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TITLE: **Effects of Hepatitis B Surface Antigen on Virus-specific and Global T cells in Patients with HBV Infection**

SHORT TITLE: Long-term HBsAg deletes HBs-specific T cells

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ABBREVIATIONS:

AIC, Akaike's Information Criterion; BTLA, B and T lymphocyte attenuator; CHB , chronic hepatitis B; CPG, clinical practice guidelines; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CyTOF, cytometry by time-of-flight; FoxP3, forkhead box P3; HBc, core; HBeAg, hepatitis B e antigen; HBpol, polymerase; HBs, envelope; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBx, X; IFN, interferon; KLRG1, killer-cell lectin like receptor G1; MAIT cells, mucosal-associated invariant T cells; NA, nucleos(t)ide-analoge; NK cells, natural killer cells; PD-1, Programmed cell death protein 1; PMA, phorbol-12-myristate-13-acetate; qHBsAg, quantitative hepatitis B surface antigen; siRNA, small interfering RNA; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; UMAP, Uniform Manifold Approximation and Projection

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DISCLOSURES:

AB participates in advisory boards on hepatitis B virus immune therapy for Gilead Sciences, Spring Bank Pharmaceuticals, Vir Biotechnology, Abivax, and Jiangsu Simcere Pharmaceutical. He is also a cofounder of Lion TCR, a biotech company developing T cell receptors for treatment of virus-related cancers. PTFK has collaborative grant funding from Gilead Sciences, participates on the advisory board of and provides consultancy to Gilead Sciences and Janssen, and is an investigator for industry-led trials with Gilead Sciences, Janssen, Alere, and Assembly Biosciences. All other authors declare that there is no conflict of interest regarding the publication of this article.

AUTHORS CONTRIBUTIONS:

NLB, SP, PTFK and AB conceived the overall study. NLB, MH and AB designed experiments, analysed and interpreted the data, and wrote the manuscript. NLB, USG, MH, LR, KK, DZMT, and RA performed experiments. USG, PTFK, NH, MZ, SX and YuC recruited patients, performed clinical monitoring, and provided clinical expertise. YaC and EWN provided expertise for CyTOF experiments. CAD contributed to UMAP analysis. AH performed multivariate analysis. AT advised on data presentation. USG, PTFK and SP revised the manuscript.

Abstract

Background & Aims: Chronic hepatitis B virus (HBV) infection is characterized by the presence of defective viral envelope proteins (hepatitis B surface antigen, HBsAg) and the duration of infection – most patients acquire the infection at birth or during the first years of life. We investigated the effects of these two factors on patients' global lymphocytes and HBV-specific T cells.

Methods: We collected blood samples and clinical data from 243 HBV-infected patients of different age (3 – 75 y) and of various ethnicities in the United Kingdom and the People's Republic of China. We measured virological (HBV DNA, HBV genotype, HBsAg, HBeAg) and clinical parameters (ALT) and isolated peripheral blood mononuclear cells (PBMC). In 48 chronically infected patients with varying levels of serum HBsAg we analyzed global T- and natural killer (NK)-cell expression of 40 markers by mass cytometry. PBMC of 189 chronically infected and 38 patients with resolved infection were incubated with overlapping HBV-peptide libraries to identify HBV-specific T cells by interferon gamma ELISpot assays or by flow cytometry. To analyze which covariates are associated with HBV-specific T cell responses, we fit a multivariate linear regression model and performed variable selection using Akaike's Information Criterion (AIC).

Results: Although global T and NK cell phenotypes and functions did not change with level of serum HBsAg, numbers of HBs-specific T cells were positively correlated with serum levels of HBsAg (r=0.3367; $P<.00001$). However, after performing variable selection using AIC, the final multivariate linear regression model identified the age of the patients as the only significant covariate associated with numbers of HBs-specific T cells ($P=.000115$). In patients younger than 30 y, HBs-specific T cells constituted

28.26% of the total HBV-specific T cells; this value decreased to 7.14% in patients older than 30 y.

Conclusions: In an analysis of immune cells from patients with chronic HBV infection, we found the duration of HBsAg exposure, rather than the absolute quantity of HBsAg, alters the profile of HBV-specific T cell responses. Although the presence of HBs-specific T cells might not be essential for the clearance of HBV infection in all patients, our study indicates that therapeutic intervention designed to restore anti-HBV immunity should consider patients younger than 30 y.

