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Original Article

Higher hepatitis B core-specific T cell response is associated with a lower risk of clinical relapse after discontinuation of oral antiviral treatment

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Abstract *Background:* Hepatitis B virus (HBV)-specific T cell response is a major host immune response to control the virus. However, it is still unclear how it affects long-term outcomes of chronic hepatitis B patients, especially those who stop nucleos(t)ide analogue (NA) therapy. We aimed to explore whether the HBV-specific T cell response at the end of treatment (EOT) was associated with clinical outcomes.
Methods: In a prospective cohort study, 51 HBeAg-negative patients who discontinued NA therapy were enrolled.

Abbreviations: HBV, hepatitis B virus; NA, Nuclos(t)ide-analogue; HBeAg, hepatitis B e antigen; HBcrAg, hepatitis B core-related antigen; EOT, end of treatment; ETV, entecavir; TDF, tenofovir disoproxil fumarate; HBsAg, hepatitis B surface antigen; PBMC, peripheral blood mononuclear cells; CHB, chronic hepatitis B; ALT, alanine aminotransferase; ELISPOT, enzyme-linked immunosorbent spot; SFC, spot-forming cells; IQR, interquartile range; HBc, hepatitis B core; HBx, hepatitis B X protein; Pol, polymerase; HR, hazard ratio; CI, confidence interval.

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Results: In a mean follow-up of 25.3 months, 25 patients developed clinical relapse. We found that a stronger hepatitis B core (HBc)-specific T cell response at EOT was associated with a lower risk of clinical relapse. Compared to the low-response group, the high-response group had a lower risk of clinical relapse with hazard ratio of 0.21 (95% CI: 0.05—0.88). The high HBc-specific T cell response was associated with reduced surge of HBV DNA and HBcrAg during the first year of follow-up. The T cell response at EOT was comparable between different NA treatments. Notably, the overall HBV-specific T cell response could be partially restored along with clinical relapse; however, such re-invigorated T cell response was not associated with HBsAg seroclearance.
Conclusions: A higher HBc-specific T cell response at EOT was associated with lower risk of clinical relapse and reduced surge of HBV DNA and HBcrAg levels off NA therapy.

Introduction

Chronic infection of hepatitis B virus (HBV) is an important global health issue, which leads to adverse outcomes of cirrhosis and hepatocellular carcinoma.[1] Nucleos(t)ide analogue (NA) therapy is effective to suppress viral replication, reduce liver necroinflammation, and then minimize the risk of disease progression. However, the optimal treatment duration of NA is still debatable as the majority of patients develop viral relapse and a significant proportion of them experience severe hepatitis flare after stopping NA,[2] which puts them at various risks of developing liver decompensation or even mortality.[3]

The interactions between virus and host are important to determine the prognosis after stopping NA.[4] On one hand, viral markers, which reflect the residual amount of replication templates in the liver, have been shown as predictors for clinical relapse. On the other hand, HBV-specific immunity also plays an important role in controlling the virus.[5] However, only a few studies adopted HBV-specific T cell response as an immune marker to identify patients who can stop NA safely.[4,6—8] Notably, these studies included a small number of patients, with less than 50 participants in each study, and the predominant viral genotype was genotype D virus. In addition, the study by Peña-Asensio et al. explored limited MHC class I-restricted epitopes.[9] As a result, there is a lack of comprehensive exploration of the role of HBV-specific T cell response in Asian patients infected with genotype B or C virus.

Another interesting and important question is whether HBV-specific T cell response is affected by different NA or restored by stopping NA. For example, recent data showed a delayed clinical relapse was observed in entecavir (ETV)-treated patients, but not in tenofovir disoproxil fumarate (TDF)-treated patients.[10—12] Whether ETV could better restore HBV-specific T cell response, and further delay or lower the risk of clinal relapse remains largely unknown. In addition, several studies suggested that clinical relapse after withdrawal of NA may facilitate clearance of hepatitis B surface antigen (HBsAg), especially in Whites.[13,14] The effect has been speculated through the reinvigoration of HBV-specific T cell response, which could be associated with ALT flare.[13] However, both hypotheses need to be explored since neither of them has been supported by immunological data.

