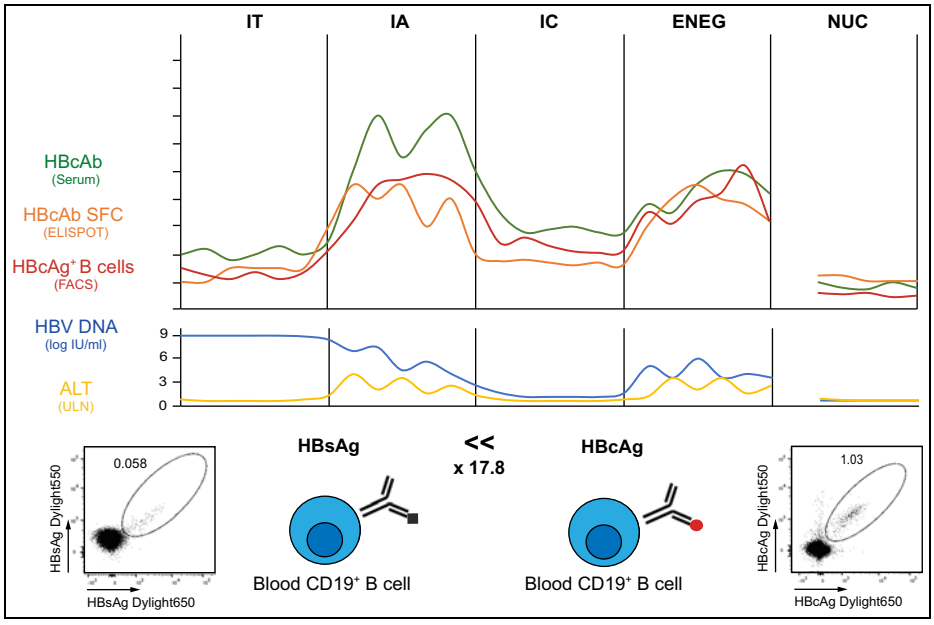
Research Article
Viral Hepatitis

JOURNAL
OF HEPATOLOGY

Hepatitis B core-specific memory B cell responses associate with clinical parameters in patients with chronic HBV

Graphical abstract



Highlights

- B cell responses in chronic HBV are predominantly directed against HBcAg, not HBsAg.
- HBcAg-specific memory B cells associate with HBV's clinical phases.
- HBcAg-specific B cell responses are reduced during antiviral treatment.

Authors

Thomas Vanwolleghem, Zwier M.A. Groothuismink, Kim Kreefft, Magdeleine Hung, Nikolai Novikov, Andre Boonstra

Correspondence

thomas.vanwolleghem@uza.be (T. Vanwolleghem).

Lay summary

In recent years, studies examining the role of B cells during chronic hepatitis B virus infection have regained interest. We show that circulating B cells more often target the hepatitis B core antigen than the hepatitis surface antigen. Moreover, these hepatitis B core-specific B cells associate with the natural history of chronic HBV, and their responses decline during effective antiviral treatment.

https://doi.org/10.1016/j.jhep.2020.01.024
© 2020 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Research Article
Viral Hepatitis

JOURNAL OF HEPATOLOGY

Hepatitis B core-specific memory B cell responses associate with clinical parameters in patients with chronic HBV

Thomas Vanwolleghem[1,2,*], Zwier M.A. Groothuismink[1], Kim Kreefft[1], Magdeleine Hung[3], Nikolai Novikov[3], Andre Boonstra[1]

[1]*Department of Gastroenterology and Hepatology, Erasmus University Medical Center, Rotterdam, The Netherlands;* [2]*Laboratory of Experimental Medicine and Pediatrics, Faculty of Medicine and Health Sciences, University of Antwerp and Department of Gastroenterology and Hepatology, Antwerp University Hospital, Antwerp, Belgium;* [3]*Department of Biology, Gilead Sciences, Foster City, United States of America*

Background & Aims: Little is known about the frequency, phenotype and function of HBV-specific B cells during chronic infection. Here we study HBcAg and HBsAg-specific B cells in different clinical phases of a chronic HBV infection.

Methods: We included 118 treatment naïve and 34 nucleos(t)ide analogue-treated patients with chronic HBV and 23 healthy HBsAg-vaccinated controls. Global and HBV-specific B lymphocytes were examined by FACS using fluorescently labeled HBsAg and HBcAg as baits. Functional HBV-specific B cell responses were quantified in B cell ELISPOT assays. Anti-HBs and anti-HBc antibodies were measured in serum and in ELISPOT supernatant by ELISA.

Results: Higher HBcAg-directed B cell responses were found in HBV clinical phases with elevated *vs.* low serum alanine aminotransferase (ALT) levels, irrespective of the HBeAg-status. In contrast, HBsAg-directed responses were lower and did not significantly fluctuate. In individual patients a mean 17.8-fold more circulating B cells target HBcAg than HBsAg baits. These HBcAg-specific B cells present a classical memory B cell profile and have slightly higher CD69 expression levels compared to global memory B cells. Viral suppression and ALT normalization upon treatment led to a numeric and functional reduction of HBcAg-specific B cell responses, accompanied by progressive decreases in serum anti-HBc antibodies.

Conclusion: HBcAg-specific memory B cells present a classical memory B cell phenotype, vary in number and function throughout HBV's natural history and are significantly reduced during antiviral treatment.

Lay summary: In recent years, studies examining the role of B cells during chronic hepatitis B virus infection have regained interest. We show that circulating B cells more often target the hepatitis B core antigen than the hepatitis surface antigen. Moreover, these hepatitis B core-specific B cells associate with the natural history of chronic HBV, and their responses decline during effective antiviral treatment.

© 2020 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Keywords: Hepatitis B core antigen; B cell; Hepatitis B virus; ELISPOT.
Received 12 February 2019; received in revised form 27 January 2020; accepted 30 January 2020; available online 13 February 2020

* Corresponding author. Address: Nc-1017, Department of Gastroenterology and Hepatology, Erasmus University Medical Center, Dr Molewaterplein 40, 3015GD, Rotterdam, The Netherlands. Tel.: +31107032786; fax: +31107032793.
E-mail address: thomas.vanwolleghem@uza.be (T. Vanwolleghem).

https://doi.org/10.1016/j.jhep.2020.01.024

Introduction

Globally 257 million people remain chronically infected with the hepatitis B virus (HBV) and at risk of developing decompensated liver disease and hepatocellular carcinoma. The natural course of a chronic HBV infection (CHB) follows distinct clinical phases determined by antiviral immune responses and changes in viral diversity.[1]

While virus-specific T cell exhaustion is a hallmark of a CHB, insight into the phenotype and function of B cells that specifically target HBV antigens is limited. The B cell ELISPOT assay is most widely used to analyze HBV-specific memory B cells, which need to be differentiated *in vitro* into antibody secreting cells before they can be quantified as spot-forming cells (SFCs).[2–5] HBsAg B cell ELISPOT assays detect low numbers of anti-HBsAg-antibody (HBsAb)-producing B cells in the blood of patients with CHB, despite the presence of vast amounts of HBsAg in their serum.[4–6] Recently, HBsAg-specific B cells could be directly isolated *ex vivo* from the liver and blood of patients with CHB by using fluorescently labeled HBsAg as baits.[7,8] These HBsAg-specific cells had an impaired anti-HBs antibody production capacity and were enriched for an atypical memory (ATM) B cell phenotype, expressing low levels of both the classical human memory B cell marker CD27 and complement receptor CD21, and high levels of co-inhibitory markers, such as PD-1 and FcRL5,[7,8] suggesting that the HBsAg-specific B cells are exhausted.

However, several observations are difficult to reconcile with a concept of HBV-specific B cell exhaustion during CHB. First, it is likely that a certain level of immune control is exercised by HBV-specific B cells, given the existence of fulminant flares after B cell depleting treatments with anti-CD20 antibodies. Second, we have previously identified B cell-related transcriptomic changes in blood and liver of patients with CHB paralleling clinical parameters in subsequent HBV disease phases.[1,9] HBsAg-specific B cell studies do not reflect this dichotomized disease presentation of CHB, as the numbers or function of HBsAg-specific B cells remain stable irrespective of HBV DNA or alanine aminotransferase (ALT) levels.

To examine whether the observed HBV-specific B cell impairment applies to other HBV proteins, we applied a fluorescently labeled HBcAg bait to study frequency, phenotype and function of HBcAg-specific memory B cells in a well-characterized cohort of untreated and virally suppressed patients with CHB. Our

Table 1. Demographics and clinical characteristics of patients with CHB and HBsAg-vaccinated healthy controls.