Keywords: Hepatitis B virus; HBsAg; T cell; immune response

Introduction

An estimated 250 million people worldwide are infected with HBV, a non-cytopathic hepatotropic DNA virus resulting in nearly 1 million deaths per year from the complications of chronic hepatitis[1]. A peculiar feature of HBV is its ability to produce not only a progeny of infectious virions, but also truncated forms of the nucleocapsid protein (HBeAg) and defective non-infectious particles comprised of different forms of surface proteins (HBsAg)[2]. These non-infectious viral proteins (HBeAg and HBsAg) are secreted into the serum and have been shown to impact HBV host immunity. For example, HBeAg has been shown, in animal models, to play a role in the suppression of nucleocapsid-specific T cells in vertically HBV-infected new-borns[3,4].

The defective particles made by surface proteins (~10^{14}/ml) may have a broader effect on patients' immune cells. It is likely that they act primarily as a decoy for protective anti-HBs antibodies, since they outnumber the infectious virions 10^3 to 10^6-fold and are collectively measured in patients' sera as HBsAg in concentrations that can exceed 10-20 µg/ml (~10,000 IU/ml)[5]. Besides, HBsAg is associated with functional defects of HBs-specific B cells[6,7], and may also have an impact on HBV-specific T cells. Antigens presented by hepatocytes modulate the priming of T cells[8] and the continuous stimulation by antigens presented by both hepatocytes and professional antigen presenting cells could exhaust and subsequently delete T cells. This seems to occur in HBV-transgenic mice[9] and recent detailed analysis of HBV-specific T cells in adult patients with chronic hepatitis B (CHB) showed a preferential detection of core- and polymerase-specific T cells, but seldom HBs-specific T cells[10-12]. HBsAg has also been suggested to have an impact on innate immune responses[13], especially on dendritic cell function[14], although this last hypothesis has not been confirmed by other

data[15].These lines of evidence have generated the hypothesis that HBsAg has a generic immunosuppressive activity and a therapeutic reduction of quantitative HBsAg (qHBsAg) is a necessary step to restore HBV-specific immune responses and to reach functional cure[16]. This idea has garnered great popularity and therapeutic efforts to reduce HBsAg have led to the development of compounds with the ability to inhibit HBsAg production[17,18], despite the fact that a direct proportional relationship between immunological defects and qHBsAg has never been demonstrated; moreover only complete HBsAg loss is associated with recovery of HBV-specific T and B cell responses[6,19].

A robust understanding of the duration of HBsAg exposure and its impact on anti-HBV host cellular immunity is also lacking. This is surprising given that CHB infection is typically acquired at birth and the lifelong persistence of high quantities of HBV antigens is a peculiar trait of CHB infection[5]. Moreover, evidence from animal models showed that duration of viral antigen exposure progressively deletes antiviral T cells[20]. This could be explained by the general consensus that an awakening of the patients' immune response against HBV was only present in adulthood (immune active phase). However, the demonstration that HBV-specific immune responses[21] and the pathological consequences of CHB[22] are already detectable in young patients with chronic infection, provide the rationale to analyse both the effect of HBV antigen quantity and its duration of exposure on patients' global lymphocytes and HBV-specific T cells.

Material and Methods

Study approval: Clinical assessment and peripheral blood sampling was performed at the Royal London Hospital (London, UK) and at the Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, China), in accordance with the Declaration of Helsinki. Written informed consent was obtained, and the study was approved by the London Harrow Research Ethics Committee (reference 10/H0715/239), the East London & The City Research Ethics Committee (reference P/01/023) and the Clinical Trial Research Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University (reference [2015]2-206).

Patients: 205 patients with chronic HBV infection were selected for this study. Furthermore, 27 individuals with resolved acute HBV (anti-HBc positive and anti-HBs positive), and 11 patients with a clinical history of resolved chronic HBV infection were included. Figure 1 and Tables 1 and 2 summarize the clinical and virological parameters of all patients.

