg-IFN treatment at 72 weeks

Variables	CHBV (n = 55)				p			
	CHBV (n = 55)	NREL-CHBV (n = 33)	REL-CHBV (n = 22)	PEG-CHBV (n = 13)	CHBV vs. REL-CHBV	CHBV vs. PEG-CHBV	REL-CHBV vs. NREL-CHBV	REL-CHBV vs. PEG-CHBV
Demographics								
Age (y)	46 (19–65)	46 (24–65)	46 (19–60)	54 (19–60)	0.319	0.826	0.460	0.322
Male: female	52/3	31/2	21.0/1.0	13/0	—	—	—	—
Liver function test								
S. albumin (g/dL)	4 (4–5)	4 (4–5)	4 (4–5)	4 (4–5)	0.923	0.433	0.498	0.498
ALT (IU/L)	37 (19–39)	49 (26–69)	133 (85–1024)	51 (14–87)	0.004	0.109	0.007	0.008
AST (IU/L)	32 (21–42)	35 (21–58)	80 (37–714)	37 (17–75)	0.010	0.186	0.057	0.017
S. bilirubin direct (mg/dL)	0.2 (0.1–1)	0.2 (0.1–1)	0.2 (0.1–0.8)	0.1 (0.1–0.2)	0.778	0.081	0.478	0.025
S. bilirubin indirect (mg/dL)	0.7 (0.1–1.4)	0.8 (0.1–1.4)	0.7 (0.1–1.7)	0.7 (0.5–1)	0.889	0.625	0.388	0.802
S. Bilirubin total (mg/dL)	0.9 (0.3–2.1)	1 (0.3–2.1)	0.9 (0.4–7.6)	0.9 (0.6–1.2)	0.401	0.344	0.242	0.264
Virological parameters								
HBsAg (IU/mL)	3183 (156–73,768)	1866 (138–20,038)	16,137 (1210–160,000)	131 (0–9235)	0.028	0.029	0.031	0.027
HBsAg negative, N (%)	0	0	0	6 (27) ^a	—	—	—	—
HBV DNA (IU/mL)	ND	1086 (0–1720)	34,141 (2080–110,000,000)	0 (0–524)	0.001	0.120	0.041	0.018
HBV DNA negative N (%)	55 (100)	6 (18)	0	10 (77)	—	—	—	—
Anti-HBs (IU/mL)	ND	ND	ND	ND	—	—	—	—
N (%)	0	0	0	0	—	—	—	—
Hematology								
Hemoglobin (g/dL)	15 (12–18)	15 (13–18)	14 (12–15)	16 (16–17)	0.528	0.043	0.226	0.052
Platelets (10 ⁹ /L)	150 (105–216)	158 (112–216)	160 (150–189)	156 (133–179)	0.957	0.756	0.403	0.738
Leukocytes (10 ⁹ /L)	5 (3–10)	5 (3–10)	5 (3–7)	5 (3–6)	0.601	0.530	0.350	0.903

Note: Data represented in median and interquartile range. p-values < 0.005 (represented in bold).

^a27% is 6 of all 22 patients who relapsed.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHBV, treated chronic HBV (on day of NA stop); NREL-CHBV, chronic hepatitis B nonrelapsers (at 6 mo of NA stop); PEG-CHBV, chronic hepatitis B after PEG-IFN treatment (at 72 wk); REL-CHBV, chronic hepatitis B relapsers (at time of clinical relapse).

those who did not relapse (NREL-CHBV) (Supplemental Figure 3, <http://links.lww.com/HC9/A226>; data processing and cleaning and Supplemental Figure 4, <http://links.lww.com/HC9/A226>). With the clinical relapse, REL-CHBV had significantly increased ALT, AST, HBsAg, and HBV DNA but decreased Hb compared with NREL-CHBV (Table 1). Further, to understand the effect of clinical relapse on immune cells [natural killer (NK) cells, T cells, B cells, and Tfh cells], the immune characterization of these cells was performed. We have observed that there was no significant difference in NK/NKT cell population and T helper subsets (T helper 1/17 cells) (Supplemental Figure 4A-B, <http://links.lww.com/HC9/A226>). CD4 EM cells were increased with decreased CD8 central memory (CM) in REL-CHBV (Supplemental Figure 4C, D, <http://links.lww.com/HC9/A226>). The increase in CD4 effectors suggests that clinical relapse awakens the exhausted immune function. There was no change in B cell subsets among relapsers and non-relapsers (Supplemental Figure 4E, F, <http://links.lww.com/HC9/A226>), but Tfh cells and its subsets Tfh1/17 cells showed increased frequency in REL-CHBV group (Supplemental Figure 4G, <http://links.lww.com/HC9/A226>). These data suggest that during clinical relapse

after stopping NA, there was an increased frequency of inflammatory subsets like Th1/17 and Tfh1/17, which may be responsible for increased IFN- γ and IL-17 in REL-CHBV.^[17] Further, we assessed the immune phenotype to determine the effect of PEG-IFN treatment in REL-CHBV leading to loss of HBsAg.

Th1/17 and Th17 cells play a role in relapse and with Peg-IFN treatment loss in sAg

The balance between Th1/Th2, Th17, and Th1/17 cells plays a major role in viral clearance or persistence. To know which Th subset is important in relapse and for HBsAg loss after Peg-IFN, we stained cells with anti-CD4, CXCR5, CXCR3, and CCR6 to analyze Th1, Th2, Th17, and Th1/17 subsets (Figure 2A, Supplemental Figure 3, <http://links.lww.com/HC9/A226>). We concatenated CD4 T cells flow cytometry data with UMAP and analyzed the related phenotypes (Figure 2B). In between N-CHBV and CHBV patients, N-CHBV showed significantly increased Th1, Th2, and Th17 (Supplemental Figure 5A, <http://links.lww.com/HC9/A226>).