	Immune tolerant	Immune active	Inactive carrier	HBeAg- hepatitis	Nucleos(t)ide treatment	Healthy control
N =	23	31	38	26	34	23
Age, years	31.2 ± 9.8	31.1 ± 8.2	37.5 ± 11.8	40.7 ± 10.3	49.1 ± 15.5	40.6 ± 8.9
Gender						
Male (%)	4 (20)	19 (61.3)	19 (50)	18 (69.2)	26 (76.5)	13 (56.5)
Ethnicity						
African	1	2	13	5	3	2
Asian	20	26	13	14	16	8
Caucasian	0	1	1	3	6	13
Other	2	2	11	4	9	0
ALT, U/L	25.1 ± 7.8	149.4 ± 325.0	29.6 ± 15.3	78.5 ± 52.5	24.5 ± 10.6	n.a.
Fibrosis						
F0- <F2	23 (100)	20 (64.5)	32 (84.2)	18 (69.2)	26 (76.5)	n.a.
F2	0 (0)	3 (9.7)	1 (2.6)	4 (15.4)	1 (2.9)	
F3	0 (0)	4 (12.9)	1 (2.6)	0 (0)	2 (5.9)	
F4	0 (0)	1 (3.2)	0 (0)	2 (7.7)	0 (0)	
Unknown	0 (0)	3 (9.7)	4 (13.3)	2 (6.6)	5 (14.7)	
HBV genotype						
A	0	3	4	4	9	n.a.
B	8	5	8	2	4	
C	8	15	4	6	7	
D	2	1	7	5	5	
E	0	0	4	0	1	
Unknown	5	7	11	9	8	
HBV DNA, log IU/ml	8.55 ± 0.66	7.85 ± 1.31	2.50 ± 0.98	5.53 ± 1.47	1.47 ± 0.50	n.a.

Values presented are mean ± SD or n/n (%). ALT, alanine aminotransferase; n.a., not applicable; CHB, chronic HBV infection.

study coincides with a recently published study by Le Bert *et al.*, who undertook a similar approach by performing a comparative analysis of HBcAg -and HBsAg-specific B cells.[10] In line with their findings, we demonstrate that HBcAg-specific B cells are functional and present at higher frequencies than HBsAg-specific B cells in patients with CHB. In addition, we now show that HBcAg-specific memory B cell responses associate with the natural history of a CHB and that the numbers and function of HBcAg-specific B cells are reduced upon treatment-induced viral suppression and ALT normalization in CHB.

Materials & methods

Study population

Serum and/or peripheral blood mononuclear cells (PBMCs) were obtained from patients with CHB (n = 152, Table 1) attending the outpatient clinic of the Erasmus Medical Center, Rotterdam, the Netherlands or the Antwerp University Hospital, Antwerp, Belgium and categorized into 4 clinical HBV phases: immune tolerant (IT) or "HBeAg-positive chronic infection" – HBeAg+, HBeAb-, repetitive normal ALT (<upper limit of normal [ULN]); immune active (IA) or "HBeAg-positive chronic hepatitis" – HBeAg+, repetitive or intermittent high ALT (>ULN); inactive carrier (IC) or "HBeAg-negative chronic infection" – HBeAg-, HBeAb+, low ALT, HBV DNA <20,000 IU/ml; HBeAg- hepatitis (ENEG) or "HBeAg-negative chronic hepatitis" – HBeAg-, HBeAb+, repetitive or intermittent high ALT (>ULN). Categorization was performed as previously described[1,9,11] with minor modifications: i) no restrictions regarding the level of liver fibrosis, although most patients had ≤F2 fibrosis; ii) cohorts of HBsAg-vaccinated healthy controls (HCs, n = 23) and virally suppressed nucleos(t)ide analogue (NUC)-treated patients with CHB (n = 34) were included; iii) For some IC patients intermittent ALT ≤2 ULN was allowed if there was documented liver steatosis without histological steatohepatitis and no other signs of liver disease.[12] ALT levels of IC patients remained significantly lower compared to ENEG and IA patients (Table 1; p <0.001). ULN was 33 and 44 IU/L for females and males, respectively. Written informed consent was obtained from all patients. The study protocol was approved by the institutional ethics committees and conducted in accordance with the guidelines of the Declaration of Helsinki.

Fluorescently labeled HBsAg and HBcAg

DyLight550 and DyLight650 fluorescently double-labeled HBsAg (genotype C) and HBcAg (genotype A) were prepared by Gilead Sciences, in part as described before.[8] For HBcAg production, the full length WT46 core (1-185) encoding sequence with a stop codon was contract-synthesized by GeneArt. The *E. coli* codon optimized open reading frame sequence was cloned into NdeI-HindIII linearized pET30a vector (Agilent, Santa Clara, CA). pET30a-FL GTA HBcAg DNA was transformed into BL21-CodonPlus(DE3) cells (Agilent, Santa Clara, CA). The produced HBcAg particles in *E. coli* cell lysates were purified by a HiTrap Q HP anion exchange column (5 ml, GE Healthcare), eluted and dialysed into 1xPBS. Labeling of HBcAg with DyLight dyes (Thermo Scientific, Waltham, MA, USA) was performed according to the manufacturer's protocol.

Assessment of the phenotype of global and HBV-specific B lymphocytes

Three to four million PBMCs were thawed from 77 patients, incubated on ice with AmCyan live/dead staining (Thermofisher), a panel of antibodies against CD3, CD19, CD27, CD21, CD38, CD69, CD10 and FcRL5 (CD307e) (Table S1), and DyLight550 and DyLight650 fluorescently double-labeled HBsAg or HBcAg at a final concentration of 1 μg/ml and 600 ng/ml, respectively. Due to limited availability of PBMCs for some patients, HBcAg-bait stainings were performed on 28 untreated and 16 NUC-treated patients with CHB and 11 HCs. Analyses of CD69, CD38 and

FcRL5 surface markers on HBcAg-specific B cells were restricted to untreated patients with CHB and absolute HBcAg-specific B cell counts >150. Due to a lower number of HBcAg-specific B cells in NUC-treated patients, only CD38 expression was examined in those patients with absolute HBcAg-specific B cell counts >30. Samples were evaluated by flow cytometry (FacsCanto II, BD) and analyzed using FlowJo software.

In vitro HBsAb and HBcAb production by ELISPOT

ELISPOT assays were performed on PBMCs from 109 patients as previously described with some modifications.[13] Thawed PBMCs were incubated in a 24 well-plate at 2×10^E6 PBMC/well for 5 days at 37°C with 20 U/ml IL-2 and 1 μg/ml R848, counted with trypan blue and analyzed by FACS for CD19, CD3, CD56 and Live dead AmCyan. ELISPOT round bottom 96-well plates were coated overnight at 4°C with 1 μg/ml HBsAg (serotype adw, kind gift from Dynavax) and 0.5 μg/ml HBcAg (kind gift from Roche) in a volume of 50 μl PBS. Control wells were coated with anti-human IgG antibodies as per the manufacturer's instructions (U-CyTech, Utrecht, The Netherlands). PBS coated wells served as background controls. Stimulated PBMCs were added in triplicate at 500,000; 10,000 or 1,000 live cells per well for HBsAg-, HBcAg- or anti-human IgG-coated wells, respectively, and incubated for another 24 h at 37°C. Thereafter IgG-SFCs were developed according to the manufacturer's protocol (U-CyTech, Utrecht, The Netherlands) and counted on a BioReader 3000 ELISpot reader (Bio-Sys, GmbH, Karben, Germany). Since the input PBMC numbers in the HBcAg –and HBsAg ELISPOT differed, we normalized the SFC to 10^E6 PBMC, to 10^E4 IgG SFC and to 10^E6 CD19+ B cells. To determine the *in vitro* anti-HBcAg-antibody (HBcAb) production capacity of HBcAg-bait binding B cells, a similar number of total B cells, HBcAg-bait double positive and bait-negative B cells were FACS sorted from 2 patients with CHB and 2 HCs and expanded on irradiated 3T3 CD40L expressing cells (kind gift from Prof Cees van Kooten, LUMC, the Netherlands) in the presence of IL-2 (200 IU/ml) and IL-21 (50 ng/ml) in medium supplemented with insulin-transferrin-selenium (ThermoFisher), as described previously.[8] After 13 days, supernatant was collected, cells were resuspended in IL-2 (200 IU/ml), IL-21 (50 ng/ml) and IL-6 (50 ng/ml) and added to HBcAg or anti-human IgG-coated ELISPOT plates as described above.

Analysis of serum samples and supernatant of *in vitro* polyclonally stimulated PBMCs

Serum samples of 86 patients and ELISPOT supernatant were analyzed with a quantitative ELISA for total HBsAb (lower limit of quantification 10 mIU/ml) and/or total HBcAb as per the manufacturer's instructions (Beijing Wantai Biological, China). For HBcAb, depending on the applied dilution, the lower limit of quantification for serum was 1,000 or 3,000 IU/ml and for supernatant 0.6 IU/ml. Serial dilutions of supernatant of FACS sorted and *in vitro*-expanded HBcAg bait-positive and bait-negative B cells were measured by a qualitative monolisa anti-HBc plus ELISA, according to manufacturer's instructions (Bio-Rad, Veenendaal, the Netherlands).

