Review

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Humoral immunity in hepatitis B virus infection: Rehabilitating the B in HBV

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Summary

Insights into the immunopathogenesis of chronic HBV infections are fundamental in the quest for novel treatment approaches aimed at a functional cure. While much is known about the ineffective HBV-specific T-cell responses that characterise persistent HBV replication, B cells have been left largely understudied. However, an important role for humoral immunity during the natural history of HBV infections, as well as after functional cure, has been inadvertently revealed by the occurrence of HBV flares following B cell-depleting treatments. Herein, we review our current understanding of the role of the humoral immune response in chronic HBV, both at the level of HBV-specific antibody production and at the phenotypic and broader functional level of B cells. The recent development of fluorescently labelled HBV proteins has given us unprecedented insights into the phenotype and function of HBsAg- and HBcAg-specific B cells. This should fuel novel research into the mechanisms behind dysfunctional HBsAg-specific and fluctuating, possibly pathogenic, HBcAg-specific B-cell responses in chronic HBV. Finally, novel immunomodulatory treatments that partly target B cells are currently in clinical development, but a detailed assessment of their impact on HBV-specific B-cell responses is lacking. We plead for a rehabilitation of B-cell studies related to both the natural history of HBV and treatment development programmes.

Keywords: B cells; hepatitis B virus; antibodies; global B cells; hepatitis B-specific B cells; flares

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Introduction

Globally 257 million people face a lifetime risk of decompensated liver disease or hepatocellular carcinoma due to chronic HBV infection. First-line treatment with nucleos(t)ide analogues (NAs) suppresses viral replication and improves clinical outcomes, but seldom leads to serological clearance of HBsAg.[1]

Over the last 2 decades, most studies on HBV pathogenesis have focused on T-cell responses and the lack of clearance in the chronic phase caused by virus-specific T-cell exhaustion. B cells have long been neglected, although several observations indicate humoral responses to be relevant in chronic HBV: i) HBsAg-seroconversion is regarded as a successful treatment endpoint[2]; ii) HBeAg seroconversion heralds the transition between clinical phases and comes with improved immune control[3]; and iii) B cell-depleting treatments, such as rituximab, may lead to HBsAg seroreversion and fatal HBV flares, even in patients with a resolved infection.[4]

Insight into the phenotype and function of B cells that specifically target HBV antigens is however limited. Quantification of HBV-specific B cells has long depended on their *in vitro* secretion of HBV-binding antibodies which can be detected by ELISA or ELISPOT assays.[5] However, this technique does not allow for direct *ex vivo* enumeration or phenotypic characterisation, as memory B cells need to be differentiated *in vitro* into antibody-secreting cells. Recently, fluorescently labelled HBsAg and HBcAg baits have been developed that specifically bind to their cognate B-cell receptor (BCR) on memory B cells. For the first time since the discovery of HBV, this has enabled the quantification and functional characterisation of HBV-specific B cells.[6–9] In addition, several novel therapeutic approaches are being developed for chronic HBV that partly target B cells, such as programmed cell death 1 (PD-1) immune checkpoint inhibitors, and Toll-like receptor (TLR) 7 and TLR9 agonists (reviewed in[10]). Herein, we review our current understanding of the role of the humoral immune response in chronic HBV, both at the level of HBV-specific antibody production and at the phenotypic and broader functional level of B cells. Finally, we offer a perspective on the future therapeutic implications of these recent insights.

Antibodies against hepatitis B viral antigens

Studies on serum antibodies specific for different HBV antigens have been the first to provide insight into antiviral B-cell responses. Antibody produc-

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Key points

- Serum HBV-specific antibodies against the HBV surface (HBsAg), e (HBeAg) and core (HBcAg) protein correlate with immune protection, with the transition between clinical phases and with nucleos(t)ide analogue treatment responses.
- Extrafollicular memory B-cell formation may take place in the livers of patients with chronic HBV.
- Global memory B cells, in chronic hepatitis B (CHB), are activated and present some level of exhaustion, suggesting a process of overstimulation, but not hindering humoral immune responses to other infections or vaccines.
- HBsAg-specific B cells are defective at antibody secretion early after HBV exposure and remain so until viral clearance.
- HBcAg-specific B cells remain abundant, fully functional and associate with clinical disease phases.
- Some germline-encoded HBcAg-directed B-cell responses can lead to fulminant liver failure via antibody-dependent cytotoxicity.
- Novel treatments aimed at functional cure should include a thorough characterisation of hepatitis B-specific B cell responses.

tion against some HBV antigens correlates with immune control by neutralising (sub)viral particles but may also result in antibody-dependent cellular cytotoxicity (ADCC) by binding to surface expressed viral epitopes.[11,12]

The HBV genome (Fig. 1) is organised into 4 partially overlapping open reading frames (ORFs): the S ORF, encoding HBsAg; the X ORF, encoding the HBx protein; the P ORF, encoding HBV polymerase; and the C ORF, encoding HBeAg, HBcAg and a precore protein (p22cr). Together, the latter 3 can be detected by a single hepatitis B core-related antigen (HBcrAg) assay.[13] As a decoy antigen, HBsAg is secreted at much higher concentrations – up to 10^5 times higher – than infectious HBV virions. The HBeAg is secreted as a dimer, early in the course of wild-type HBV infections. The kinetics, intracellular trafficking and possible secretion of HBcAg as a naked capsid remain a matter of debate.[14] The HBx protein is suggested to play a role in hepatocarcinogenesis in patients with chronic hepatitis B (CHB).[15,16] Antibodies against HBcAg, HBeAg and HBsAg bear diagnostic importance in the clinical characterisation of CHB infections and are therefore a focus of this review. Antibodies to the HBx protein and HBV polymerase,[16,17] can be detected as well, but little is known about their clinical relevance. Antibodies against the HBV polymerase may reflect ongoing viral replication, whereas antibodies against the HBx protein are mostly found in patients with hepatocellular carcinoma. Eventually the latter may also serve as a surrogate marker for cirrhosis development.[18] An overview of the different HBV-specific antibodies is provided in Fig. 1.

