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HBeAg seroconversion is associated with a more effective PD-L1 blockade during chronic hepatitis B infection

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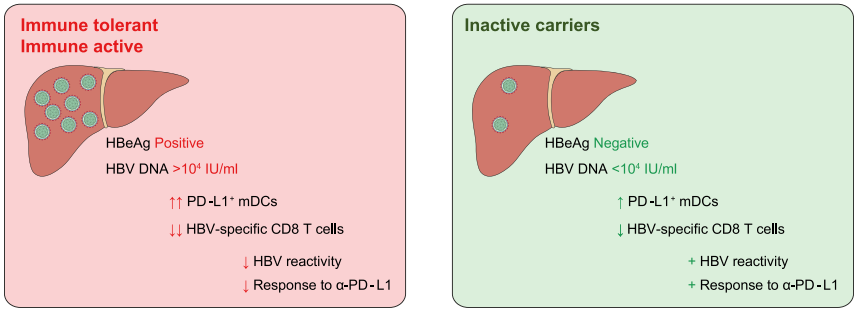
Graphical abstract



Highlights

- Upregulation of the PD-1:PD-L1 axis is more profound in HBeAg-positive samples.
- This upregulation does not normalize in HBeAg-negative patients, or patients under antiviral therapy.
- HBV-specific T cell reactivity is higher in HBeAg-negative patients with low HBV DNA levels.
- 97% of HBV-reactive patients respond to anti-PD-L1 blockade with MEDI2790 irrespective of their clinical status.

Lay summary

Hepatitis B virus (HBV)-specific T cell responses during chronic infection are weak due to the upregulation of inhibitor molecules on the immune cells. In this study we show that the inhibitory PD-1:PD-L1 axis is upregulated during chronic HBV infection and successful antiretroviral therapy does not restore normal levels of PD-1 and PD-L1 expression. However, in HBV e antigen-negative patients, treatment with an anti-PD-L1 antibody can increase the functionality of HBV-specific T cell responses by an average of 2-fold and is a promising new therapy for patients with chronic HBV infection.

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Research Article

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HBeAg seroconversion is associated with a more effective PD-L1 blockade during chronic hepatitis B infection

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Background & Aims: Current therapies for chronic hepatitis B virus (HBV) infection control viral replication but do not eliminate the risk of progression to hepatocellular carcinoma. HBV-specific CD8 T cells are necessary for viral control, but they are rare and exhausted during chronic infection. Preclinical studies have shown that blockade of the PD-1:PD-L1 axis can restore HBV-specific T cell functionality. The aim of this study was to analyze how the clinical and treatment status of patients impacts the ability of HBV-specific T cells to respond to PD-L1 blockade.

Methods: Expression patterns of the PD-1:PD-L1/PD-L2 axis were analyzed in healthy donors and chronically infected patients in different clinical phases of disease. A functional assay was performed to quantify baseline HBV-specific T cell responses in chronically infected patients. Baseline responses were then compared to those attained in the presence of an anti-PD-L1 monoclonal antibody (MEDI2790).

Results: Chronically infected patients were characterized by the upregulation of PD-1 within the T cell compartment and a concomitant upregulation of PD-L1 on myeloid dendritic cells. The upregulation was maximal in HBV e antigen (HBeAg)-positive patients but persisted after HBeAg negativization and was not restored by long-term treatment. HBV reactivity, measured as frequency of HBV-specific T cells, was significantly higher in HBeAg-negative patients with lower HBV DNA levels, independently of HBV surface antigen or alanine aminotransferase levels. Anti-PD-L1 blockade with MEDI2790 increased both the number of IFN-γ-producing T cells and the amount of IFN-γ produced per cell in 97% of patients with detectable HBV reactivity, independently of patients' clinical or treatment status.

Conclusion: Patients with lower levels of HBV DNA and the absence of HBeAg have more intact HBV-specific T cell immunity and may benefit the most from PD-L1 blockade as a monotherapy.

Introduction

Hepatitis B virus (HBV) infection, especially in adulthood, can be spontaneously resolved without evident liver inflammation or after an acute, but limited, inflammatory episode[1]. During the acute immune response to HBV infection the critical antiviral role of cytotoxic CD8 T cells has been demonstrated in the chimpanzee model[2], and viral clearance is associated with vigorous, broad and polyclonal T cell responses.[2–4] However, in most mother to child transmission and some adult patients, HBV establishes a chronic infection after eliciting a weak, narrow, and delayed T cell response that is insufficient to eliminate the virus.[5–7]

According to World Health Organization reports[8] there are 257 million people – a striking 3.5% of the worldwide population – living with chronic HBV infection. Persistent viral replication leads to continuous necroinflammation and patients are at higher risk of cirrhosis, end-stage liver disease, hepatic decompensation and hepatocellular carcinoma.[9,10] Current therapies for chronic HBV infection provide long-term benefits by suppressing viremia but most patients will never lose the expression of hepatitis B surface antigen (HBsAg), which indicates that persistent infection is active at a subclinical level within the liver.[11,12] Accordingly, it has recently been reported that the 8-year cumulative risk of developing hepatocellular carcinoma for successfully treated patients is still almost 6% for patients with complete viral suppression and over 8% if the achieved viral suppression is only partial.[13] Altogether, HBV-related deaths have increased by 22% in the last decade and account for close to a million deaths every year.[8]

Since HBV is non-cytopathic, the pathogenesis of the liver disease is dependent on the necroinflammation due to the constant immune response driven by viral replication.[14] Together with the naturally immune suppressive environment of the liver (*e.g.* interleukin (IL)-10 and transforming growth factor beta),[15,16] high levels of virus and viral antigens and the accumulation of regulatory T cells (Tregs),[17] contribute to a dysfunctional immune response to HBV[18] and drive the exhaustion of HBV-specific T cells. However, functional HBV-specific CD8 T cells are needed to control hepatic flares and the resurgence of viral replication

Keywords: Chronic HBV infection; PD-L1 blockade; PD-1 blockade; HBV-specific T cells; checkpoint inhibitor; immunotherapy; MEDI2790; HBV cure.
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after cessation of long-term successful antiviral therapy.[19] Therefore, restoring HBV immunity through immunotherapy is currently being investigated as a promising approach to treat patients with chronic HBV infection.[20,21]