Clinical and virological parameters: On enrolment, viral serology and HBV DNA levels were tested in all patients (COBAS AmpliPrep/COBAS TaqMan HBV test v2.0; Roche Molecular Diagnostics). HBsAg, HBeAg, and anti-HBe levels were measured with the chemiluminescent microparticle immunoassay (CMIA; Abbott Architect, Abbott Diagnostics), and HBV genotype was recorded where available. All patients included were HCV, HDV and HIV negative.

PBMC isolation: PBMCs were isolated from peripheral blood by Ficoll gradient and cryopreserved.

Staining for CyTOF: To avoid possible batch effects from running multiple experiments, PBMCs of all 48 CHB patients were only divided into 2 sets of 24; thawed and stained at the same time. Cells were washed and each sample was divided to perform 2 assays; either stained directly or stimulated for 4 hours with phorbol-12-myristate-13-acetate (PMA; 150 ng/ml) and ionomycin (1 μM) in the presence of brefeldin A and monensin (eBioscience). Staining for cell viability, surface and intracellular makers with metal tagged antibodies (Table S1), as well as sample labelling with dual mass-tag cellular barcodes and DNA staining were performed as described in detail previously[23]. As only a limited number of samples can be acquired on the same day, stained samples were frozen in 3 batches to be thawed and acquired over the subsequent weeks as described in detail previously[23]. For each batch, the same internal control PBMCs of a healthy donor were included as well as 8 experimental samples (equal distribution of CHB patients with high and low HBsAg level, HBeAg positive/negative and treatment naïve/nucleos(t)ide-analogue [NA] therapy). On the day of mass cytometry acquisition, on a CyTOF2 instrument, one batch of stained cells was thawed at room temperature, washed, diluted and mixed with EQ Four Element Calibration Beads (Fluidigm).

CyTOF data analysis: After acquisition, parameters were normalized with EQ beads (Fluidigm) and 0 values were randomized using a uniform distribution of values between –1 and 0 in an R script. Cells were manually de-barcoded using FlowJo version 10 (BD). Live lymphocytes from all samples were down-sampled to 100,000 events to normalize the contribution between donors. High-dimensional data analysis

was performed with the nonlinear dimensionality reduction technique Uniform Manifold Approximation and Projection (UMAP)[24] and FlowJo.

Flow cytometry: PBMCs were thawed and stained for live cells followed by surface staining for 30 minutes on ice. For global T cell analysis, cells were then permeabilized with FoxP3 staining kit and stained for intracellular markers. All antibodies are listed in Table S2. Cells were acquired on an LSR-Fortessa cytometer (BD) and analysed with FlowJo version 10 (BD).

HBV peptide libraries: Libraries of 313 fifteen-mer peptides overlapping by 10 amino acids were used to identify HBV-specific T cells. The peptides covered the whole proteome of HBV genotypes B, C, and D (GenBank AF121243, AF 112063, AF 21241, respectively) and were purchased from Mimotopes. Their purity was above 80%, and their composition was confirmed by mass spectrometry. Peptides were pooled as described previously[25]. The peptide libraries were matched to the closest HBV genotype of each patient, as indicated in Table 2.

T cell culture: For the in vitro assays, T cell lines were generated as follows: 20% of PBMCs were pulsed with 10 µg/ml of each of the overlapping HBV peptides for 1 hour at 37°C, subsequently washed, and co-cultured with the remaining cells in AIM-V medium supplemented with 2% AB human serum (both Gibco; Thermo Fisher Scientific). T cell lines were cultured for 10 days in the presence of 20 U/ml of recombinant IL-2 (R&D Systems).

ELISpot: ELISpot assays for the detection of IFNγ–producing cells were performed on in vitro-expanded T cell lines using HBV peptides pooled into the following mixtures: X, core, envelope 1 and 2, polymerase 1, 2, 3 and 4. T cell lines were incubated overnight at 37°C with pools of HBV peptides (5 µg/ml of each peptide), where final DMSO concentrations did not exceed 0.5%. IFNγ ELISpot assays (Millipore) were performed as described previously[25].

