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PD-1 blockade partially recovers dysfunctional virus-specific B cells in chronic hepatitis B infection

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Conflicts of interest:

AB receives research support from Gilead Sciences to test the effect of HBV antigens on immune cell function. He acted as a consultant and served on the advisory boards of Gilead Sciences, MedImmune, Janssen-Cilag, Abivax, and HUMABS BioMed. He is also a cofounder of Lion TCR, a biotech company developing T cell receptors for treatment of virus-related cancers. P.T.F. Kennedy has collaborative grant funding from Gilead Sciences, participates in advisory board/provides consultancy to Gilead Sciences and Janssen, and is an investigator for industry-led trials with Gilead Sciences, Janssen, Alere, and Assembly Biosciences. CF, MH, NN and SF are employed by Gilead Sciences. All other authors declared that no conflict of interest exists.

Abstract

Chronic HBV (CHB) infection suppresses virus-specific T cells, but its impact on humoral immunity has been poorly analyzed. Here, we developed a dual staining method, which utilizes HBsAg labelled with fluorochromes as “baits”, for specific ex vivo detection of HBsAg-specific B cells and analysis of their quantity, function and phenotype. We studied healthy vaccinated subjects (n=18) and patients with resolved (n=21), acute (n=11) or chronic (n=96) HBV infection and observed that frequencies of circulating HBsAg-specific B cells are independent of the HBV infection status. In contrast, serum HBsAg presence affects function and phenotype of HBsAg-specific B cells that were unable to mature in vitro into antibody-secreting cells and displayed an increased expression of markers linked to hyperactivation (CD21low) and exhaustion (PD-1). Importantly, B cell alterations were not limited to HBsAg-specific B cells but affected the global B cell population. HBsAg-specific B cell maturation could be partially restored by a method involving the combination of IL-2, IL-21 and CD40L-expressing feeder cells, and further boosted by addition of anti-PD-1 antibodies.

In conclusion, HBV infection has a marked impact on global and HBV-specific humoral immunity, yet HBsAg-specific B cells are amenable to a partial rescue by B cell maturing cytokines and PD-1 blockade.

Introduction

The coordinated activation of different components of the immune system is crucial for protection from pathogens. While innate immunity controls the initial response to infection, specific humoral and cellular immunity are required to eliminate the pathogen and prevent re-infection. The progressive functional inactivation of adaptive immunity characterizes persistent infections with alterations in both global and antigen-specific T and B cell compartments(1-5). A similar situation is evident during HBV infection where virus control is associated with the presence of functional antiviral T cells and to the serological detection of anti-HBs antibodies(6). However, while the quantity and function of HBV-specific T cells has been clearly defined in CHB patients(7-12) detailed characterization of the anti-HBV specific B cell response is lacking. In order to fill this knowledge gap, we developed a novel system, which utilizes recombinant HBsAg labelled with two different fluorochromes, for ex vivo detection of HBsAg-specific B cells by flow cytometry. This method is independent to the secretory function of antibodies and allows precise measurement of the frequency and function of HBsAg-specific B cells.

Studies in CHB patients have reported activation(13) and reduced functional capacity(14) of the global B cell population and have typically being unable to detect anti-HBs secreting B cells(15-17), which were suggested to be deleted by HBsAg-specific CD8 T cells(18). However, the presence of HBsAg-anti-HBs immune complexes in the sera of CHB patients(19) and detection of rare anti-HBsAg antibodies producing cells in ELISpot assays performed on PBMCs of CHB patients(14, 20) suggests that deletion or functional inactivation of HBsAg-specific B cells is not complete(21). The current study focused on HBsAg-specific B cells, because even though humoral responses target all HBV proteins(19), only anti-HBs specific antibodies are associated with the concept of functional HBV cure (serological negativity of HBV-DNA and HBsAg) and protection(22, 23). Anti-HBs antibodies target different epitopes located in the small surface protein of HBV(24) and have a protective role by blocking the interaction of HBV with heparan sulfate molecules(25),

but additional mechanisms, like direct suppression of HBV production from infected hepatocytes(26, 27), have been suggested.

An unusual feature of the HBV replication cycle is that virus-infected hepatocytes secrete a large quantity of subviral particles (HBsAg) in addition to infectious virions. These non-infectious subviral particles vastly outnumber HBV virions in the circulation of CHB patients (by 10^3-10^6 fold), and can reach concentrations as high as 50-100 μg/ml(24). Importantly, the small S HBV protein is present in both viral and subviral particles, and thus anti-HBs antibodies target both HBV virions and HBsAg. The evolutionary reason why HBV has developed its ability to produce large quantity of subviral, non-infectious particles is still not clear. One attractive hypothesis is that HBsAg functions as a decoy to saturate anti-HBs antibodies and so prevents virus neutralization(28). It has also been proposed that HBsAg can suppress innate, humoral or cellular immunity(29). In this study we developed a method to directly detect HBsAg-specific B cells ex vivo, which allows us to analyze the impact that HBsAg has on the quantity, phenotype and function of virus specific B cells and on strategies that can boost their functionality.

Results

Direct ex vivo detection of HBsAg-specific B cells

Direct visualization of HBsAg-specific B cells utilizing HBsAg conjugated microbeads has been performed in HBsAg vaccinated individuals(30), but not in patients with HBV infection where anti-HBV-specific B cells have so far been evaluated using functional assays that might underestimate their size(15-17). In order to detect low frequency antigen-specific B cells(31), two batches of HBsAg (genoytpe A) were chemically labelled with two distinct fluorochromes, Dylight550 and Dylight650 (Figure 1A). The fluorochrome labelled HBsAg reagents were designed to act a “bait” to bind specifically to anti-HBsAg-B cell receptors (BCR) on the cell surface of $CD19^+$ B cells.

To test this strategy, we evaluated peripheral blood samples from a healthy subject who received an HBV vaccine boost.

Serum levels of IgG anti-HBs following the booster vaccination increased by day 7 and remained high until day 60 (Figure 1B). In addition, the frequency of total plasmablasts in the peripheral blood of this vaccinated subject reached maximum levels on day 7-post vaccination (Figure 1C), confirming that the booster vaccination worked successfully. We stained PBMCs isolated from this subject with Dylight550 and Dylight650 labelled HBsAg (HBsAg-D550 and HBsAg-D650) and antibodies that allowed gating on total memory B cells (MBCs) (Supplementary Figure 1). Figures 1D and 1E show the frequency of double positive HBsAg-$D550^+$/$D650^+$ MBCs (naïve $CD21^+CD27^-$ excluded) within the total $CD19^+$ B cell population from the day of boost (day 0) to day 60 after HBV vaccination.

In parallel with the kinetics of total plasmablasts and antibody production, the frequency of double positive HBsAg-$D550^+$/650^+ MBCs peaked at day 7, reaching a frequency of 0.4% of total $CD19^+$ B cells (Figure 1E) and then gradually declined. This HBsAg-double positive B cell population was principally comprised of $CD27^{high}CD38^{high}$ plasmablasts at day 7 to 9 post vaccination while HBsAg-$D550^+$/650^+ plasmablasts were not detectable 15 days after the booster vaccination (Figure 1D). Interestingly, the mean

fluorescence intensity (MFI) of HBsAg-D550^{+}/650^{+} double positive B cells increased progressively over time (Figure 1F) as expected from MBCs that express higher levels of surface BCR than plasmablasts. Finally, no changes in the frequency of HBsAg-D550^{+}/650^{+} B cells were detected on naïve (CD21^{+}CD27^{-}) B cells (Supplementary Figure 1). These data suggest that our fluorochrome conjugated HBsAg is able to directly detect ex vivo HBsAg-specific B cells.

To confirm this, HBsAg-D550^{+}/650^{+} and HBsAg-D550^{-}/650^{-} MBCs were sorted and cultured in vitro after polyclonal stimulation to induce expansion, plasmablast differentiation and antibody production. Only HBsAg-D550^{+}/650^{+} B cells secreted anti-HBs antibodies as measured by ELISA and ELISpot assays (Figure 1G). Thus, HBsAg-D550 and HBsAg-D650 can identify HBsAg-specific B cells directly ex vivo at a single cell level.

Frequency of HBsAg-specific B cells during HBV infection

We first utilized HBsAg-D550 and HBsAg-D650 to measure the frequency of HBsAg-specific B cells in the peripheral blood of HBV vaccinated subjects, and patients acutely or chronically infected with HBV. The clinical and virological profiles of the tested subjects are summarized in Table 1. We studied, 18 healthy vaccinated subjects, 11 acute (HBsAg+, IgM anti-core+, high ALT), 21 resolved (anti-HBs+ and anti-HBc+) as well as 96 CHB patients (HBsAg+) divided into the following clinical phases: 22 HBeAg+ chronic infection, 24 HBeAg+ chronic hepatitis, 24 HBeAg- chronic hepatitis, 26 HBeAg- chronic infection(32). In addition, PBMCs of 5 healthy non-HBV-vaccinated subjects (anti-HBs and anti-HBc negative) were included as controls.

Figure 2A shows the frequency of HBsAg-specific B cells in the different cohorts. HBsAg-D550^{+}/650^{+} B cells were detected at variable frequencies in all HBV vaccinated subjects and HBV infected patients tested. In contrast, they were not detected in 4 out of the 5 healthy non-vaccinated controls, and only at a very low frequency (0.01% of total B cells) in one of these controls, demonstrating the high specificity of the dual staining detection method.

The median (interquartile range) frequency of HBsAg-specific B cells in the different cohorts was remarkably similar [vaccinated 0.065 (0.035-0.088)%, acute 0.053 (0.026-0.094)%, resolved 0.041(0.025-0.074)%, chronic 0.079 (0.048-0.12)%] even though it was slightly higher in patients with CHB versus resolved. There was large variability of HBsAg-specific B cell frequencies between different subjects with CHB, where HBsAg-specific B cells could be detected at levels ranging from to 0.01% to 0.43% of total B cells. However, these different frequencies were not associated with distinct clinical or virological profiles of HBV infection. Deconvolution of HBsAg-specific B cell frequency in different categories of CHB patients showed comparable variability in all phases of CHB (Figure 2B) with the median frequency (approximately 0.1% of total B cells) was identical in all cohorts of CHB patients.
There was also no statistically significant association between HBsAg-specific B cell frequency and serum levels of HBsAg, HBV-DNA and ALT (Figure 2C).

We hypothesized that the marked variability of HBsAg-specific B cells detected in CHB patients may be related to the genotype of the infecting virus. Since our fluorochrome conjugated HBsAg reagents are based on HBV genotype A, a possibility is that our reagents would preferentially bind B cells specific for HBsAg genotype A or D (that are genetically closely related) but not B cells specific for other genotypes (B, C and E that are more distant).
Accordingly, the HBV genotypes for 51 out of 96 CHB patients were determined. As shown in Figure 2D, a similar frequency of HBsAg-specific B cells was detected in CHB patients irrespective of the genotype of the infecting virus. Thus, the frequency of HBsAg-specific B cells is comparable in patients irrespective to their natural history stage. This contrasts with the feature of HBV-specific T cells, which are present in higher frequency in patients who resolve HBV than in those with chronic HBV infection(33).

In order to further analyze the relationship between HBsAg-specific B cell frequency and viral control, we studied patients with acute hepatitis B infection from the time of onset of clinical symptoms to functional cure (i.e. HBV-DNA negativity, HBsAg loss and detection of anti-HBs).

First, we investigated whether the B cell compartment is activated during acute hepatitis B. We thus measured the frequency of plasmablats ($CD19^+$, $CD10^-$, $CD21^-$, $CD27^{high}$, $CD38^{high}$) in 6 acute hepatitis B patients and, as controls, in 5 patients with acute dengue infection (Figure 3A). Frequency of plasmablasts was extremely low in 5 out of the 6 patients analyzed at all the different time points. A single subject showed a frequency of 6.5% of plasmablasts out of total B cells at the onset of acute hepatitis. In contrast, high frequency of plasmablasts was easily detected (16-37% of total B cells) in 4 of the 5 acute dengue patients studied within a week from the onset of dengue symptoms. Their detection was transient since 2 weeks after the acute phase the plasmablast frequency fell below 1.5% of total B cells. Thus, during the symptomatic phase of acute hepatitis B, the initiation of a B cell response is not detectable in the circulating compartment. In line with the lack of a robust B cell response, the frequency of HBsAg-specific B cells remained remarkably stable in all the 6 acute HBV patients analyzed over time despite the changes in HBV-DNA, ALT and the development of anti-HBs antibody (Figure 3B). HBsAg-specific B cell frequency during acute hepatitis B was similar to the frequency detected in resolved patients (anti-HBs+ and anti-HBc+ patients with a history of hepatitis).

Thus, the B cell immunity profile appears remarkably stable during the acute symptomatic phase of acute hepatitis B and the quantity of circulating HBsAg-specific B cells does not correlate with HBV control.