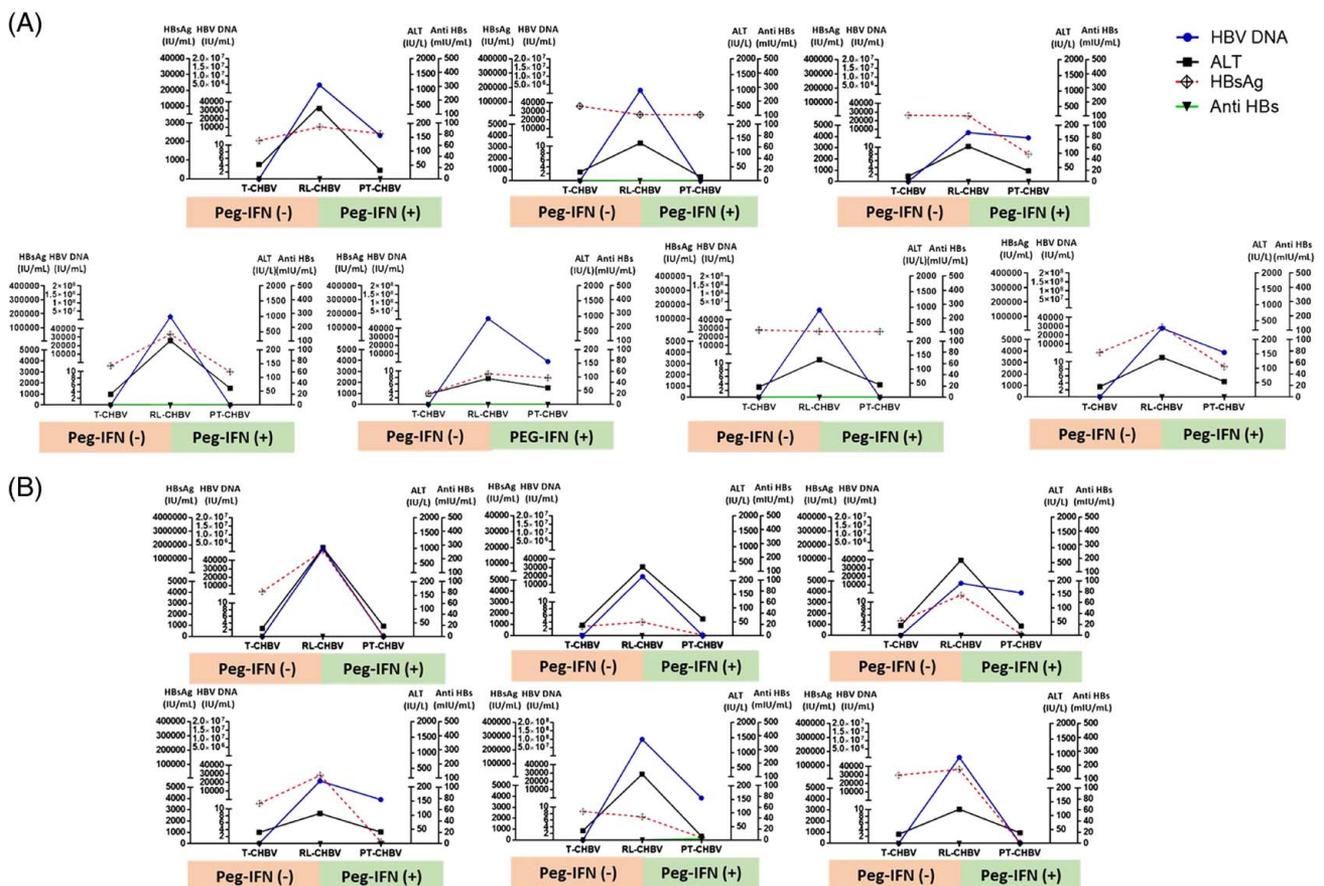


FIGURE 1 Viral and serological marker kinetics in patients. (A) Subjects unable to clear HBsAg. (B) Subjects with HBsAg loss at 72 weeks. Abbreviations: ALT, alanine transaminase; CHBV, chronic HBV-infected patients on antiviral treatments; PEG-CHBV, Peg-IFN–treated CHBV; REL-CHBV, Relapsers in CHBV.

There was no significant difference in Th1, Th2, and Th17 during a relapse, but Th1/Th17 population was increased in REL-CHBV compared with CHBV, suggesting an important role of Th1/Th17 in HBV relapse (Figure 2C).^[18] Cytokine bead array data revealed that REL-CHBV patients had significantly increased IL-6, IL-10, and IFN- γ compared with CHBV (Supplemental Figure 6A, <http://links.lww.com/HC9/A226>, red dots).

After viral relapse, patients were treated with Peg-IFN α 2b, which is a potent immune modulator. However, no significant difference was observed in Th1 and Th2 populations, and there was a decline in Th1/Th17 but a rise in Th17 cells in PEG-CHBV patients (Figure 2C). Th1/17 cells were increased during relapse in patients who had a loss of HBsAg after Peg-IFN therapy (Figure 2C). Further, the median fluorescent intensity (MFI) of CCR6 and CXCR3 was also increased in Th1/17 in REL-CHBV and PEG-CHBV, also evident in the UMAP clusters (Figure 2D).

Correlation of cellular data with clinical parameters and cytokine levels revealed that Th1 and Th2 cells were positively correlated with HBsAg, soluble inhibitory molecules (sBTLA, sIDO, sTIM3, sPD1, and GITR), and total bilirubin, respectively, in CHBV (Figure 2E). However, on relapse, Th17 cells were significantly correlated with soluble CD80, BTLA, IDO, PD1, PDL2, CD137, and GITR, whereas Th1/17 had significant correlation with ALT, IFN- α , sPD1, and sPDL2 (Figure 2E). On comparison of REL-CHBV and PEG-CHBV, along with the downregulation of inhibitory molecules like sGITR, sPD1, sBTLA, and IDO, there was a > 1.5-fold upregulated difference in IFN- α and IFN- λ levels (Supplemental Figure 6A, <http://links.lww.com/HC9/A226>, yellow dots).

Although IFN- α is secreted by pDC, it is shown to be a strong signal for IFN- γ production in viral infections.^[19] In our data, a strong correlation of IFN- α with Th1/17 (Figure 2F) suggests that in REL-CHBV presence of increased IFN- γ , IL17A, and Th1/17 cells could have been from IFN- α induction in response to HBV^[17,19] (Supplemental Figure 6A, B, <http://links.lww.com/HC9/A226>). In PEG-CHBV, most of the Th1/Th2 cytokines were lowered except IL-12p40, IL-1RA, and IL-18 (Supplemental Figure 6B, C, <http://links.lww.com/HC9/A226>). Further, PEG-CHBV patients showed a positive correlation of Th17 and Th1/17 cells with a decrease in IL7 and sPDL2 (Figure 2E). Declined inhibitory molecules in PEG-CHBV are suggestive of diminished inflammation in these subjects. It is well documented that IFN- α also induces NK,^[20] and IFN- γ is being produced by NK/NKT,^[17,20,21] therefore, it was important to analyze the role of NK/NKT cells in viral clearance and HBsAg loss in relapsers on Peg-IFN treatment. However, we have observed no significant change in NK/NKT cells in REL-CHB and PEG-CHB (Supplemental Figure 7, <http://links.lww.com/HC9/A226> with results).

Restoration of T-cell effector cells during relapse and after Peg-IFN treatment improved memory T cells

Next, we performed a comparative evaluation of CD4⁺CD8⁺; T Naive (CD45RO-veCCR7+ve), CM (CD45RO+veCCR7+ve), effector memory (EM) (CD45RO+veCCR7lo), and TEMRA (CD45RO-veCCR7-ve) subsets as shown in gating strategy (Figure 3A, Supplemental Figure 3, <http://links.lww.com/HC9/A226>). We concatenated CD8 T cells flow cytometry data obtained from these groups and performed UMAP analysis (Figure 3B).

Treated CHBV patients showed significantly increased naive T cells, T_{CM}, and T_{EM} compared with N-CHBV suggesting the effect of NA treatment (Supplemental Figure 5B, <http://links.lww.com/HC9/A226>). On comparing CHBV with REL-CHBV, there was no difference in frequencies of CD8⁺T cells or their subsets at the time of relapse compared to CHBV (Figure 3C). Further, CCR7 and CD45RO MFI revealed that effector memory T cells had low CCR7 and high CD45RO expression in REL-CHBV, suggesting a more potent effector population during relapse, which is also evident in UMAP (Figure 3D).