Attempts to modulate the innate immune response of chronic HBV-infected patients have shown limited results *in vivo*,[22] suggesting that stimulation of innate cells alone may be insufficient to positively alter the clinical status of chronic HBV infection. In contrast, preclinical studies have shown the function of cells of the adaptive immune system, namely CD8 T cells, can be enhanced with immunotherapies that target an inhibitory pathway.[23] *In vitro* studies have shown that in chronic HBV infection, blockade of the programmed cell death 1 (PD-1): programmed cell death 1 ligand 1 (PD-L1) axis can increase both the production of HBV antibodies[24] and the numbers and functionality of HBV-specific T cells.[18,25] Similarly, *in vivo* PD-L1 blockade in the woodchuck model of chronic hepatitis showed sustained antiviral effects without liver damage.[26] As preclinical evidence supports targeting of the PD-1:PD-L1 axis as a therapeutic strategy to treat patients with chronic HBV infection, our aim was to determine how the clinical and treatment status of patients affects HBV-specific T cell reactivity in the absence or presence of blockade of the PD-1:PD-L1 axis with the anti-PD-L1 monoclonal antibody MEDI2790.

Patients and methods

Patients

Sixty-five adult patients with chronic HBV infection (23 were female [35.4%]; median age 44 years old) in follow-up at the Toronto General Hospital Liver Center, University Health Network in Toronto, Canada were included in this study. All patients had chronic HBV infection documented by the presence of HBsAg for at least 12 months, had available historical and clinical laboratory data related to HBV infection for at least 6 months preceding enrollment and were willing and able to provide consent. Exclusion criteria included: i) known coinfection with hepatitis C virus, hepatitis delta virus and/or HIV, ii) known active autoimmune disease including autoimmune hepatitis, iii) renal dialysis, iv) known cirrhosis, hepatocellular carcinoma or liver transplantation, v) prior use of an HBV therapeutic vaccine, vi) use of systemic corticosteroids or other immune suppressive agents within 4 weeks of screening or anticipated need for periodic use of systemic steroids during the study, vii) current treatment with immune modulators or immune suppressors and viii) patients under acute flare or reactivation of HBV infection (defined as symptoms of acute hepatitis and alanine aminotransferase [ALT] >10x the upper limit of normal [ULN] or elevated bilirubin levels). The study protocol was approved by the Ethics Committee of the Toronto General Hospital, University Health Network. Healthy controls were obtained from the MedImmune, LLC Research Specimen Collection Program (RSCP). All healthy controls are adults who tested negative for HBsAg, HIV-1, HIV-2, HTLV-1, HTLV-2 and rapid plasma reagin. Informed consent was obtained from each individual at enrollment. Peripheral blood samples were obtained by venipuncture, anonymized and processed to obtain peripheral blood mononuclear cells (PBMCs) and plasma samples. All samples were cryopreserved until further use. Characteristics of the cohort are summarized in Table 1.

Polychromatic flow cytometry

PBMC samples from 7 healthy donors and 25 patients with chronic HBV, selected according to sample availability, were thawed, washed in CTL Anti-Aggregate™ medium (Cellular Technology Limited, CTL) and rested overnight at 37°C in CTL Test Medium™ (Cellular Technology Limited, CTL). Cells were then washed and stained in 96-well plates at 1 million cells per well with LIVE/DEAD™ Fixable Aqua Dead cell stain kit (Invitrogen). Surface markers were stained with titrated amounts of monoclonal antibodies in the presence of Super Bright Staining Buffer (eBiosciences). The gating strategy is shown in Fig. S1A: anti-CD3 BUV661 clone UCHT1, anti-CD20 BUV805 clone 2H7, anti-CD16 BUV496 clone 3G8, anti-CD4 BV570 clone SK3, anti-CD8 BUV563 clone RPA-T8 and anti-PD-L2 BUV395 clone MIH18 from BD Biosciences; anti-CD14 Brilliant Violet (BV) 785 clone M5E2, anti-PD-1 BV605 clone EH12.2H7 and anti-PD-L1 BV421 clone 29E.2A3 from Biolegend and anti-CD11c PE Cyanine5.5 clone 3.9 from eBiosciences. After a final wash at least 5E5 cells were acquired in a BD FACSymphony™ cell analyzer. Analysis was performed in FlowJo V10. Cytokine production and polyfunctionality were analyzed with the Simplified Presentation of Incredibly Complex Evaluations (SPICE v5) software provided by Dr. M. Roederer and the National Institute of Allergy and Infectious Diseases (NIAID, National Institutes of Health) as previously described.[27]

T cell expansion and functionality assay

PBMC samples from the 65 patients with chronic HBV were thawed, washed in CTL Anti-Aggregate™ medium (Cellular Technology Limited, CTL) and left overnight at 37°C in complete RPMI with 10% human serum. Cells were plated at 1E7/ml and overlapping peptide pool for the HBV-capsid protein (>90% purity, JPT) was added to the culture. IL-2 at a final concentration of 2 IU/ml was added to the cells at day 2 and maintained until day 5. After a 5-day stimulation, quadruplicates of 2.5E5 expanded cells were re-stimulated with the same HBV-capsid peptide pool (or actin peptide pool as irrelevant peptide, when appropriate) in the presence of MEDI2790 or a control IgG isotype. Staphylococcal enterotoxin B was included as a positive control in every plate. HBV-specific T cell responses were quantified by ELISpot using ELISpot^{PLUS} interferon (IFN)-γ pre-coated plates (MabTech) according to manufacturer's instructions. Quantification was performed using the ImmunoSpot® reader and images were analyzed with the ImmunoSpot® software (Cellular Technology Limited, CTL).

Multiplex cytokine analysis of the assay supernatants

Supernatants from the T cell ELISpot assays were recovered, frozen down in single assay aliquots and stored at -80°C. After thawing, supernatants were analyzed in duplicate for the presence of different inflammatory cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and tumor necrosis factor [TNF]) using the MSD MultiSpot Assay System Proinflammatory Panel 1 (human) kit (Mesoscale, MSD) according to the manufacturer's instructions. Proper standard curves, positive and negative controls were included in every assay.