Function of HBsAg-specific B cells during HBV infection

The lack of correlation between HBsAg-specific B cell frequency and HBV control might indicate that HBsAg-specific B cells present in different groups of HBV infected subjects have functional differences. Indications that anti-HBs producing B cells are functionally altered in CHB infection have been already suggested by data derived from analysis of bulk B cells(15-17).

To further decipher the functionality of HBsAg-specific B cells, we sorted HBsAg-specific B cells from 14 CHB patients and measured their ability to proliferate and mature into antibody secreting B cells. HBsAg-specific B cells of healthy vaccinated (4 subjects) were also sorted as controls. HBsAg-

specific B cells of all four vaccinated individuals expanded in vitro and produced anti-HBs antibodies (Figure 4A). However, microscopic inspection of the cell culture of HBsAg-specific B cells of CHB patients revealed that in most of the cases B cells expanded poorly or disappeared. As such ELISpot assays to analyze the frequency of HBV-specific B cells were performed only in 5 chronic patients and anti-HBs antibody spots were detected in a single culture (Figure 4A). Parallel analysis of anti-HBs antibody titers in supernatants of all cultured HBAg-specific B cells of CHB patients confirmed that anti-HBs antibodies were detected at very low titers only in 1 of the 14 HBsAg-specific B cell cultures tested (Figure 4B).

We reasoned that HBsAg-specific B cells present in CHB patients might require help from feeder cells to properly expand. We thus adopted a different protocol of B cell maturation that utilizes fibroblasts over-expressing CD40L as feeder cells and specific cytokines (IL-2 and IL-21)(34). Similar numbers of HBsAg-specific B cells were again sorted from 16 CHB patients and from 8 healthy controls, expanded for 13 days and then analyzed for the presence of anti-HBs secreting cells by ELISpot assay.

Figure 4C shows that we could recover the ability of HBsAg-specific B cells to mature into anti-HBs secreting B cells in 11 out of 16 tested CHB patients. With this method, the sorted cells survived and expanded well, but their maturation capacity was still compromised (mean spot counts: healthy = 240 spots, CHB = 22 spots).

Taken together these data directly demonstrate that functional impairment affects HBsAg-specific B cells of CHB patients in comparison to the ones present in healthy vaccinated individuals.

In addition, we analyzed the functionality of HBsAg-specific B cells present in acute hepatitis B patients. HBsAg-specific B cells were sorted at the indicated time points from PBMC of 5 acute patients (Figure 5) and were expanded using the two protocols (with or without feeder cells) utilized to expand HBsAg-specific B cells of CHB patients. Note that the limited sample availability precludes us to expand the HBsAg-specific B cells from the same patient/time point with both protocols. Anti-HBs titers in the supernatants (Figure 5A) and quantity of anti-HBs-producing B cells (Figure 5B) were

measured after 7 or 13 days of expansion. Surprisingly, HBsAg-specific B cells of acute HBV patients sorted during the acute phase of hepatitis B (HBsAg+ phase), similar to CHB patients, did not differentiate to antibody secreting cells and did not produce antibodies irrespective of their expansion method. In contrast, HBsAg-specific B cells sorted from PBMC collected after HBsAg seroconversion in 3 out of the 3 tested patients expanded and produced anti-HBs (Figure 5A,B).

Thus, HBsAg-specific B cells present during acute HBV infection display functional impairment similar to those detected in CHB patients but recover their functionality after HBsAg+ seroconversion. Conjointly, the data obtained in chronic and acute HBV patients suggest that HBsAg modulates the functionality of HBsAg-specific B cells irrespective to the outcome of HBV infection.

Phenotype of B cells during HBV infection

To understand the possible causes of the impaired functionality of HBsAg-specific B cells, we analyzed and compared the phenotype of HBsAg-specific and total B cells present in patients with acute and chronic HBV, as well as in those with resolved infection along with vaccinated subjects. In other chronic infections (e.g. HCV, HIV and malaria), B cells, with reduced expression of the complement receptor type 2 (CD21) and of the memory marker CD27, are present in augmented frequencies. They are defined as Atypical Memory (AtM) B cells and show a reduced ability to proliferate and produce antibodies after stimulation with B cell activators(3-5).

To characterize the phenotype of HBsAg-specific B cells, we performed analysis of flow cytometric data using the algorithm Uniform Manifold Approximation and Projection (UMAP). This algorithm enables visualization of high-dimensional cell parameters in a two-dimensional map by plotting cells with similar phenotype in close proximity(35). Live total MBCs ($CD19^+CD10^-$; $CD21^+CD27^-$ naïve B cells excluded) and HBsAg-$D550^+/650^+$ MBCs from a total of 141 samples were concatenated and analyzed for the expression of markers that define MBC subsets (CD21, CD24, CD27, CD38, IgG) and their

activation status (CD19, CD23, CD39, CD69, CD73, CD95, HLA-DR, PD-1). UMAP analysis automatically separated plasmablasts ($CD24^{-}CD38^{high}$), classical IgG^{+} and IgG^{-} MBCs and AtM B cells ($CD21^{-}CD27^{-}$) (Figure 6A).
Next, we analyzed the phenotypic profile of HBsAg-specific B cells from 15 healthy vaccinated and 76 CHB patients with different disease phases by overlaying them onto the concatenated UMAP of total MBCs (Figure 6B). This revealed that HBsAg-specific B cells have generally a heterogeneous phenotype, however only in CHB patients we detected an accumulation of HBsAg-specific cells within the cluster of AtM B cells (red). While only 5.5% of HBsAg-$D550^{+}/650^{+}$ B cells from healthy vaccinated subjects were present within the AtM cluster defined by UMAP, it was 14.7% for CHB patients (Figure 6C).

Since UMAP did not separate the classical $CD21^{+}CD27^{+}$ resting (RM) and $CD21^{-}CD27^{+}$ activated (AM) MBC subsets, and only allows to compare samples stained with the exact identical antibody panel, we also quantified the phenotypic distribution of HBsAg-specific and total B cells using manual gating strategies (Supplementary Figure 2). This showed clearly that most HBsAg-specific B cells of healthy vaccinated and CHB patients display a phenotype of RM B cells and confirmed again that a significantly higher proportion of HBsAg-specific B cells of CHB patients display an AtM phenotype (16.6% CHB, 8.4% HC vac; $p = 0.01$; Supplementary Figure 2).
The frequency of HBsAg-specific B cells with an AtM phenotype was identical in different cohorts of CHB patients (Figure 6C) and was not correlated with HBsAg quantity, HBV-DNA or ALT (Supplementary Figure 3). Importantly, the modification of the B cell phenotype was not selectively present in the HBsAg-specific B cell compartment, but an enriched frequency of AtM was also detected in total B cells, which declined with progressing phases of chronic HBV infection (Figure 6D). In line with this decline, we found a positive correlation between HBV-DNA values and frequency of global AtM B cells, but not with serum HBsAg levels (Figure 6E).

In addition, we analyzed if HBsAg-specific B cells present during the HBsAg+ serological phase of acute hepatitis were associated with a preferential AtM

phenotype (Figure 6F,G). Despite the significant decline of HBV-DNA, ALT and HBsAg values during the course of acute hepatitis B, we failed to detect any consistent pattern in term of AtM phenotype frequency of HBsAg-specific B cells at different time points during acute hepatitis (Figure 6G). One patient had a very high frequency (67%) of HBsAg-specific B cells with an AtM phenotype at the onset of acute HBV infection and in this patient the HBsAg-specific B cells with an AtM phenotype decreased sharply over time (Figure 6G). Yet, the overall frequency of AtM in HBsAg-specific B cells of patients with acute HBV infection was not statistically different than in resolved or vaccinated subjects (Figure 6F). However, the global B cell compartment of acute HBV patients was significantly enriched for AtM in comparison with healthy vaccinated subjects (Figure 6H). Again, we found no consistent pattern of global AtM frequency during acute hepatitis B (Figure 6I).

Alteration of the global B cell compartment during HBV infection

The increased frequency of total B cells with an AtM phenotype supports the notion that HBV infection might influence not only the HBV-specific but also the global B cell compartment.

Evidences on the impact of HBV infection on total B cells have been previously reported(13, 36). We analyzed the transcriptional profile of the four different mature B cell subsets of healthy vaccinated and CHB patients with high viral replication and absence of inflammation (HBeAg+ chronic infection). $CD19^{+}CD10^{-}$ B cells were sorted based on their expression of CD21 and CD27 (Figure 7A). In parallel, naïve and memory populations of CD4 and CD8 T cells, using anti-CCR7 and anti-CD45RA, were also sorted to test whether differential gene expression is more pronounced on the B or T cell compartment (Figure 7B). Firstly, to better understand the properties of AtM B cells present in CHB patients, we compared their gene expression profile to classical MBCs, RM and AM (Supplementary Figure 4). This revealed that they are similar to AtM B cells found enriched in patients with persistent HIV, HCV and *Plasmodium falciparum* infection, characterized by up regulation of e.g. *TBX21*, *FCRL5*, *FCRL3*, *LILRB2* and *SIGLEC6* and down regulation of *CCR7*, *SELL* and *IL13RA*(3, 4, 37-39). Moreover, after sorting the different

global MBC subsets, we confirmed that AtM B cells enriched in CHB patients are similarly defective in expansion and differentiation into antibody secreting cells (total IgG) upon polyclonal CpG activation (Supplementary Figure 4B).

Then we analyzed if the four B cell subsets (naïve, RM, AM and AtM) of healthy and CHB patients display differences in their gene expression profile. In all the four B cell subsets combined, a total of 55 genes were differentially regulated (>2 fold different, $p<0.5$; total of 588 immune genes measured), but the AtM B cells are the more diversified by HBV infection with 32 genes differentially regulated. Strikingly, the gene expression profile of naïve and memory CD4 and CD8 T cells of CHB patients and healthy were more homogeneous with only 12 genes differentially regulated in the seven T cell populations compared.

Among the most up regulated genes in B cells of CHB patients were some related to B cell activation, such as CD83, a co-stimulatory molecule induced on B cells by activated T cells through CD40 engagement(40). CD83 was up regulated on naïve, RM and AtM B cells of CHB patients. Also all four B cell populations present in CHB patients up-regulate NFKBIZ, which is induced following BCR or TLR stimulation(41). We also noted differential expression of genes involved in B cell differentiation. DUSP4, a tumor suppressor gene in B cell lymphoma, was up regulated by 9.8-fold in AtM of CHB patients compared to healthy controls. It has a role in inhibiting cell proliferation and differentiation through the inhibition of MAP-kinase.

We also observed that *ICAM-2* was down regulated on AM and AtM B cells (CD21low B cells) of CHB patients. ICAM-2, is essential for long-lasting cognate T follicular helper (Tfh)-B cell interactions and efficient selection of low-affinity B cell clones for proliferative clonal expansion(42).

Importantly, we detected an increased expression of PD-1 within AtM B cells of CHB infected patients. A hallmark of CHB infection is the presence of exhausted HBV-specific T cells(8), but whether HBV infection can affect the global B and T cell populations remains controversial. In the 5 CHB patients studied here, differently than in AtM B cells, the activation inhibitor PD-1 was not over expressed in any of the tested T cell subsets of CHB patients.

Collectively, these data show that HBV infection appears to exert a more pronounced impact on the global B than T cell population and that functional modulation is not restricted to HBsAg-specific but to the global B cell compartment.

Rescue of HBsAg-specific B cell function with anti-PD-1 antibodies

Increased mRNA expression of *PD-1* in AtM B cells of CHB patients led us hypothesize that PD-1 blockade can further boost the HBsAg-specific B cell differentiation into antibody secreting cells. PD-1 is a negative regulator of T cell activation but it is also expressed by B cells and it can regulate human B cell maturation(43). Importantly, siRNA down regulation of PD-1 expression recovers the HIV-specific exhausted B cell response(44) and anti-PD-1 treatment of SIV infected macaques boosts functionality of antiviral T and B cells(45).

We detected that mRNA and protein expression of PD-1 are elevated on AM and specifically AtM B cells compared to the classical RM B cells (Figure 8A-C). In addition, the global AtM B cell population is enriched by HBV infection (Figure 8D) and also among HBsAg-specific B cells of chronically infected patients (Figure 8E). Since HBsAg-specific B cells express higher levels of PD-1 compared to global MBCs (Figure 8F), we tested in vitro whether PD-1 blockade can impact on the antibody secreting cell maturation of HBsAg-specific B cells present in CHB patients.

Sorted HBsAg-specific B cells of four CHB patients (3 HBeAg+ infection, 1 HBeAg+ hepatitis) and three healthy vaccinated subjects were cultured with CD40L-expressing fibroblasts with or without anti-PD-1 antibody. Figure 8G shows that anti-PD-1 treatment was able to increase the number of anti-HBs producing B cells preferentially in the CHB patients tested. The ability to mature into antibody secreting cells increased in average by 9.7-fold in the CHB patients and by 1.6-fold in healthy vaccinated subjects. Although the number of antibody producing cells was still lower in CHB patients, our data demonstrate that PD-1 blockade can partially restore their anti-HBs secretory

ability.

Discussion

We performed here a comprehensive ex vivo analysis of the frequency, function and phenotype of HBsAg-specific B cells in patients with different profiles of HBV infection.