However, a comparison between REL-CHBV and PEG-CHBV revealed a significant increase in total CD8T cells with lower naive T cells (Figure 3C). Although frequencies of T_{EM} also increased at week 72, it did not achieve significance (Figure 3C). Similarly, no significant change was observed on comparison of patients who resolved and who did not resolve HBsAg by 72 weeks. However, patients who did not clear HBsAg showed an increased number of T_{EMRA} cells at 72 weeks Peg-IFN therapy, suggesting more exhausted CD8 T cells at the end of therapy (Figure 3C, line plot). Further, CCR7 and CD45RO MFI were increased in both REL-CHBV and PEG-CHBV. Increase in CD45RO MFI of T_{CM} in PEG-CHBV also suggests the decline of effector cells and inflammation with the establishment of better memory cells compared with N-CHBV and CHBV (Figure 3D). This is also evident in UMAP, as the major T_{CM} contour falls under the PEG-CHBV cluster (Figure 3D).

Correlation analysis revealed the positive and negative correlation of naive CD8 T cells with TGF- β and effector CD8 cells with sPDL2 in CHBV (Figure 3E). But, in relapse, CD8 T cells were negatively correlated with IFN- α , and CD8 memory cells were associated with an increase in total bilirubin (Figure 3E). In PEG-CHBV, T_{EM} and T_{EMRA} cells were positively and negatively associated with IL-12p40. IL-12p40 being an inflammatory cytokine may suggest its effect on CD8 effector cells functioning (Figure 3F).

We did not observe much difference in CD4⁺ T cells during relapse and PEG-CHBV except the increase in

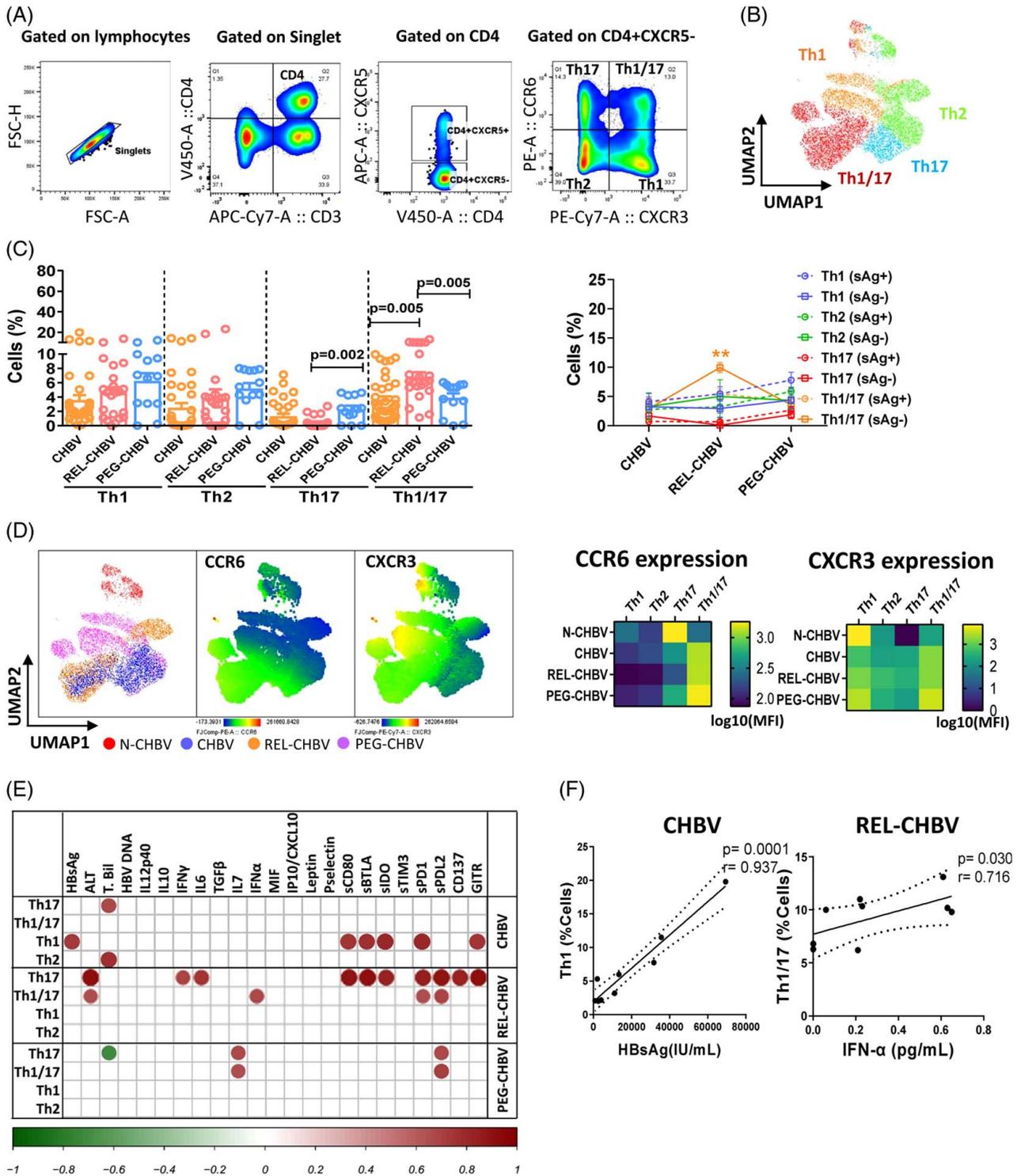


FIGURE 2 Th cells subset during relapse and after Peg-IFN therapy. (A) Gating strategy of Th subsets. (B) UMAP distribution of Th1, Th2, Th17, and Th1/17. (C) Representative % frequency of Th1, Th2, Th17, and Th1/17 in lymphocytes of CHBV, REL-CHBV, PEG-CHBV, and HBSAg-positive and negative patients. (D) Expression profile of CCR6 and CXCR3 in representative UMAP and heatmap showing Th1, Th2, Th17, and Th1/17. (E and F) Correlation analysis between cytokines, clinical parameters, and T-cell subsets. Parameters with p -value > 0.05 and correlation coefficient (ρ) > -0.6 to < 0.6 were omitted. The Mann-Whitney U test and Kruskal-Wallis test were applied wherever applicable ($p < 0.05$ are considered) ($n = 35$, CHBV, $n = 22$, REL-CHBV, $n = 13$, PEG-CHBV). Abbreviations: CHBV, chronic HBV-infected patients on antiviral treatments; MFI, median fluorescent intensity; PEG-CHBV, Peg-IFN-treated CHBV; REL-CHBV, Relapsers in CHBV; UMAP, uniform manifold approximation and projection.