Statistics

Statistical analyses were performed in Prism (Graphpad version 7 for Windows). Differences between groups were tested with one-way ANOVA or Kruskal-Wallis test where appropriate. Paired samples were analyzed with Wilcoxon matched pairs or repeated measures ANOVA. If p values were ≤0.05, pairwise comparison between clinical phases was done with the unpaired t test or the Mann-Whitney U test where appropriate. Two-tailed p tests were used to explore differences in clinical phases and the impact of NUC treatment. Based on the observed difference in serum profiles of virus-specific antibodies, one-tailed p tests were used to examine functional and numerical differences between selected clinical phases in ELISPOT and FACS analyses. Spearman's Rank correlation coefficient r was applied to examine the correlation of clinical parameters with HBcAg-specific ELISPOT results and FACS stainings. Differences were considered significant at $^*p$ ≤0.05, $^{**}p$ ≤0.01, $^{***}p$ ≤0.001.

Results

HBcAb levels in serum and PBMC cultures are higher in HBV clinical phases with elevated ALT

The total cohort consists of 152 patients with CHB, of which 34 were virally suppressed with NUC, and 23 age-matched HBV-vaccinated HCs (Table 1). As shown in Fig. 1A, the levels of total serum HBcAb show a fluctuating pattern with higher levels in clinical phases with elevated serum ALT (IA and ENEG) and lower HBcAb levels in phases with relatively low serum ALT (IT and IC). HBsAb in the same patients with CHB are hardly measurable, except in healthy individuals that received the HBsAg vaccine. We next examined whether the fluctuating HBcAb levels in serum were reflected by the number of HBcAb -producing B cells in blood with an optimized HBcAg B cell ELISPOT assay (Fig. 1B). The numbers of SFCs against HBcAg were normalized to the number of leukocytes present in the culture, to the number of control IgG SFCs, or to the number of CD19+ B cells after 5 days of *in vitro* expansion (Fig. 1C). Interestingly, we observed that the numbers of HBcAb SFCs were higher in blood of HBeAg-positive and HBeAg-negative patients with elevated ALT levels, compared to patients with low serum ALT levels. This difference reached statistical significance when data were normalized to the control IgG SFCs or to CD19+ B cells. In addition, the HBcAb levels in supernatant of the stimulated PBMCs were significantly higher in cultures from HBeAg-negative patients with high ALT *vs.* low ALT, confirming the observed difference at the cellular level (Fig. 1C).

Fluorescently labeled HBcAg baits reveal differences in HBV-specific memory B cells during the natural history of CHB

We next questioned whether the number of HBcAg-specific memory B cells in blood reflected the same pattern of fluctuating HBcAb levels in serum and PBMC cultures of patients with CHB, as PBMC culture conditions promote the differentiation of CD27+ memory B cells into antibody-secreting cells.[2,14] To examine this, we applied HBcAg proteins fluorescently labeled with Dylight650 or Dylight550, and optimized the protocols to use this reagent for flow cytometric analysis. By gating on CD3-CD19- lymphocytes, we show that these baits specifically bind to CD19+ B cells (Fig. S1). To diminish non-specific binding, we exclude naïve B cells from the gating strategy and examine the HBcAg Dylight650 and Dylight550 dual positive B cells in memory B cells, as recently proposed by Le Bert *et al.* (Fig. S1; Fig. 2B left panel).[10] To further determine the specificity of the HBcAg bait, we analyzed the HBcAb production capacity of bait-positive and bait-negative B cells from 2 patients with CHB and 2 HCs in a HBcAg ELISPOT. HBcAb were exclusively produced by

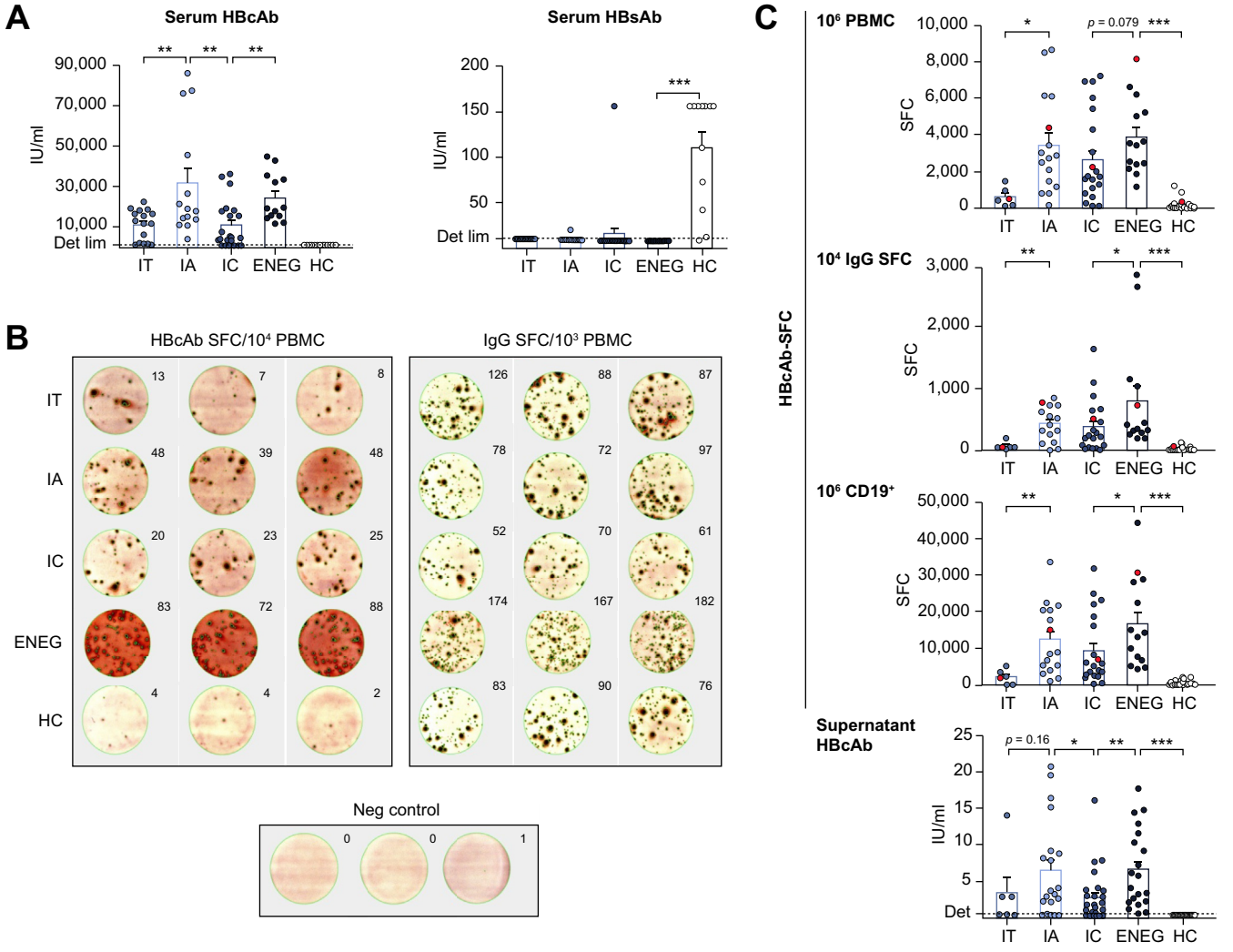


Fig. 1. Serum and *in vitro* produced anti-HBc antibodies follow a distinct pattern in subsequent clinical disease phases. (A) HBcAb and HBsAb were measured by ELISA in serum of untreated patients with CHB in different clinical phases (n = 79) and HBV-vaccinated healthy controls (HC, n = 13). (B and C) HBcAg B cell ELISPOT: PBMCs of untreated patients with CHB in different clinical phases (n = 68) and HCs (n = 20) were cultured and transferred to wells coated with HBcAg. *In vitro* produced HBcAb were quantified as SFCs or in supernatant by ELISA. Control wells were coated with anti-IgG. Uncoated wells served as background controls. (B) Representative pictures of triplicate wells of 1 patient for each group are shown, with the respective number of input PBMCs per well. (C) SFCs are normalized to the numbers of input PBMCs, control IgG SFCs, and CD19+ B cells as indicated next to the respective graphs. Samples shown in (B) are red. For the indicated immune tolerant sample the red dot is positioned at the mean SFC of 2 runs. Differences were tested with 1-way ANOVA. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. CHB, chronic HBV infection; HBcAb, anti-HBcAg-antibody; HBsAb, anti-HBsAg-antibody; HCs, healthy controls; PBMCs, peripheral blood mononuclear cells; SFCs, spot-forming cells.

HBcAg-binding B cells from patients with CHB, but not from HCs (Fig. S2A). We finally performed a titration study and found a bait concentration of 0.6 μg/ml to result in minimal background staining in HCs, while retaining an optimal identification of HBcAg-specific memory B cells in patients with CHB (Fig. S2B).