This study allows us to begin to define the features of humoral immunity against HBV that has previously been largely neglected.

The first observation is that the quantity of circulating HBsAg-specific B cells is remarkably similar in vaccinated subjects and in patients with acute or CHB infection. We did not find any associations between the frequency of HBsAg-specific B cells and HBsAg or HBV-DNA quantity in chronic infection. Furthermore, the numbers of circulating HBsAg-specific B cells are stable during the symptomatic phase of acute HBV infection despite the robust changes in HBV-DNA and HBsAg levels characteristic of acute hepatitis B.

These findings differ from circulating HBV-specific T cell frequency that is inversely proportional to the level of HBV-DNA in adult patients with CHB infection(33) and fluctuate vigorously during acute hepatitis B(46). At first sight, this discrepancy could be interpreted as a sign that B cells have a limited role in controlling HBV, but the ability of HBV-specific MBCs to suppress HBV is indirectly supported by data from Rituximab treated subjects(47, 48). The CD20-expressing B cell depletion treatment is known to trigger HBV reactivation in a high proportion of anti-HBc+ subjects and the importance of HBsAg-specific B cells during HBV infection is confirmed by our functional data.

Indeed, the second clear observation of our work is that, while the frequency of circulating HBsAg-specific B cells is independent of the stage of HBV infection, their function is influenced by the presence of HBV infection itself and in particular by the presence/absence of serum HBsAg.

HBsAg-specific B cells of vaccinated individuals and of HBV infected patients that have resolved HBV infection (anti-HBs+ and anti-HBc+) efficiently mature in vitro with different expansion/differentiation protocols. In contrast, HBsAg-specific B cells sorted from both chronic and acute (still in the HBsAg+ phase)

HBV infected patients demonstrate clear functional defects. First, they were substantially unable to expand without the presence of feeder cells expressing CD40L. Furthermore, even though HBsAg-specific B cells were not completely exhausted and could be recovered in some CHB patients, their ability to expand/differentiate in the presence of CD40L interaction and cytokines (IL-2 and IL-21) remains defective in comparison to HBsAg-specific B cells present in HBsAg negative subjects. These data confirm the historical work performed with bulk B cells that have indicated specific functional defects of HBsAg-specific B cells in CHB patients(15-17).

However, our demonstration that HBsAg-specific B cells of CHB patients can be partially rescued in vitro suggests that a residual functionality of HBsAg-specific B cells could be maintained in vivo in the presence of T helper and Tfh cells or in the proper anatomical location (lymph node)(49). This could explain why anti-HBs antibodies can still be measured in form of immunocomplexes in CHB patients(19).

At the moment the causes of the partial functional defects of HBsAg-specific B cells are not entirely clear. HBsAg-specific B cells are likely to directly interact with the circulating HBsAg via their specific BCRs and this might provide prolonged stimulatory signals to specific B cells and result in their terminal differentiation and functional exhaustion as seen in other persistent infections(3-5). Such scenario fits with the maturation defects of HBsAg-specific B cells present in patients with HBsAg positivity, but it is somehow surprising that we could not find any associations between HBsAg quantity and functional defects. For example, the ability of CD40L feeder cells to rescue HBsAg-specific B cell function was detected in CHB patients characterized by different clinical and virological profiles (HBsAg and HBV-DNA quantity).

Similarly, the phenotype of HBsAg-specific B cells present in CHB patients only partially fits with their functional defects. An enrichment of HBsAg-specific B cells with a phenotype of atypical memory B cells is clearly present in CHB patients (our data and Burton et al. (50)). These B cells were characterized by low CD21 and high CD69 and PD-1 expression, compatible with overstimulation and exhaustion. However, in all the CHB patients studied here such phenotypic features affect on average only ~15% of the HBsAg-

specific B cells. Furthermore, HBsAg-specific B cells detected during acute hepatitis B are unable to expand/differentiate in vitro, but they did not display the phenotypic features detected in cells of CHB patients.

Analysis of gene expression at single cell level will be necessary to understand if multiple mechanisms able to suppress the in vitro maturation of the different populations of HBsAg-specific B cells exist in vivo. At the moment, the technical challenge to sort a sizeable number of HBsAg-specific B cells of different phenotype (AtM, RM and AM) preclude this analysis.

In addition, an in-depth analysis of HBV-specific T helper cells and especially Tfh cells, which provide help for B cell maturation via CD40L and IL-21 in the germinal center, in association with B cells will be important to better understand the causes of HBsAg-specific B cell defects. Recent data in mouse models have shown the importance of boosting the T helper and Tfh cell response for HBsAg seroconversion (51, 52), but data on Tfh cells in humans remain limited (53).

One other limitation of our analysis needs to be highlighted: the circulating compartment could not be the correct anatomical location for B cell quantity and functional analysis. The vast majority of B cells resides in the lymph nodes where the process of IgG somatic maturation takes place(49). In addition, HBsAg-specific B cells can also be compartmentalized not only in spleen and lymph nodes but also in HBV chronic infected livers as recently detected by Burton et al. (50). However, the finding that both circulating and intrahepatic B cells of CHB patients are enriched of atypical memory B cells (50) demonstrate that the circulating compartment is representative of the global B cell features of HBV infected patients.

We should also consider the fact that our method based on the use of recombinant HBsAg likely underestimated the real number of total HBsAg-specific B cells in HBV infected patients. Antibodies recognize not only linear but also conformational epitopes. The recombinant HBsAg used in our approach, although produced in mammalian cells(54), may have a different conformation than the natural envelope protein synthesized by infectious hepatocytes and present on the surface of the virions and of the defective HBsAg particles. In addition, circulating HBsAg could mask some HBsAg-specific B cells. Both issues make it likely that in HBV infected patients the

absolute quantity of HBsAg-specific B cells is higher than what we can measure.

Perhaps this inability to visualize the totality of HBsAg-specific B cells might partially explain the other interesting finding of our work, the fact that global, non-HBV specific B cell populations, display a gene expression profile that was altered by HBV infection and share activation of genes involved in hyperstimulation and suppression of differentiation (*CD83, DUSP-4*) that support the B cell maturation defects of HBsAg-specific B cells. It will certainly be important to precisely measure the overall size of the HBV-specific B cell repertoire. This will allow understanding whether HBV infection alters the B cell compartment irrespective of their antigen specificity or whether the analysis with fluorochrome conjugated HBsAg underestimates the HBV-specific B cell quantity. At the moment, our data indicate that both HBsAg-specific and total B cells are altered and that the total B cell alteration is more pronounced than what we detected on total T cells. This observation is in line with data showing that only few total T cells express more exhaustion markers in CHB patients than healthy individuals(55) and also with reports of preferential modifications of gene signatures of B cells induced by CHB infection(13, 36). The unanswered question is whether the modifications of both HBsAg-specific and total B cells are caused only by circulating HBsAg, other viral products or by mechanisms not linked to virological products such as an altered intrahepatic environment present in CHB patients, as suggested by Burton et al.(50).

The full understanding of the contribution of these mechanisms on B cell functionality in CHB infection will be important to select the new therapeutic strategies that aim to recover the faulted HBV-specific immunity present in CHB patients and that are now entering clinical trials(23, 29, 56).

Agents designed to reduce secretion or synthesis of HBsAg might indeed restore HBV immune function since, to our knowledge, our observation that full HBsAg-specific B cell functionality is recovered only at the time of complete negativity of serological HBsAg, represents the first clear observation that circulating HBsAg acts, in HBV infected patients, not only as decoy of anti-HBs antibodies(28), but as a modulator of HBsAg-specific B cell function. On the other hand, the lack of associations between HBsAg quantity

and HBsAg-specific-B cell defects might indicate that partial suppression of HBsAg quantity cannot be sufficient to recover B cell immunity.

In contrast, the findings that anti-PD-1 antibodies augment HBsAg-specific-B cell recovery in all CHB patients tested suggest that anti-PD1 therapy in CHB patients might be able to improve both HBV-specific T and B cell functionality. This hypothesis is supported not only by our in vitro data but also by the report of the ability of anti-PD-1 treatment to recover B cell functionality in SIV–infected macaques(45). In line with the B cell profile of HIV infected individuals, humoral immunity in SIV-persistent infection should share the functional, phenotypic and transcriptomic alteration detected by us (and by the accompanying Burton et al. paper(50)) in the HBsAg-specific and global B cell compartment of CHB patients. Thus, we believe that anti-PD-1 therapy might be able to indeed act also on the altered B cell compartment of CHB patients.

In conclusion, dual staining with fluorochrome conjugated HBsAg allowed us to directly visualize HBsAg-specific B cells in different categories of HBV infected and vaccinated subjects and decipher the impact that HBV has on humoral adaptive immunity. We observed that while HBV infection status does not impact on the frequency of circulating HBsAg-specific B cells, the functional state of global and HBsAg-specific B cells is robustly affected by HBsAg presence. The demonstration that the compromised HBsAg-specific B cells of CHB patients are amenable to functionally recover both after HBsAg clearance or through utilization of anti-PD-1 antibodies opens new therapeutic possibilities for achieving HBV functional cure and call for a precise monitoring of B cell function during future clinical trials with new agents designed to boost HBV-specific immunity.

Methods

Patients

Healthy (non HBV-infected) group comprised 18 subjects who completed a standard HBsAg vaccination (Engenirix B, 20μg HBsAg protein, (GlaxoSmithKline, Brentford, UK)) scheduled at least 5 years before entering the study with the exception of a subject who performed a new boost HBV vaccination and 5 healthy non-HBV vaccinated subjects (anti-HBs negative and anti-HBc negative). Furthermore, 21 subjects with a clinical history of resolved acute hepatitis (anti-HBc+ and anti-HBs+), 11 patients with acute hepatitis B (HBsAg+, IgM anti-HBc+, high ALT; longitudinal samples were obtained from 6 of them) and 96 treatment naïve patients with CHB infection (HBsAg+) were included. Table 1 summarizes clinical and virological parameters. CHB patients were categorized into standard phases using the criteria outlined in the EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection(32). Thus classification and denomination of CHB patients used in this work is; a) HBeAg+ chronic infection (eAg+CInf): normal ALT (< 40 IU/L), HBeAg positive and high HBV DNA; b) HBeAg+ chronic hepatitis (eAg+CHep): elevated ALT, HBeAg+; c) HBeAg- chronic hepatitis (eAg-CHep): elevated ALT, anti-HBe+; d) HBeAg- chronic infection (eAg-CHep): normal ALT, anti-HBe+, low HBV DNA(32).

Clinical and virological parameters

On recruitment to the study, anti-HBs titers (in vaccinated subjects) or viral serology and HBV DNA levels (in HBV infected subjects) were tested. HBsAg, HBeAg and anti-HBe levels were measured with a chemiluminescent microparticle immunoassay (CMIA; Abbott Architect, Abbott Diagnostics, IL) and, where available, HBV genotype was recorded. HBV DNA levels in serum were quantified by real-time PCR (COBAS AmpliPrep/COBAS TaqMan HBV test v2.0; Roche Molecular Diagnostics).

Production of fluorescently labelled HBsAg

Recombinant HBsAg (genotype A) was produced by Gilead Sciences as described(54). For labeling, purified recombinant HBsAg proteins were individually conjugated with DyLight-550 or DyLight-650 fluorochromes (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Briefly, 5 mg HBsAg (1 mg/mL) was mixed with 50 µg of each dye and incubated at room temperature (RT) for 1 hour. The labeling was neutralized by adding 50 µL of 1M Tris pH 7. Excess fluorochrome was removed by dialysis against PBS. Labeling efficiency was determined as described in the manufacturer's protocol (on average seven dye molecules per HBsAg particle).

PBMC isolation and flow cytometry analysis of total and HBsAg specific B cells

PBMCs were isolated from peripheral blood by density centrifugation using Ficoll-Hypaque Plus (GE Healthcare) and were then cryopreserved. Cells were thawed and then directly used for ex vivo flow cytometry. For staining experiments, first viability assay was performed by incubation of the cells for 10 min at RT with Live/Dead Fixable Blue Stain reagent (L23105; Life Technologies). A panel of 15 mouse anti-human monoclonal antibodies against CD3, CD10, CD19, CD21, CD23, CD24, CD27, CD38, CD39, CD69, CD73, CD95, HLA-DR, IgG and PD-1 (details of antibodies in Supplementary Table 1) in combination with HBsAg-DyLight-550 and HBsAg-DyLight-650 was used. After live/dead staining and washing steps, cells (~3 million) were incubated with a mixture containing optimum concentration of the antibodies together with the 2 labelled HBsAg reagents in Brilliant Stain Buffer (Becton, Dickinson & Company) for 30 min, on ice (50 µl in V-shaped bottom 96 well plate). Following incubation, cells were washed and fixed with 1% formaldehyde before acquisition on LSR-Fortessa flow cytometry (Becton, Dickinson & Company).