Antibodies against HBsAg

Located on HBV's envelope, the HBsAg consists of a mixture of 3 proteins encoded by a single ORF. Three in frame standing start codons define the pre-S1, pre-S2 and S region. The latter region is translated into the small surface protein (S-protein). The middle surface protein contains the S domain and an additional pre-S2 domain, while the large surface protein encompasses the pre-S1, pre-S2 and S domains (Fig. 1).[19] Epitopes not present on the middle or small S proteins can be recognised by specific monoclonal antibodies, but their clinical significance needs to be established.[20] Therefore current routine clinical assays do not discriminate between antibodies specific for any of the 3 surface proteins and are reported as anti-hepatitis B surface antibodies (HBsAb).[21]

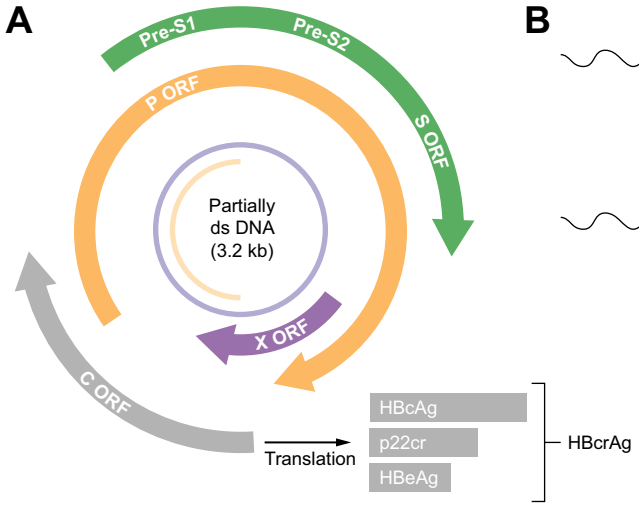
HBsAb have been a focus of intense research ever since the early discovery of HBV, given their association with a protective immune response. Antibodies against the so-called "a" determinant, which is part of the major hydrophilic loop of the S-protein, inhibit binding to heparan sulfate proteoglycans on the surface of hepatocytes and thereby have virus neutralising capacity.[22] Antibodies against a highly conserved motif in the pre-S1 domain have additional strong neutralising activity by inhibiting the interaction with the sodium taurochlorate cotransporting polypeptide (NTCP) hepatocyte entry receptor[23] (Fig. 1). In addition, *in vitro* and mouse model studies suggest that intracellular expressed or internalised HBsAb may also block the release of HBV particles from infected hepatocytes.[24,25]

In HBsAg vaccinees, HBsAb levels >10 mIU/ml are considered a correlate of clinical protection.[26] Importantly, vaccine-induced HBsAb alone do not provide sterilising immunity, as the current recombinant HBV vaccine is exclusively composed of the S-protein, thereby eliciting antibodies that may inhibit the binding of the "a" determinant, but not the interaction with the NTCP-receptor (Fig. 1).[22,27] In a study of 90 vaccinated healthcare workers, occupational exposure to HBV 10-28 years after vaccination was associated with HBV core- and polymerase-specific T-cell responses despite protective HBsAb levels, suggestive of self-limiting HBV breakthrough infections.[28] Furthermore, HBsAb may not protect against infection with HBsAg mutants.[29] Engineered viruses with a HBV polymerase mutation, resulting in changes in the overlapping HBsAg protein, were able to infect previously vaccinated chimpanzees. Although these HBV S gene mutants can be selected by first generation NAs, no significant spread among the HBV-vaccinated population has been observed thus far.[30]

Following seroclearance of HBsAg, free HBsAb mostly appear in plasma as a hallmark of resolved CHB infection. While routine assays are not capable of detecting HBsAb-HBsAg immune complexes, PEG precipitation enabled the quantification of circulating immune complexes in a cohort of 25 patients with CHB, the kinetics of which seemed to correlate with ALT peaks and ultimate HBsAg loss.[31,32] In addition, a proportion of chronic HBsAg carriers have concurrent free HBsAb in their plasma.[33,34] These findings indicate that the HBsAg-directed humoral response persists throughout a CHB infection but is of inadequate quality and/or magnitude to neutralise the overwhelming amount of subviral HBsAg particles. Similarly, attempts to clear a CHB infection using exogenous HBsAb have been disappointing, only resulting in temporary reductions in HBsAg levels.[35] However, in a preventive clinical setting, passive immunisation with HBsAb-enriched plasma preparations is the cornerstone to circumvent HBV (re)infections in children born to CHB-infected mothers or in patients with CHB who receive a liver transplant.[36,37] Exogenous HBsAb may therefore represent an attractive addendum to novel therapies that already substantially reduce the production or secretion of HBsAg.