Intracellular cytokine staining: Expanded T cell lines were stimulated overnight at 37ºC with or without HBV peptide pools (5 µg/ml of each peptide), in the presence of 2 µg/ml Brefeldin A (Sigma-Aldrich). As a positive control, cells were stimulated with PMA (2 ng/ml) and ionomycin (1 µg/ml). Cells were stained with the LIVE/DEAD fixable far red dead cell stain kit (ThermoFisher) and surface antibodies (Table S3). Cells were subsequently fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen), stained with intracellular antibodies (Table S3), acquired on an LSR-Fortessa cytometer (BD) and analysed with FlowJo version 10 (BD).

Multivariate analysis: To analyse which covariates are associated with HBs-specific T cell responses, we fit a multivariate linear regression model and performed variable selection using Akaike's Information Criterion (AIC). T cell responses have been transformed as log(SFU+1) to make the distribution of the response closer to the Normal. We included a set of 9 potential covariates in the model: Gender, HBeAg, Age, HBsAg, HBV-DNA and ALT, as well as the interactions between Age and HBeAg, HBV-DNA and HBeAg, HBsAg and HBeAg.

Statistical Analysis: Statistical significance was determined by GraphPad Prism using tests as stated in the figure legends. $P \leq 0.05$ was considered significant.

Results

Patient selection and experimental procedures.

We selected 243 HBV-infected patients. Global circulating T and NK cells were studied in 48 patients (**Table 1**), while HBV-specific T cells were analysed in 189 patients with CHB (HBsAg+) and in 38 patients with resolved infection (anti-HBs+; 27 after acute HBV infection, 11 following documented CHB infection; **Table 2**). **Figure 1A** depicts the number of individuals who were studied with different immunological techniques. CHB patients were characterized by a wide range of qHBsAg (negative to 10^6 IU/ml) with a preponderance of levels between 10^3 and 10^5 IU/ml. A broad age distribution was represented (3 to 75 years), with a median age of 29. Notably, our study included a considerable number of patients above 50 years of age (n=30) and equally a sizeable number of under 20-year-olds (n=34). As expected[5], HBsAg levels declined with age (**Fig 1B**). In accordance with the natural profile of HBV infection, CHB patients with HBeAg positive chronic infection (n=34) or chronic hepatitis (n=47) were younger (median age 24) than patients classified with HBeAg negative chronic infection (n=35; median age 25) or chronic hepatitis (n=89; median age 44; **Fig 1C**). The CHB cohort was comprised of patients with no (alanine aminotransferase [ALT] <40 IU/mL), mild (ALT 40-80 IU/mL) and severe (ALT >80 IU/mL) hepatitis equally distributed across age (**Fig 1D**).

The impact of HBsAg quantity on global T and NK cells.

We first analysed the impact of varying levels of HBsAg on global non-antigen-specific T and NK cells. Forty-eight patients with CHB infection were selected based on their serum qHBsAg (2 to 365,623 IU/ml). Equal numbers of HBeAg positive and negative patients were represented, including treatment-naïve patients and those undergoing

NA therapy (**Table 1**). CyTOF technology was utilized to simultaneously analyse their PBMCs for the expression of 40 markers (**Table S1)** involved in T and NK cell activation, differentiation, exhaustion, in addition to the production of different cytokines and cytotoxicity-associated molecules, both spontaneously and following stimulation.

CyTOF data were analysed with the high-dimensionality reduction algorithm UMAP, which maps cells with related phenotypes in close proximity, and thus automatically separated all major lymphocyte subsets[24] (**Fig 2A, S1A**). UMAP density plots (**Fig 2B**) and gated lymphocyte subsets (**Fig S1B**) displayed no difference in the frequencies of CD4 and CD8 T cells of naïve, memory and effector phenotypes; similarly, no difference in MAIT, NK, B and mononuclear phagocyte cell subsets was detected in CHB patients with varying levels of HBsAg. **Figure 2C** shows the median expression level of 33 markers in 20 different lymphocyte subsets in patients with low (<1,000 IU/ml; n=14) and high (>10,000 IU/ml; n=13) levels of HBsAg; notably no significant difference was observed. Also, applying correlation analysis of all markers in all subsets with qHBsAg, we did not find any association (data not shown).