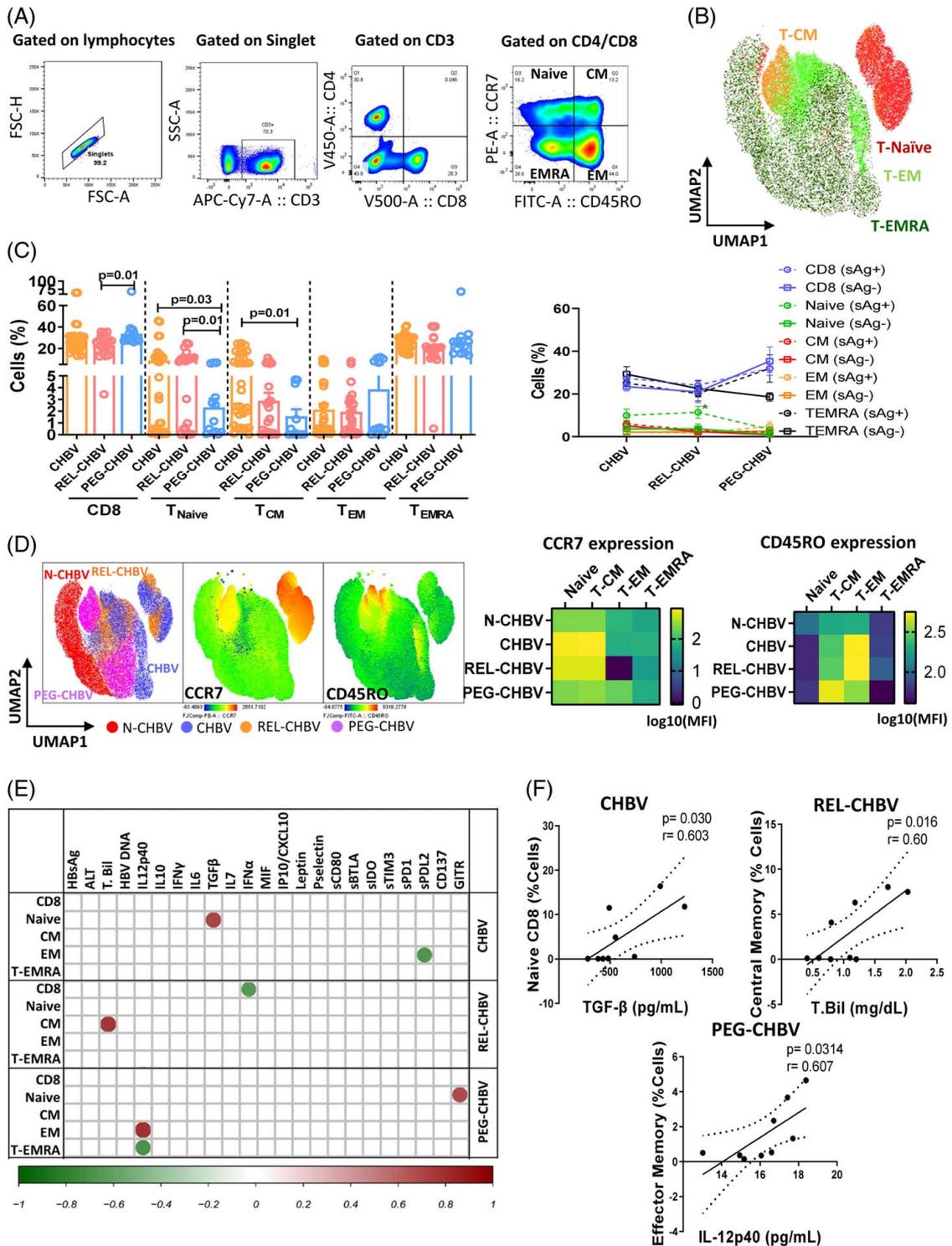


FIGURE 3 Immune modulation and its effects on CD8 T-cell naive, effector, memory, and TEMRA subsets. (A) T_{Naive} , T_{EM} , T_{CM} , T_{EMRA} subsets gating strategy in CD8 cells. (B) UMAP distribution of CD8 T_{Naive} , T_{EM} , T_{CM} , and T_{EMRA} . (C) Percentage frequency representation of CD8 T_{Naive} , T_{EM} , T_{CM} , and T_{EMRA} cells in lymphocytes of CHBV, REL-CHBV, PEG-CHBV, and HBsAg-positive and negative patients. (D) UMAP representation, distribution, and expression of CCR7 and CD45RO in CD8 T_{Naive} , T_{EM} , T_{CM} , and T_{EMRA} cells. (E and F) Correlation of significant cytokines with clinical parameters and CD8 T_{Naive} , T_{EM} , T_{CM} , and T_{EMRA} cell subsets in CHBV patients at baseline, relapse, and 72 weeks. Parameters with p -value > 0.05 and correlation coefficient (ρ) > -0.6 to < 0.6 were omitted. Mann-Whitney U test and Kruskal-Wallis test were applied wherever applicable ($p < 0.05$ are considered) ($n = 35$, CHBV, $n = 22$, REL-CHBV, $n = 13$, PEG-CHBV). Abbreviations: CHBV, chronic HBV-infected patients on antiviral treatments; CM, central memory; EM, effector memory; EMRA, terminally differentiated effector memory cells re-expressing CD45RA; MFI, median fluorescent intensity; PEG-CHBV, Peg-IFN-treated CHBV; REL-CHBV, Relapsers in CHBV; UMAP, uniform manifold approximation and projection.

T_{EM} cells (Supplemental Figure 8, <http://links.lww.com/HC9/A226>, with results in Supplementary Data).

However, we realized that relapse and Peg-IFN treatment had a major effect on the B-cell compartment and increased B cells during relapse, and post-Peg-IFN treatment may be instrumental in the loss of HBsAg (Supplemental Figure 9, <http://links.lww.com/HC9/A226>, with results in Supplementary Data).

Increase in plasma B cells and Tfh17 cells in post-Peg-IFN

Mature B cells were also subcategorized with CD21 and CD27 markers expression into naive, classical memory, plasma cells, and atypical memory (ATM) B cells (Figure 4A, Supplemental Figure 3, <http://links.lww.com/HC9/A226>). Flow cytometry data of CD19+ B cells were concatenated and analyzed with UMAP for the comparative, phenotypic evaluation of B cells and their subset in all subjects (Figure 4B).

In viral relapse, there was no significant difference in naive, plasma, and ATM B cells during relapse from CHBV (Figure 4C) though classical memory showed a decline in REL-CHBV compared with CHBV. Treated CHBV patients showed higher ATM B cells than N-CHBV (Supplemental Figure 5C, <http://links.lww.com/HC9/A226>).

After Peg-IFN treatment, there was no significant change in B cells except an increase in naive B cells of PEG-CHBV compared with CHBV and REL-CHBV (Figure 4C). Further, patients who have cleared HBsAg and who did not cleared HBsAg also revealed no significant change between any B-cell subset. However, a typical spike in naive B cells could be observed in HBsAg-positive subjects at 72 weeks (PEG-CHBV) (Figure 4C). Although there was no significant difference in the frequencies of plasma B cells in REL-CHBV and PEG-CHBV, but CD27 and CD21 MFI heatmap and UMAP revealed increased protein expression of CD21 in naive, classical, and CD27 in plasma B cells in PEG-CHBV compared with REL-CHBV suggesting more efficient and robust plasma B cells after Peg-IFN treatment (Figure 4D).