To tackle these critical issues, we conducted a prospective study enrolling Asian patients with HBV genotype B or C infection, who were non-cirrhotic and HBeAg-negative, and had discontinued NA therapy. Peripheral blood mononuclear cells (PBMC) at the end of treatment (EOT) and at the time of clinical relapse were collected to examine HBV-specific T cell response. We first explored whether HBV-specific T cell response at EOT was associated with different clinical outcomes. Second, we explored whether the HBV-specific T cell response was affected by different NA or the emergence of clinical relapse.

Methods

Patients

In this prospective cohort study, we have consecutively enrolled non-cirrhotic HBeAg-negative chronic hepatitis B (CHB) patients who discontinued NA therapy at the National Taiwan University Hospital since 2017. All the patients started NA due to the presence of HBeAg-negative hepatitis according to the Asian Pacific Association for the Study of the Liver (APASL) treatment guidelines and received at least 3-year NA therapy according to the National Health Insurance reimbursement guidelines in Taiwan.[15] Patients with cirrhosis, human immunodeficiency virus, hepatitis C virus co-infection, autoimmune hepatitis, or alcoholism were excluded from this study.

The patients stopped NA treatment after achieving undetectable serum HBV DNA for more than 12 months according to the APASL guidelines.[15] The reasons for discontinuing NA treatment included completing the reimbursement duration of 3 years or by the preference of the patients, such as achieving low levels of HBsAg.[16]

Following the discontinuation of NA therapy, patients were monitored at the first and third month, and subsequently every 3 months over a span of 2 years. This monitoring aimed to assess alanine aminotransferase (ALT) levels and HBV DNA levels. Additional visits were arranged by the clinical physicians if their HBV DNA/ALT levels rose. The virological relapse was defined as an HBV DNA level $>$2000 IU/mL.[15] The clinical relapse was defined as virological relapse with a two-fold elevation of ALT level. The study was approved by the institutional review board of the

National Taiwan University Hospital and all patients gave their written informed consents before enrollment.

Virological assays

Serum levels of HBV DNA, HBsAg, and HBcrAg were determined using the commercial kits. The details are summarized in supplementary material.

PBMC isolation and T cell culture

Human peripheral blood mononuclear cells (PBMCs) were collected at EOT and when clinical relapse developed. For the patients without clinical relapse, the PBMC was collected at the end of follow-up if available. The PBMC was isolated using a Ficoll gradient technique and cryopreserved. Cells were thawed on the day of the experiment to generate T cell lines.[6] Briefly, 20% of PBMC was first pulsed with 10 μg/ml of all overlapping HBV peptides (15-mer peptides overlapping by 10 residues), including hepatitis B core (HBc) protein, 1 for hepatitis B X protein (HBx), 2 for HBsAg, and 4 for hepatitis B polymerase (Pol), for 1 h 37 °C (Supplementary material). After washing, it was co-cultured with 80% of PBMC in AIM-V medium (Gibco; Thermo Fisher Scientific) with 2% AB human serum (Gibco; Thermo Fisher Scientific). T cell lines were expanded for 10 days in the presence of 20 U/ml of recombinant IL-2 (R&D Systems).

Enzyme-linked immunosorbent spot (ELISPOT)

We utilized the ELISPOT assay to identify cells producing interferon-gamma (IFN-γ). In brief, we mixed 100,000 in-vitro expanded T cell line with each of the 8 HBV peptide pools separately. The T cell lines were then incubated overnight with individual pools of HBV peptides in 96-well plates coated with anti-human IFN-γ antibody.[6] After adding biotinylated anti-human IFN-γ, the streptavidin-alkaline phosphatase, and the alkaline-phosphatase substrate, the spots in each well were counted using an automated ELISPOT reader (Immunospot; Cellular Technology Limited). The detail is summarized in supplementary material.