The optimum concentration of the labelled HBsAg for staining was defined by testing samples obtained from healthy vaccinated and unvaccinated donors. Different concentrations of the reagents were tested for binding to memory B cells to select a concentration that had both reasonable staining intensity on B

cells, with no binding to T cells and minimal background staining in unvaccinated subjects (Supplementary Figure 1). 1 µg of each reagent/50 µl staining mixture was chosen for the entire study. Analysis of flow cytometry data was performed using FlowJo software version 10 (Becton, Dickinson & Company) and the non-linear dimensionality reduction technique UMAP(35). We downsampled the global MBCs to 10000 cells per sample, and used a logicle transformation with w=0.1, t=500000, m=4.5, a=0.

Cell sorting

Cell sorting was performed for 3 purposes: 1) Transcriptional analysis of different subsets of total B cells, CD4+ and CD8+ T cells; 2) Functional analysis of AtM compared to naïve and classical memory B cells; 3) Functional analysis of HBsAg-specific B cells.

For Nanostring analysis on subsets of total B and T cells, PBMCs were thawed and directly stained (no enrichment) using the following mixture of Abs: a) T cell staining: anti-CD3, anti-CD19, anti-CD4, anti-CD8, anti-CD197 (CCR7) and anti-CD45RA; b) B cell staining: anti-CD3, anti-CD10, anti-CD19, anti-CD21 and anti-CD27. For sorting of HBsAg- specific B cells, B cells were pre-enriched using EasySep human B cell isolation kit (STEMCELL Technologies) to a purity of >95 %. Following live/dead staining, cells were stained with anti-CD3, anti-CD10, anti-CD19, anti-CD21, and anti-CD27 plus HBsAg-Dylight550/650. An average of 300 HBsAg-specific B cells for the experiments without feeder cells and 150 specific cells for co-culture with feeder cells were sorted. All sorts were performed with FACS Aria III machine (Becton, Dickinson & Company).

Functional analysis of HBsAg-specific B cells in vitro

All cultures were performed in Iscove modified Dulbecco medium (IMDM, Invitrogen) and 10% ultra-low IgG fetal bovine serum (Gibco, Thermofisher) supplemented with 50 µg/mL human transferrin and 5 µg/mL human insulin (Sigma-Aldrich) (now called IMDM complete medium) in 96 wells U shape-

bottomed culture plates. For functional characterization of HBsAg-specific B cells, cells were cultured in two different settings as follow:

A) Sorted HBsAg-specific B cells from pre-enriched B cells of healthy vaccinated, acute and chronic HBV infected patients were cultured in vitro with optimized conditions for B cell expansion and differentiation to antibody secreting cells reported by M. Jordan(57) with few modifications. Briefly, sorted HBsAg-specific B cells from each subject were seeded in 100 μl/well IMDM complete medium containing IL-2 (20 U/mL), IL-10 (50 ng/mL), IL-15 (10 ng/mL), 10 μg/mL phosphorothioate CpG oligodeoxynucleotide 2006 (ODN) (Invivogen) and monomeric soluble recombinant human CD40L (50 ng/ml) (all cytokines and s-CD40L were purchased from R&D Systems). Cells were cultured 4 days in this condition (step 1 to stimulate/expand B cells), then pelleted and re-suspended in new medium containing IL-2 (20 U/mL), IL-10 (50 ng/mL), IL-15 (10 ng/mL), and IL-6 (50 ng/mL). Cells were cultured for another 3 days in this condition (step 2) to enhance their differentiation to plasmablasts producing antibody. After 7 days of culture in these two steps, supernatants were collected and cells were washed and re-suspended in fresh medium as in step 2 and seeded into ELISpot plates.

B) Sorted HBsAg-specific B cells were co-cultured with irradiated 3T3-msCD40L feeder cells (3T3-mouse fibroblast cell line over-expressing CD40L) (kind gift from Prof. Mark Connors, NIH/NIAID, Bethesda, MD, USA) according to a protocol explained in detail in Jinghe Huang et al.(34). Briefly, irradiated 3T3-msCD40L cells were cultured in 96 wells U shape bottom plates to 70% confluency in DMEM medium containing 10% FBS. After 1 day, sorted HBsAg-specific B cells were re-suspended in 200ul IMDM complete medium containing IL-2 (200 U/mL) and IL-21 (50 ng/mL) and transferred to wells containing feeder cells. For PD-1 blockade experiments, 5 μg/ml anti-PD-1 (kind gift of Prof. Cheng-I Wang, Singapore Immunology Network, Singapore(58)) was added into the culture medium during the entire experiment. After 13 days, cultured cells were washed and re-suspended in fresh medium with IL-2, IL-21, and IL-6 (50 ng/mL) and then seeded into ELISpot plates.

Functional analysis of AtM compared with naïve, and classical MBCs in vitro

2000 sorted total naïve, RM, AM and AtM B cells were cultured 7 days in 2 steps with CpG polyclonal stimulation as described above. Cell culture supernatants were collected after both steps and tested to measure total IgG concentration using Human IgG total ELISA kit (eBioscience) according to the manufacturer's instructions.

ELISpot assays

ELISpot assays for the detection of anti-HBs producing cells was performed on expanded B cells based on a protocol by M. Jahnmatz et al.(59) with some modifications. Briefly, ELISpot plates (Millipore) were pre-treated with 35% ethanol, washed and coated with 1 µg/well (10 µg/ml) r-HBsAg (produced by Gilead Sciences(54)) overnight at 4°C. Plates were washed, blocked, then expanded B cells were added and incubated for 18 hours. IgG anti-HBs secreting B cells were detected by addition of biotin-conjugated goat Abs specific for human IgG-Fc (Mabtech, MT78/145), then ALP-conjugated streptavidin (BD), followed by development of plates using BCIP/NBT (Pierce, Cat. no: 34034) and counting by the ImmunoSpot image analyzer 3.2 (Cellular Technology, Ltd.).

Anti-HBs ELISA

Monolisa anti-HBs Plus kit (Bio-Rad Laboratories, Inc) was used for measuring concentrations of anti-HBs antibody in the serum of the healthy vaccinated subject, as well as in culture supernatants of sorted specific B cells after expansion in vitro, based on the kit instructions.

NanoString gene expression analysis

Four different subsets of B cells and CD4+ and CD8+ T cell subsets obtained by cell sorting, were lysed in RTL lysis buffer Qiagen (supplemented with 2-Mercaptoethanol at 1:100), at a ratio of 1 µl RTL:10,000 cells. Cell lysates from a minimum of 10,000 cells were analyzed using the preassembled

nCounter GX Human Immunology Kit and the nCounter system (NanoString Technologies, Seattle, WA), according to the manufacturer's instructions. Data analysis and heat map representation were performed with nSolver Analysis Software (version 3) provided by NanoString Technologies. Expression of 588 different genes were normalized based on the geometric means of both the supplied positive controls and the panel of 15 reference genes, as recommended by the manufacturer. Only genes that were significantly different ($p<0.05$, t-test FDR adjusted) and at least 2-fold differentially expressed between the two groups of patients or two cell subsets were considered.

Statistical analysis

Statistical analyses were performed in Prism (GraphPad) using the non-parametric two-tailed Mann-Whitney test, the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test, Spearman's Rank correlation, or Wilcoxon paired t test as stated in the figure legends. Differences were considered significant at $* = p \leq 0.05$, $** = p \leq 0.01$, $*** = p \leq 0.001$ and $**** = p \leq 0.0001$.

Study approval

Blood donors were recruited from the viral hepatitis clinic at The Royal London Hospital and acute dengue samples from Tan Tock Seng Hospital in Singapore. Written informed consent was obtained from all subjects. The study was conducted in accordance with the Declaration of Helsinki and approved by the Barts and the London NHS Trust local ethics review board and the NRES Committee London–Research Ethics Committee (reference 10/H0715/39) and by the Singapore National Healthcare Group ethical review board (DSRB 2008/00293).

Author contributions

LS performed experiments. LS, NLB and AB designed experiments, analyzed and interpreted the data, wrote the manuscript. CAD and EN contributed to

UMAP analysis. CF, MH, NN and SF prepared and provided materials. USG performed some experiments and with PTFK recruited patients, performed clinical monitoring and provided clinical expertise. All authors provided critical review and approved the manuscript.

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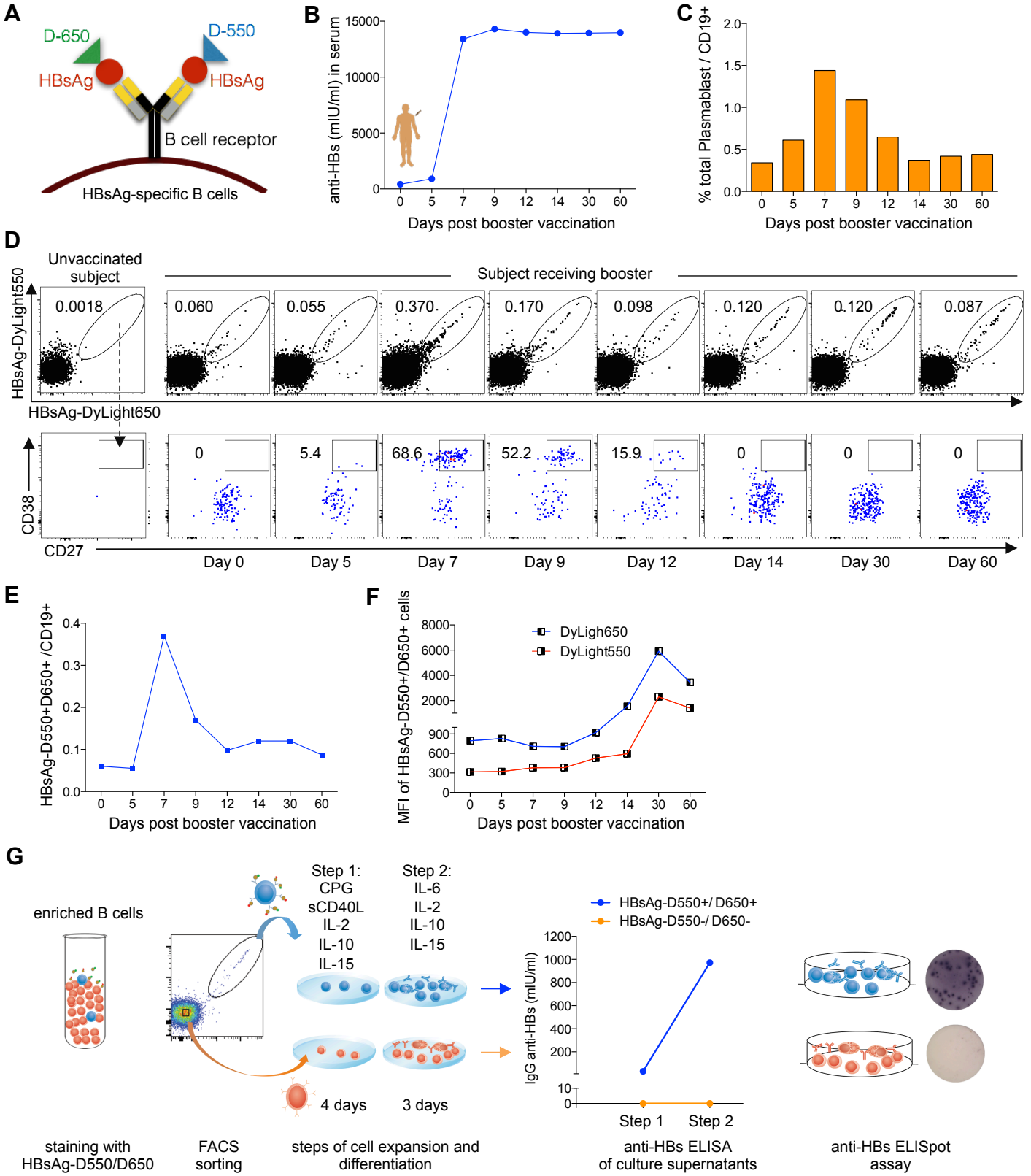


Figure 1: Fluorescently labeled HBsAg-baits bind specifically to HBsAg-specific B cells

A) Schematic representation of fluorescently labeled HBsAg-baits binding to the B cell receptor (BCR) on the surface of HBsAg-specific B cells. A healthy subject received an HBV-booster vaccination. Serum and blood samples were analyzed from day 0 to day 60 post-vaccination. **B)** Anti-HBs titers in the serum from day 0 to day 60 post-vaccination. **C)** Frequency of total plasmablasts ($CD19^+CD10^-CD21^{-/low}CD27^{++}CD38^{++}$) out of total B cells measured longitudinally. **D)** Flow cytometry plots show the frequency of HBsAg-double binding memory B cells (MBCs) (top panel) and their percentage displaying a plasmablast phenotype (bottom panel). The first plot at the left shows data of a healthy unvaccinated subject. The other plots are data of a healthy vaccinated subject at the indicated time points before and after the HBV-booster vaccination. **E)** Frequency of HBsAg-double binding MBCs over time. **F)** MFI of HBsAg-DyLight550 and HBsAg-DyLight650 on HBsAg-specific B cells at the different time points. **G)** Equal numbers of $HBsAg\text{-}D550^+D650^+$ and $HBsAg\text{-}D550^-D650^-$ MBCs were FACS sorted from PBMCs of day 60 post booster vaccination, and triggered for antibody production by CpG and sCD40L polyclonal activation. Cells were cultured in 2 different steps with different cytokine mixtures. Subsequently, anti-HBs ELISA and anti-HBs ELISpot assays were performed on culture supernatants and on the cells, respectively.