Moreover, the production of different cytokines and cytotoxicity-associated molecules was not altered by qHBsAg in different T and NK cell subsets (**Fig 2D, 2F**), and no correlation with the expression of several immunoregulatory receptors was identified[26], including PD-1, CTLA-4, TIM-3, KLRG1 and CD160 (**Fig 2E**). To confirm that qHBsAg is not affecting global T cell exhaustion or inhibition, we analysed regulatory T cell frequency ($CD4^+FoxP3^+CD25^+$) and additional immunoregulatory receptors, including

BTLA and TIGIT, by flow cytometry. Similarly, these markers did not correlate with qHBsAg (**Fig S1C**).

Comparative analysis of the T and NK cell features was performed either by considering only qHBsAg as a distinct parameter among all the 48 CHB patients or by separating the patients based on HBeAg and/or NA-treatment status, which importantly did not reveal any significant correlation (data not shown). However, our method was able to identify phenotypic alterations in immune cell subsets that are known to be associated with age, serving as an internal positive control (**Fig S1D**). Taken together, these data show that qHBsAg does not impact any generic immunomodulatory function on T and NK cells.

The impact of HBsAg quantity and patient age on HBV-specific T cells.

Having analysed the effect of HBsAg on global T and NK cells, we studied the impact of qHBsAg and patients' age on HBV-specific T cells in terms of frequency and fine-specificity. We stimulated PBMCs of 189 CHB patients with a pool of overlapping peptides covering the whole HBV proteome, using peptide libraries matching the HBV genotype of each patient. After 10 days of in-vitro expansion, the ability of T cells to produce IFNγ following stimulation with distinct peptide pools covering single HBV proteins (envelope, core, polymerase and X; HBs, HBc, HBpol and HBx respectively) was tested using ELISpot assays (**Fig 1A**).

We first quantified HBV-specific T cells in CHB patients classified according to the nomenclature defined in the EASL 2017 clinical practice guidelines (CPG), as shown in **Figure 3A**. No significant differences were detected on total HBV-specific T cells.

Yet, we found significantly more HBs-specific T cells in patients with HBeAg positive chronic infection versus HBeAg negative chronic hepatitis, and a trend towards an increase in the quantity of HBcore-specific T cells in HBeAg negative CHB patients. A previous study of HBV-specific T cells in adult CHB patients[27] similarly showed an increased HBcore-specific T cell response in HBeAg negative patients, but did not reveal any significant association of HBs-specific T cells with CHB disease phase. However, distinct from the study by Park et al.[27], our patient population includes a broader age-range and is not restricted to adult patients (**Fig 1B, 1D**). We then directly correlated the quantity of HBV-specific T cells with qHBsAg (**Fig 3B**) and age (**Fig 3C**). The quantity of T cells specific to the whole HBV proteome did not show any significant correlation (**Fig 3B, 3C**). However, when we decipher T cells specific to the individual proteins, the magnitude of HBc-, HBpol- and HBx-specific T cells appeared unaffected (**Fig 3D**), whereas the quantity of HBs-specific T cells correlated positively with HBsAg levels (**Fig 3B**) and negatively with increasing age (**Fig 3C**). Importantly, after performing variable selection using AIC, the final multivariate linear regression model (described in Material and Methods) identified the age of the patients as the only significant covariate associated with numbers of HBs-specific T cells (P = 0.000115; **Table S4**). Quantity of HBs-, HBc-, HBpol- and HBx-specific T cells in individual CHB patients is displayed in **Figure 3D**. While HBc-, HBpol- and HBx-specific T cell responses were uniformly distributed across age, robust HBs-specific T cell responses were detectable almost exclusively in the first three decades of infection (**Fig 3D**), when CHB patients displayed high qHBsAg (**Fig 1B**). Intracellular cytokine staining of CD4 and CD8 T cells in CHB patients with an ELISpot response to HBs-peptides showed that such responses were mediated by both CD4 (11/20) and/or CD8 (13/20) T cells (**Fig S2**).

Remarkably, a reduction of HBs-specific T cells was observed in patients over the age of 30, despite a greater number of patients in this group being treated with NA therapy (**Table 2**). This is at odds with the concept that NA therapy should induce partial recovery of HBV-specific T cell responses[28]. Importantly, HBV-specific T cells were not increased in CHB patients with liver inflammation; on the contrary, we observed a trend towards a negative association with ALT levels, which reached significance for HBcore-specific T cells that were reduced by 3.9-fold in patients with severe hepatitis (**Fig. S3**).