MEDI2790 antibody generation

MEDI2790 was generated as previously described.[28] Briefly, IgG2 and IgG4 XenoMouse animals were immunized with human PD-L1-Ig or Chinese hamster ovary cells expressing human PD-L1. Hybridomas were established and supernatants screened for binding to human PD-L1-transfected HEX 293 cells and inhibition of PD-1 binding to PD-L1 expressing Chinese hamster ovary cells. MEDI2790 was selected based on affinity, activity and specificity

Table 1. Characteristics of the cohort.

	All cohort	HBV non-reactive (NR)[1]	HBV reactive (R+)[1]	*p* NR *vs.* R+
n	65	29	36	
Sex (% Female)	23/65 (35.4%)	13/29 (44.8%)	10/36 (27.80%)	n.s.[2]
Age, years	44 [32–54]	44 [31–57]	44 [32–54]	n.s.
Race (% Asian)	53/65 (81.5%)	28/29 (96.6%)	25/36 (69.4%)	0.0470
IFN history (% yes)	4/65 (6.2%)	3/29 (10/3%)	1/36 (2.8%)	n.s.
HBeAg[3]				0.0072
HBeAg (+)	13/65 (20%)	10/29 (34.5%)	3/36 (8.3%)	
>1.3x ULN of ALT	4/13 (30.8%)	2/10 (20%)	2/3 (67%)	
HBeAg (-)	40/65 (61.5%)	12/29 (41.4%)	28/36 (77.8%)	
>1.3x ULN of ALT	9/40 (22.5%)	3/12 (25%)	6/28 (21.4%)	
Antiviral therapy[4]	12/65 (18.55)	7/29 (24.1%)	5/36 (13.9%)	
HBeAg (+)	2/12 (16.75)	2/7 (28.6%)	0/5 (0%)	
ALT (IU/ml)	28 [20–42]	25 [19–38]	31 [23–51]	0.1240
AST (IU/ml)	27 [21–34]	27 [20–35]	28 [21–33]	n.s.
ALT/AST >1	24/65 (36.9%)	15/29 (51.7%)	9/36 (25%)	0.0265
HBsAg (IU/ml)	2,228 [687–9,526]	2,077 [816–12,718]	2,277 [327–7,250]	n.s.
HBV DNA	1,220 [20–84,050]	23,340 [20–1.7E7]	817 [69–13,975]	0.1444
IFN-γ SFU/1E6 cells [Isotype]	65 [12–201]	20 [9–57]	186 [122–397]	<0.0001
Average spot intensity [Isotype]	0.41 [0.08–1.61]	0.07 [0.04–0.18]	1.46 [1.06–3.51]	<0.0001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, HBV e antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; IFN, interferon; SFU, spot-forming units; ULN, upper limit of normal.

[1]HBV-reactivity was defined as ≥100 SFUs per million cells after re-stimulation in any of the conditions tested.

[2]n.s. = not significant; p >0.15. Mann-Whitney U test.

[3]All HBeAg-negative patients in this category spontaneously seroconvert.

[4]Antiviral therapy – 9/12 (75%) tenofovir, 2/12 (16.7%) entecavir and 1/12 (8.3%) lamivudine. Dichotomic variables are expressed as number/total number (frequency). Continuous variables are expressed as median [Interquartile range, IQR].

profile. The constant domain of the antibody was then exchanged for a human IgG1 triple-mutant domain containing 3 point mutations that reduce binding to C1q and Fc gamma receptors, resulting in reduced antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity.

Statistical analysis

GraphPad Prism statistical analysis program (GraphPad Software v7.04) was used to perform the statistical analyses and to create the graphs. Data are shown as individual points or bars depicting the mean ± standard error (SEM). Variables were analyzed using the following non-parametric tests when appropriate: Mann-Whitney U test for unpaired variables, Wilcoxon matched-pairs signed rank test for paired variables, Chi-square test for dichotomic variables and Kruskal-Wallis test to compare 3 or more experimental groups. Associations were analyzed by linear regression and 95% CI. P values <0.05 were considered significant.

Results

The PD-1:PD-L1/PD-L2 axis is upregulated in chronic HBV infection

To determine whether PD-L1 blockade would be a suitable strategy to treat chronic HBV infection, we analyzed the expression of the different components of the PD-1:PD-L1/PD-L2 axis (gating strategy shown in Fig. 1A and Fig. S1A) in healthy donors (n = 7) and chronically infected patients (n = 25). Analysis of immune populations in peripheral blood showed similar frequencies of T cells, B cells and classical monocytes between healthy donors and chronically infected patients (Fig. S1B). However, chronic HBV infection was characterized by lower frequency of myeloid dendritic cells (mDCs) and a concomitant increase of CD16-expressing inflammatory monocytes (Fig. 1B). Furthermore, PD-1 expression was upregulated on both CD8 and CD4 T cells (Fig. 1C and Fig. S1C) while PD-L1 and PD-L2 were both dysregulated in the myeloid populations (Fig 1D and Fig. S1D). Inflammatory monocytes, despite being highly expanded in chronic infection, expressed lower frequencies of both PD-1 ligands. Strikingly, the monocyte population expressing only PD-L1 ($PD\text{-}1^{-}PD\text{-}L1^{+}PD\text{-}L2^{-}$) was almost completely lost in chronically infected patients (Fig. S1D). Conversely, PD-L1 expression was significantly increased on mDCs. While we also observed an increased frequency of $PD\text{-}L1^{+}PD\text{-}L2^{+}$ mDC double positives, chronic HBV infection favored the expansion of PD-L1-expressing mDCs in the peripheral blood (Fig. 1D). Thus, the immune system of HBV-infected patients can be characterized by high levels of PD-1-expressing CD8 T cells and high levels of PD-L1 expression on the mDC population. This suggests that PD-L1 blockade could be a suitable strategy to treat chronic HBV infection.