Calculation of HBV-specific T cell response

The number of IFN-γ-producing cells of each peptide pool was calculated by subtracting the spot number in the control well without peptide from the spot number in the corresponding well. The value was expressed as spot-forming cells (SFC) relative to 1×10^5 PBMCs. For the viral proteins containing multiple pools, including HBsAg and Pol, the protein-specific T cell response was calculated by adding the spot numbers of each pool. For the overall HBV-specific T cell response, we summed up spot number of 8 pools.

Statistical analysis

The continuous variables were reported as median and Interquartile range (IQR) and categorical data as number (percentage). The HBV DNA, HBsAg, and HBcrAg levels were logarithmically transformed for statistical analysis. Differences between groups were evaluated by Mann Whitney U Test and Chi-square statistic as appropriate. The difference of HBV-specific T cell response between the EOT and clinical relapse or end of follow-up for a given patient was assessed using paired t-test. The entry date was defined as the EOT time. The time at risk was measured from the entry date until the development of clinical relapse, the last hospital visit, or the time of antiviral therapy retreatment, whichever came first. The cumulative incidence of clinical relapse was estimated by Kaplan-Meier analysis and compared using the log-rank test. The risk of clinical relapse was modeled using Cox proportional hazards regression analysis including age, sex, ALT, HBsAg, and HBcrAg levels as adjusting variables. The statistical analysis was performed by STATA (version 13.0; Stata Corp, College Station, TX, USA). All tests were 2-sided and a P value < 0.05 was considered statistically significant.

Results

Baseline characteristics and follow-up results

Table 1 compares the characteristics at the end of treatment (EOT) between patients with and without clinical relapse. Among the 51 patients, most of them were male (74.5 %) and the median age at enrolment was 52.0 years. We further categorized the patients with HBsAg level of 100 IU/mL and HBcrAg level of 1000 U/mL, respectively, as both cutoffs had been adopted to stratify the post-NA risk of clinical relapse.[17–19] Patients without clinical relapse, compared to those with clinical relapse, tended to have higher proportions of HBsAg <100 IU/mL (26.9% vs. 8.0%, $P = 0.076$) and HBcrAg <1000 U/mL (57.7% vs. 32.0%, $P = 0.65$). In addition, more patients with undetermined HBV genotypes were noted in those without clinical relapse (15.3% vs. 0%), as they tended to have lower HBV DNA levels after withdrawal of NA. Additionally, all patients were negative for anti-HDV antibody in serum, and none had received immunosuppressants during the follow-up.

HBV-specific T cell response at EOT by ELISPOT in patients with and without clinical relapse

The distribution of the overall and the viral protein-specific T response is summarized in the box plot (Suppl Fig. 1). We compared the overall T cell response and the response specific to HBc, HBx, HBsAg, and Pol between the patients with and without clinical relapse (Fig. 1A). In contrast to the T cell response specific to other viral proteins, only a higher HBc-specific T cell response was associated with a lower risk of clinical relapse (Mean ± SD: 58.4 ± 88.6 vs. 14.5 ± 21.3 SFC/10^5 cells, $P = 0.03$).

We then decided to adopt HBc-specific T cell response determined by ELISPOT to stratify the risk of clinical relapse. When stratifying the patients by the 3rd quartile of HBc-specific T cell response (35 SFC/10^5 cells), the high HBc-specific T cell response was associated with lower risk of clinical relapse with HR of 0.21 (95% CI: 0.05—0.88) (Fig. 1B). Multivariable analysis, including age, sex, ALT level, HBsAg, and HBcrAg levels as adjusting variables, consistently

Table 1 Host and viral characteristics in 51 HBeAg-negative chronic hepatitis B patients who stop oral antiviral treatment.