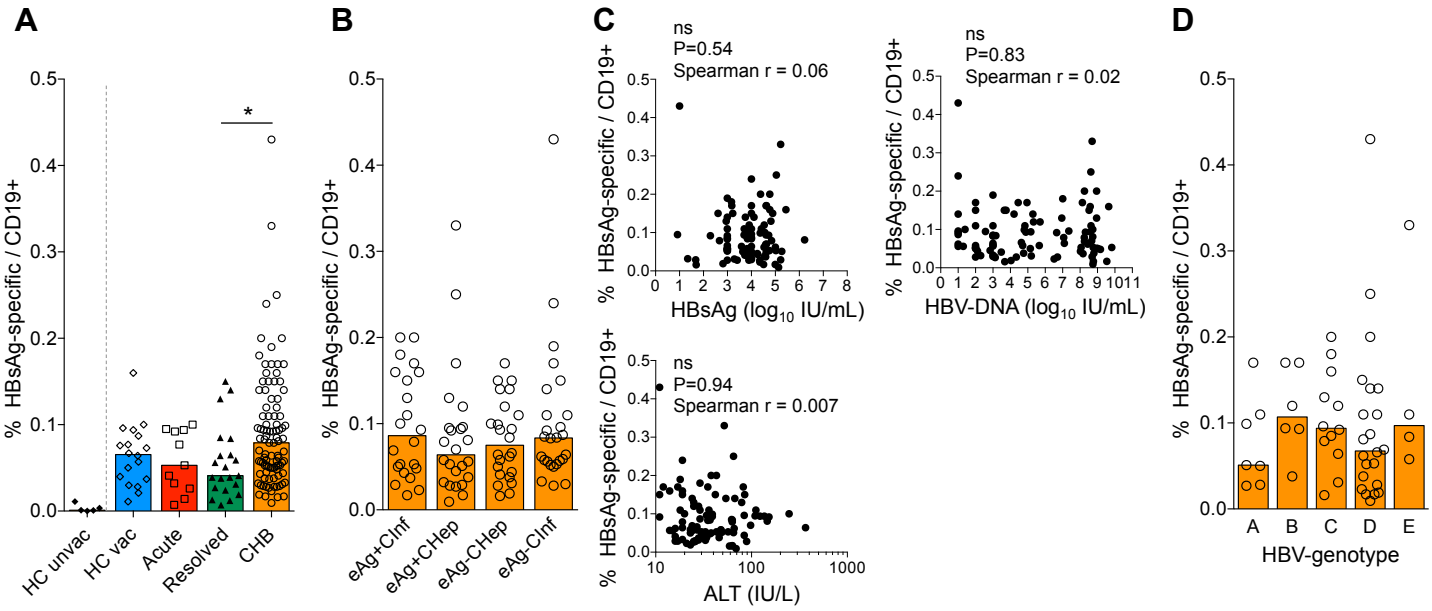


Figure 2: Similar frequency of HBsAg-specific B cells in diverse cohorts of HBV infected patients
A) Frequency of HBsAg-specific B cells in 5 healthy HBV-unvaccinated (HC unvac), 18 healthy HBV-vaccinated (HC vac), 11 acute HBV, 21 resolved HBV and 96 chronic HBV (CHB) patients out of total CD19+ B cells. **B)** Frequency of HBsAg-specific B cells out of total CD19+ B cells in different phases of CHB: 22 HBeAg+ chronic infection (eAg+CInf), 24 HBeAg+ chronic hepatitis (eAg+CHep), 24 HBeAg- chronic hepatitis (eAg-CHep) and 26 HBeAg- chronic infection (eAg-CInf). **C)** No correlation between frequency of HBsAg-specific B cells and serum levels of HBsAg, HBV-DNA and ALT. **D)** Frequency of HBsAg-specific B cells among 51 CHB patients infected with five different HBV-genotypes. Data are presented as median and statistical analysis was performed by the Kruskal-Wallis test followed by Dunn's multiple comparisons test, *p < 0.05 (A, B, D); Spearman's Rank correlation (C).

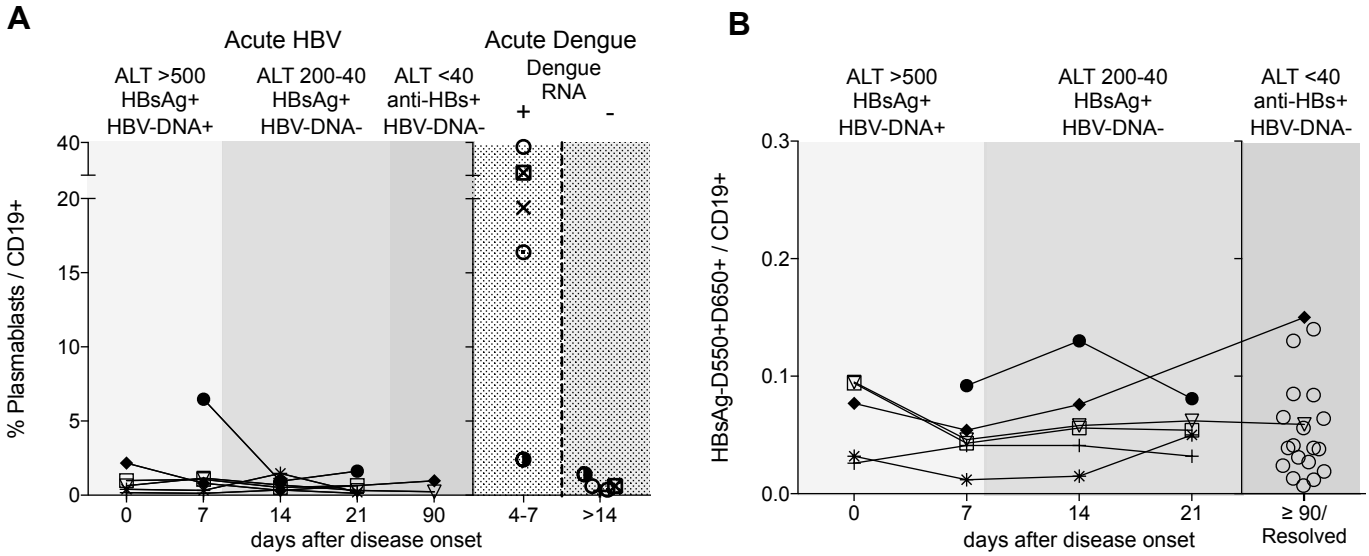
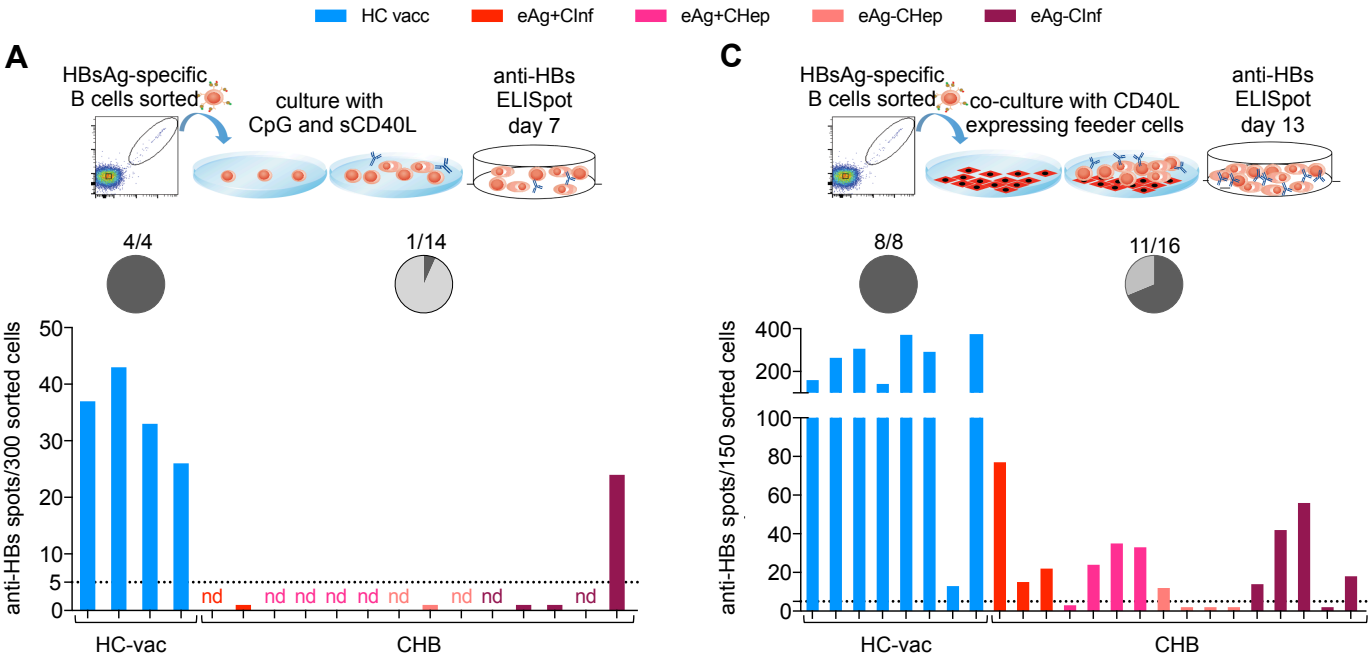


Figure 3: Longitudinal profile of B cell responses in acute HBV patients

A) Frequency of global plasmablasts ($CD19^{+}CD10^{-}CD21^{-}CD27^{++}CD38^{++}$) out of total B cells ($CD19^{+}$) were analyzed at the indicated different time points in 6 patients with acute hepatitis B (left) and in 5 patients with acute dengue infection (right). Time points are indicated as days after onset of clinical symptoms. Virological features of acute HBV and dengue are indicated at the top of the figures. **B)** Longitudinal frequency of HBsAg-specific B cells in the 6 acute hepatitis B patients compared to the frequency obtained in 21 subjects with resolved HBV infection (anti-HBc+, anti-HBs+; open circles). HBsAg-specific B cells were calculated as the frequency of memory double positive HBsAg-$D550^{+}/D650^{+}$ B cells out of total $CD19^{+}$ B cells.



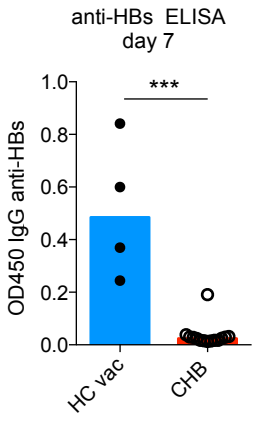


Figure 4: HBsAg-specific B cells from chronic HBV patients are dysfunctional and require co-culture with CD40L-expressing feeder cells for survival, expansion and anti-HBs production.

A) HBsAg-specific B cells from 4 healthy vaccinated donors and 14 chronic HBV patients were FACS sorted and cultured in the presence of CpG, sCD40L, IL-2, IL-10, IL-15 for 4 days and subsequently with IL-2, IL-6, IL-10 and IL-15 for another 3 days before the anti-HBs ELISpot assays were performed (nd=not done due to low cell number). The chart shows the percentage of anti-HBs-producers (dark grey=positive). **B)** Supernatants of the cultured specific B cells (from Figure 4A) were taken after 7 days culture and anti-HBs levels were measured by ELISA. Data are presented as median and statistical analysis was performed by Mann-Whitney test; ***p < 0.001. **C)** HBsAg-specific B cells from 8 healthy vaccinated donors and 16 chronic HBV patients were FACS sorted and co-cultured with CD40L expressing feeder cells in the presence of IL-2 and IL-21 for 13 days before the anti-HBs ELISpot assays were performed. The chart shows the percentage of anti-HBs-producers (dark grey=positive).