***HBs-specific T cell dominance in CHB patients of differing age*.**

We then analysed the composition and dominance of HBV-specific T cells and calculated, for individual patients with a detectable HBV-specific T cell response (164/189), the percentage of T cells recognizing the distinct HBV proteins (**Fig 4**). The bar graphs show the results of each individual patient (**Fig 4A**) and the pie charts display the median composition of HBV-specific T cells in patients of different age groups (**Fig 4B**). The percentage of HBs-specific T cells diminishes with age: whilst over 30-40% of HBV-specific T cells target HBs protein in patients aged 3-24 years, this is reduced to less than 10% in patients over the age of 35. Moreover, HBs-specific T cells constitute the dominant HBV-specific T cell response almost exclusively in under 25-year-olds, while it was never found to be dominant in patients over the age of 45.

Characterisation of HBV-specific T cells in patients who resolved HBV infection after acute or chronic infection.

The influence that the duration of infection can exert on the HBV-specific T cell composition was then studied in two different populations with prior exposure to HBV. We collected PBMCs from patients who controlled HBV (anti-HBs positive and anti-HBc positive) either after documented chronic infection (n=11, likely infected at birth and with at least >5 years clinically documented infection) or after an acute infection (n=27, HBsAg positive for <3 months). Importantly, the individuals in these two groups were age-matched.

The majority of HBsAg-negative anti-HBc positive individuals with no history of CHB demonstrated, as expected, a robust expansion of HBV-specific T cells (22/27), and almost all (20/22) have T cells that recognize more than a single HBV protein (**Fig 5A**). Notably, HBs-specific T cells represent on average 33% of the total HBV-specific T cells detected and are the dominant response in 8/22 individuals (**Fig 5B**). This pattern was completely divergent from patients who became HBsAg negative after long-term CHB infection (**Fig 5A, 5B, right panels**). In these patients, HBV-specific T cell expansion was not only quantitatively lower, detected in fewer patients (6/11), and strikingly, no patient (0/11) demonstrated the presence of IFNγ producing HBs-specific T cells. These data show that even in HBsAg negative patients, the duration of prior HBV infection affects the antigenic profile of HBV-specific memory T cells.

Lastly, we compared the median quantity of HBs-specific T cells (**Fig 5C upper panel**) and their median percentage among total HBV-specific T cells (**Fig 5C lower panel**) in all patients studied, subdivided by acute resolved and chronic resolved infection and by the age of CHB patients, thus consequently by the duration of HBV infection. Our data show that only during the first three decades of chronic infection, thus with

persistent HBsAg exposure, HBV-infected patients can still mount a HBs-specific T cell response, similar to that detected in patients who experienced transient short-term acute infection. However, in chronically infected patients above the age of 35, thus after long-term exposure to high quantity of HBsAg, the frequency and dominance of HBs-specific T cells is significantly diminished and does not recover after resolution of infection.

Discussion

In this study, we first analysed the impact of HBsAg quantity on global T and NK cell phenotype and function. We did not find any direct experimental evidence linking HBsAg quantity with the suppression of global cellular immunity (T and NK cells). Different levels of HBsAg did not modulate proportionally the level of cytokines and cytotoxicity-associated molecules present in different T and NK cell subsets. Likewise, we did not observe any correlation with expression of several immunoregulatory receptors, including PD-1, CTLA-4, TIM-3, KLRG1, CD160, BTLA4 and TIGIT that have been shown to modulate the function of HBV-specific T cells.

Our results are not unexpected. Despite global defects of NK and T cell function that have been reported in HBV infected patients[29-31], these alterations were found in individuals with concomitant liver inflammation and are primarily caused (at least with regard to T cells) by arginine depletion[30,31]. When we subdivide the CHB patient cohort into patients with no, mild and severe hepatitis and compare them with healthy donors, we find a trend towards increased exhaustion/activation of T and NK cells linked with liver inflammation, but not with HBsAg levels (**Fig. S4**). Further supporting evidence that HBsAg does not affect global T cells is clear from our recent data, where we compared the immune gene expression profiles of multiple T and B cell subsets in age-matched healthy donors and CHB patients[6]. In order to detect differences induced only by HBV and not by liver inflammation, T and B cells were sorted from the blood of CHB patients in the HBeAg positive chronic infection phase, characterised by high levels of HBsAg and normal levels of transaminases. We could not find any significant alterations in the T cell compartment between CHB patients and healthy donors[6]. Finally, epidemiological data of CHB patients have never shown any evidence of

immunological defects. On the contrary, HBsAg presence has been linked to better control of malaria or increased maturation of general immunity in new-borns[32], results that are clearly not compatible with the idea of HBsAg-mediated generic immunosuppression.