PD-1 and PD-L1 expression levels are similar across different HBV clinical profiles

We then sought to analyze PD-1 and PD-L1 expression across the different phases of HBV chronic infection to determine if there is an optimal HBV-infected patient population for treatment with PD-L1 blockade. Immune populations and PD-1:PD-L1 frequencies

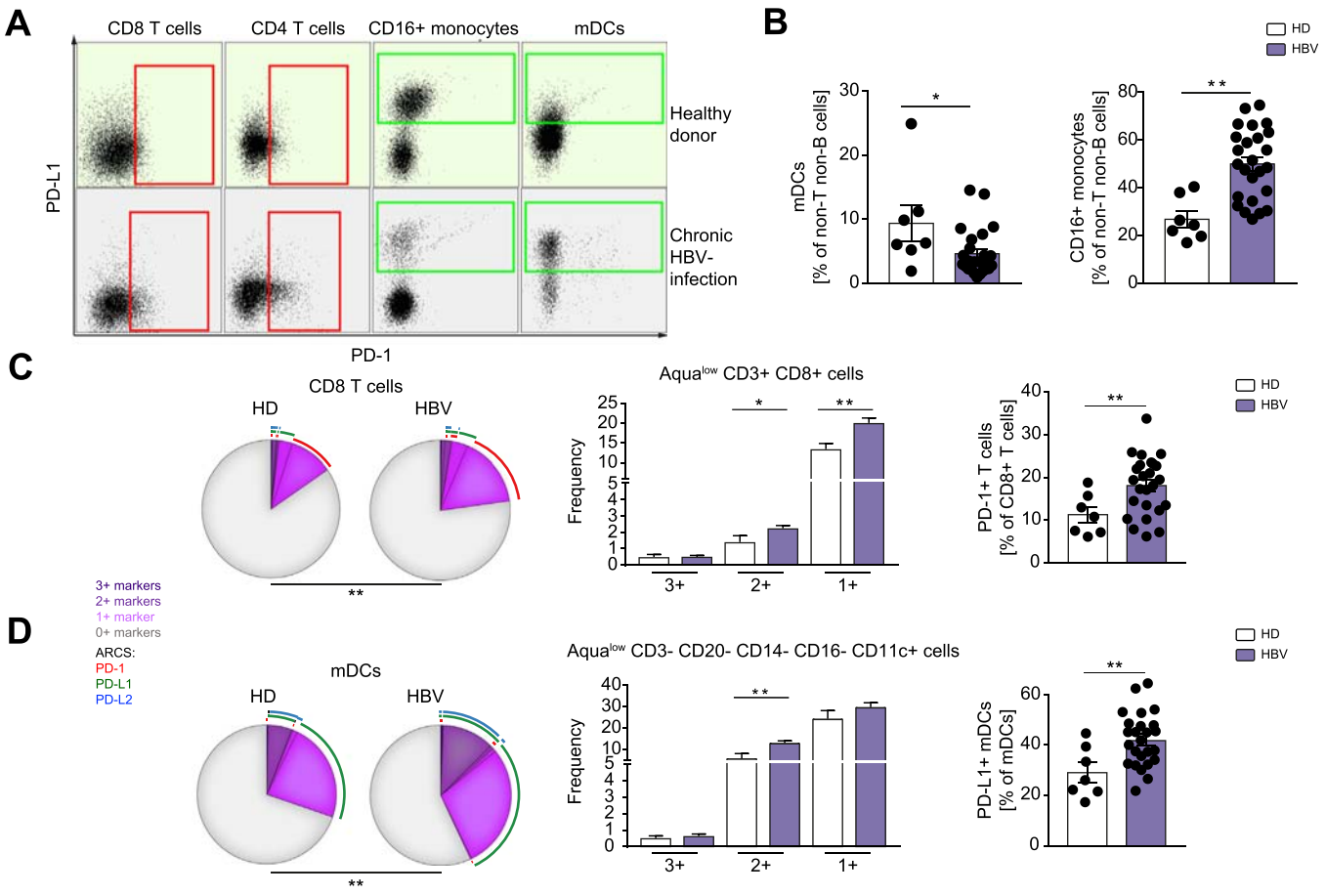

Fig. 1. Upregulation of the PD-1:PD-L1/PD-L2 axis in chronic HBV. (A) Representative flow plots for an HD and a chronic HBV-infected donor showing the PD-1 and PD-L1 expression in T cells, monocytes and mDCs (B) Pooled data showing the frequency of mDCs (left panel) and inflammatory monocytes (right panel) in HDs and individuals with chronic HBV infection. (C) Dysregulation in the PD-1:PD-L1/PD-L2 axis in CD8 T cells (upper panel) and mDCs (lower panel). SPICE data (left and middle panels) show the frequency of cells co-expressing 3 ($PD\text{-}1^{+}PD\text{-}L1^{+}PD\text{-}L2^{+}$), 2, 1 or no markers ($PD\text{-}1^{-}PD\text{-}L1^{-}PD\text{-}L2^{-}$) at the same time. Arcs indicate cells expressing each individual marker. Pooled data (right panel) show the significant increase of PD-1-expressing CD8 T cells and PD-L1-expressing mDCs in chronic HBV infection. Mann-Whitney *U* test. $^{*}p$ <0.05, $^{**}p$ <0.001. HBV, hepatitis B virus; HD, healthy donor; mDCs, myeloid dendritic cells.

were analyzed according to the hepatitis B e antigen (HBeAg) status, indicative of active viral replication, and HBsAg and HBV DNA levels. We found a major imbalance on the frequencies of peripheral mDC and inflammatory monocytes in patients on antiviral therapy and untreated patients positive for the HBeAg (Fig. 2A) compared to healthy donors. HBV-infected patients negative for the HBeAg antigen, even if they are untreated, have partially recovered – but not yet normal – mDC and monocyte frequencies (Fig. 2A). However, the frequency of PD-1 expressing T cells and PD-L1-expressing mDCs and monocytes were similar among HBeAg-positive/negative patients (Fig. 2B and Fig. S2A) and potentially independent of HBsAg or HBV DNA levels (Fig. 2C and Fig. S2B). Thus, the upregulation of the PD-1:PD-L1 axis persists during all clinical phases of HBV chronic infection and it is not restored to homeostatic levels after successful antiviral therapy.