As we reported previously,[1] we show in Fig. 2A that the frequency of total B cells within the lymphocyte population does not change during the natural history of CHB ($p = 0.29$); nor are there differences in the overall frequency of CD27+CD21- activated memory (AM) ($p = 0.87$; Fig. 2A middle panel) and CD27+CD21+ resting memory (RM) B cells ($p = 0.20$; Fig. 2A right panel; gating strategy Fig. S1).

However, after applying the fluorescently labeled HBcAg as bait at the optimal concentration of 0.6 μg/ml, the highest fractions of HBcAg-binding memory B cells are seen in IA and ENEG patients compared to IT and IC patients (Fig. 2B). A mean 0.050 ± 0.030% of CD19+CD10- memory B cells isolated from HCs bound HBcAg Dylight650 and Dylight550, which was significantly lower than the HBcAg-specific fraction in IA, IC and ENEG patients (Fig. 2B). Of the 4 IT patients available for analysis, only 1 patient had a fraction HBcAg-binding memory B cells (0.039%) that fell below this 0.05% cut-off seen in HCs, again corroborating specific HBcAg-binding in patients with CHB, with the applied bait concentration and gating strategy. In addition, the fluorescently labeled HBcAg bait equally detected B cells from patients infected with HBV genotype A through E, excluding an HBV genotype bias ($p = 0.49$; Fig. S2C).

To get more insight in the variations of the HBcAg-specific B cells during HBV's natural history, we gated on the respective CD27+ memory B cells (Fig. 2B, right panel). A significant higher fraction of CD27+CD21- AM B cells targeted the HBcAg in IA compared to IT patients (Fig. 2B right panel). Overall, a lower

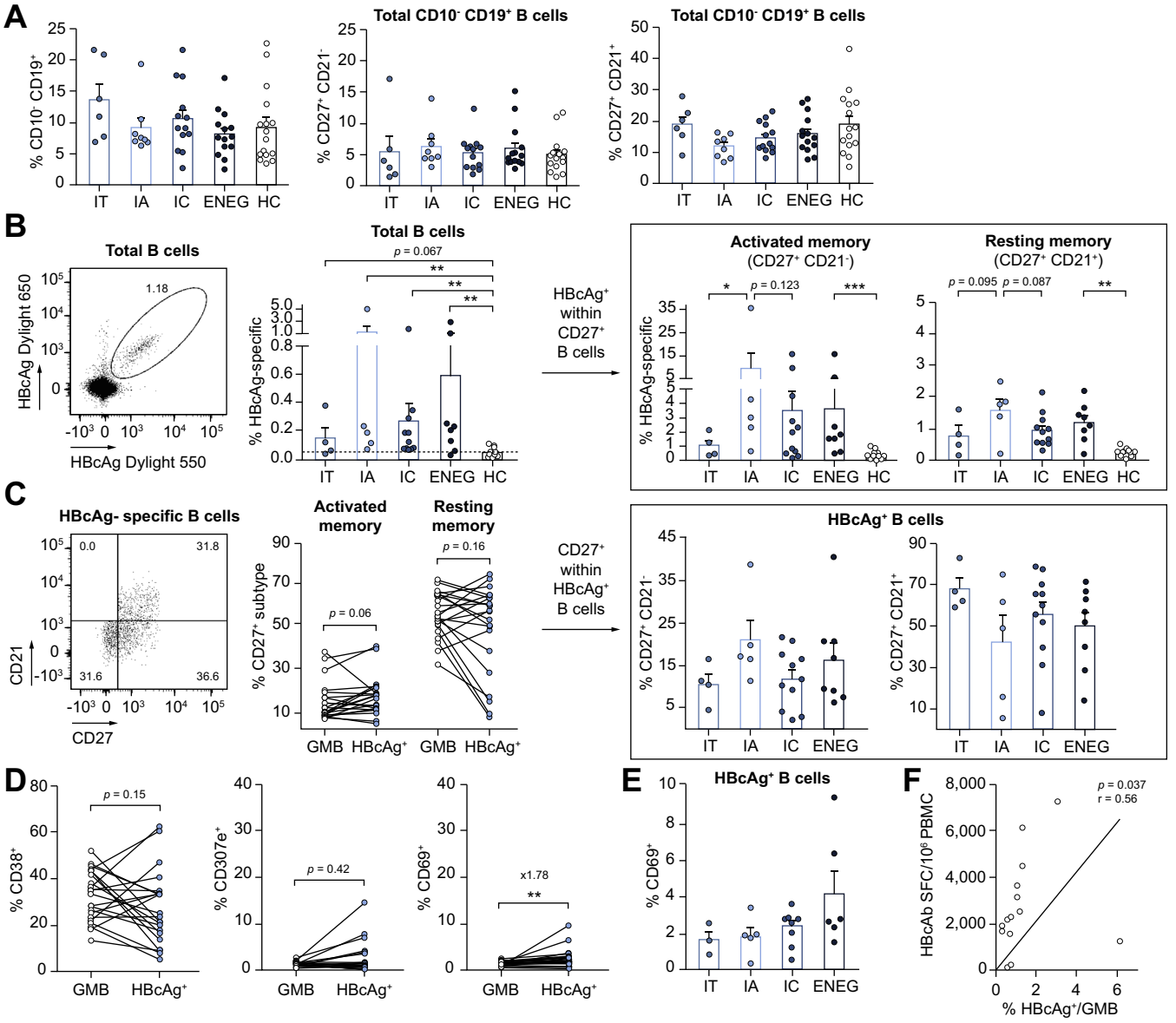


Fig. 2. HBcAg-specific memory B cells associate with HBV's clinical phases. (A) CD10-CD19+ B cell frequencies and the fraction of CD27+CD21- activated or CD27+CD21+ RM B cells are depicted for the HBV clinical phases (n = 41) and HCs (n = 15). For gating strategy: see Fig. S1. (B) Gating for dual HBcAg bait-positive total B cells is illustrated and quantified for the HBV clinical phases in left and middle panes. The grey line shows the mean of HBcAg-binding B cells in HCs at 0.05%. Right panels show the fraction of HBcAg-specific B cells within the CD27+ memory B cell subtypes depicted for the HBV clinical disease phases. (C) Gating for CD27+ memory B cell subtypes within the HBcAg-specific B cells is depicted, and quantified for the GMB cells and HBcAg-specific B cells from the same patient (left and middle pane). Right panels show the fraction of CD27+ memory B cell subtypes within the HBcAg-specific B cells for the respective HBV phases. (D) Frequencies of CD38, CD307 and CD69 expressing GMB cells and HBcAg-specific B cells are presented for the same patient (E) and within the HBcAg-specific B cells for the HBV clinical phases. (F) Correlation between HBcAb SFCs (normalized to 10^E6 input PBMCs) and the fraction HBcAg-specific GMB cells (Spearman's Rank correlation r). Differences were tested with 1-way ANOVA, except for the pairwise comparisons in C and D, which were tested with a Wilcoxon matched pairs test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. GMB, global memory B cells; PBMCs, peripheral blood mononuclear cells; RM, resting memory; SFCs, spot-forming cells.

fraction of CD27+CD21+ RM B cells bound HBcAg compared to CD27+CD21- AM B cells (all HBV phases combined: 1.1% *vs.* 4.2%, $p = 0.0005$). Nevertheless, a similar trend towards higher frequencies of HBcAg-specific RM B cells in IA compared to IT patients was observed (Fig. 2B right panel).

We next analyzed the different memory subsets of HBcAg-specific B cells in patients with CHB. Due to our gating strategy no HBcAg-specific B cells with a naïve phenotype were identified (Fig. 2C left panel, Fig. S1). HBcAg-specific CD27+ memory B cells had a predominant CD21+ resting memory profile (53.5 ± 21.9% RM *vs.* 14.5 ±9.2% AM, $p < 0.0001$), similar to the global memory subpopulations observed within the same patients (Fig. 2C middle panel). Although the highest mean fractions of HBcAg-specific B cells with an AM phenotype were observed in the IA (20.9 ± 10.5%) and ENEG (16.3 ± 11.3%) disease phases, this did not reach statistical significance ($p = 0.26$ for AM and $p = 0.39$ for RM between clinical phases respectively; Fig. 2C right panel). Compared to global memory B (GMB) cells, HBcAg-specific B cells had 1.8-fold higher expression levels of the activation marker CD69 ($p = 0.0039$), with a trend towards higher levels