	ALL (N = 51)	Non-Relapse (N = 26)	Relapse (N = 25)	P value
Gender				
Male (%)	38 (74.5)	19 (73.1)	19 (76.0)	0.881
Age at EOT years, Median (IQR)	52.0 (11.7)	51.1 (8.2)	52.4 (14.3)	0.720
Drug				
ETV (%)	34 (66.6)	18 (69.2)	16 (64.0)	0.392
TDF (%)	17 (33.3)	8 (30.8)	9 (36.0)	
Pre-treatment HBV DNA levels, logIU/mL, Median (IQR)	6.3 (2.3)	5.8 (1.9)	6.6 (2.4)	0.263
Serum ALT level at EOT, U/L				
Median (IQR)	20.0 (8.0)	18.5 (7.75)	20.0 (11.00)	0.180
HBV genotype (%)				
B	32 (62.8)	14 (53.9)	18 (72.0)	0.103
C	15 (29.4)	8 (30.8)	7 (28.0)	
Undetermined	4 (7.8)	4 (15.3)	0 (0.0)	
HBsAg at EOT <100 IU/mL	9 (17.6)	7 (26.9)	2 (8.0)	0.076
HBcrAg at EOT <1000 U/mL	23 (45.1)	15 (57.7)	8 (32.0)	0.065

NOTE.
EOT, end of treatment; IQR, Interquartile Range; ETV, entecavir; ALT, alanine transaminase; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBcrAg; hepatitis B core-related antigen; ETV, entecavir; TDF, tenofovir disoproxil fumarate.

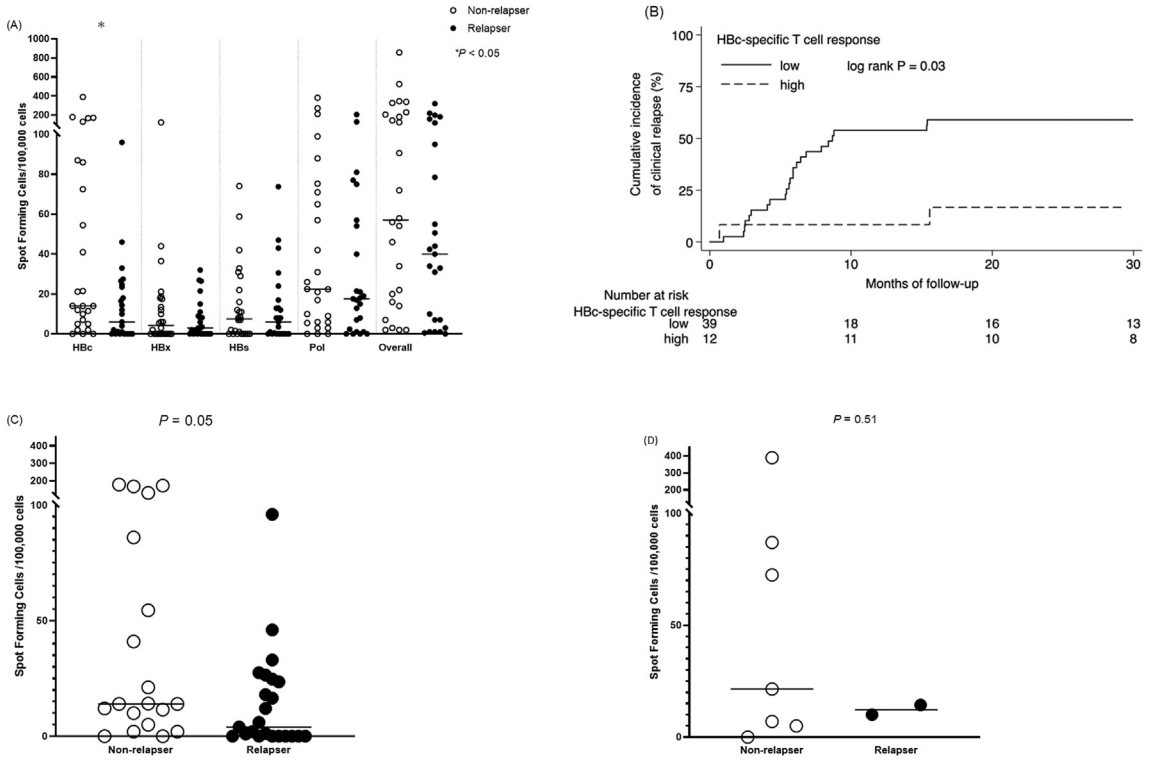

Figure 1. (A) HBc-specific T cell response was higher in patients who did not experience clinical relapse compared to those who did. (B) Patients with a higher HBc-specific T cell response ($\geq$35 SFC/10^5 cells, 3rd quartile) had a lower risk of clinical relapse. (C) Stratification by HBsAg level of 100 IU/mL showed a trend towards higher HBc-specific T cell response in the non-relapse group compared to the relapse group in patients with HBsAg >100 IU/mL, (D) but not in those with HBsAg <100 IU/mL.