Antibodies against HBeAg

HBeAg is a small polypeptide not needed for viral replication or infection. Secreted HBeAg acts as a tolerogen capable of down-regulating HBcAg-specific T-cell responses in transgenic mice.[3,38,39] Similarly, in a large cohort of patients with CHB, weaker HBcAg-specific T-cell responses were observed in HBeAg+ compared to HBeAg- patients.[40]



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ORF	S	C		X	P
Antibodies	HBsAb	HBcAb	HBeAb	HBxAb	Polymerase antibodies
Presence	Complexed or free circulating at low concentrations during CHB infections	In all patients exposed to HBV	After HBeAg seroconversion	In some patients with hepatocellular carcinoma	In patients with active viral replication
	After functional cure				
Protective	Yes	No	No	Unknown	Unknown
Clinical implication	Hallmark of functional cure	High levels are protective for viral rebound after treatment cessation	HBeAg seroconversion correlates with host immune control	May correlate with liver cirrhosis and HCC	
	Prevention of mother to child transmission				
	Prevention of reinfection after liver transplantation	High levels in phases with active liver disease	Improved clinical outcomes in HBeAg seroconverted patients		
	Passive immunization				

Fig. 1. Hepatitis B virus. (A) Overview of the structure of the HBV genome, (B) the structure of HBsAg and (C) characteristics of antibodies against HBV's viral antigens (C). ds, double stranded; HBcAb, antibodies against HBcAg; HBeAg, antibodies against HBeAg; HBsAb, antibodies against HBsAg; NTCP, sodium taurocholate cotransporting peptide; ORF, open reading frame.

HBeAg seroconversion, defined as the seroclearance of HBeAg and the appearance of antibodies against HBeAg (HBeAb) occurs gradually in the natural history of CHB infection, and is aided by the selection of precore stop codon mutant viruses that lose the ability to secrete HBeAg. This process coincides with an increasing diversity in HBV quasispecies, as was shown in a longitudinal follow-up study of 15 HBeAg-positive patients with CHB, 8 of whom went on to develop HBeAg seroconversion.[41] HBeAb cannot neutralise HBV virions. Instead, passive immunisation with HBeAb in chimpanzees was shown to induce prolonged hepatitis, hinting towards a detrimental effect.[42] Nonetheless, HBeAg seroconversion and the transition to the HBeAg-negative CHB infection phase coincides with a substantially improved immune control, resulting in a strong transcriptional suppression of covalently closed circular DNA (cccDNA).[43] However, HBeAg-seroconverted patients may develop chronic HBeAg-negative hepatitis (ENEG), characterised by increased cccDNA transcriptional activity, viral replication, liver inflammation and alanine aminotransferase (ALT) levels. The risk hereto varies with HBV genotype and inherently also with geographical region, due to the differential distribution of HBV genotypes globally.[43,44]

Antibodies against HBcAg

The inner nucleocapsid of HBV virions is composed of 240 copies of the viral capsid, termed the HBcAg, which is not actively secreted in blood.[45] HBcAg was found to be a superantigen capable of eliciting both T cell-dependent and -independent immune responses,[46] suggesting a direct interaction between the protein and B cells.

Antibodies against the HBcAg (HBcAb) are the first to appear upon infection and persist for decades even after cure. Like HBeAb, HBcAb do not neutralise viral particles and passive immunisation may even prolong episodes of hepatitis.[42] Levels of

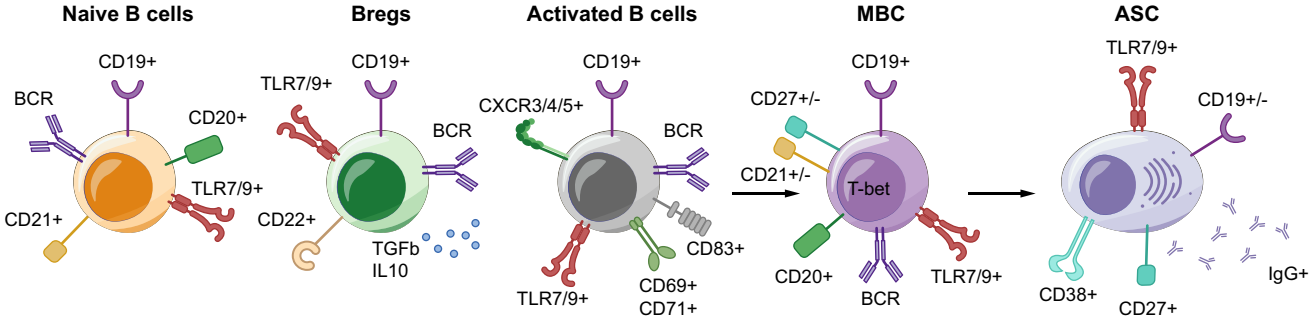
core-specific IgM and IgG antibodies bear clinical relevance, as they can differentiate between acute HBV infection and flares of CHB,[47] and may be associated with NA treatment responses.[48,49] Furthermore, HBcAb levels tend to be higher in disease phases with high levels of viral replication, inflammation and signs of liver damage, the so-called immune active and ENEG clinical phases.[9] Unlike HBsAb, high levels of HBcAb persist throughout and after a CHB infection and can circulate as HBV RNA-containing capsid-antibody complexes.[9,50]

Phenotype and function of B cells in CHB

Until recently, our knowledge on the role of B cells during CHB was limited to the study of global B cells and HBV-specific antibody production. As only a minority of B cells are HBV-specific, changes in the phenotype and function of global B cells in CHB are ascribed to indirect effects of secreted viral proteins and the accompanying inflammatory state. With the introduction of novel bait-based approaches, it is now possible to directly examine memory B cells that bind to HBsAg or HBcAg.[6-9] In the next section, we give a high-level overview of the heterogeneity of B cells and their function, after which we focus on B cells during CHB, both on a global level as well as at the HBV-specific level. A broad overview of the phenotype and function of the different B-cell subsets and HBV-specific B cells is depicted in Fig. 2.