HBV-specific responses can be robustly detected after T cell expansion

Due to the low frequency of HBV-specific T cells in PBMCs from chronically infected patients[18,25,29] we developed a 5-day expansion protocol followed by short-term re-stimulation and ELISpot analysis to evaluate the presence and responsiveness of HBV-reactive T cells. A subset of 10 patients was first analyzed to confirm our ability to robustly detect HBV-reactive T cell responses. IFN-γ spot-forming units (SFUs) per million cells were quantified after re-stimulation with either irrelevant peptide (actin) or HBV-capsid peptide pool (Fig. S3A). We found a significant increase in both the number of IFN-γ-producing cells and the IFN-γ production per cell, as measured as mean spot intensity in chronic HBV-infected patients whose cells were re-stimulated with the HBV-capsid peptide pool (Fig. 3A). HBV reactivity upon re-stimulation with capsid peptide pool was confirmed by analyzing the concentration of different cytokines in the ELISpot supernatant. Significantly higher concentrations of Th1 cytokines (IFN-γ, TNF and IL-2) were found in the supernatants of HBV-reactive patients (Fig. 3B). As previously described[16] we also found higher levels of IL-10 immunosuppressive cytokine upon re-stimulation, while IL-4 levels remained unchanged (Fig. S3B). The rest of the inflammatory cytokines analyzed remained unchanged (data not shown).

We then analyzed the full cohort of 65 chronically HBV-infected patients (Table 1). Following this protocol, we were able to detect robust responses in 55.4% (36/65) of the analyzed patients (Fig. 3C). HBV reactivity was further confirmed by quantification of the supernatant cytokines. Concentration of IFN-γ, IL-10 (Fig. 3D), IL-2 and IL-6 (Fig. S3C) were significantly higher upon re-stimulation in HBV-reactive patients (HBV-R+) when compared to non-reactive patients (HBV-NR). TNF concentration tended to be higher in HBV-reactive patients but did not reach significance while

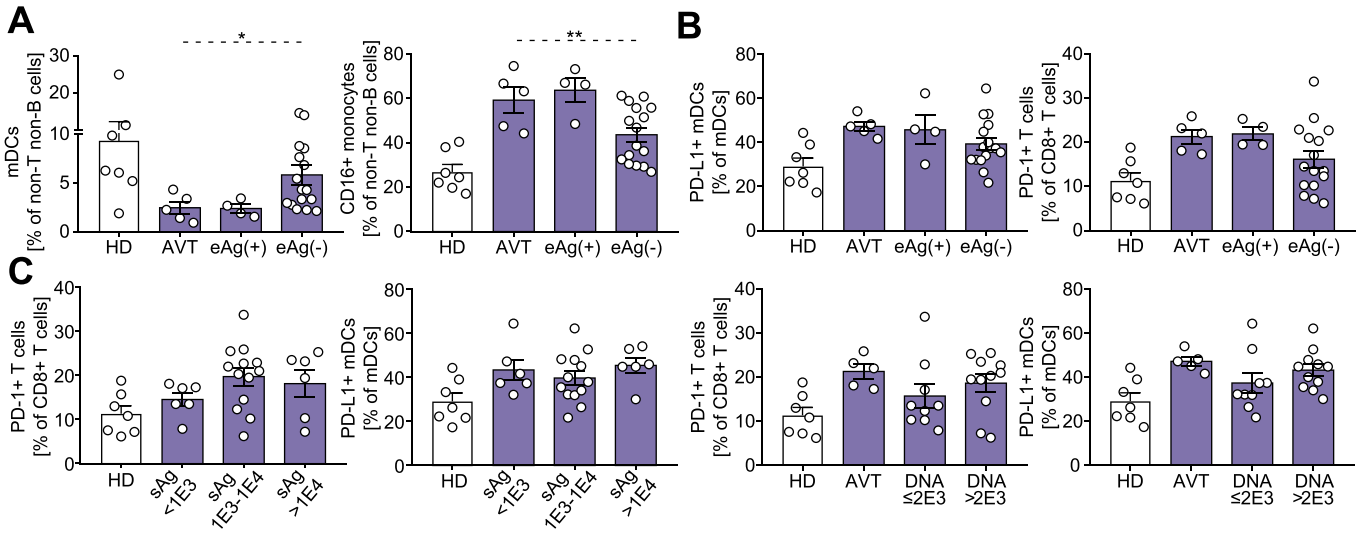

Fig. 2. Clinical correlates for the PD-1:PD-L1 axis dysregulation. Pooled data showing the frequency of (A) mDCs and inflammatory monocytes and (B) PD-1-expressing CD8 T cells and PD-L1-expressing mDCs in HDs, patients with chronic HBV infection under antiviral therapy, and untreated HBV-infected patients positive (eAg(+)) or negative (eAg(-)) for the HBeAg. Pooled data showing the frequency of PD-1-expressing CD8 T cells and PD-L1 expressing mDCs in HDs and chronically infected HBV patients with (C) different levels of HBsAg and (D) different levels of HBV DNA. Kruskal-Wallis test. *p <0.05, **p <0.001. HBeAg, HBV e antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; HD, healthy donor; mDCs, myeloid dendritic cells.

the concentration of IL-4 (Fig. S3C) and the rest of cytokines tested (data not shown) was similar between non-reactive and HBV-reactive patients. Thus, despite the low frequency of HBV-specific T cells detected *ex vivo* during chronic infection,[18] this system allows for a robust quantification of HBV-specific IFN-γ-producing cells in more than half of the tested donors.

HBV reactivity is more prevalent after HBeAg seroconversion and is associated with a preserved mDC population

We then analyzed whether any of the biological parameters measured were associated with the clinical stage of disease (Table 1). Results showed that HBV reactivity was more prevalent in patients negative for HBeAg (20% *vs.* 66%, Fig. 4A, left panel). When only HBV-reactive patients were analyzed, the magnitude of the response (as quantified as IFN-γ SFUs per million cells) was higher in HBeAg-negative patients (Fig. S4A, left panel). However, the low numbers of HBeAg-positive patients analyzed should be taken into consideration. Low levels of HBV DNA were also associated with higher HBV reactivity and a higher magnitude of response (Fig. 4A and Fig. S4A). These results were consistent when treated patients were excluded from the analysis (data not shown). The frequency of HBV-reactive patients was similar in HBeAg-negative patients with normal (≤1.3x ULN) or high (>1.3x ULN) ALT levels, but the magnitude of the response was higher in treatment naive patients with normal levels of ALT (Fig. 4B). Surprisingly, the reactivity was not associated with HBsAg levels (Fig. S4B). HBV-reactive patients had intermediate frequencies of mDC and inflammatory monocytes (closer to the levels shown in healthy samples) while the lack of reactivity to HBV re-stimulation was associated with significantly lower levels of