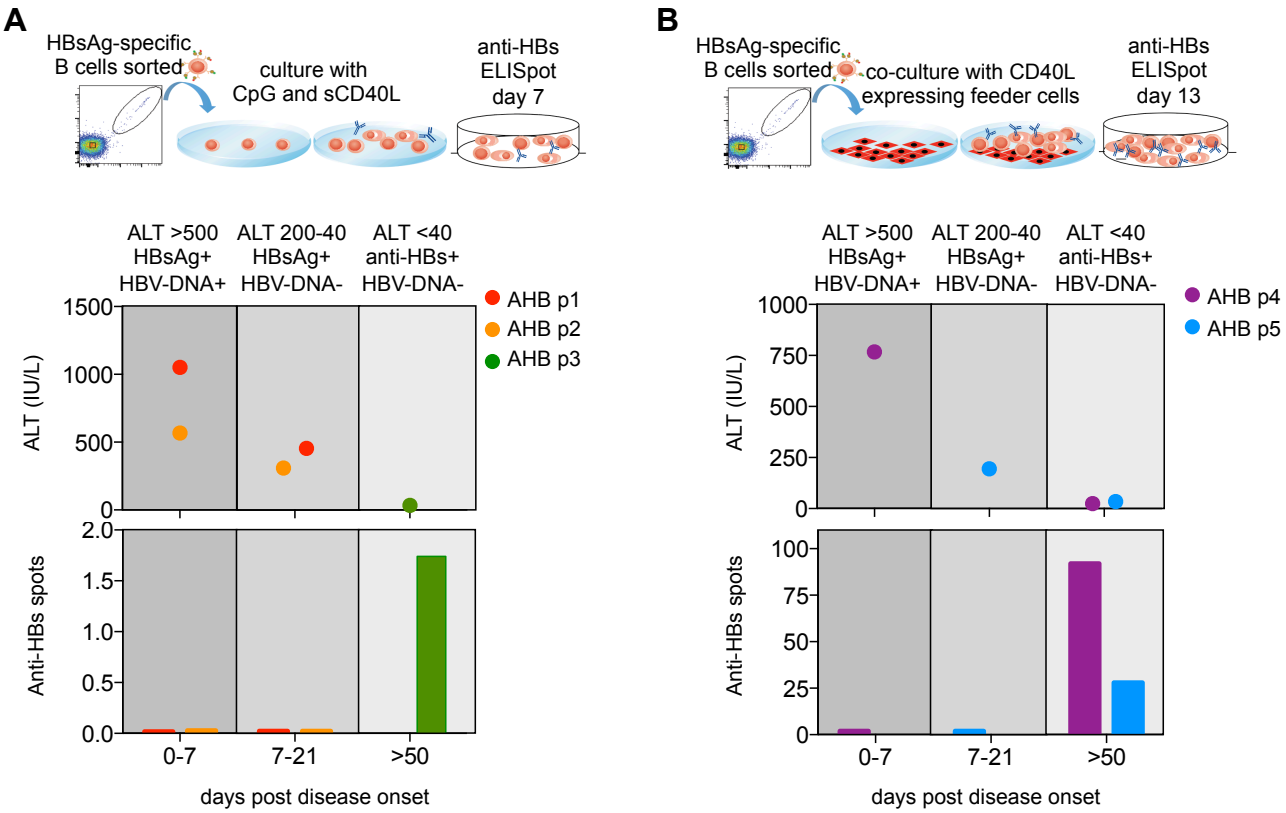


Figure 5: Functional characterization of HBsAg-specific B cells during acute hepatitis B
A) HBsAg-specific B cells were sorted from PBMCs of 3 patients at different time points after onset of acute hepatitis B (AHB). The schematic graph on the top indicates the different serological and clinical parameters (ALT, HBsAg and HBV-DNA) at which PBMCs were collected. Sorted HBsAg-specific B cells were polyclonal stimulated with CpG, sCD40L, IL-2, IL-10, IL-15 for 4 days and subsequently cultured with IL-2, IL-6, IL-10 and IL-15 for another 3 days. After 7 days, culture supernatants were collected and tested in an anti-HBs specific ELISA. Bars indicate the optical density of detected anti-HBs antibody. **B)** HBsAg-specific B cells were sorted from PBMCs of 2 additional AHB patients at the indicated 3 different time points. Sorted HBsAg-specific B cells were expanded on CD40L expressing fibroblasts with the addition of IL-2 and IL-21 for 13 days. Expanded cells were tested on anti-HBs B cell ELISpot. Bars indicate the numbers of spots obtained.

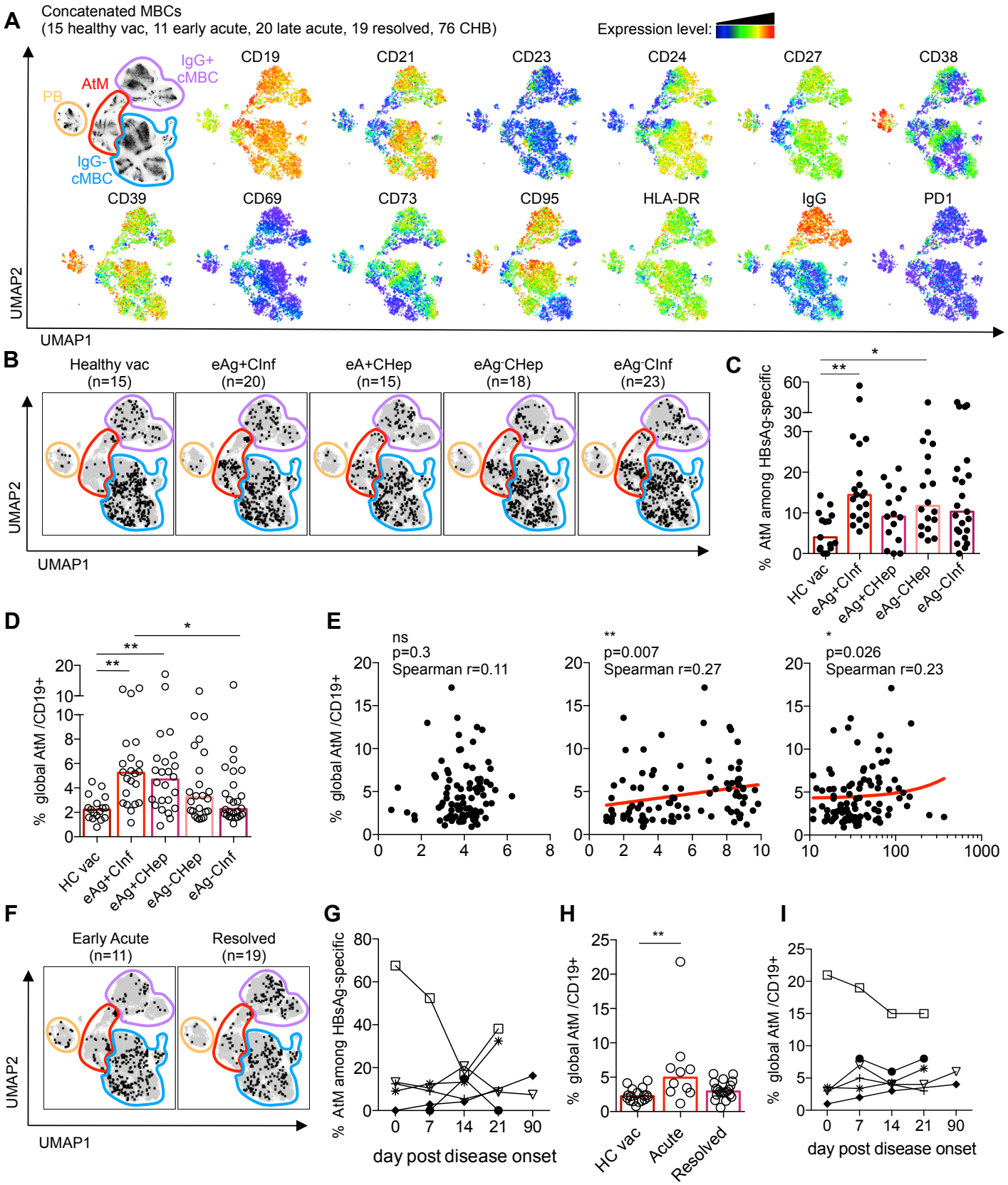


Figure 6: HBsAg-specific and global B cells of chronic HBV patients are enriched for an AtM phenotype
A) Flow cytometric data of memory B cells (MBCs) from 15 healthy vaccinated, 11 early acute, 20 late acute, 19 resolved and 76 chronic HBV patients were analyzed by the dimensionality reduction algorithm UMAP and concatenated. Four different MBC subsets were delineated (left panel) based on the expression heat maps of 13 markers (right panels). **B)** HBsAg-specific B cells from 15 healthy vaccinated, 20 HBeAg+ chronic infection, 15 HBeAg+ chronic hepatitis, 18 HBeAg- chronic hepatitis and 23 HBeAg- chronic infection patients were concatenated, normalized to their correct frequency and overlaid onto the UMAP plot of global concatenated MBCs. **C)** Frequency of atypical memory (AtM) B cells among HBsAg-specific B cells within the different cohorts. **D)** Frequency of global AtM B cells among total B cells (CD19+) present in the subjects of the different cohorts. **E)** Correlation of frequency of global AtM among total B cells with serum HBsAg (left), HBV-DNA (middle) and ALT (right) levels. **F)** Overlay of HBsAg-specific B cells on global MBCs of 11 concatenated acute (left) and 19 resolved (right) HBV patients. **G)** Percentage of HBsAg-specific B cells with an AtM phenotype in 6 acute HBV patients at different time points from disease onset. **H)** Percentage of global AtM B cells among total B cells (CD19+) of healthy vaccinated, acute and resolved HBV patients. **I)** Percentage of global AtM B cells in 6 acute HBV patients at different time points from disease onset. Bar graphs are presenting median and statistical analysis was performed by the Kruskal-Wallis test followed by Dunn's multiple comparisons test (C,D,H); Spearman's Rank correlation (E); *, P < 0.05; **, P < 0.01.

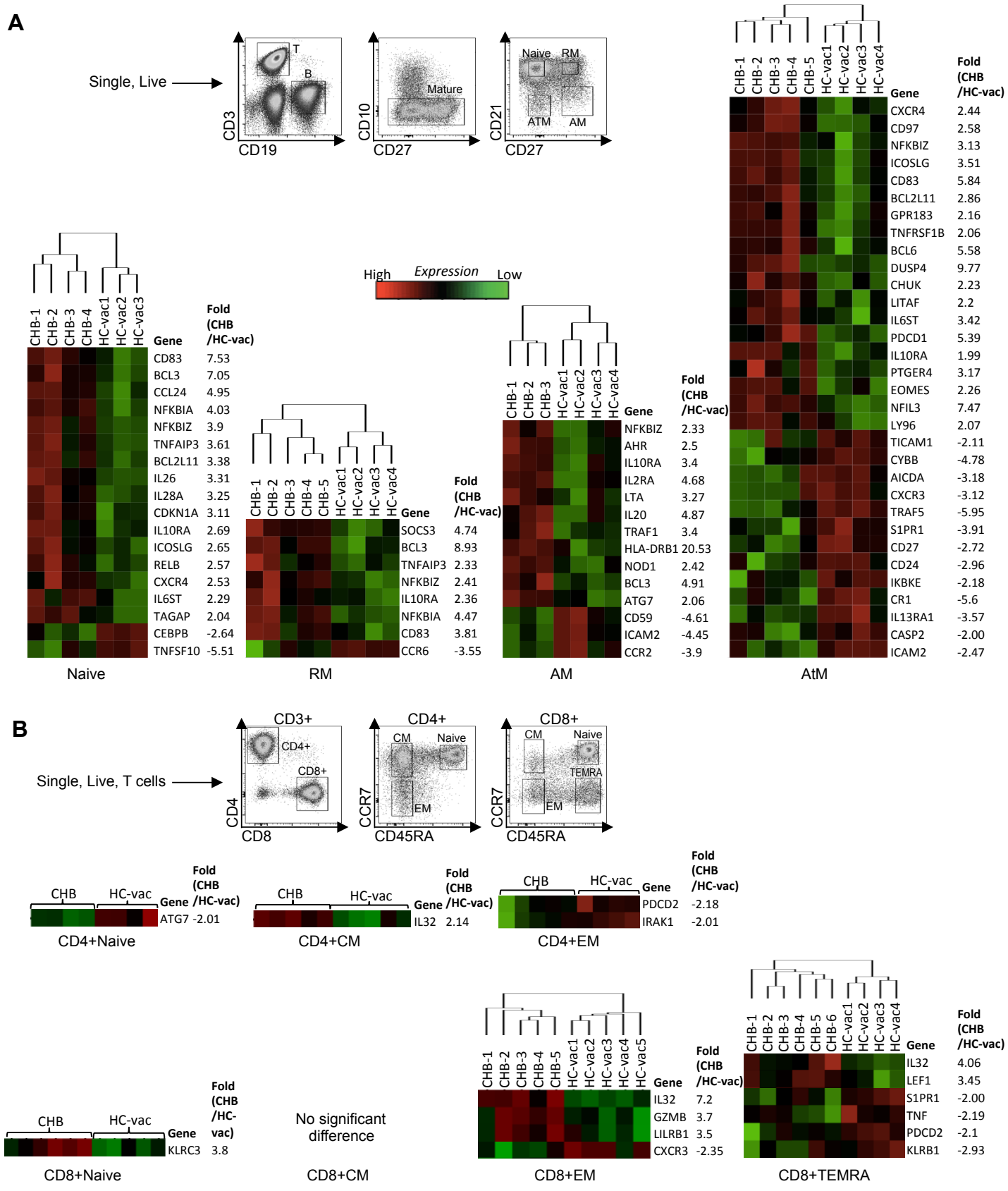


Figure 7: Transcriptional alteration of global B cell subsets by chronic HBV infection

A) Four different mature ($CD19^{+}CD10^{-}$) B cell populations from 5 chronic HBV patients (CHB; eAg+Cinf) and 4 healthy vaccinated (HC-vac) subjects were FACS sorted based on their expression of CD21 and CD27 (see gating strategy). Cells were lysed and mRNA expression levels of 588 immune related genes were measured by NanoString. Heat maps showing immune genes that are significantly different between CHB vs HC-vac ($p<0.05$, ≥2-fold different) within the four different B cell subsets, naïve, resting memory (RM), activated memory (AM) and atypical memory (AtM). **B)** Four different subsets of global CD4+ and CD8+ T cells (CD3+) were sorted from 6 chronic HBV patients and 5 healthy vaccinated controls based on their expression of CCR7 and CD45RA (see gating strategy) and analyzed by NanoString (EM=effector memory; CM=central memory). Heat maps showing immune genes that are significantly different between CHB vs HC-vac ($p<0.05$, ≥2-fold different) within the different T cell subsets.