Correlation analysis of B cells with clinical parameters and plasma cytokines depicted significant association of TGF- β with plasma cells in CHBV, suggesting the inhibition of plasma B cells through TGF- β and CXCL10 association with ATM B cells in REL-CHBV suggesting the recruitment of short-lived or exhausted B cells.^[22] Further, an increase in IL7 was associated with increased plasma B cells in PEG-CHBV, suggesting a pivotal role of IL7 in B cell development (Figure 4E, F).

The maturation of B cells, memory, and plasma B cells directly depends on Tfh cells. Tfh cells (Figure 5A, Supplemental Figure 3, <http://links.lww.com/HC9/A226>) flow cytometry data were concatenated to show

separate populations of Tfh1, Tfh2, Tfh17, and Tfh1/17 with UMAP analysis (Figure 5B). We observed that antiviral-treated CHBV patients showed higher percentage of Tfh, Tfh1/17 cells compared with naive CHBV (Supplemental Figure 5D, <http://links.lww.com/HC9/A226>).

During a viral relapse, Tfh17 was decreased, but Tfh1/17 was significantly increased, suggesting its link with the viral load during relapse. Increased Tfh was also observed in PEG-CHBV from CHBV (Figure 5C).

A decrease in Tfh1/17 but regain of the Tfh17 population after Peg-IFN treatment was observed (Figure 5C). There was no significant gain in Tfh17 cells in patients who have cleared the HBsAg. However, a sustained increase of Tfh1/17 was observed in patients during relapse and PEG-CHBV who have cleared HBsAg. Moreover, an increase in CCR6 and CXCR3 MFI evident on heatmap and UMAP of Tfh1/17 subsets in PEG-CHBV strengthened our data (Figure 5D).

Correlation analysis on Tfh cell subsets with clinical parameters and plasma cytokine revealed a strong association of total bilirubin with Tfh2 in CHBV, suggesting the role of bilirubin in Tfh2 differentiation. In REL-CHBV, HBV DNA, IL-10, and CXCL10 were positively correlated with Tfh1 subsets. In PEG-CHBV, Tfh17 cells were positively correlated with IL7, but Tfh1/17 and Tfh1 were negatively correlated with IL7 (Figure 5E, F).

Improved T-cell functionality during relapse and increased HBV-specific IFN- γ -producing T cells after Peg-IFN

PBMCs were stimulated with overlapping pooled surface (HBsAg) and core (HBcAg) peptides for 10 days (Supplemental Figure 10A, B, <http://links.lww.com/HC9/A226>, experimental design) and IFN- γ , TNF- α , IL-21, and IL-17 cytokines secreting T cells were analyzed (Supplemental Figure 10C, <http://links.lww.com/HC9/A226>, gating strategies).

CHBV patients showed low frequencies of surface and core-specific IL-21-secreting CD4 T cells compared with REL-CHBV (Figure 6A). During a relapse, both surface and core-specific CD4 T cells secreting IL-21 cells frequencies, as well as IL-21 MFI, were significantly increased. Although other cytokines secreting CD4 T cells were also increased in REL-CHBV compared with CHBV but did not achieve significance. However, PEG-CHBV patients had more frequency, as well as MFI of HBs-specific IFN γ -secreting CD4⁺T cells (Figure 6A).

We did not observe any significant change in surface or core-specific IFN- γ , IL-17, IL-21, and TNF- α -secreting CD8⁺ T cells in CHBV, REL-CHBV, and PEG-CHBV patients (Supplemental Figure 11A, <http://>