Heterogeneity of memory B cells and other B cell subsets

After binding of a cognate antigen, naïve B cells differentiate into memory B cells (MBCs), resulting in a novel BCR of mostly switched isotype, a higher affinity and a faster recall response upon antigen re-encounter.[51] The diversity of affinity-matured BCRs allows MBCs to respond to a virtually infinite number of different antigens. Once differentiated into plasma cells, the antibody secretory rate is astonishingly high at an estimated



A	B Function	C	Ag-specific B cells in CHB	D Phenotype	E Function	
Ag-Naive CD19+ CD20+ CD27- CD21+ → Depends on BCR signal	- **IL-10 and TGFb: secretion:** inhibit effector T cells and enhance regulatory T cell function	- Co-stimulatory molecule production - Ag presentation - Expansion & maturation into antibody secreting cells - Antiviralcytokine production (IL-6, TNFα)	**HBsAg**	**AtMBC** → CD19+ CD20+ CD27− CD21−	- Defective co-stimulatory molecule production - Defective Ag presentation - Defective expansion & maturation into antibody secreting cells - Defective antiviral cytokine production - Exhaustion associated membrane markers (PD1+, FcRL3/4/5+)	Reduced HbsAb production
			HBcAg	**cMBC** → CD19+ CD20+ CD27+ CD21+	- Effective co-stimulatory molecule production - Ag presentation - Effective expansion & maturation into antibody secreting cells - Effective antiviral cytokine production - Effective Ab production	Increased HbcAb production

Fig. 2. Phenotype and function of global and HBV-specific B-cell subsets in CHB. (A) Naïve B cells. (B) Bregs inhibit effector T cells via cytokine production. (C) Global activated B cells are involved in immune activation,antigen presentation, antiviral cytokine production and mature efficiently in ASCs. (D+E) Phenotype (D) and Function (E) of HBV-specific B cells. HBsAg-specific B cells have a predominant AtMBC phenotype, are dysfunctional and produce a reduced amount of HBsAb; HBcAg-specific B cells have a predominant cMBC phenotype, are fully functional and produce an increased amount of HBcAb. ASCs, antibody-secreting cells; AtMBCs, atypical memory B cells; BCR, B-cell receptor; Bregs, regulatory B cells; CHB, chronic hepatitis B; cMBC, conventional memory B cells; CXCR3/4, C-X-C motif chemokine receptor type 3/4; FcRL3/4/5, Fc receptor-like protein 3/4/5; IL-10, interleukin 10; MBCs, memory B cells; PD1, programmed cell death protein 1; TGF-β, transforming growth factor-β; TLR7/9; toll-like receptor 7/9.

$5x10^7$ molecules per hour.[52] Both this antibody secretory speed and the BCR diversity underscore the tremendous power of humoral immunity.

According to the classical view, the process of BCR somatic hypermutation during which MBCs mature, exclusively takes place in the germinal centres of the secondary lymphoid organs, such as the spleen and lymph nodes. However, it is now accepted that adaptive immune responses can also occur outside the spleen or lymph nodes, driven by constant antigen exposure. This extrafollicular maturation of naïve B cells into MBCs is increasingly recognised in infections where germinal centre formation is delayed or inhibited. In fact, most of the early antibody formation upon pathogen encounter will be extrafollicular in origin, before genuine germinal centres are formed.[53] If the infection persists (as in CHB) or is pervasive (as in influenza or COVID-19 pneumonia), aggregates of lymphoid cells in the infected parenchyma might organise into structures resembling the T- and B-cell zones of lymph nodes.[54] In the liver, such periportal lymphoid follicles are characteristic of chronic HCV infections, but are also present in up to a quarter of CHB infections.[55]

Affinity-matured MBCs recirculate and patrol the body, and therefore can be studied in peripheral blood. Importantly, while extrafollicular B-cell responses might be especially relevant in chronic viral infections, there remains a lot to be learned about their phenotype and function, given the paucity of human tissue sampling studies. In animal models, extrafollicular MBC responses were found to yield generally lower affinity BCRs compared to germinal centre MBCs.[56] These observations call for further studies on intrahepatic B cells in CHB and caution against the generalisation of peripheral blood studies.

Apart from their ontogeny and BCR affinity, subtypes of MBCs have been identified based on their BCR isotype, the surface marker expression of CD27 and the complement receptor type 2 (CD21), whose reduced expression is a marker of activation. CD27 is regarded as the classical MBC marker, but in certain inflammatory diseases, such as chronic infections and autoimmune pathology, CD27-negative MBCs have been identified.[51] Non-isotype switched MBCs express IgM and/or IgD, while IgG, IgA or IgE expression defines a fully mature, isotype switched BCR. Additional surface markers, such as CD24 and CD38, are required for a complete subtyping of circulating B cells, although consistent classification remains challenging.[57]

Next to their indispensable antibody-producing role, B cells are also capable of presenting antigens to T cells,[58] are required for the development of lymphoid tissues, and can secrete cytokines that may incite or downregulate secondary immune responses.[59,60] One specific subtype of cytokine-producing B cells, so-called regulatory B cells, suppress cytotoxic T cells and induce regulatory T cells, via the production of interleukin (IL)-10 and transforming growth factor-β.[61–63] Elevated levels of IL-10 have been observed in patients with HIV or HCV and in mouse studies of chronic lymphocytic choriomeningitis virus infections. These were found to correlate with diminished T-cell activity and the failure to control viral replication.[64,65]

Global B cells in CHB: activated *vs.* exhausted?