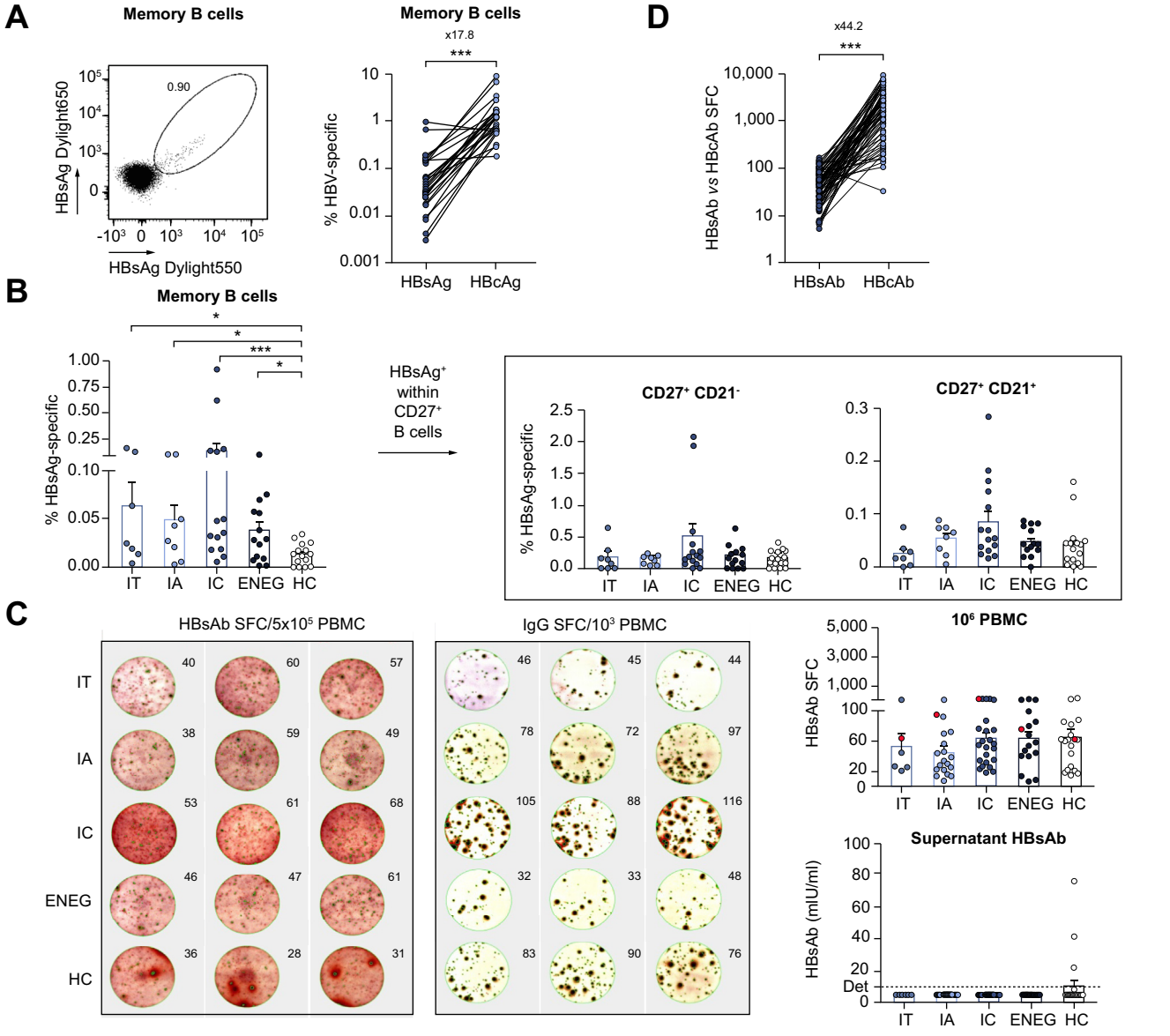


Fig. 3. HBsAg-specific B cells are outnumbered by HBcAg-specific B cells and do not fluctuate with HBV's clinical phases. (A) Gating for dual HBsAg bait positive memory B cells is presented. Paired HBsAg-specific and HBcAg-specific B cells are quantified in global memory B cells of untreated patients with CHB. (B) The fraction of HBsAg-specific B cells is depicted for HBV clinical phases (n = 43) and vaccinated HCs (n = 15). Right panels show the fraction of HBsAg-specific cells within the CD27+ memory B subtypes for the HBV clinical phases. (C) PBMCs of untreated patients with CHB in different clinical phases (n = 68) and HCs (n = 20) were cultured and transferred to triplicate wells coated with HBsAg. *In vitro* produced HBsAb were quantified in supernatants of these cultures or as SFCs and normalized to 10^E6 of input PBMCs. Control wells were coated with anti-IgG antibodies. Representative pictures of triplicate wells of 1 patient for each group are shown, with the respective number of input PBMCs per well. Pictured samples appear red in the ELISPOT graph. For the indicated immune tolerant and ENEG samples the red dot is positioned at the mean SFC of 2 runs. (D) Paired HBsAb- and HBcAb-SFCs (normalized to 10^E6 input PBMCs) in PBMC cultures of untreated patients with CHB. Differences were tested using a Wilcoxon matched pairs test (panel A and D), a Kruskal-Wallis test (panel B) and 1-way ANOVA (panel C). $^*p \leq 0.05$, $^{***}p \leq 0.001$. CHB, chronic HBV infection; ELISPOT, enzyme-linked immune absorbent spot; HBcAb, anti-HBcAg-antibody; HBsAb, anti-HBsAg-antibody; HCs, healthy controls; PBMCs, peripheral blood mononuclear cells; SFCs, spot-forming cells.

among subsequent clinical HBV phases ($p = 0.28$; Fig. 2E); but not of CD38 or the inhibitory IgG co-receptor FcRL5 (Fig. 2D). Next, we analyzed whether HBcAg-specific B cells, functionally as measured in the ELISPOT assay and numerically as measured by FACS, correlated with clinical parameters in untreated patients. While HBcAb SFCs correlated with ALT levels ($p = 0.022$, Spearman r=0.31; Fig. S3A), no correlation was observed between ALT or HBV DNA levels and HBcAg-specific memory B cells (Fig. S3B). However, in paired analyses, HBcAb production by cultured PBMCs showed a good correlation with the fraction of HBcAg-specific memory B cells in blood from untreated patients with CHB ($p = 0.037$, Spearman r = 0.56; Fig. 2F).

Overall, these data demonstrate that HBcAg baits are able to identify HBcAg-specific and HBcAb-producing B cells in patients with CHB that present a classical memory B phenotype, comparable to the global memory B cell population, with slightly higher CD69 expression levels. Moreover, in clinical disease phases with high ALT, similar to serum HBcAb levels and HBcAb SFCs, HBcAg-specific B cells showed numerical (Fig. 2B) and phenotypical (Fig. 2C) variations with trends towards a higher

number and a more AM phenotype compared to phases with low ALT. These data support the observed differences in HBcAb levels in serum and PBMC cultures and point to an association of HBcAg-specific memory B cell responses with the natural history of CHB.

HBsAg-specific B cells show no clear functional or numerical association with HBV clinical phases and are outnumbered by HBcAg-specific B cells

To determine whether the findings obtained for HBcAg-specific B cells also apply for HBsAg, we used the same approach with fluorescently Dylight650 or Dylight550 labeled HBsAg bait (Fig. 3A, Fig. S1). As shown in Fig. 3A, HBsAg-specific memory B cells in the peripheral blood of patients with CHB are significantly outnumbered by their HBcAg-specific counterparts in matched samples (17.8-fold, p <0.0001). In addition, although the highest levels of overall, AM or RM HBsAg-specific B cells are noted in HBeAg-negative patients with normal ALT levels, there is no clear fluctuating pattern between subsequent clinical phases (Fig. 3B). We next analyzed the capacity of *in vitro* expanded PBMCs to produce HBsAb in an optimized ELISPOT assay. Importantly, although a lower number of circulating HBsAg-specific B cells are present in vaccinated HCs compared to patients with CHB (Fig. 3B), the net number of HBsAb SFCs in cultures of PBMCs is not different between both cohorts, suggesting a higher HBsAb production at the individual B cell level in HCs compared to patients with CHB, as recently described[7,8] (Fig. 3C; p = 0.49 for PBMC normalized results). With the lower limit of quantification of 10 mIU/ml of our Wantai HBsAb ELISA, HBsAbs could be detected in 4 supernatants of vaccinated HCs only (Fig. 3C; p = 0.01). It is important to note that in line with the low frequencies of HBsAg-specific B cells, a mean 44.2-fold lower number of SFCs were seen in paired HBsAg and HBcAg B cell ELISPOT assays (Fig. 3D; p <0.0001). Overall, these results indicate an HBV-specific humoral immune response that is predominantly directed against the HBcAg and not the HBsAg in untreated patients with CHB.

HBcAg-specific B cells with a CD27-CD21- ATM phenotype are unaffected by clinical parameters in patients with CHB

Recently, 2 research groups have demonstrated a HBsAg-specific and global B cell dysfunction in CHB, with an accumulation of CD27-CD21- ATM B cells.[7,8] In our cohort, overall ATM B cells do not significantly differ between subsequent clinical phases and HCs (p = 0.12; Fig. 4A). Furthermore, we did not observe significant differences in the number of B cell ELISPOT IgG SFCs between treatment-naïve patients with CHB, a cohort of NUC-treated patients with CHB (normal ALT levels and low level of liver fibrosis), or vaccinated HCs (p = 0.40; Fig. 4B). This indicates that the global B cell population is still able to effectively produce antibodies despite the presence of high amounts of HBV proteins in blood. In addition, the fraction of HBcAg-specific B cells with an ATM phenotype does not differ between NUC-treated virally suppressed and untreated patients with CHB, nor are there significant differences throughout clinical phases (p = 0.85 and p = 0.40 respectively, Fig. 4C). Finally, in our combined NUC-naïve and -treated cohort there is no significant correlation between HBcAg-specific ATM B cells on the one hand and HBV DNA or ALT levels on the other hand (Fig. 4D). Overall these data demonstrate that the number of HBcAg-specific B cells with a CD27-CD21- ATM phenotype are unaffected by viral parameters or disease stage in both untreated and virally suppressed patients with CHB.