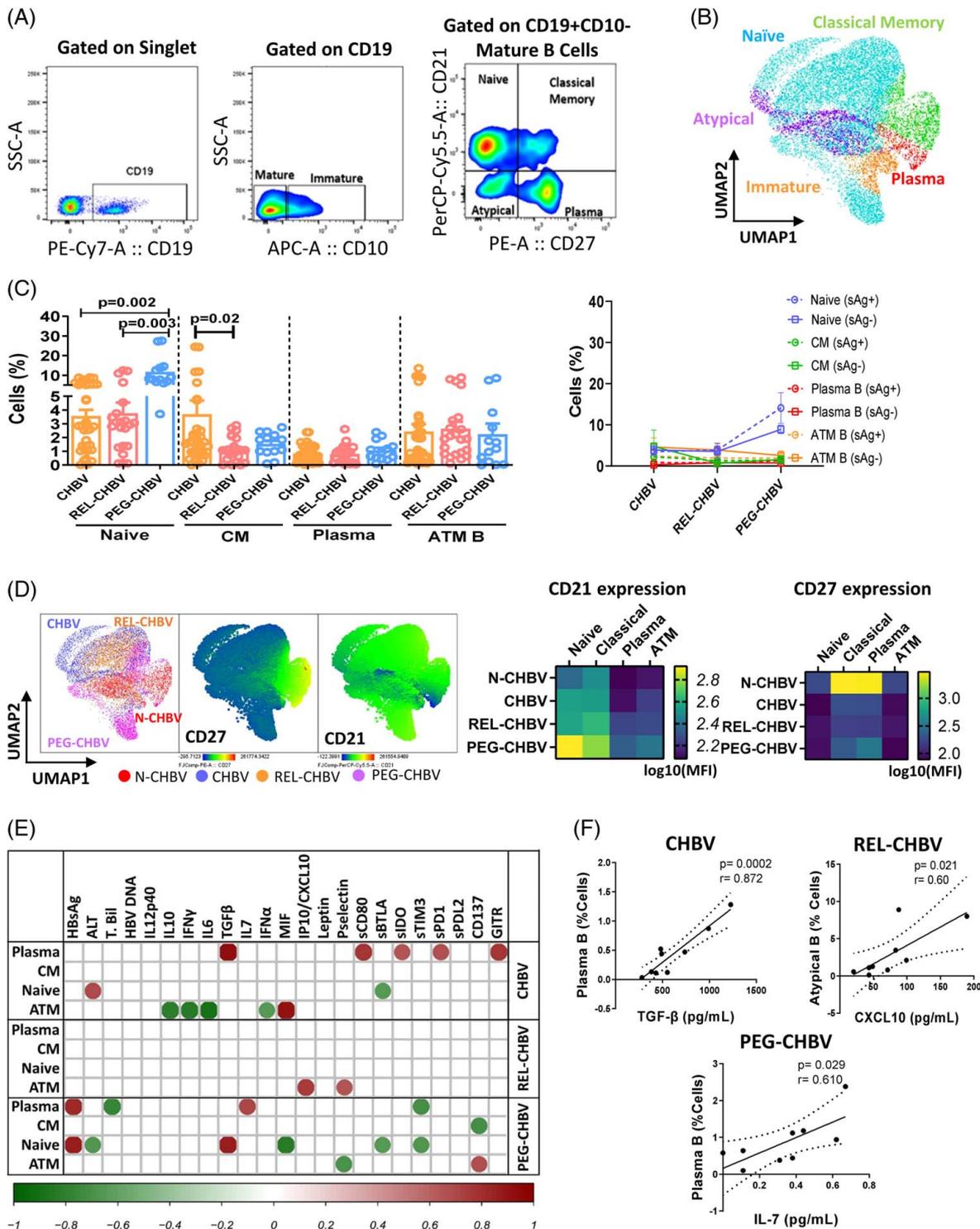


FIGURE 4 Peg-IFN modulates B cell and its subsets by increased plasma B-cell expression (A) gating strategy of B-cell subsets. (B) UMAP distribution of B cells and subsets. (C) Representative dot plots of B cell subsets % frequencies in lymphocytes of CHBV, REL-CHBV, PEG-CHBV, and HBsAg-positive and negative patients (D) Distribution and expression of CD27 and CD21 in B-cell subsets on UMAP. (E and F) Correlation of significant cytokines with clinical parameters and B-cell subsets at baseline, relapse, and 72 weeks. Parameters with p -value > 0.05 and correlation coefficient (ρ) > -0.6 to < 0.6 were omitted. Mann-Whitney U test and Kruskal-Wallis test were applied wherever applicable ($p < 0.05$ are considered) ($n = 35$, CHBV, $n = 22$, REL-CHBV, $n = 13$, PEG-CHBV). Abbreviations: ATM, atypical memory; CHBV, chronic HBV-infected patients on antiviral treatments; CM, central memory; EM, effector memory; PEG-CHBV, Peg-IFN-treated CHBV; REL-CHBV, Relapsers in CHBV; UMAP, uniform manifold approximation and projection.

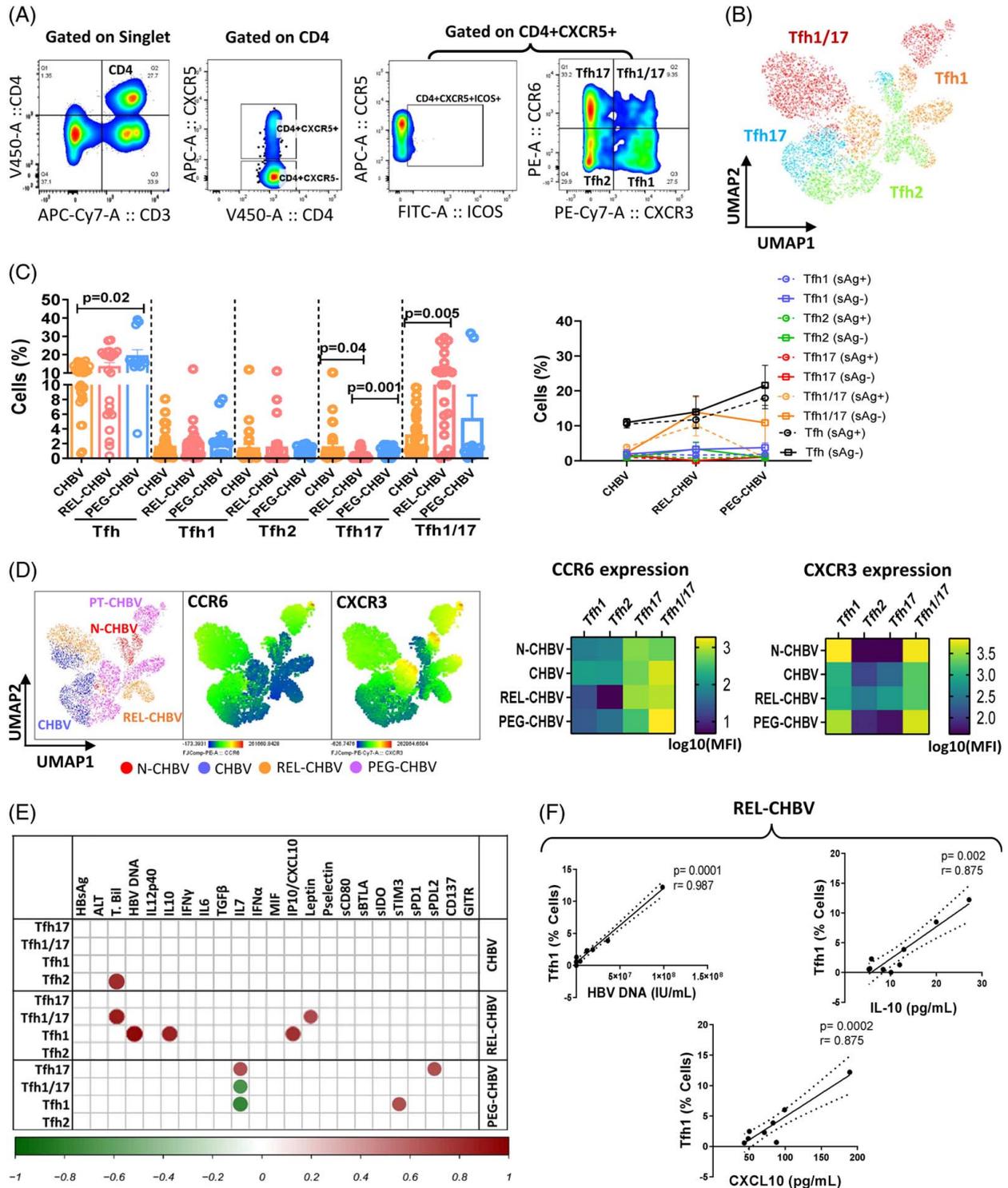


FIGURE 5 Effect of immune modulation by drug withdrawal therapy followed by Peg-IFN treatment on Tfh cells and subsets (A) gating strategy of Tfh subsets (B) UMAP distribution of Tfh subsets. (C) Dot plots representing the % frequency of Tfh cell subsets in lymphocytes of CHBV, REL-CHBV, PEG-CHBV, and HBSAg-positive and negative patients. (D) UMAP expression distribution of CCR6 and CXCR3 in Tfh cell subsets. (E and F) Correlation of significant cytokines with clinical parameter and Tfh cell subsets in CHBV patients at baseline, relapse, and 72 weeks. Parameters with p -value > 0.05 and correlation coefficient (ρ) > -0.6 to < 0.6 were omitted. Mann-Whitney U test and Kruskal-Wallis test were applied wherever applicable ($p < 0.05$ are considered) ($n = 35$, CHBV, $n = 22$, REL-CHBV, $n = 13$, PEG-CHBV). Abbreviations: CHBV, chronic HBV-infected patients on antiviral treatments; MFI, median fluorescent intensity; PEG-CHBV, Peg-IFN-treated CHBV; REL-CHBV, Relapsers in CHBV; UMAP, uniform manifold approximation and projection.

links.lww.com/HC9/A226). Further, surface antigen stimulation shows drastically hampered Tfh cell responses in CHBV compared with relapsers (Figure 6B). During relapse, HBs-specific frequencies of TNF- α , IL-21, IFN- γ , and IL-17-secreting Tfh cells and their MFIs were dominant, but HBc raised only IL-21 expression. However, in PEG-CHBV patients, these responses were diminished (Figure 6B) with surface antigen, but core antigen-induced IFN- γ -secreting Tfh cells (Figure 6B).

These data suggest that during relapse (REL-CHB), patients do gain T-cell functionality against both HBV-specific surface and core antigens as compared with CHBV. Further, with Peg-IFN, they tend to maintain these functionalities at 72 weeks by producing IFN- γ through CD4 and CD4 Tfh cells. However, at 72 weeks, it cannot be clearly stated whether the effect is due to PEG-IFN or NA stoppage.