Early studies found circulating B cells to be hyperactivated in patients with CHB compared to healthy controls (HCs), with higher expression levels of the early activation marker CD69, the transferrin receptor CD71 and the liver-homing marker CXCR3 (CD183); higher *in vitro*-induced antibody production, but marginally lower proliferation capacities.[66,67] Importantly, these phenotyping studies did not apply the different surface markers that would enable a full subtyping of MBCs, such as CD21 or IgD. Since the discovery, in patients with HIV, of a population of exhausted circulating human MBCs that proliferate poorly, are deficient at antibody production and lack CD21 and CD27, more attention has been given to these so-called atypical MBCs (AtMBCs) in chronic infections.[68,69]

Chronic immune activation and inflammation are suggested to drive the expansion of these AtMBCs, which upregulate an array of inhibitory genes including Fc receptor-like 5 (FcRL5), FcRL3 and Siglec6 and contribute to deficient virus-specific immune responses.[68] Recent multiparameter FACS studies applying this subtyping found the fraction of AtMBCs to reach 5-6% of global B cells in patients with acute or chronic HBV infection, compared to 2-4% in healthy vaccinees.[6,7,70] The AtMBC fraction of global B cells tended to decline with progressing clinical phases and showed a slight positive correlation with HBV DNA levels.[6,7,70] Phenotypically, AtMBCs in CHB fairly closely resembled those initially described in HIV, with the expression of T-bet (T-box 21 transcription factor), CD11c, and other members of the FcRL-family.[7] Compared to conventional MBCs, these AtMBCs differentiated less well into plasma cells, had a lower calcium flux upon BCR engagement and produced less antiviral cytokines (IL-6 and tumour-necrosis factor-α); all pointing towards an exhausted phenotype.[6,7]

However, the overall functional consequence of global AtMBC enrichment might be subtle, as the *in vitro* IgG production capacity of peripheral blood mononuclear cells (PBMCs) is preserved in patients with CHB,[9,66,67] and vaccine-induced hepatitis A seroconversion rates in patients with CHB are comparable to HCs.[71] Furthermore, COVID-19 mortality is not higher in patients with past or current HBV infection, despite recent evidence on the importance of neutralising antibody responses.[72,73] Nevertheless, effects might be found in other B-cell functions, such as antigen presentation or cytokine production.

Phenotypically, global B cells do not differ between clinical phases of a CHB infection.[6,7,9,74] However, in bulk transcriptome studies, we identified an immune gene signature in the blood and liver of patients with CHB consisting of many B cell-related genes that correlated with distinct clinical phases.[74,75] Specifically, the transition from the immune tolerant to the immune active phase was characterised by a more pronounced activity of the B-cell compartment, compared to the T-cell compartment. Whereas this role is not entirely unexpected during HBeAg seroconversion, subsequent clinical phases showed significant B-cell activities as well.[74,75] We recently corroborated this by performing RNA sequencing on sorted intrahepatic and peripheral global CD19+ B cells. The global B-cell transcriptome of CHB showed an activated status in the immune active and inactive carrier phase, with upregulated CD83, CD300c, CXCR4, CD69 levels and various innate stimulating genes.[76] An activating signature was also found by Salimzadeh *et al.* in sorted MBC subsets from patients with CHB, *e.g.* upregulated CD83 levels.[6] In addition, we found gene expression profiles of intrahepatic B cells from patients with CHB to be very different from their paired peripheral counterparts. The former upregulated the BCR and several immune signalling pathways compared to peripheral blood B lymphocytes, suggesting a process of extrafollicular MBC formation.[53,76] Conversely, Burton *et al.* found intrahepatic B cells to be enriched in AtMBCs.[7]

Overall, the combination of activated global B cells and AtMBC enrichment suggests a process of overstimulation resulting in some level of phenotypic and functional exhaustion. A similar mechanism has been described for viral-specific T cells during chronic antigen exposure.[77] However, this inhibition can be overcome via strong non-BCR signals and PD-1 blockade *in vitro*.[6,7] Furthermore, global B-cell function remains intact based on overall *in vitro* antibody production levels and humoral immune responses to other infections or vaccines.

In addition, the observed phenotypic and transcriptome differences between liver and blood samples suggest that B-cell reprogramming during CHB is tissue specific and caution against the overinterpretation of peripheral blood studies.

HBsAg-specific B cells: defective and partially rescuable

Early after the discovery and clinical application of the neutralising capacity of HBsAb, isolated PBMCs from patients with CHB were found to be defective at producing these antibodies in patients affected by CHB.[5,37] Since then, numerous studies have confirmed these findings, and showed that *in vitro* HBsAb production was significantly higher in vaccinated HCs, restored following HBsAg-seroconversion and correlated with serum HBsAb titres.[6,7,9,67,78] As these assays depend on *in vitro* secretion of HBsAb after polyclonal stimulation of total PBMCs, until very recently, it was not clear whether the circulating MBC pool specific for HBsAg was depleted or dysfunctional during CHB. The advent of fluorescently labelled HBsAg baits that bind to their cognate HBsAg BCR on MBCs illustrated for the first time that the number of circulating HBsAg-specific MBCs is astonishingly similar between acute, chronic and resolved HBV infections and even in successfully vaccinated HCs.[6,7] Furthermore, the frequency of these HBsAg-specific MBCs did not differ across a range of different clinical parameters, such as HBsAg, HBV DNA or ALT levels.[6,7] On average these cells accounted for 0.1% to 0.3% of total CD19 B cells in CHB.[6–9] Importantly, 2 different HBsAg bait stainings have been applied, accounting for the wide range of circulating HBsAg-specific B cells between reports. A dual staining strategy with DyLight550- and DyLight650-labelled HBsAg baits resulted in negligeable background staining (up to 0.01% of total B cells), with a median 0.1% of circulating B cells found to be dual HBsAg bait-positive in patients with CHB.[6,8,9] The use of a single AF488-HBsAg bait was associated with unspecific staining of up to 0.2% of B cells in unexposed individuals. Still a clearly higher fraction of single bait HBsAg-positive B cells could be discerned above background in both vaccinated HCs and patients with CHB.[7] Furthermore, the specificity of the staining was corroborated after sorted HBsAg bait-positive B cells, but not sorted HBsAg bait-negative B cells, were shown to produce HBsAb *in vitro*.[6,7] Importantly, the *in vitro* HBsAb production per sorted HBsAg-binding B cell was significantly lower in patients with CHB compared to vaccinated HCs, and required co-culture with at least IL-2, IL-21 and CD40 ligand-expressing cells to differentiate the anergic HBsAg-specific B cells into HBsAb-secreting plasma cells.[6,7]