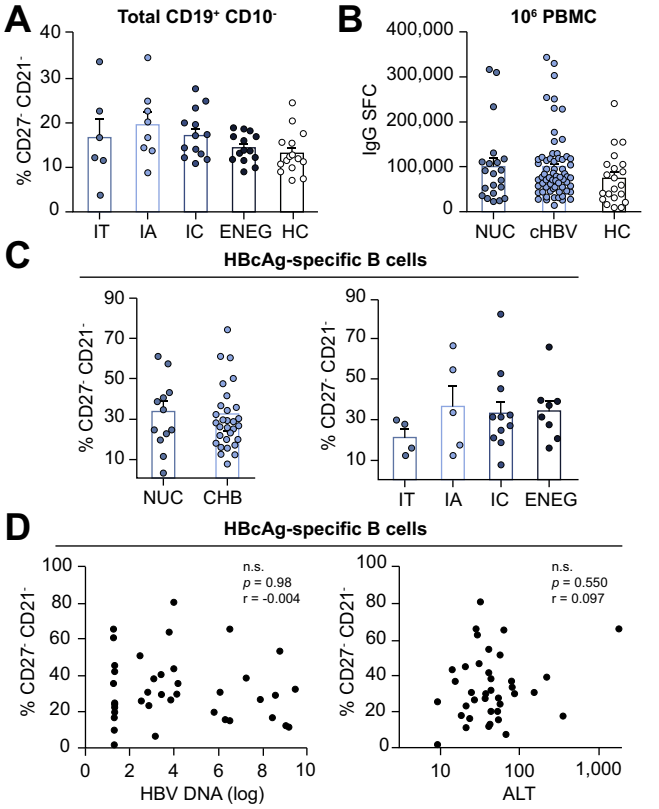


Fig. 4. HBcAg-specific B cells with a CD27-CD21- ATM phenotype are unaffected by clinical parameters. (A) CD27-CD21- ATM B cell frequencies are depicted for the HBV clinical disease phases (n = 41) and HCs (n = 15). For gating strategy: Fig. S1. (B) PBMCs of NUC-treated (n = 21), untreated patients with CHB in different clinical phases (n = 68), and HCs (n = 20) were cultured and transferred to triplicate wells coated with anti-IgG. *In vitro* produced IgG antibodies were quantified as SFCs and normalized to 10^E6 of input PBMCs. (C) The fraction of CD27-CD21- ATM B cells within the HBcAg-specific B cells is depicted for NUC-treated (n = 12) and untreated patients with CHB (n = 28) and respective HBV clinical disease phases. (D) No correlation between CD27-CD21- HBcAg-specific B cell frequencies and serum HBV DNA or ALT levels (Spearman's Rank correlation r). Differences were tested using the Kruskal-Wallis (panel A and C), and 1-way ANOVA (panel B). ALT, alanine aminotransferase; ATM, atypical memory; HCs, healthy controls; NUCs, nucleos(t)ide analogues; PBMCs, peripheral blood mononuclear cells; SFCs, spot-forming cells.

HBcAg-specific B cells are numerically and functionally reduced after NUC-induced viral suppression and upregulate CD38 expression

To further examine the impact of treatment-induced viral suppression and ALT normalization on HBcAg-specific B cells, both HBcAg-bait staining and HBcAg ELISPOT were applied to a cohort of NUC-treated patients with CHB, with demographic characteristics and liver fibrosis degrees similar to the IA and ENEG phase patients (Table 1). *In vitro* HBcAb production of PBMCs was drastically reduced in NUC-treated patients compared to the IA and ENEG phase patients, both at the level of SFCs as in the supernatant (Fig. 5A). Lower *in vitro* HBcAg-specific B cell responses were paralleled by a lower number of circulating HBcAg-specific B cells overall (Fig. 5B). In addition, the HBcAg-specific

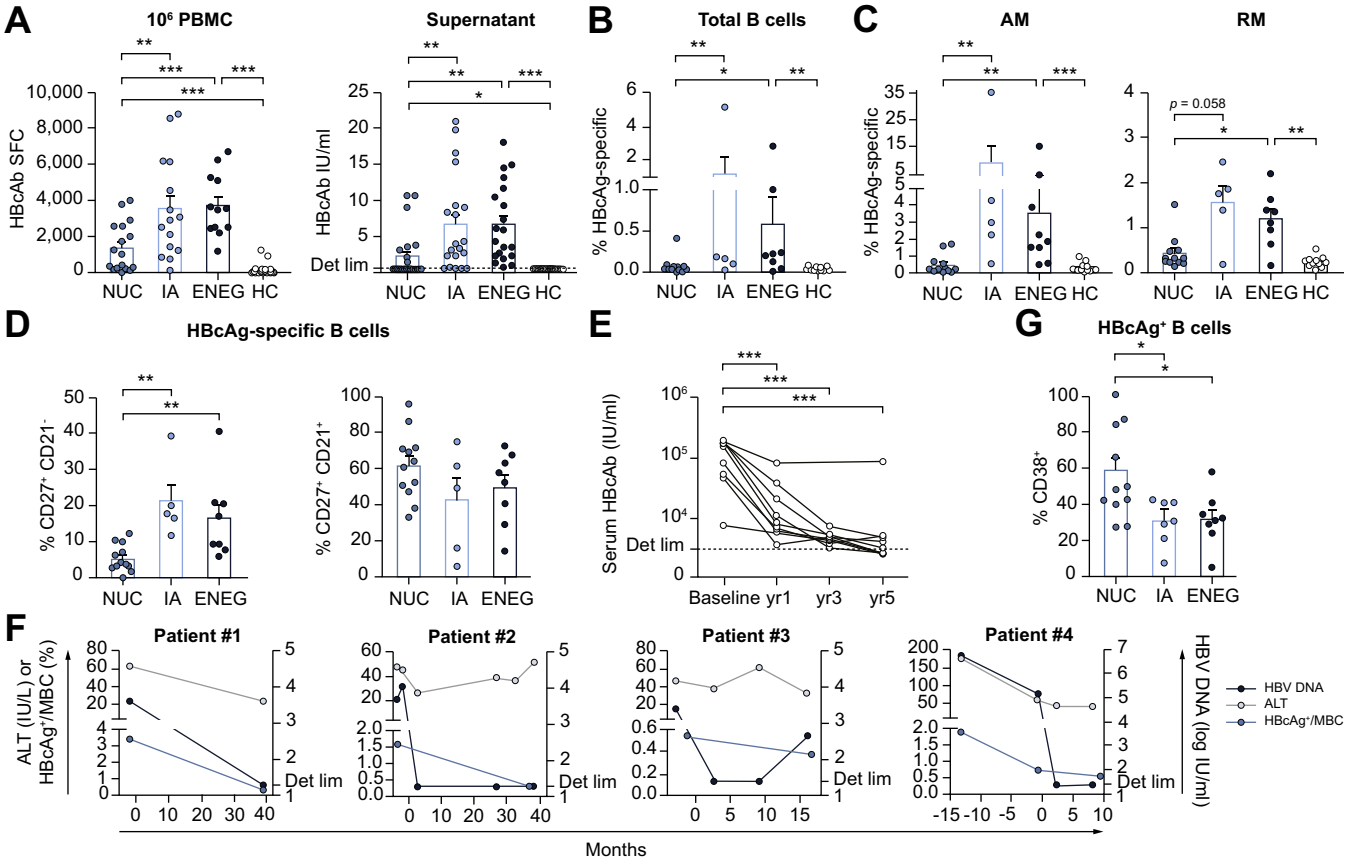


Fig. 5. Antiviral treatment leads to numeric and functional reduction of HBcAg-specific B cell responses. (A) HBcAg B cell ELISPOT: PBMCs of NUC-treated patients with CHB (n = 21), untreated patients with CHB (IA, n = 19 and ENEG, n = 19) and HCs (n = 20) were cultured and transferred to wells coated with HBcAg. *In vitro* produced HBcAbs were quantified as SFCs and normalized to 10^E6 input PBMCs or by ELISA in supernatant. (B) The fraction of HBcAg-specific total B cells is depicted for NUC-treated patients with CHB (n = 12), untreated patients with CHB (IA, n = 5 and ENEG, n = 8) and HCs (n = 11). (C) The fraction of HBcAg-specific B cells was quantified within the CD27+ memory B cell subtypes for the HBV clinical disease phases. (D) The fraction of CD27+ memory B cell subtypes within the HBcAg-specific B cells was quantified for NUC-treated and untreated patients with CHB. (E) Serum HBcAbs were quantified by ELISA before and up to 5 years during NUC treatment in patients with CHB (n = 9). (F) Longitudinal analysis of the frequency of HBcAg-specific memory B cells, serum HBV DNA and ALT levels of 4 patients with CHB with respect to NUC treatment start (=time 0). (G) CD38 expression is quantified on HBcAg-specific B cells of NUC-treated and untreated patients with CHB. Differences were tested using 1-way (panel A) and repeated measures (panel E) ANOVA and a Kruskal-Wallis test (panels B, C, D and G). $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. ALT, alanine aminotransferase; CHB, chronic HBV infection; ELISPOT, enzyme-linked immune absorbent spot; ENEG, hepatitis B e antigen negative hepatitis; HBcAb, anti-HBcAg-antibody; HBsAb, anti-HBsAg-antibody; HCs, healthy controls; IA, immune active, hepatitis B e antigen positive hepatitis; NUCs, nucleos(t)ide analogues; PBMCs, peripheral blood mononuclear cells; SFCs, spot-forming cells.