Checkpoint blockades further improved T-cell functionality in post-Peg-IFN-treated patients

Though our data indicated that during relapse T cells achieved better functionality and post-Peg-IFN treatment, IFN- γ secreting CD4, and Tfh cells were maintained. However, we observed increased PD1 and CTLA4 inhibitory molecules expressing T cells during relapse in REL-CHBV (Supplemental Figure 12, <http://links.lww.com/HC9/A226>). Whether we can increase the HBV-specific T-cell functionality ex vivo in REL-CHBV and Peg-IFN-treated patients by checkpoint blockade using anti-PD1, anti-CTLA4, and anti-Lag3 antibodies; PBMCs were stimulated with surface and core overlapping peptides with or without checkpoint inhibitors, and T-cell responses were analyzed. With PD1 blockade, on sAg stimulation, there was no significant difference in CHBV and REL-CHBV, but PEG-CHBV patients showed increased IL-21-secreting CD4⁺ T cells and IL-21 MFI (Figure 6C). With cAg stimulations, CHBV patients showed a significant reversal in exhaustion by increasing TNF- α , IL-21, and IL-17-secreting CD4⁺T cells on CTLA4 but not on PD1 blockade (Figure 6C). In REL-CHBV, IL-21-secreting CD4T cells were also significantly increased by PD1 blockade but not by CTLA4 blockade. REL-CHBV also had better TNF- α responses with CTLA4 and PD1 blockade, but PEG-CHBV did not show any changes with core stimulations (Figure 6C).

We observed better effects of checkpoint inhibitors in CHBV patients' CD8 T cells rather than relapsers and PEG-CHBV. sAg-stimulated CD8 T cells have shown more of IL-17 and IL-21 with PD1 blockade. Further, CHBV patients showed more TNF- α , IL-17, and IFN- γ secreting core-stimulated CD8 T cells with CTLA4 blockade (Supplemental Figure 11B, <http://links.lww.com/HC9/A226>).

CD8 T cells in relapsers did not show any difference with any checkpoint blockade. But PEG-CHBV patients showed increased IL-21-secreting CD8 T cells on CTLA4 blockade (Supplemental Figure 11B, <http://links.lww.com/HC9/A226>).

In CHBV patients, sAg-stimulated Tfh cells also had more TNF- α on Lag3 and PD1 blockade with no effect on relapsers. But PEG-CHBV again showed increased IL-21-secreting Tfh cell on PD1 blockade. Core stimulations had no impact on Tfh cells in any group with any blockade (Figure 6D). These data suggest that with HBsAg stimulation, PD1 and CTLA4 checkpoint blockers can further scale up IL-21 secretions in PEG-CHBV patients, which may facilitate more HBsAg loss.

DISCUSSION

We have found that during relapse after stopping NA therapy, there is a high level of IL-6 and IFN- γ in plasma (Supplemental Figure 6, <http://links.lww.com/HC9/A226>), which is suggestive of more differentiation of Th17 and Th1/17 cells.^[23] As a result, we observed an increase in Th1/17 cells at the time of relapse. We know that discontinuation of NAs therapy in HBeAg-negative CHB patients alters the cytokine milieu and immune compartment for viral clearance and sAg decline.^[14] Clinical relapse could also be beneficial in terms of driving the immune-mediated elimination of infected hepatocytes to achieve immune control.^[12] However, complete HBsAg loss and seroconversion were achieved in only a very small proportion of patients, and that too after a prolonged period.^[15] Earlier, Lim et al^[15] observed sAg loss in 10.1% and 7.7% of patients, either treated with Peg-IFN and NA or overlap therapy of Peg-IFN and NA. However, we have observed sAg loss in 6 patients out of 22 relapsers who have cleared sAg revealed good immune response and host immune dominant flare, and those who were unable to clear sAg showed viral dominant flare (Figure 1A, B).^[5,24]

Peg-IFN is a strong immune modulator likely to work in synergy with the host immune restorative mechanisms. We have shown for the first time the effect of Peg-IFN on T cells (naive, effector, memory, TEMRA, Tfh) and B cell differentiation in NA withdrawal subjects. Among these, we found that Peg-IFN drives dominantly Th17 and Th1/Th17 cell differentiation (Figure 2), and increased Th1/17 cells are likely associated with sAg clearance.^[18] Earlier, Peg-IFN treatments have demonstrated overall lymphopenia but increased in activated TRAIL-expressing NK cells with impaired IFN- γ secretion.^[25] We did not observe any pertinent response of lymphopenia or on NK cells with respect to relapse or after IFN treatment (Supplementary Figure 7, <http://links.lww.com/HC9/A226>).