In patients with CHB a mean 15-30% of HBsAg-specific B cells presented a CD27- CD21- AtMBC phenotype and expressed high levels of PD-1 and other inhibitory markers. In contrast, a conventional MBC phenotype (CD27+/CD21+) was the principal component of HBsAg-specific B cells in vaccinated HCs. Importantly however, the HBsAb secretory function of HBsAg-specific B cells could be partially restored *in vitro* by co-culture with PD-1 blocking antibodies.[6,7]

Overall, these studies demonstrated that the HBsAg-directed humoral immune response early after HBV exposure is defective at producing HBsAb and remains so, irrespective of the outcome of the infection. This defect can be restored partially *in vitro* by B cell-maturing cytokines and PD-1 blockade and completely after HBsAg clearance *in vivo*. The onset and mechanisms driving this exhaustion are currently unclear and further studies into the kinetics and determinants of this process are warranted.

HBcAg-specific B cells: abundant and associated with disease phase

Using a similar dual DyLight550 and DyLight650 bait staining strategy, we and others recently demonstrated that HBcAg-specific B cells circulate at a roughly 10-fold higher frequency than HBsAg-specific B cells in the peripheral blood of patients with CHB and, unlike HBsAg-specific B cells, mature efficiently into antibody-secreting cells.[8,9] These dual HBcAg bait-positive cells present a CD27+ classical memory, IgG+ class-switched, MBC profile and have slightly higher CD69 expression levels compared to global memory B cells. HBcAg-specific MBCs are phenotypically more activated compared to HBsAg-specific B cells, with a higher expression of CD95 and a lower expression of IL-10Rα. Furthermore, the fraction of HBcAg-specific B cells with a CD27- CD21- AtMBC profile was significantly lower compared to HBsAg-specific B cells.[8,9]

Interestingly, the frequency of circulating HBcAg-specific MBCs increased during hepatitis disease flares and was drastically reduced upon ALT normalisation and HBV DNA suppression during antiviral treatment.[8,9] In untreated CHB infections, HBcAb levels (both IgM and IgG) have been found to correlate with ALT kinetics.[79–81] With the application of HBcAg bait stainings, it became clear that the number of circulating HBcAg-specific MBCs follow the fluctuating serum HBcAb patterns. Indeed, using ELISPOT assays, it was shown that *in vitro* HBcAb production by total PBMCs correlated with ALT levels on the one hand and the number of circulating HBcAg MBCs on the other hand.[9] Furthermore, in clinical disease phases with higher ALT levels, HBcAg-specific MBCs showed a higher activated memory phenotype, compared to patients with low ALT. Overall, this corroborates an association between HBcAg-specific humoral immunity and the natural history of CHB and opens avenues for the use of these B-cell responses as biomarkers of immune activity.

Nevertheless, these associations do not allow us to draw a conclusion on the causal relationship between HBcAg-specific humoral immune responses and disease activity. As HBcAg is predominantly expressed intracellularly, HBcAg-specific B cells might encounter their cognate antigen less frequently, until HBcAg is released from lysed hepatocytes during cytotoxic T-cell responses.[82] This would imply that HBcAg-specific B-cell responses are bystanders and do not incite the initial round of cell lysis. The contrary has however been observed in HBV-induced acute liver failure (HBV-ALF). In an elegant study of 4 cases with HBV-ALF, an intrahepatic dominant B-cell gene signature was found, together with an extensive infiltration of CD27+ B cells, producing germline IgM and IgG antibodies specific for HBcAg, accompanied by complement deposition. Liver T-cell infiltration was low, supporting the notion of a T-cell independent germline-encoded B-cell response towards HBcAg in HBV-ALF which leads to ADCC and massive necrosis.[83,84] Recently this phenomenon was also found to occur in experimental

fulminant HBV infections with precore HBV mutants in chimpanzees.[85] It is unclear whether a similar mechanism may be involved – albeit partly – in ALT flares during CHB infections, which have been ascribed to CD8 T-cell and innate immune responses but are still not fully understood.[86] Earlier studies have documented HBcAg-HBcAb-complement colocalisation in the livers of patients with CHB, suggesting that complement-mediated cytotoxicity may also occur in this setting.[87] Using newly available HBV bait techniques and longitudinal samples from patients with CHB should help to unravel the contribution of humoral immunity to CHB flares.