showed that a higher HBc-specific T cell response was associated with a lower risk of clinical relapse (Table 2).

We also restricted the study population to 42 patients with HBsAg level >100 IU/mL, who were beyond the recommendation criteria to stop NA. There was a trend that HBc-specific T cell response was still stronger in patients without clinical relapse than those with clinical relapse (Median (IQR): 21.2 (118.5) vs. 6.0 (27.5) SFC/10^5 cells, $P = 0.05$, Fig. 1C). Only 9 patients with HBsAg level <100 IU/mL, we did not find a significant difference in HBc-special T cell response between the low-HBsAg patients with and without clinical relapse ($P = 0.51$, Fig. 1D).

Table 2 Factors associated with clinical relapse after withdrawal of antiviral treatment in 51 patients with HBeAg-negative chronic HBV infection.

	Months of follow-up	Clinical relapse (N)	Crude HR (95% CI)	Adjusted HR (95% CI)
Age (per year increase)			1.01 (0.97—1.05)	1.04 (0.99—1.09)
Gender				
Female	543.82	6	1.0	1.0
Male	1070.50	19	0.97 (0.39—2.42)	1.07 (0.40—2.86)
Serum ALT level (per 1 U/L increase)			1.01 (0.98—1.05)	1.00 (0.96—1.04)
Serum HBsAg level (IU/mL)				
< 100	285.04	2	1.0	1.0
≧100	1329.29	23	2.81 (0.66—11.94)	2.35 (0.52—10.58)
HBcrAg (U/mL)				
< 1000	833.43	8	1.0	1.0
≧1000	780.89	17	2.03 (0.87—4.71)	2.06 (0.76—5.60)
HBc-specific T cell response (SFC/10^5 cells)				
< 35	1028.32	23	1.0	1.0
≧35	586.00	2	0.21† (0.05—0.88)	0.23 ‡ (0.05—0.99)

Note.
†P = 0.03, ‡P = 0.04.
Abbreviations: HR, hazard ratio; CI, confidence interval; ALT, alanine transaminase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; HBc hepatitis B core protein; SFC: spot-forming cells.

HBV-specific T cell response at EOT associated with different surges of viral load and viral antigen

In terms of virological relapse, a higher HBc-specific T cell response was associated with a lower risk of virological relapse but not statistically different (P = 0.08). However, when looking at the peak level of viral rebound, it was lower in patients with high HBc-specific T cell response when compared to those with low HBc-specific T cell response (Fig. 2A). We also stratified the patients with the 3rd quartile of overall HBV-specific T cell response and the T cell response specific to other viral proteins. The peak viral load was comparable between patients with high and low response to other HBV proteins and overall HBV protein (Suppl Fig. 2A—D).

In addition to the viral loads, we also explored the relationship between peak viral antigens within 1 year after stopping NA and HBV-specific T cell response at EOT. We first found that there was a rebound of HBcrAg levels (Suppl Fig. 3A), but not HBsAg levels (Suppl Fig. 3B) during the follow-up. When correlating with HBV-specific T cell response at EOT, the peak level of HBcrAg surge was lower in patients with high HBc-specific T cell response than those with low T cell response (Fig. 2B). In terms of other viral protein-specific T cell response, we found that the patients with higher pol-specific and overall HBV-specific T cell response were also associated with lower peak levels of HBcrAg (Suppl Fig. 4A—D). None of the HBV-specific T cell response was shown to be associated with HBsAg rebound (Fig. 2C, Suppl Fig. 5A—D).