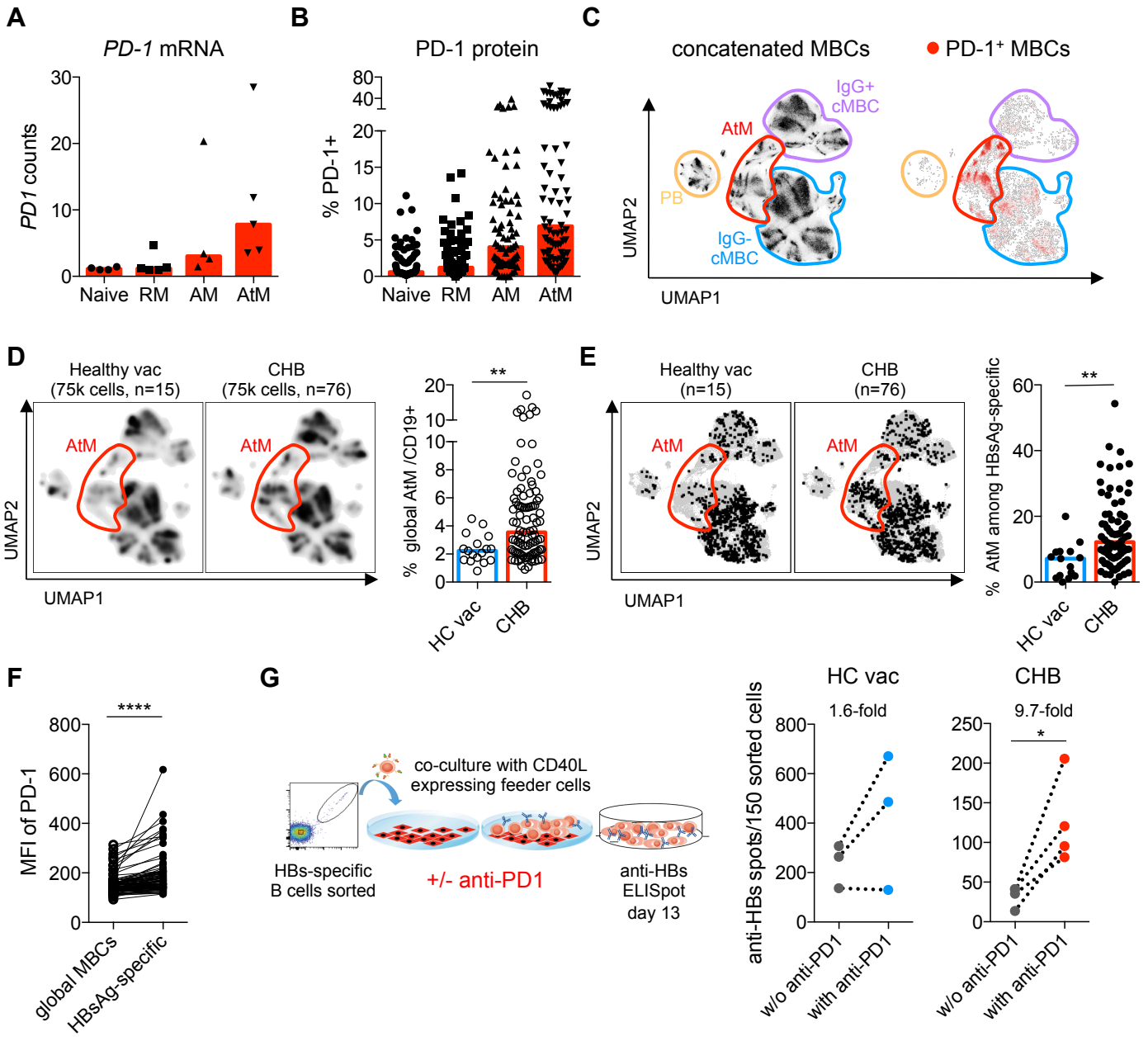
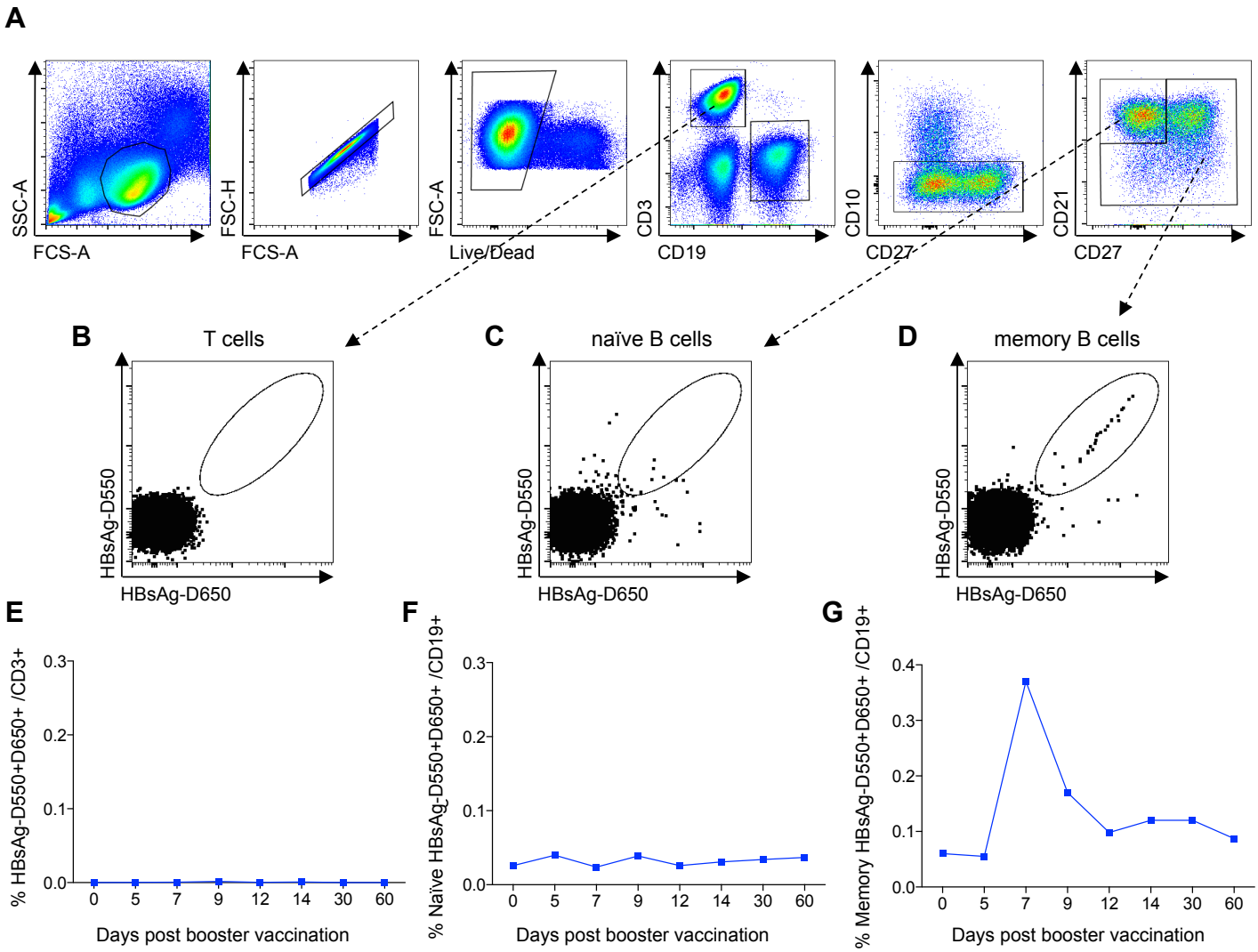


Figure 8: PD-1 blockage partially recovers dysfunctional HBsAg-specific B cells of CHB patients
A) mRNA expression of *PD-1* in the indicated B cell subsets of 5 CHB patients measured by NanoString. **B)** Surface PD-1 expression on the B cell subsets of 96 CHB patients measured by flow cytometry. **C)** Flow cytometric data of MBCs from 141 samples analyzed by UMAP and concatenated. 4 subsets of MBCs were delineated (left, plots reshown from Fig 6A) and PD-1+MBCs are shown (right). **D)** MBCs of 15 healthy-vaccinated (HC-vac; left) and 76 CHB patients (right) were downsampled to equal cell numbers and the density UMAP plots are shown; the cluster of AtM B cells is highlighted in red. Right bar graph shows percentage of global MBCs with an AtM phenotype in 17 HC-vac and 96 CHB patients. **E)** Double positive HBsAg-D550^{+}D650^{+} B cells from 15 HC-vac (left) and 76 CHB patients (right) were concatenated, downsampled to normalized frequencies and overlaid onto the UMAP plot of global concatenated MBCs; the cluster of AtM B cells is highlighted in red. Right bar graph shows percentage of HBsAg-specific B cells with an AtM phenotype in HC-vac and CHB patients. **F)** Mean fluorescence intensity (MFI) of PD-1 on global MBCs and HBsAg-specific MBCs of 96 CHB patients. **G)** Double positive HBsAg-D550^{+}D650^{+} MBCs from 4 CHB patients and 3 HC-vac subjects were FACS sorted, and co-cultured for 13 days with CD40L-expressing feeder cells in the presence of IL-2 and IL-21, with or without anti-PD-1 antibody (schematic left). Subsequently, anti-HBs secreting cells were detected by ELISpot assay (right). Average fold change in the number of anti-HBs spots are shown above the plots. Bar graphs are presenting median and statistical analysis was performed by the Mann-Whitney (D,E) and Wilcoxon paired t-test (F,G); *, P < 0.05; **, P < 0.01; ****, P < 0.0001.

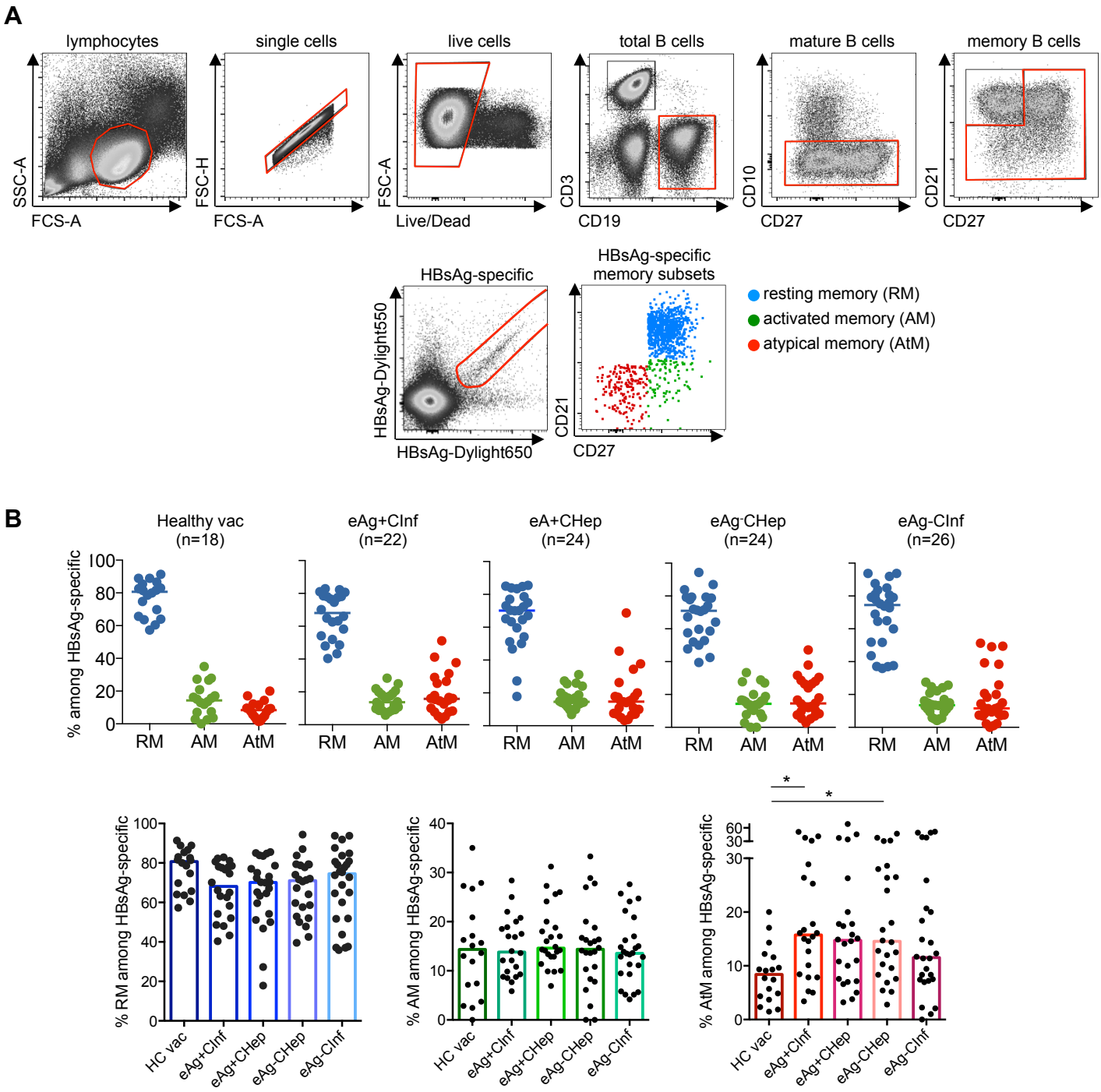
Table 1: Details of the cohorts of chronic, acute and resolved HBV patients and healthy vaccinated and unvaccinated subjects from this study