fraction of CD27+ CD21- AM (Fig. 5C left panel) and CD27+CD21+ RM B cells were also reduced upon NUC treatment (Fig. 5C right panel). Finally, HBcAg-specific B cells with an CD27+ CD21- AM B cell phenotype were significantly reduced in virally suppressed compared to IA and ENEG disease patients (Fig. 5D left panel).

We next corroborated the observed cross-sectional findings in a longitudinal cohort of patients with CHB for which serum (n = 9) and PBMCs (n = 4) were available before and after NUC treatment initiation: serum HBcAb levels (Fig. 5E; $p = 0.0001$) and the fraction of HBcAg-specific memory B cells declined after treatment start in all patients, despite a viral blip in 1 patient (pt #3, Fig. 5F). Finally, this functional and numeric reduction upon viral suppression was associated with higher CD38 expression levels of HBcAg-binding B cells (overall $p = 0.048$, Fig. 5G). Importantly, as can be appreciated from Fig. 5F and Table 1, HBV DNA suppression was accompanied by ALT normalization in NUC-treated patients, so both the reduction of liver inflammation and the inhibition of viral replication, could have contributed to lower HBcAg-specific B cell responses in these patients.

Combined, these data demonstrate that HBcAg-specific memory B cells present a classical memory B cell phenotype, vary in number and function throughout HBV's natural history and are significantly reduced during NUC-induced HBV DNA suppression and ALT normalization.

Discussion

Little is known about the frequency, phenotype and function of HBV-specific B cells during CHB, although antiviral antibody responses mark the transition between clinical disease phases and correlate with the risk of viral flares upon treatment discontinuation or B cell depletion.[15–17] We have recently shown that at the transcriptome level, B cell-related genes are expressed more in disease phases with elevated ALT, both in blood and livers of untreated patients with CHB, suggesting a direct or indirect involvement in the immunopathogenesis.[1,9]

Here we applied fluorescently labeled HBcAg as baits to *ex vivo* track HBcAg-specific B cells in blood samples of a well-characterized cohort of patients with CHB. We also examined

functional differences in B cell ELISPOT assays. Similar to our transcriptomic data,[1,9] we find that HBcAg-specific B cell responses are higher in patients with CHB and elevated serum ALT, compared to patients with low ALT. In contrast, HBsAg-specific responses are at least an order of magnitude lower and do not fluctuate throughout HBV's natural history. Similar to the Le Bert paper, non-specific binding of HBcAg baits in healthy unexposed individuals is minimized, by gating out naïve B cells and titrating the HBcAg bait to an optimal concentration of 0.6 μg/ml.[10] In addition, HBcAg-binding B cells in patients with CHB are able to produce HBcAb in expanded B cell cultures and correlate with HBcAg PBMC ELISPOT responses. Furthermore, both ELISPOT and FACS bait approaches show similar predominant HBcAg-directed humoral immune responses in paired samples of patients, corroborating recent data.[10] Our findings further substantiate that the HBcAg-directed humoral immune response is modulated during subsequent clinical HBV disease phases. Recently, using a similar HBsAg bait approach, 2 groups demonstrated that HBsAg-specific B cells are defective at anti-HBs production and enriched for an ATM B cell phenotype in patients with CHB.[7,8] Here we show that HBcAg-specific humoral responses are not impaired: a steady fraction of HBcAg-specific B cells display an ATM B cell phenotype, without variation throughout HBV's clinical disease phases and no changes upon NUC-induced viral suppression. Indeed, similar to the data of Le Bert *et al.*, we find that HBcAg-specific B cells phenotypically resemble classical memory B cells.[10] At the transcriptome level however the same group found global memory B cells to differ from HBcAg-specific B cells in patients with CHB,[10] as well as from their global memory B cell counterparts in vaccinated HCs,[8] suggesting a differential impact of HBV infection and its proteins on the global and HBV-specific B cell population.[8,10] The functional consequence of these differences remains unclear. We observed no difference in the *in vitro* IgG production capacity of PBMCs of untreated or virally suppressed patients with CHB and vaccinated HCs, while earlier studies using bulk B cells have pointed towards a hyperactivated state with high expression levels of activation markers, such as CD69, CD71, CD80 and CD86 and a lower proliferative capacity in patients with CHB.[4,18] Phenotypically, HBcAg-specific B cells had slightly higher CD69, but similar expression levels of CD38 and FcRL5 compared to the global memory B cell population.

Why do B cell responses against HBsAg and HBcAg profoundly differ? The HBV capsid is known to be a remarkable immunogenic structure,[19,20] so it is puzzling why HBsAg-specific B cells responses are exhausted as suggested by recent studies,[7,8] but not HBcAg-specific B cells. Differences in antigen-specific follicular T cell help[6] or different degrees of antigen exposure are likely to contribute. While HBsAg is known to be secreted in high excess to HBV virions, the kinetics of HBV capsid expression and its presence in serum remain unclear. Recently, antibody-HBV capsid complexes have been found in sera of patients with CHB.[21] Whether these capsids are actively secreted or released during cell-lysis or hepatocyte turnover remains to be determined. Besides differences in release of both antigens, localization in the infected liver might also contribute to the observed difference in B cell responses. Relative levels of HBsAg and HBcAg in the infected hepatocyte differ substantially with hardly any colocalization by immunohistochemistry studies.[22,23] In addition, intracellular HBcAg expression shifts from the nucleus to cytoplasm upon hepatocyte regeneration, possibly triggered by inflammation.[22] Combined, it is tempting to speculate that the immune system is exposed to the immunogenic HBV capsids during cell-lysis, either directly via HBcAg-specific immune responses or indirectly. The association of HBcAg-specific B cell responses with clinical phases that we observed, does not allow for a conclusion to be drawn on the role of B cells in HBV's immunopathogenesis. Nevertheless, earlier studies showed that patients with CHB and elevated ALT also had deposits of complement fixing HBcAg-immune complexes in their liver.[24,25] In addition, some patients harbor HBcAg-specific B cell clones that can induce acute cell-lysis through a T cell-independent antibody-complement mediated mechanism.[26,27] Future mechanistic studies should therefore aim at clarifying the putative pathogenic potential of HBcAg-specific B cell responses.

Another important novel finding of our study is that treatment-induced viral suppression and ALT normalization lead to a numeric and functional reduction of HBcAg-specific B cell responses. Indeed, serial sampling showed a decline of serum HBcAb and circulating HBcAg-specific B cells in a longitudinal NUC-treated cohort. Interestingly, CD38 expression increased on HBcAg-specific B cells upon NUC treatment, suggesting a more active state. HBV capsid-specific T cells have on the contrary been shown to inversely correlate with HBV viral load,[28] and also associate with viral control following NUC withdrawal.[29,30] NUC-induced viral suppression only transiently restores HBV-specific T cells.[30,31] These opposing HBV capsid-specific T and B cell responses imply that immunomodulatory treatments aimed at a functional HBV cure would also need to differentially target both cell types.

In conclusion, by applying fluorescently labeled HBcAg baits, we demonstrate that the humoral immune response is predominantly directed against the HBcAg and modulated during subsequent clinical HBV disease phases. HBcAg-specific B cells present a classical memory B cell phenotype and are significantly reduced during antiviral treatment. Future studies should aim at unravelling the pathogenic potential of these HBV capsid directed responses.

Abbreviations

ALT, alanine aminotransferase; AM, activated memory; ATM, atypical memory; CHB, chronic HBV infection; ELISPOT, enzyme-linked immune absorbent spot; ENEG, hepatitis B e antigen negative hepatitis; GMB, global memory B cells; HBcAb, anti-HBcAg-antibody; HBsAb, anti-HBsAg-antibody; HCs, healthy controls; IA, immune active, hepatitis B e antigen positive hepatitis; IC, inactive carrier; IT, immune tolerant; NUCs, nucleos(t)ideanalogues; PBMCs, peripheral blood mononuclear cells; RM, resting memory; SFCs, spot-forming cells; ULN, upper limit of normal.