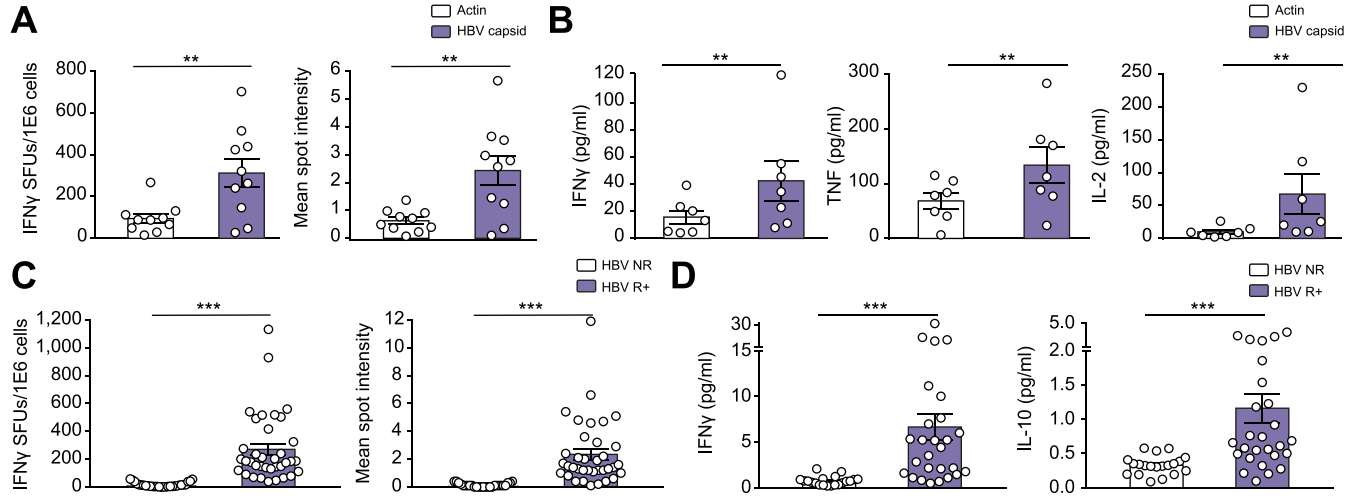

Fig. 3. HBV-specific responses in chronic HBV infection. Pooled data showing (A) IFN-γ SFUs per million cells (left panel) and average intensity (right panel) and (B) Cytokine concentration in the assay supernatant after re-stimulation of the expanded PBMCs with either irrelevant peptide (actin) or HBV-capsid peptide pool. (C) IFN-γ SFUs per million cells (left panel) and spot intensity (right panel) after re-stimulation with HBV-capsid peptide pool in non-reactive (HBV-NR) and HBV-reactive (HBV-R+) patients. (D) Cytokine concentrations in the assay supernatant in non-reactive (HBV-NR) and HBV-reactive (HBV-R+) patients after re-stimulation. Mann-Whitney *U* test. **p <0.001, ***p <0.0001. HBV, hepatitis B virus; PBMCs, peripheral blood mononuclear cells; SFUs, spot-forming units.

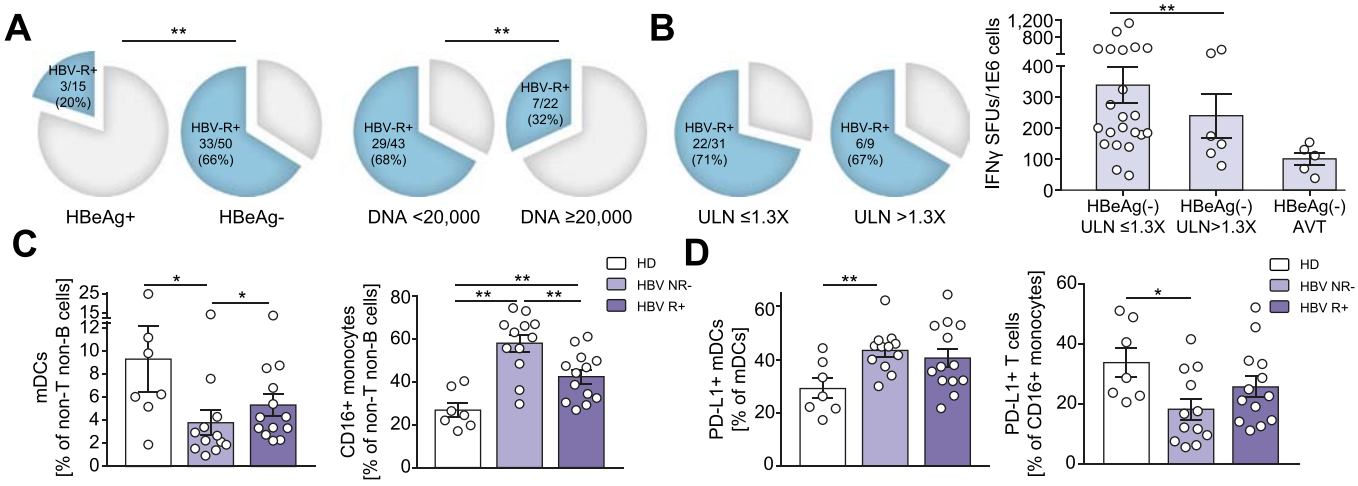

Fig. 4. Clinical and biological correlates to HBV reactivity. (A) Frequency of HBV-reactive patients in patients positive or negative for HBeAg (left panel) and with low or high HBV DNA (right panel). Chi-square test. $^{**}p$ <0.001. (B) (Left panel) Frequency of HBV-reactive patients in patients negative for HBeAg with normal (Phase 3) or increased (Phase 4) ALT levels. (Right panel) IFN-γ SFUs per million cells among HBV-reactive patients, negative for HBsAg, with normal or altered ALT levels. Mann-Whitney *U* test. $^{**}p$ <0.001. Pooled data showing the frequency of (C) mDCs and inflammatory monocytes or (D) PD-L1 expression levels among these cell types in HDs, non-reactive (HBV-NR) and HBV-reactive (HBV-R+) patients. Mann-Whitney *U* test. $^{*}p$ <0.05, $^{**}p$ <0.001. ALT, alanine aminotransferase; HBeAg, HBV e antigen; HBV, hepatitis B virus; HD, healthy donor; mDCs, myeloid dendritic cells; SFUs, spot-forming units.

mDCs and higher levels of inflammatory monocytes (Fig. 4C). PD-1 expression on T cells was similar in HBV-reactive and non-reactive patients (Fig. S4C). PD-L1 expression on myeloid cells from HBV-reactive patients, despite still being upregulated, tended to be closer to that observed on myeloid cells from healthy donors (Fig. 4D). Altogether, these results show that HBV reactivity is more prevalent in patients with stronger immune responses (after HBeAg seroconversion) but reactive patients still show upregulation of inhibitor molecules and could benefit from PD-L1 blockade to strengthen the HBV-specific T cell immune response.