Importantly, the quantification of HBcAg-specific MBCs required the exclusion of CD27-CD21+ naïve B cells, as HBcAg can bind to a conserved motif of the naïve BCR in a non-canonical manner.[88,89] Indeed, naïve B cells of non-exposed vaccinated HCs could bind HBcAg baits but were unable to secrete HBcAb upon *in vitro* culture.[8,9] HBcAg particles are known to behave as a T-cell independent superantigen, capable of cross-linking the naïve BCR.[46] Elegant mouse studies have demonstrated that HBcAg activates naïve murine B cells without T-cell help, but is also efficiently cross presented to T cells, thereby surpassing the ability of other professional antigen-presenting cells, such as dendritic cells.[46,90,91] In this respect, it is relevant to note that the transcriptome of sorted HBcAg-specific B cells showed an upregulation of genes involved in antigen presentation.[8]

The study of the different functions of HBcAg-specific B cells is still in its infancy. Further co-culturing experiments are expected to unravel whether HBcAg-specific B cells have a predominant antibody secretory, antigen presenting or regulatory effector function.

Heterogeneity in HBcAg- and HBsAg-specific adaptive immune responses

As is evident from the aforementioned studies, HBsAg- and HBcAg-specific B-cell responses differ profoundly in magnitude, phenotype and function. Contrary to this, their transcriptome was found to be very similar with only 34 differentially expressed genes (DEGs) out of 348 detected genes. Furthermore, in comparison to sorted global MBCs, the vast majority of DEGs were shared between both HBV-specific B-cell populations. Amongst others, genes linked to innate immune activation, the IFN-response and antigen cross-presentation were upregulated in both HBsAg- and HBcAg-specific B cells.[8] These findings point towards important antibody-independent functions of HBV-specific B cells in CHB. However, these results should be interpreted with caution, as the sole binding of the HBV bait to its BCR might induce changes to the transcriptome of the sorted bait-binding B cells, which would not be observed in sorted global MBCs.

In support of the differences between HBsAg- and HBcAg-specific B cells, a similar heterogeneity in virus-specific CD8 T-cell phenotype and responsiveness was identified in recent studies. Compared to HBV-specific T cells targeting the HBV polymerase, core-specific T cells presented a less pronounced exhaustion state and were better equipped to proliferate following antigen stimulation.[92-94] Notably, similar to the humoral response against HBsAg, these studies also found a low HBsAg-specific T-cell responsiveness. The reason for these heterogeneous virus-specific adaptive immune responses can primarily be ascribed to different expression levels of viral surface, core and polymerase antigens in infected hepatocytes, in addition to distinct secretory routes for subviral particles and proteins.[14,95] A large excess of HBsAg is known to be secreted by infected hepatocytes, but the secretion of HBcAg as naked viral particles is still widely debated and anticipated to be an artifact of hepatoma cell cultures.[13,50] The current view is that capsid-antibody complexes in the serum of patients with CHB would result from release of HBcAg from lysed hepatocytes and not from secretion of *bona fide* HBcAg particles devoid of a viral envelope.[50,82] Apart from heterogeneity in viral antigen expression, a differential antigen presentation by professional antigen-presenting cells, like Kupffer cells, *vs.* hepatocytes might contribute to the observed differences in HBcAg- and HBsAg-specific adaptive immune responses, as shown recently in mouse studies.[96] Finally, the intrinsic properties of HBcAg, as a superantigen, might explain why pronounced immune responses are incited even after limited antigen exposure, as is expected after the lysis of a limited number of infected hepatocytes.[46,82]

Overall, these findings, support the notion of a humoral immune response in CHB that is predominantly directed towards the core and not the envelope of the virion.

HBV flares after B-cell depletion

Of all immunosuppressive therapies, the highest HBV reactivation rates (of ≥10%) are reported following B cell-depleting treatments, even in patients with a resolved infection. The exact mechanism by which waning B-cell responses lead to HBV flares remains to be elucidated, although most B cell-depleting treatments are combined with other immunosuppressive medications, thereby also partially inhibiting T-cell and innate responses.[97] The anti-CD20 monoclonal antibody rituximab induces a depletion of circulating naïve and memory B cells via FcR-mediated ADCC, which is carried out by the monocyte-macrophage system, leading to impaired recall antibody responses.[98,99] Importantly, anti-CD20 treatment is fairly well tolerated, with no apparent increase in infectious complications, as serum antibody concentrations are maintained by persisting long-lived plasma cells.[99-101] In patients with a resolved HBV infection, serum HBsAb seem to protect against HBV reactivation, but do wane following rituximab treatment.[102,103] Counterintuitively, higher baseline HBcAb levels may increase the risk of an HBV flare following B-cell depletion, which adds to the described dichotomy in HBsAg- *vs.* HBcAg-directed humoral immune responses.[104] Nevertheless, mouse studies revealed the importance of antibody-independent B cell functions on the outcome of rituximab treatment.[105,106] Indeed, a reduced antigen-specific CD4 T-cell response was observed following B-cell depletion, while CD8 T-cell responses remained unaffected. In addition, B-cell antigen presentation was required for optimal antigen-specific CD4+ T-cell priming in settings of low antigen burden, such as late in a disease course.[105] A similar mechanism might be relevant after functional cure, when HBV antigen burden is low.