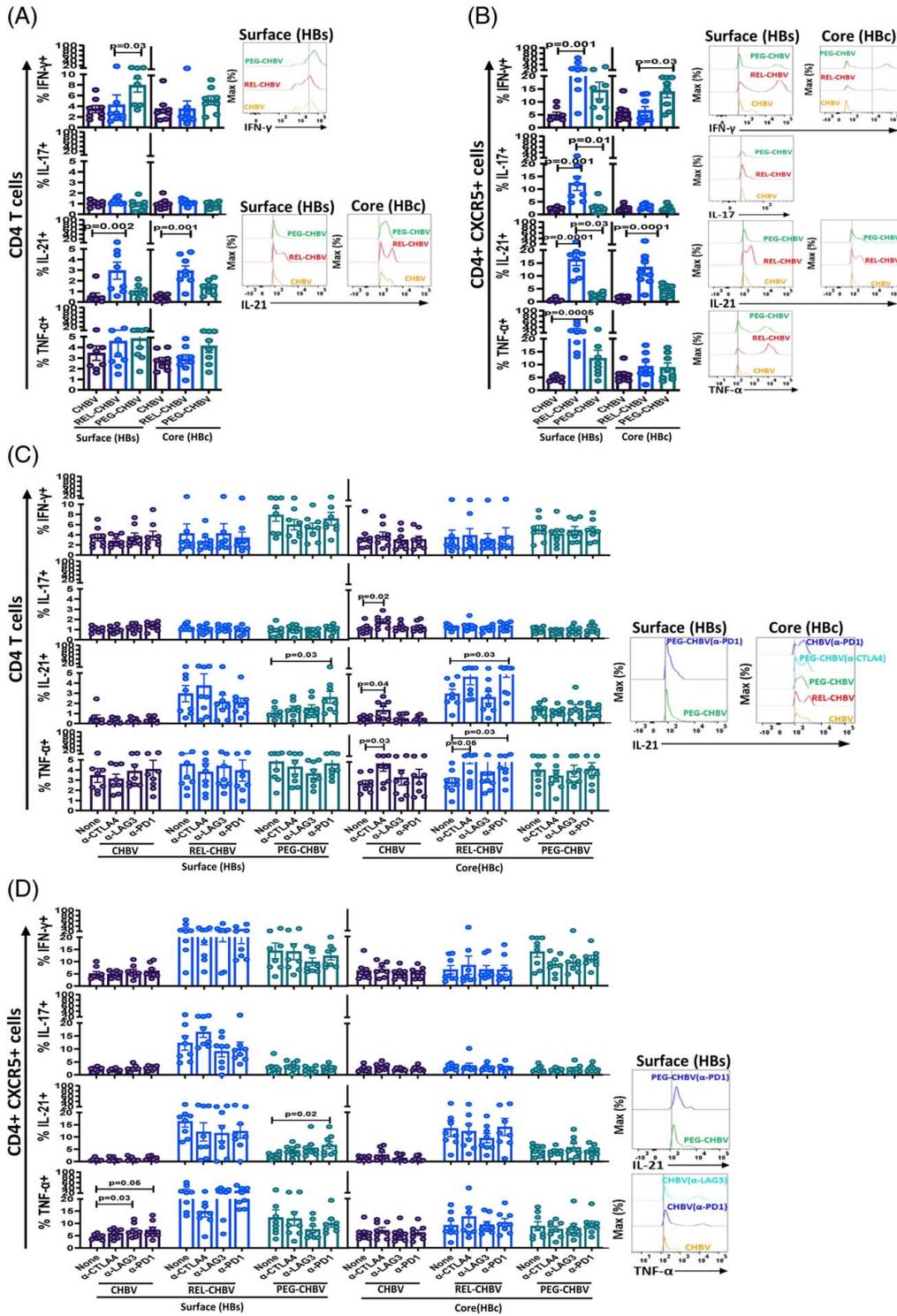


FIGURE 6 HBV-specific T-cell functionality of CD4, CD8, and Tfh cells: T-cell response after HBsAg and HBcAg stimulation in CD4 T cells (A) and Tfh cells (B). T-cell responses after stimulation (HBsAg and HBcAg) and immune checkpoint blockade (anti-CTLA4, anti-LAG3, and anti-PD1) in CD4 (C) Tfh cells (D). Mann-Whitney *U* test and Kruskal-Wallis test were applied wherever applicable ($p < 0.05$ are considered) ($n = 8$, subjects in each group). Abbreviations: CHBV, chronic HBV-infected patients on antiviral treatments; MFI, median fluorescent intensity; PEG-CHBV, Peg-IFN-treated CHBV; REL-CHBV, relapsers in CHBV.

CHBV patients who relapsed on NA withdrawal showed an increase in effector CD4 and CD8 T cells but declined in T_{CM} pool (Figure 4). These data also support earlier findings, which showed that NA

withdrawal increases the effector T cells and helps in resetting the immune system by inducing the memory cells to form more effector cells leading to the decline of memory pool and increased effector pool.^[26] Further,

patients who have shown sAg loss had increased CD4 but decreased CD4 /CD8 T_{EMRA} during relapse, as well as after Peg-IFN therapy. T_{EMRA} cells are considered to be terminally differentiated, senescent and exhausted cells in chronic infections. Our study showed that patients who have cleared sAg had a decline in T_{EMRA} cells suggesting the depletion of senescent T cells. It is documented earlier that after stopping NA therapy, there was more chemokine production,^[14,27] especially CXCL-10, which was associated with sAg loss.^[28] After 3 years of entecavir treatment, an increase in CXCL-10 levels was considered a promising predictor for HBsAg decline.^[29] Further, CXCL10 chemokine, after Peg-IFN treatment, was helpful in the recruitment of T cells, monocytes, NK, DC, and macrophages.^[30] Our results supported the earlier findings, as an increase in CXCL10 was observed in response to elevated IFN- γ in PEG-CHBV patients (Supplemental Figure 6, <http://links.lww.com/HC9/A226>).^[31] In addition, there were increased levels of IFN- α and IFN- λ in PEG-CHBV patients (Supplemental Figure 6, <http://links.lww.com/HC9/A226>). All these changes in cytokine and chemokine milieu suggest a beneficial resetting of immune response for HBsAg clearance. Relapse is considered good for achieving functional cure, but it may behave as a double-edged sword; on one hand, T-cell responses may increase, but with persistently high antigen levels, it may induce more T-cell exhaustion with increased expression of PD1, CTLA4, LAG3, TIM3, TIGIT, CD244, and CD160.^[32,33] We have seen increased inhibitory molecules expression during relapse (Supplemental Figure 12, <http://links.lww.com/HC9/A226>). On checkpoint inhibition with anti-PD1 and CTLA4, IL-21, IL-17, and TNF- α -secreting CD4 and CD8 T cells were further increased in PEG-CHBV. Stopping NA certainly enhanced the HBV core-specific T-cell responses but our data showed that T-cell functionality was further modulated by Peg-IFN treatment, as IFN- γ -producing CD4 and Tfh cells were increased at 72 weeks (Figure 6). Further, during relapse and after IFN treatment, mature B cells (CD19⁺CD10^{-ve}) were increased (Supplemental Figure 9, <http://links.lww.com/HC9/A226>) but with no change in plasma B cells, which endorses the lack of antibody against sAg at 72 weeks. However, increased CD27 and CD21 MFI in plasma B cells suggest the presence of more efficient and robust plasma B cells in PEG-CHBV (Figure 4D), which may affect sAg clearance after a few years. Further, during relapse and after Peg-IFN treatment, increase in Tfh1/17 cells supported their role in B-cell maturation and IFN- γ production (Figure 5). Functional analysis reliably supported the IFN- γ source from Th1/17 and Tfh1/17 (Figure 6A, B).

In conclusion, our study supports the use of peg-IFN as an immune modulator after stopping NA with clinical relapse to achieve a functional cure. As in this study, Peg-IFN treatment helped achieve HBsAg seroclearance

in more patients than usual and can act as a treatment regimen for patients who are nonresponders to NA therapy and are unable to clear HBsAg.

However, this study is limited with the fact that we were only able to analyze the circulating immune compartment for 72 weeks. We know that Peg-IFN therapy shows delayed response and has been reported to show seroconversion after 5–10 years.^[34–37] Long follow-ups of 3–5 years are required to see the exact effect of Peg-IFN treatment.^[15,24] We can assume that long-term assessment and follow-up of these patients could have answered more of our queries.

AUTHOR CONTRIBUTIONS

Mojahidul Islam: performed all experiments, data acquisition, analysis, interpretation, and manuscript writing; Karan Kumar: performed all clinical assessments, intervention of Peg-IFN, patient recruitment and follow-up, and manuscript editing; Jayesh K. Sevak: performed some experiments, data acquisition, analysis, and interpretation with Mojahidul Islam, Ankur Jindal: patient stratification and manuscript editing; Ashish K. Vyas: initial experimental design; Gayatri Ramakrishna: provided inputs; Shyamasundaran Kottlil: conceptualization of experiments, data, and manuscript editing; Manoj K. Sharma: all clinical data review, editing, assessment, stratification, and manuscript editing; Shiv K. Sarin: conceptual design of study, clinical data review, editing, assessment, stratification, and revision of the article; Nirupama Trehanpati: conceptual design of study, designing of experiments and analysis, critical revision of the article for important intellectual content, and finalized the manuscript.

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CONFLICT OF INTEREST

The authors have no conflicts to report.

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