PD-L1 blockade with MEDI2790 can significantly increase the HBV-specific T cell response

Next, we analyzed whether disrupting the PD-1:PD-L1 inhibitory axis by anti-PD-L1 blockade with MEDI2790 could increase HBV-specific T cell responses. First, we confirmed that PD-L1 expression in mDCs was still present in our samples after the 5-day expansion protocol (Fig. S3A and Fig. S5A). Then, we analyzed the full cohort (Table 1) for reactivity to HBV-capsid peptides in the presence or absence of the anti-PD-L1 monoclonal antibody (MEDI2790). When the donors with detectable reactivity to the HBV pool were analyzed (36/65, 55.4%), all but 1 sample (35/36, 97%) showed a significant increase in the frequency of IFN-γ-producing cells (186 [125-379] SFUs per million cells *vs.* 379 [230-573] SFUs per million cells, p <0.0001) in the presence of MEDI2790 (Fig. 5A and Fig. S5B). On average, the presence of MEDI2790 induced a 2-fold increase in the frequency of HBV-specific IFN-γ-producing T cells (Fig. 5A). In addition, concomitantly, there was a significant increase in the production of IFN-γ per cell (1.5 [1.2–3.4] average intensity *vs.* 3.7 [2.4–5.7] average intensity, p <0.0001) (Fig. 5A). To confirm that PD-L1 blockade was increasing the overall immune response beyond IFN-γ we quantified the cytokines present in the supernatant of the assay. We showed that Th1 and inflammatory cytokines IFN-γ, IL-2, TNF or IL-6, as well as the immunosuppressive cytokine IL-10, were all significantly increased in the presence of MEDI2790 (Fig. 5B). Moreover, the number of IFN-γ SFUs per million cells gained in the presence of MEDI2790 was inversely associated with the frequency of peripheral blood PD-L1-expressing mDCs (Fig. 5C).

Because of the potential concomitant use of checkpoint inhibitors and antivirals we separately analyzed the effect of MEDI2790 in patients on suppressive antiviral therapy. Despite the low number of treated patients in this cohort (n = 12) we could observe a significant increase in the number of the overall IFN-γ SFUs per million cells (p = 0039, Wilcoxon paired test) after MEDI2790 treatment. Interestingly, 2 out of the 12 patients (2/12, 16.7%) were HBV non-reactive at baseline but showed detectable HBV reactivity (IFN-γ SFUs per million cells >100) after MEDI2790 treatment (data not shown).

It is also worth noting that when analyzing patients determined to be HBV non-reactive by ELISpot we still observed a significant increase in the concentration of IFN-γ in the culture supernatants after HBV-peptide stimulation in the presence of MEDI2790 (Fig. S5C). This result suggests that PD-L1 blockade is influencing HBV-specific T cell responses even when these responses are undetectable in the absence of treatment using the current experimental methodology. In sum, our results show that PD-L1 blockade by MEDI2790 may be a promising approach to restore the function of HBV-reactive T cells in patients with chronic HBV infection.

Discussion

Chronic HBV infection leads to life-threatening conditions and increases by 100-fold the risk of developing hepatocellular carcinoma.[9,30] Several antiviral drugs (PEGylated-IFN-α and different nucleos(t)ide reverse transcriptase inhibitors) are currently used in monotherapy as a life-long treatment for chronic HBV infection. Since viral replication is key to liver injury and disease progression, current treatments provide long-term benefits by suppressing HBV viremia and reducing hepatic necroinflammation. However, successfully treated patients have a sustained persistent infection within the liver and maintain an 8-year cumulative risk of developing hepatocellular carcinoma of 6%,[13] even when achieving persistent and complete viral suppression.[11–13]

The loss of HBsAg, which indicates complete immune control and suppression of the virus, is regarded as a functional cure and the optimal endpoint of therapy.[31] However, using current antiviral strategies, HBsAg loss is rarely achieved and therapy