We then characterized HBV-specific T cells, which are present at very low frequency in the blood of CHB patients and thus cannot influence the global T cell profile. Similarly, we did not find any evidence that HBsAg quantity could proportionally suppress the magnitude or multi-specificity of HBV-specific T cells. Conversely, to our surprise, we found that HBs-specific T cells were more likely to be detected in the blood of patients with higher levels of HBsAg. These findings, however should not be interpreted as a demonstration that HBsAg does not play a role in the modulation of anti-HBV immunity. On the contrary, when we analysed our results in relation to the age of the patients, which corresponds to the duration of infection, we find clear evidence, equally in CHB and in patients with resolved infection, that HBsAg persistence affects both the magnitude and dominance of their HBs-specific T cells. Importantly, only HBs-specific T cells declined with age, while T cells specific for the other HBV antigens persisted, and we confirm an increase of HBcore-specific T cell responses in HBeAg negative patients as observed by Park et al.[27]. Taken together, our results show that the impact of HBsAg on host immunity is not global but exclusively antigen-specific. Our data suggest that the duration of HBsAg exposure, more than the quantity of HBsAg-derived epitopes presented by hepatocytes or professional antigen presenting cells, has the ability to increasingly delete HBs-specific T cells. Importantly, the progressive attrition of HBs-specific T cells in older patients detected in our study complements recent evidence from two other

independent groups showing, with different experimental procedures, the presence of only HBcore- and HBpol-specific T cells in adult HBeAg negative CHB patients[10,11]. The selective attrition of HBs-specific T cells and not of those specific for the other HBV antigens likely results from the prevailing expression of HBsAg. Although it declines with age, its overall expression is much higher than the other HBV antigens, and persists over time, as it is derived not only from infected hepatocytes, but also progressively from hepatocytes with HBV-DNA integration[33]. One other possibility is that the presence of HBcore- and HBpol-specific T cells remains, because of an increased immunogenicity at helper T cell level. It was demonstrated that HBcAg induced a much stronger helper T cell response than HBsAg in acute HBV patients[34]. However, a precise quantification of CD4 and CD8 T cells specific for the individual HBV antigens in different phases of CHB infection needs to be performed to confirm this hypothesis.

A limitation of our study is the complete reliance on the circulating compartment, despite our acceptance that HBV-specific T cells can preferentially be localized in the liver[35,36]. Nevertheless, since there is no evidence demonstrating that intrahepatic recruitment is selective only for HBs- and not for HBcore- or HBpol-specific T cells or that it occurs only after a certain age, we believe that our cross-sectional data, analysing the peripheral compartment of patients, depicts a true progressive modification of the HBV-specific T cell repertoire. Certainly, a paired analysis of the global repertoire of HBV-specific T cell responses in blood and liver of patients at different ages would be ideal to confirm unequivocally our findings, but the quantity of intrahepatic lymphocytes necessary for such an analysis impose severe practical limitations. Moreover, the low frequency of HBV-specific T cells in CHB patients made

it necessary to expand them in vitro, which may not fully represent the in vivo frequency, yet quantifies T cells with proliferative and cytokine producing capacity, likely more relevant for biological function.

We believe that our results can have important therapeutic implications. First, based on the lack of correlation between qHBsAg and global HBV-specific T cell immunity, we doubt that any reduction of serum HBsAg level, utilizing siRNA or antibodies specific for HBsAg, can trigger the restoration of HBV-specific immunity as often hypothesized[17]. Notwithstanding, we should consider that the modulation of HBsAg presentation induced by siRNA has clear kinetic differences in comparison to the one derived from the progressive and slow natural decline of HBsAg[37]. Thus, therapeutic strategies designed to recover the host's ability to control HBV through a sharp reduction of HBV antigen production should be tested, but perhaps focusing on patient populations in the first three decades of life. Our data clearly show a disappearance of HBs-specific T cells relative to the increasing age of CHB patients, with a sharp decline in patients over the age of 35.