Therapeutic implications and future perspectives

Several new treatment approaches are in (pre-)clinical development that may support B-cell function in CHB. Decreasing the serum HBsAg load is one of the major goals, given its role in driving B- and T-cell exhaustion. Both intracellular approaches (*e.g.* nucleic acid polymers, S-antigen transport-inhibiting oligonucleotide polymers [STOPS^{TM}] or short-interfering RNA targeting the HBV mRNA[107-109]) and neutralising antibodies are under study. However, the latter approach will need to target different HBsAg epitopes to avoid HBsAg escape mutations and

might result in serum sickness from a high amount of insoluble immune complexes.[35,110]

Reversal of immune exhaustion by immune checkpoint inhibitors is an established treatment approach with proven clinical benefit in patients with cancer, predominantly via a T-cell dependent mechanism. In chronic viral infections, such as CHB and HIV, treatment successes have been less clear and data on its effect on B cells are scarce. *In vitro* studies on isolated B cells from HIV- and HBV-infected patients have demonstrated enhanced HIV-and HBsAg-specific antibody responses after siRNA-mediated knockdown of inhibitory receptors, including FcRL4, Siglec-6 and PD-1, or anti-PD-1 treatment, respectively.[6,7,111] PD-1 inhibition *in vivo* resulted in a 2-fold increase in neutralising antibody responses with a concomitant decrease in viral titres and improved survival in a macaque model for HIV infection, while viral clearance and seroconversion was observed in a woodchuck model of HBV infection.[112,113] Functional cure was observed in 1 out of 10 patients in a phase Ib clinical pilot study combining a therapeutic HBV vaccine with PD-1 inhibitor therapy, which led to an enhanced HBV-specific T-cell response with a concomitant ALT rise prior to HBsAg loss.[114] Thus, the contribution of restored B-cell responses to the observed clinical benefit requires further exploration.

Immunomodulatory treatment with TLR agonists, is an interesting avenue to specifically target MBCs, given their selective expression of TLR 6, 7, 9 and 10.[115] The combined engagement of BCR and TLR7 or TLR9 receptors by RNA- or DNA-containing antigens, results in strong (auto-)antibody responses and is exploited by a variety of auto-immune diseases.[116] B-cell responses may be elicited in a similar way during genuine HBV infections, as both HBV pre-genomic RNA and DNA are present in the capsid of circulating HBV virions. This may further contribute to the core-directed humoral responses described above. In preclinical TLR7 agonist studies in HBV-infected chimpanzees, HBV DNA suppression was associated with the formation of intrahepatic periportal lymphoid aggregates of CD8+ T cells and B cells, similar to those observed in patients with CHB and HCV.[55,117] Notably, these lymphoid aggregates could not be regarded as fully differentiated tertiary follicles because of the absence of follicular dendritic cells, but likely represent extra-follicular maturation of naïve B cells into MBCs.[117] However, in virally suppressed patients with CHB, 12 weeks of an oral TLR7 agonist did not result in significant serum HBsAg decreases but did enhance HBV-specific T-cell and natural killer cell responses, via plasmacytoid dendritic cell-secreted IFNα.[118] Contrastingly, the addition of a TLR9 adjuvant to therapeutic vaccines in animal models of CHB led to control of viral replication via intrahepatic proliferation of cytotoxic CD8 T cells in so-called intrahepatic myeloid-cell aggregates (iMATEs).[119,120] Importantly, these iMATEs did not require the presence of B cells, as they were also observed in *Rag2* knockout mice lacking mature B cells.

Overall, it is clear that despite high expression levels of both TLR7 and TLR9 on memory B cells[121]; the antiviral response to treatment with either agonist in models of CHB infection differed considerably, pointing towards additional target cells and downstream signalling cascades. This also emphasises the need to further elucidate the modulatory effect of novel therapeutic strategies on antiviral B-cell responses.

In conclusion, the complexity of B-cell ontogeny, B-cell heterogeneity and the preferential study of peripheral blood samples, have hindered efforts to develop an overarching view on the role of B cells in human disease, and CHB in particular. With the advent of HBV bait staining techniques, combined with advanced single cell approaches and fine needle liver sampling, we can now begin to define the features of humoral immunity to HBV. This will help to predict the risk of future HBV flares after the introduction of new immunomodulatory treatments but may also lead to B cell-targeted immunotherapies that curb the AtMBC response and functional exhaustion seen during chronic viral infections.

Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; ALT, alanine aminotransferase; AtMBCs, atypical memory B cells; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; DEGs, differentially expressed genes; ENEG, HBeAg-negative chronic hepatitis; Fcrl5, Fc receptor-like 5; HBcAb, hepatitis B core antibodies; HBcrAg, hepatitis B core-related antigen; HbeAb, hepatitis B e antibodies; HBsAb, hepatitis B surface antibodies; HBV-ALF, HBV-induced acute liver failure; HC, healthy controls; IL-, interleukin-; iMATE, intrahepatic myeloid-cell aggregates; MBC, memory B cells; NA s, nucleos(t)ide analogues; ORF, open reading frame; PBMC, peripheral blood mononuclear cells; PD-1, programmed cell death 1; TLR, Toll-like receptor.

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Conflict of interest

TVW has received grants from Gilead Sciences, is a consultant for Janssen Pharmaceuticals, Gilead Sciences, Abbvie, and a sponsored lecturer for Abbvie, Gilead Sciences. HJ has received grants from AbbVie, Arbutus, Gilead Sciences, Janssen, Merck, Roche and served as consultant for: Arbutus, Arena, Enyo, Gilead Sciences, GlaxoSmithKline, Janssen, Merck, Roche, Vir Biotechnology Inc., Viroclinics. TA and SVH have nothing to disclose.

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

Authors' contributions

Conceptualization: TVW, SVH, TA. Writing – original draft: TVW, SVH, TA. Writing – review and editing: TVW, SVH, HJ.

Supplementary data

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Author names in bold designate shared co-first authorship

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