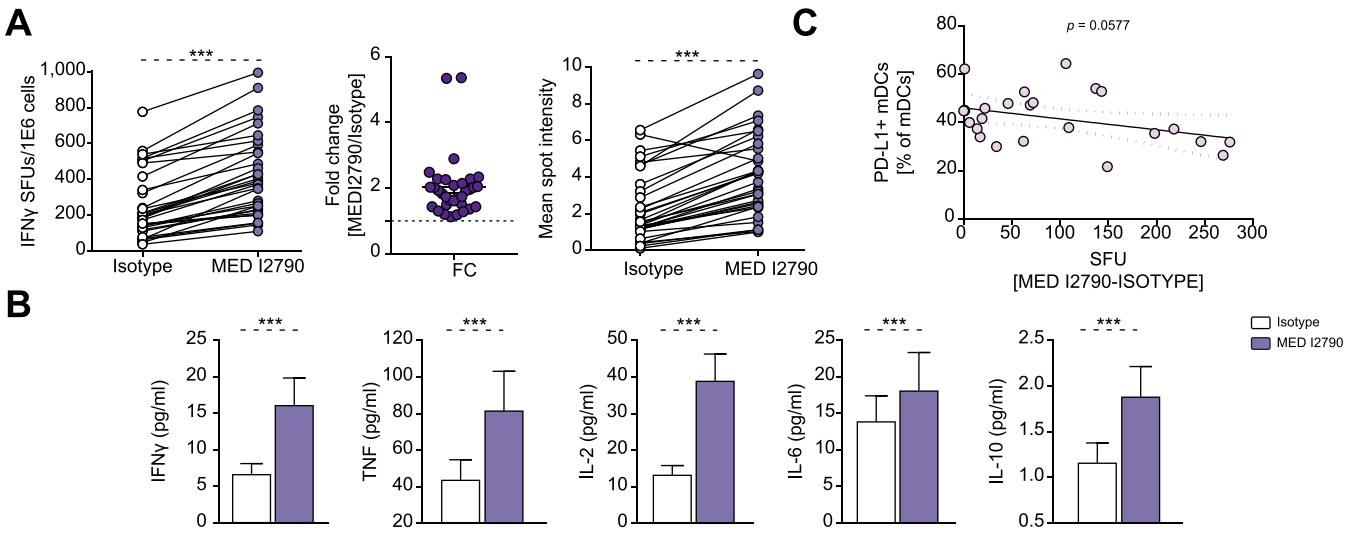

Fig. 5. PD-L1 blockade significantly increases HBV-specific responses. (A) Significant increase of both IFN-γ SFUs per million cells (left panel), IFN-γ SFUs per million cells fold-change (FC, middle panel) and spot intensity (right panel) after PD-L1 blockade with MEDI2790. Wilcoxon matched-pairs signed rank test. ***p <0.0001. (B) Cytokine concentrations in the assay supernatant in HBV-reactive patients in the presence or absence of an anti-PD-L1 blocking antibody. Wilcoxon matched-pairs signed rank test. ***p <0.0001. (C) Linear regression showing the correlation between the increase of IFN-γ SFUs per million cells (ΔSFU) in the presence of MEDI2790 and the frequency of PD-L1-expressing mDCs. Linear regression and 95% CI. p = 0.0577. HBV, hepatitis B virus; mDCs, myeloid dendritic cells; SFUs, spot-forming units.

needs to be maintained for life. Thus, a current objective for new HBV treatments is achieving HBsAg loss with finite therapy. The rationale for immune-based approaches to achieve HBsAg seroconversion and a functional cure is based on the observation that, during natural chronic HBV infection, the virus has the intrinsic ability to escape innate immune recognition, trigger a defective humoral response, and HBV-specific T cells are quantitatively and functionally defective (reviewed in Bertoletti and Bert, 2018[20]). Considering that previous studies have shown that strong HBV-specific T cell responses are enough to effectively control viral replication,[2–4] boosting HBV-specific immunity, alone or in combination with traditional antiviral therapy, is being investigated as a promising approach for achieving a functional cure.

In this study, we show for the first time that the PD-1:PD-L1 axis upregulation seen in patients with chronic HBV is not normalized in patients under antiviral therapy, despite successful viral suppression to undetectable levels for long periods of time. However, due to the cross-sectional nature of this study, these results should be further validated in a longitudinal study. In this context, we observed that HBeAg-negative patients under long-term antiviral therapy (Fig. 4B, average antiviral therapy 5 years) had a lower frequency of IFN-γ-producing cells than HBeAg-negative patients that are not under treatment. This lower frequency could be affected by the time of antiviral therapy onset or the duration of antiviral therapy, but we cannot discard the possibility that this result is due to the low number of patients (n = 5) in that group. However, altogether, these results support the growing evidence that antiviral therapy alone is not enough to eradicate viral replication within the liver. In addition, chronically infected inactive carriers, who are negative for the HBeAg, have a better immunological profile but their PD-1/PD-L1 expression levels were still not normalized. These results suggest that even low levels of hepatic necroinflammation are negatively impacting HBV-specific immunity. Failure to effectively mobilize HBV-specific adaptive immunity due to the constant expression of checkpoint inhibitors like those of the PD-1:PD-L1 axis could be contributing to the modest results exhibited by immune strategies targeting the innate branch of the immune system.[32,33]

Directly disrupting the PD-1:PD-L1 interaction with monoclonal antibodies has previously been shown to restore the functionality of exhausted CD8 T cells in both, cancer and infectious diseases.[18,23,25] In this study we show that PD-L1 blockade with MEDI2790 increases by 2-fold the HBV-specific T cell response in 97% of chronically infected patients with baseline T cell reactivity. It is worth noting that baseline reactivity was associated with a partially restored mDC subset and fewer immunological defects, suggesting that bi- or tri-specific antibodies targeting several immune inhibitors, or combination of different immune strategies may be needed to achieve a response in patients with a more compromised HBV-specific immunity. An important consideration is the risk of HBV reactivation associated with immune therapy observed in the oncology setting. Checkpoint inhibitors have shown an encouraging clinical activity across multiple cancers, but immune-mediated hepatitis is being described as a relevant cause of morbidity and mortality in cancer patients previously exposed to HBV infection.[34,35] However, most immune-mediated hepatitis B reactivation cases have been reported in patients with severe immunodeficiency (after immunosuppressive chemotherapy or bone marrow transplantation).[10,35,36] In addition, in the rare cases where immune-mediated reactivation of hepatitis B was not associated with profound immunosuppression, the episode was easily resolved after HBV antiviral treatment.[36,37] Thus, while the possibility of HBV reactivation should be considered, the risk of fatal hepatitis in immune-competent chronic HBV infection is low according to current available data.

Due to the low frequency of HBV-specific cells usually detected in chronic HBV infection, and especially in the immune tolerant phase, all our analyses were performed *in vitro* after a 5-day expansion protocol. Further studies are needed to determine whether these response levels are maintained *ex vivo* and *in vivo*. Additional analyses are also needed to quantify the exhaustion levels and response to the checkpoint blockade within the naturally suppressed environment of the liver. However, liver biopsies from clinically asymptomatic patients are not obtained routinely and HBV infection lacks a suitable animal model. Thus, PBMCs are

currently the most widely used proxy to study HBV-specific T cell responses.

In conclusion, this study provides proof-of-concept for the use of immune-based therapies to treat patients with chronic HBV infection. Our results demonstrate a role for the PD-1:PD-L1 axis in inhibiting HBV-specific T cell responses in chronic HBV-infected patients and the ability of MEDI2790, an anti-PD-L1 monoclonal antibody, to enhance HBV-specific T cell responsiveness. However, response to MEDI2790 was not significant in patients without quantifiable baseline reactivity, suggesting that combination therapies may also be needed in strategies aiming to improve HBV adaptive immunity.

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Conflict of interest
Authors work or have worked with AstraZeneca, involved in the development and production of MEDI2790.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions
SFM, KH, JAS, HLAJ and SHR designed the study. HLAJ provided the samples. SFM, KH, ASB, PS performed the experiments. SFM, ASB, SHR performed the analysis. LY provided statistical support. SFM and SHR wrote the manuscript.

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Supplementary data
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