Factors associated with HBsAg seroclearance after stopping NA

There were 5 patients with HBsAg seroclearance after stopping NA. Compared to those without HBsAg seroclearance, there was no statistical difference in terms of HBV-specific T cell response at EOT (Suppl Fig. 6). Only HBsAg <100 IU/mL (vs. > 100 IU/mL) at EOT was associated with a

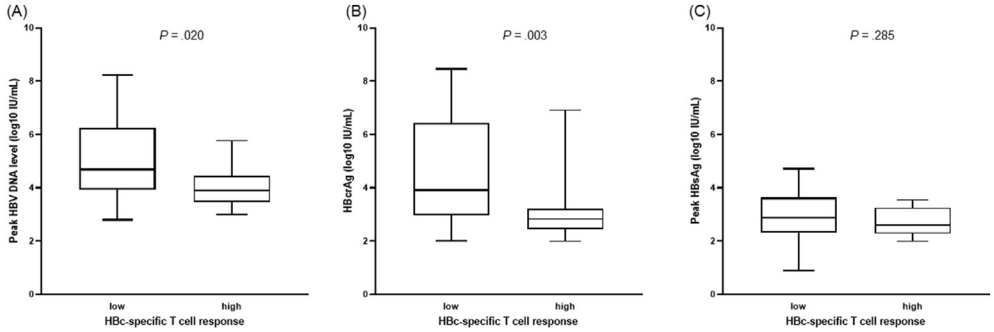

Figure 2. Patients with high HBc-specific T cell response at the end of treatment (n = 38) were associated with lower levels (n = 13) of (A) viral load surge and (B) HBcrAg rebound, but not (C) HBsAg level rebound within 1 year of nucleos(t)ide analogue withdrawal.

higher likelihood of HBsAg seroclearance after stopping NA (55.5% vs. 0%).

Comparison of HBV-specific T cell response between ETV and TDF

It has been speculated that different patterns of clinical relapse between ETV and TDF could be attributed to the different immune-modulatory effect exerted by different NA.[20] We thus compared different HBV-specific T cell response at EOT between patients receiving ETV and TDF treatment. The data showed that T cell response either overall or specific to each viral protein was comparable between ETV and TDF users (Fig. 3).

Higher HBV-specific T cell response along with clinical relapse

Early studies showed a higher probability of HBsAg seroclearance in patients with clinical relapse,[13] implying clinical relapse might be associated with restoration of HBV-specific T cell response although some data contradicts this hypothesis.[5] To test the hypothesis, we compared the HBV-specific T cell response determined at EOT and clinical relapse (N = 24) or at the end of follow-up (N = 7). Although there is a heterogenous response in patients with clinical relapse, we found the overall HBV-specific T cell response and Pol-specific T cell response at clinical relapse were higher than the response at EOT (both P = 0.03, Fig. 4A, Suppl Fig. 7A), but not other viral protein-specific T cell response. In contrast, the HBV-specific T cell response was comparable at EOT and at the end of follow-up in those without clinical relapse (Fig. 4B, Suppl Fig. 7B). To be noted, the higher HBV-specific T cell response after clinical relapse was not associated with HBsAg level reduction or HBsAg seroclearance (no patients cleared HBsAg after clinical relapse).

Discussion

Stopping NA therapy is a critical but challenging issue for the management of non-cirrhotic CHB patients. Although stopping NA might enhance HBsAg seroclearance in a small proportion of patients, the majority of them will experience clinical relapse.[3] It is well conceived that both outcomes are related to HBV-specific immune response as we observed in the natural history of HBV infection. In this study, we first showed that a higher HBc-specific T cell response at EOT was associated with a lower risk of developing clinical relapse. The surge of HBV DNA and HBcrAg levels were both lower in patients with high HBc-specific T cell response at EOT. Further analysis revealed that HBV-specific T cell response did not differ between patients with ETV and TDF therapy. Lastly, there was a moderate restoration of HBV-specific T cell response after clinical relapse. The latter finding may serve as a novel strategy to restore HBV immune response, which may facilitate HBV cure.