Clinical Phases of HBV infection		Number of subjects	Age (years)	HBsAg (log IU/mL)	HBV DNA (log IU/mL)	ALT (IU/L)
Chronic	HBeAg+ chronic infection (Immune tolerant)	22	23 ± 6	4.6 ± 0.45	8.5 ± 0.77	29.7 ± 11.8
	HBeAg+ chronic hepatitis (Immune reactive)	24	25 ± 4.7	3.9 ± 1.3	6.3 ± 2.5	65.6 ± 38.6
	HBeAg- chronic hepatitis (Immune reactive)	24	27 ± 2.5	3.6 ± 0.6	4.5 ± 1.4	68.8 ± 81.2
	HBeAg- chronic infection (Inactive carrier)	26	24 ± 4.2	3.5 ± 0.76	2.2 ± 0.7	28.9 ± 19.6
Acute		11 (6 subjects longitudinal)	33 ± 9.5	Positive	Positive	1865 ± 1141
Resolved (anti-HBs+, anti-HBc IgG+, HBsAg-)		21	?	Negative	Negative	ND
Healthy vaccinated		18	31.6 ± 4.2	Negative	Negative	ND
Healthy unvaccinated		5	45.8 ± 20.3	Negative	Negative	ND



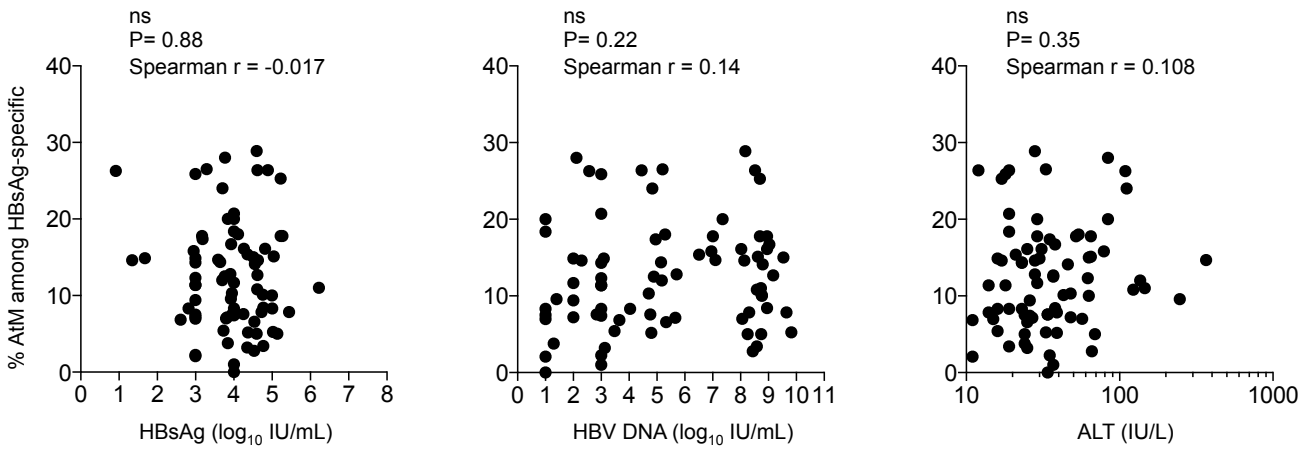
Supplementary Figure 1: Fluorescently labeled HBsAg-baits bind specifically to memory B cells after booster HBV vaccination

PBMCs of a subject receiving an HBV-booster vaccination were stained longitudinally with live/dead, anti-CD3, anti-CD19, anti-CD10, anti-CD27, anti-CD21, HBsAg-D550 and HBsAg-D650. **A)** shows the gating strategy for detection of HBsAg-specific memory B cells (MBCs); plots reshown from Fig 7A. HBsAg-D550 and HBsAg-D650 binding to T cells (**B**), to naïve B cells (**C**), and to MBCs (**D**) are shown. Longitudinal detection over 60 days post-booster vaccination of double positive HBsAg-D550^{+}D650^{+} T cells (**E**), naïve B cells (**F**) and MBCs (**G**) are shown.



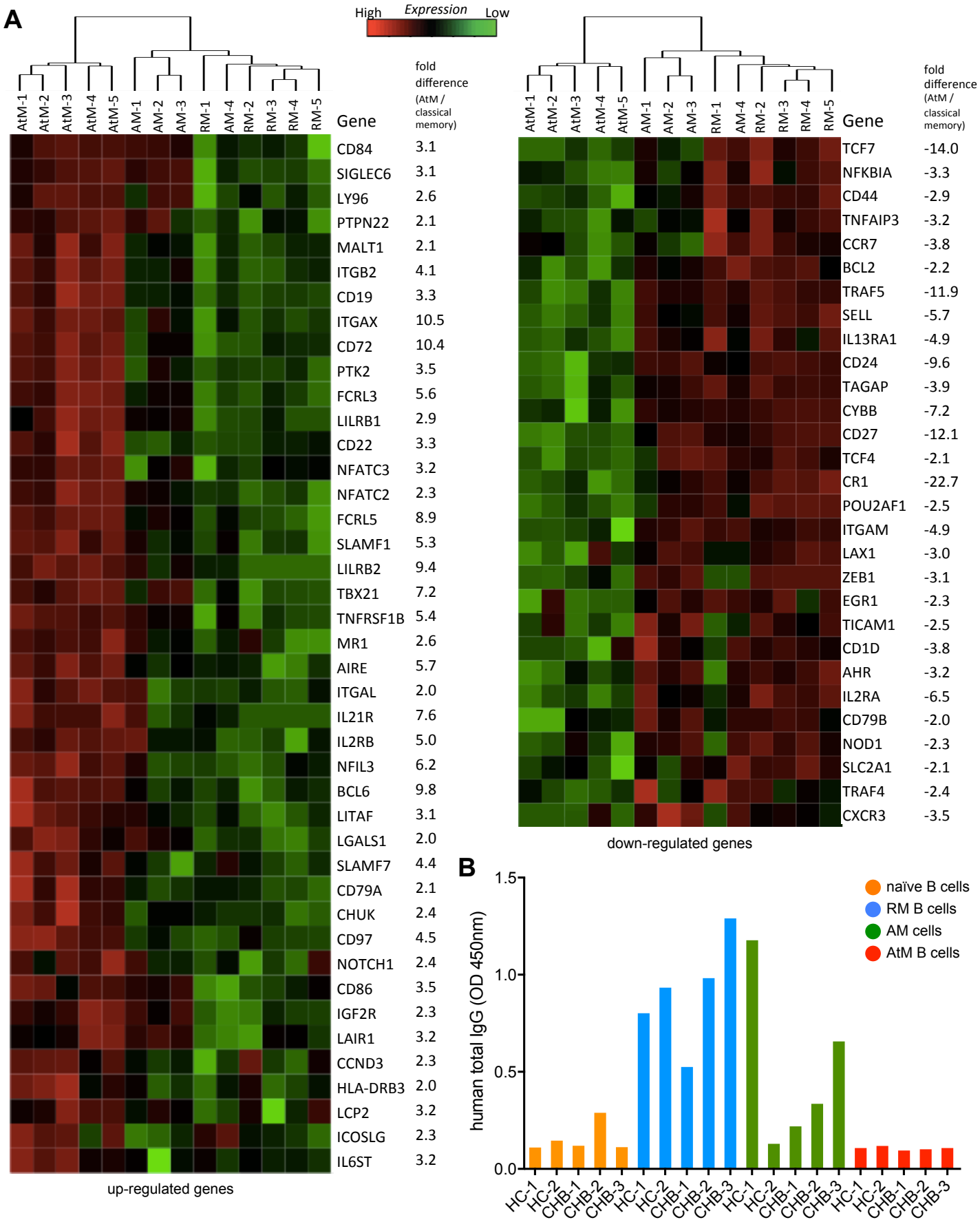
Supplementary Figure 2: Manual gating strategy and frequency of different memory B cell subsets among HBsAg-specific B cells in chronic HBV patients

A) PBMCs were stained with live/dead, anti-CD3, anti-CD19, anti-CD10, anti-CD27, anti-CD21, HBsAg-DyLight550 and HBsAg-DyLight650. Gating strategy of dual-stained HBsAg-specific B cells and their distribution into different subsets of memory B cells [resting (RM), activated (AM) and atypical (AtM) memory B cells]. Plots reshown from Fig 7A and Sfig 1A. Last two FACS plots (below; HBsAg-specific) are showing downsampled memory B cells concatenated from 96 CHB samples. **B)** Frequency of RM, AM and AtM B cells among HBsAg-specific cells in healthy and HBV patients with HBeAg+ chronic infection, HBeAg+ chronic hepatitis, HBeAg- chronic hepatitis and HBeAg- chronic infection. Data are presented as median and statistical analysis was performed by the Kruskal-Wallis test followed by Dunn's multiple comparisons test (B); *, $P < 0.05$.



Supplementary Figure 3: No correlation of HBsAg-specific B cells with AtM phenotype and virological/ biochemical parameters

Correlation of frequency of AtM among HBsAg-specific B cells with serum HBsAg (left), HBV-DNA (middle) and ALT (right) levels measured in 76 patients with chronic HBV infection. Spearman's Rank correlation.



Supplementary Figure 4: Gene expression profile and functionality of AtM B cells versus RM and AM B cells in chronic HBV infection.

A) Three different memory B cell (MBC) subsets (based on the expression of CD21 and CD27) from 5 chronic HBV patients were FACS sorted and analyzed for their immune genes expression profile by NanoString technology. A total of 588 immune genes was analyzed. **Left**: 42 genes were >2-fold up regulated ($p<0.05$) in AtM compared to the two classical MBCs. **Right**: 29 genes were >2-fold down regulated in AtM compared to the classical MBCs. **B)** 2000 naïve, RM, AM and AtM B cells were FACS sorted and cultured in the presence of CpG, sCD40L, IL-2, IL-10, IL-15 for 4 days and subsequently with IL-2, IL-6, IL-10 and IL-15 for another 3 days. Total human IgG was measured in the supernatants by ELISA assay.

Supplementary Table 1: List of antibodies used in this study

Antigen	Fluorochrome	Manufacturer	Clone	Catalog number
CD3	BV605	BioLegend	OKT3	317322
CD3	BV500	BD Biosciences	UCH-T1	561416
CD10	PE-CF594	BD Biosciences	HI10A	562396
CD19	BV510	BD Biosciences	SJ25C1	562947
CD21	BV421	BD Biosciences	B-LY4	562966
CD23	BUV395	BD Biosciences	M-L233	564203
CD24	PerCP-Cy5.5	BioLegend	ML5	311116
CD27	BV650	BD Biosciences	L128	563228
CD38	PE-Cy7	BioLegend	HB-7	356608
CD39	BV711	BD Biosciences	TU66	563680
CD69	APC-Cy7	BioLegend	FN50	310914
CD73	BV605	BD Biosciences	AD2	563199
CD95	BV737	BD Biosciences	DX2	564710
HLA-DR	AF700	BioLegend	L243	307626
IgG	BV786	BD Biosciences	G18-145	564230
CD279 (PD-1)	FITC	BD Biosciences	MIH4	557860
CD4	BV650	BD Biosciences	SK3	563876
CD8	PE-Cy7	BD Biosciences	RPA-T8	557746
CD197 (CCR7)	Biotin (plus APC-streptavidin from BD Biosciences; cat no 554067)	BD Biosciences	3D12	557648
CD45RA	FITC	BD Biosciences	HI100	555488