Financial support

This work was supported by The Foundation for Liver and Gastrointestinal Research (SLO), Rotterdam (to AB) and by a 2015 BMS Partnering for Cure Grant (number BMS AI424-565 to TV). TV is a recipient of a mandate of the Belgian Foundation against Cancer, Belgium (number 2014-087).

Conflict of interest

TV has participated in Advisory Committees or Review Panels for: Janssen Pharmaceuticals, Gilead Sciences, Abbvie, BMS, WL

Gore. He has also received grant/research support from: Gilead Sciences, Roche, BMS and speaking and teaching support from: Gilead Sciences, BMS. KK and ZG have nothing to disclose. MH and NN are employees of Gilead Sciences. AB has been in consulting or in advisory boards for Gilead Sciences and Bristol-Myers Squibb and has received research grants from Roche, Gilead Sciences, Fujirebio, and Janssen.

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

Authors' contributions

ZG and KK performed the experiments and analyzed the data. TV and AB provided patient material. MH and NN designed and provided the HBcAg and HBsAg baits. TV and AB designed experiments, analyzed and interpreted data and wrote the manuscript. TV and AB obtained funding and supervised the study. All authors provided critical review and approved the manuscript.

Acknowledgements

We would like to express our gratitude to Prof. Antonio Bertoletti and Drs. Loghman Salimzadeh and Nina Le Bert (Emerging Infectious Disease Program, Duke-NUS Medical School, Singapore) for helpful discussions on HBcAg- and HBsAg-bait gating; and to Dr Nicole van Besouw (Laboratory of Nephrology and Transplantation Medicine, Department of Internal Medicine, Erasmus MC, Rotterdam) for expert advice on ELISPOT assays.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2020.01.024.

References

Author names in bold designate shared co-first authorship

[1] Vanwolleghem T, Hou J, van Oord G, Andeweg AC, Osterhaus AD, Pas SD, et al. Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells. Hepatology 2015;62:87–100.
[2] Pinna D, Corti D, Jarrossay D, Sallusto F, Lanzavecchia A. Clonal dissection of the human memory B-cell repertoire following infection and vaccination. Eur J Immunol 2009;39:1260–1270.
[3] Wang Q, Sachse P, Semmo M, Lokhande M, Montani M, Dufour JF, et al. T- and B-cell responses and previous exposure to hepatitis B virus in 'anti-HBc alone' patients. J Viral Hepat 2015;22:1068–1078.
[4] Xu X, Shang Q, Chen X, Nie W, Zou Z, Huang A, et al. Reversal of B-cell hyperactivation and functional impairment is associated with HBsAg seroconversion in chronic hepatitis B patients. Cell Mol Immunol 2015;12:309–316.
[5] Tian C, Chen Y, Liu Y, Wang S, Li Y, Wang G, et al. Use of ELISpot assay to study HBs-specific B cell responses in vaccinated and HBV infected humans. Emerg Microbes Infect 2018;7:16.
[6] Poonia B, Ayithan N, Nandi M, Masur H, Kottilil S. HBV induces inhibitory FcRL receptor on B cells and dysregulates B cell-T follicular helper cell axis. Sci Rep 2018;8:15296.
[7] Burton AR, Pallett LJ, McCoy LE, Suveizdyte K, Amin OE, Swadling L, et al. Circulating and intrahepatic antiviral B cells are defective in hepatitis B. J Clin Invest 2018;128:4588–4603.
[8] **Salimzadeh L**, **Le Bert N**, Dutertre CA, Gill US, Newell EW, Frey C, et al. PD-1 blockade partially recovers dysfunctional virus-specific B cells in chronic hepatitis B infection. J Clin Invest 2018;128:4573–4587.
[9] Hou J, Brouwer WP, Kreefft K, Gama L, Price SL, Janssen HLA, et al. Unique intrahepatic transcriptomics profiles discriminate the clinical phases of a chronic HBV infection. PLoS One 2017;12:e0179920.
[10] Le Bert N, Salimzadeh L, Gill US, Dutertre CA, Facchetti F, Tan A, et al. Comparative characterization of B cells specific for HBV nucleocapsid and envelope proteins in patients with chronic hepatitis B. J Hepatol 2020;72:34–44.
[11] European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol 2017;67:370–398.
[12] Mellen JS, Xia VW, Hashemzadeh M, Imagawa D, Jamal M, Hoefs J, et al. The clinical presentation of chronic hepatitis B virus infection in Asian Americans: a single center retrospective study. J Clin Gastroenterol 2010;44:364–370.
[13] van Besouw NM, van Hal PT, Zuijderwijk JM, de Kuiper R, Hoek RA, van Weezel JJ, et al. Herpes zoster after lung transplantation boosts varicella zoster virus-specific adaptive immune responses. J Heart Lung Transplant 2016;35:1435–1442.
[14] Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood 2003;101:4500–4504.
[15] Bertoletti A, Ferrari C. Adaptive immunity in HBV infection. J Hepatol 2016;64:S71–S83.
[16] Yang HC, Tsou HH, Pei SN, Chang CS, Chen JH, Yao M, et al. Quantification of HBV core antibodies may help predict HBV reactivation in patients with lymphoma and resolved HBV infection. J Hepatol 2018;69:286–292.
[17] Chi H, Li Z, Hansen BE, Yu T, Zhang X, Sun J, et al. Serum level of antibodies against hepatitis B core protein is associated with clinical relapse after discontinuation of nucleos(t)ide analogue therapy. Clin Gastroenterol Hepatol 2019;17:182–191.e1.
[18] **Oliviero B**, **Cerino A**, Varchetta S, Paudice E, Pai S, Ludovisi S, et al. Enhanced B-cell differentiation and reduced proliferative capacity in chronic hepatitis C and chronic hepatitis B virus infections. J Hepatol 2011;55:53–60.
[19] Milich DR, McLachlan A. The nucleocapsid of hepatitis B virus is both a T-cell-independent and a T-cell-dependent antigen. Science 1986;234:1398–1401.
[20] Vanlandschoot P, Cao T, Leroux-Roels G. The nucleocapsid of the hepatitis B virus: a remarkable immunogenic structure. Antiviral Res 2003;60:67–74.
[21] Bai L, Zhang X, Kozlowski M, Li W, Wu M, Liu J, et al. Extracellular hepatitis B virus RNAs are heterogeneous in length and circulate as capsid-antibody complexes in addition to virions in chronic hepatitis B patients. J Virol 2018;92.
[22] **Zhang X**, **Lu W**, Zheng Y, Wang W, Bai L, Chen L, et al. In situ analysis of intrahepatic virological events in chronic hepatitis B virus infection. J Clin Invest 2016;126:1079–1092.
[23] Hu J, Liu K. Complete and incomplete hepatitis B virus particles: formation, function, and application. Viruses 2017;9.
[24] Trevisan A, Realdi G, Alberti A, Noventa F. Relationship between membrane-bound immunoglobulin and viral antigens in liver cells from patients with hepatitis B virus infection. Gastroenterology 1979;77:209–214.
[25] Rizzetto M, Bonino F, Crivelli O, Canese MG, Verme G. Complement fixing hepatitis B core antigen immune complexes in the liver of patients with HBs antigen positive chronic disease. Gut 1976;17:837–843.
[26] Chen Z, Diaz G, Pollicino T, Zhao H, Engle RE, Schuck P, et al. Role of humoral immunity against hepatitis B virus core antigen in the pathogenesis of acute liver failure. Proc Natl Acad Sci U S A 2018;115:E11369–E11378.
[27] Farci P, Diaz G, Chen Z, Govindarajan S, Tice A, Agulto L, et al. B cell gene signature with massive intrahepatic production of antibodies to hepatitis B core antigen in hepatitis B virus-associated acute liver failure. Proc Natl Acad Sci U S A 2010;107:8766–8771.
[28] Webster GJ, Reignat S, Brown D, Ogg GS, Jones L, Seneviratne SL, et al. Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. J Virol 2004;78:5707–5719.
[29] **Rivino L**, **Le Bert N**, Gill US, Kunasegaran K, Cheng Y, Tan DZ, et al. Hepatitis B virus-specific T cells associate with viral control upon nucleos(t)ide-analogue therapy discontinuation. J Clin Invest 2018;128:668–681.
[30] Boni C, Penna A, Bertoletti A, Lamonaca V, Rapti I, Missale G, et al. Transient restoration of anti-viral T cell responses induced by lamivudine therapy in chronic hepatitis B. J Hepatol 2003;39:595–605.
[31] Boni C, Laccabue D, Lampertico P, Giuberti T, Vigano M, Schivazappa S, et al. Restored function of HBV-specific T cells after long-term effective therapy with nucleos(t)ide analogues. Gastroenterology 2012;143:963–973.e9.