Whether HBV-specific T cell response could be restored after withdrawal of NA remains controversial.[4,8] For the first time, our data clearly demonstrated that only patients with clinical relapse, but not those without clinical relapse, developed stronger HBV-specific T cell response compared to the T cell response at withdrawal of NA. Even though the T cell response was not strong enough to clear HBsAg, such T cell response could theoretically be enhanced by a sequential combination of immune-modulatory agents. For example, the in vitro and human studies suggested that anti-PD-1 treatment could enhance HBV-specific T cell response.[8,21,22] As enhanced HBV-specific T cell response is

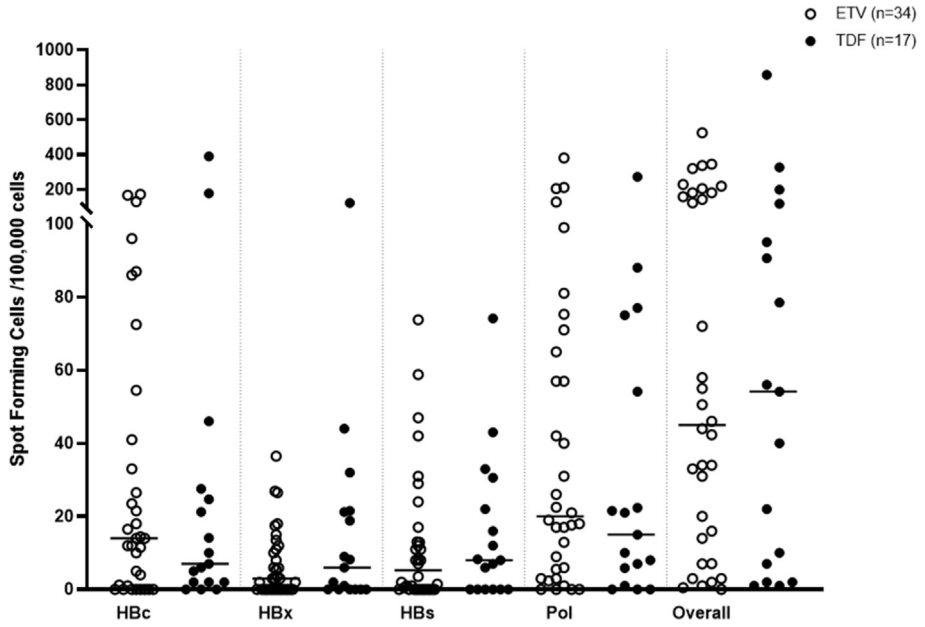

Figure 3. There was no statistical difference in HBV-specific T cell response at end of treatment between patients receiving entecavir (ETV) and tenofovir (TDF) treatment.

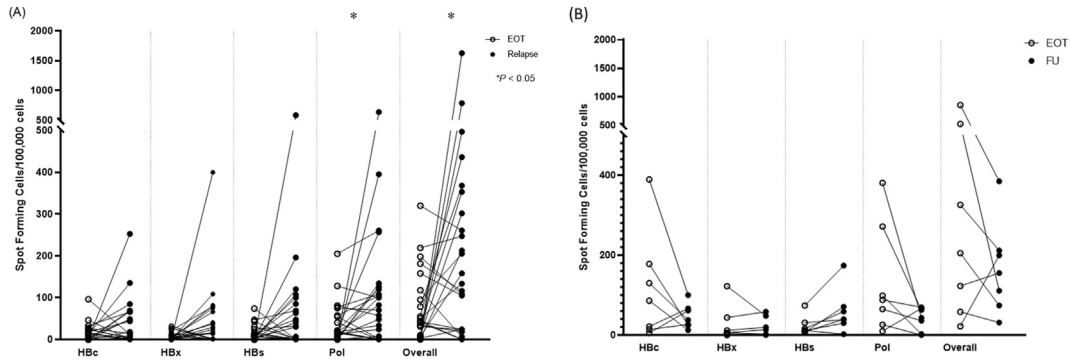

Figure 4. Different kinetic of HBV-specific T cell response in (A) 24 patients who developed clinical relapse and (B) 7 patients who did not develop clinica relapse.

the key for HBsAg seroclearance,[23] add-on anti-PD-1 in patients with partially restored HBV-specific immune response after stopping NA could be considered as a novel strategy to achieve functional cure for HBV.

The relationship between HBV-specific T cell response and clinical relapse was mostly reported in Caucasians.[4,6–8] However, off-NA prognosis seems different between Caucasian and Asian patients. For example, a higher HBsAg seroclearance rate was found in Caucasian patients than Asian patients.[24,25] In this prospective study, we enrolled the largest number of Asian patients to explore how HBV-specific immune response was associated with clinical relapse. Although plenty of viral factors have been studied,[3] the prediction model for off-NA prognosis is suboptimal. It is generally believed that immune predictors are the missing piece to complete an accurate prediction. In addition, this is the first study to examine the dynamic of HBV-specific T cell response following clinical relapse, which may provide us with a new strategy to partially reinvigorate HBV-specific T cell response. Finally, it is also the first report addressing the comparable HBV-specific T cell response between ETV and TDF users, which does not support the hypothesis that different patterns of clinical relapse between these 2 drugs are related to distinct HBV-specific T cell response.

There are a few limitations. First, the patient number is still limited. However, unlike most retrospective studies exploring viral markers alone,[24,25] it is challenging to conduct a prospective study analyzing T cell immune response with a large number of patients. A large-scale multi-center study is warranted to validate our findings in the future. Second, we did not analyze the HBV-specific T cell response using multimer-based flow cytometry due to the lack of knowledge regarding HBV genotype B or C epitopes restricted to highly prevalent class I and II human leukocyte antigen in Asians. However, we adopted the approach of T cell expansion/stimulation using overlapping peptides, which still provides a comprehensive picture of the HBV-specific T cell response. Nevertheless, this procedure is cumbersome due to its multiple steps, and the expanded T cells may not fully recapitulate the patients' HBV-specific T cell response in vivo.[26] To utilize immunological markers in clinical practice, it is necessary to develop novel assays for quantifying the HBV-specific T cell response directly ex vivo, similar to the approach used for detecting SARS-CoV-2-specific T cell functionality.[27] Third, the HBV-specific T cell dynamic is only available in 6 patients without clinical relapse. It is because we did not routinely collect the PBMC in patients without clinical relapse. Finally, HBV RNA may predict the risk of clinical relapse.[28] We did not explore its role as no standardized assay was available.

In summary, in Asian patients infected with HBV genotype B or C, a higher HBc-specific T cell response at the EOT was associated with a lower risk of clinical relapse and better viral control. The clinical relapse could partially restore the HBV-specific T cell response. These immunological findings not only help physicians optimize the clinical management of CHB patients with antiviral treatment, but also pave the path to cure HBV via partially reinvigorating HBV-specific T cell response.

Ethical approval and consent to participate

The study was conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by a priori approval by the Institutional Review Board of the National Taiwan University Hospital.

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Availability of data and material

All data produced or examined in the course of this study have been incorporated within the published article and its

accompanying supplementary materials. Requests for access to the dataset employed in this study can be directed to the corresponding authors, and such requests will be considered in a reasonable manner.

CRediT authorship contribution statement

Tai-Chung Tseng: Writing — review & editing, Writing — original draft, Project administration, Conceptualization. **Huei-Ru Cheng:** Writing — original draft, Methodology, Formal analysis, Data curation. **Tung-Hung Su:** Resources, Conceptualization. **Ping-Hung Lin:** Investigation. **Chih-Chiang Wang:** Investigation. **Hung-Chih Yang:** Project administration, Conceptualization. **Cheng-Shiue Tsai:** Investigation. **Chun-Jen Liu:** Conceptualization. **Pei-Jer Chen:** Conceptualization. **Jia-Horng Kao:** Writing — review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.07.012.