Notably, whether HBV-specific T cells of younger CHB patients can be resuscitated by immunotherapy in vivo needs to be directly tested in clinical trials, and we are not suggesting that HBs-specific T cells are indispensable to HBV control. Our data show that some CHB patients became HBsAg negative despite a lack of detection of HBs-specific T cells, indicating that we still lack a full understanding of the protective hierarchy of HBV-specific T cells. Nonetheless, our data show a clear progressive attrition of the multi-specificity of HBV-specific T cells over the duration of HBV infection, and we hypothesise that such progressive narrowing of the HBV-specific T

cell repertoire might reduce the chances of immunological control, which will have to be tested.

We[38] and others[39] have already argued that targeting young CHB patients with immune therapies might have several advantages: better access of immune cells to infected hepatocytes[40] due to their lower level of hepatic fibrosis, a liver environment without immunomodulatory cytokines present in chronic hepatitis[41], and an earlier reduction of the level of HBV-DNA integration[22]. Although the presence of HBs-specific T cells might not be essential for the clearance of CHB infection in all patients, the impact that duration of HBV infection exerts on the breadth of HBV-specific T cells demonstrated here may further suggest that earlier treatment intervention could be considered.

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Figure 1. Diagram of the experimental design and the clinical features of the patients studied

(**A**) Number of patients analyzed for global T and NK cell immunity by mass cytometry (chronic HBV [CHB], n=48; upper section), and for antigen-specific T cell responses (CHB, n=189; acute resolved, n=27; chronic resolved, n=11, lower section). (**B**) Age distribution, HBsAg level and HBeAg status of 197 CHB patients. Upper-right insert shows correlation between patient age and HBsAg quantity. (**C**) Age and number of CHB patients studied (n=205) with different clinical and virological parameters, classified according to CPG (EASL 2017). (**D**) ALT levels and age of CHB patients (n=205) divided into patients with no (ALT <40 IU/mL), mild (ALT 40-80 IU/mL) and severe (ALT >80 IU/mL) hepatitis. Spearman correlation (B).

Figure 2. Global T and NK cell profiling with CyTOF

CyTOF data of PBMCs from 48 CHB patients (ex-vivo and after PMA/ionomycin stimulation*) were analyzed by UMAP and concatenated. (**A**) Delineation of 14 different immune cell subsets based on the expression heatmaps of 40 markers (Fig S1A). (**B**) Concatenated UMAP plots of CHB patients with different levels of HBsAg. (**C**) Heatmap indicating the median frequency of 33 markers expressed by 20 immune cell subsets in patients with low (<1,000 IU/mL; n=14) and high (>10,000 IU/mL; n=13) levels of HBsAg. Correlation of indicated cytokines/chemokine (**D**) and exhaustion markers (**E**) expressed on CD4+ and CD8+ T cells with HBsAg levels (left panels), and bar graphs showing percentage of T cells expressing multiple cytokines/chemokines (**D**) or exhaustion markers (**E**) in patients with varying quantity of HBsAg. (**F**) Correlation of cytokines/chemokine and activation/inhibitory markers expressed on NK cells (left) and percentage of immature:mature NK cells (indicating

NK cell differentiation) in patients with varying levels of HBsAg (right). Spearman correlation (D, E, F).

Figure 3. Quantity of HBV-specific T cells in relation to quantitative HBsAg and age

(**A**) Quantity of T cells (spot forming units; SFU) specific for the whole HBV proteome (total) and the individual HBV proteins in CHB patient subgroups classified using EASL 2017 CPG. Correlation analyses of HBsAg levels (**B**) or age (**C**) with the quantity of T cells specific for the whole HBV proteome, HBs-, HBcore-, HBpol- and HBx-protein. (**D**) Number of T cells specific for the indicated HBV-proteins in relation to age of the CHB patients. Each bar represents one patient. ANOVA with Kruskal-Wallis test (A); Spearman correlation (B, C). $P<0.05$ considered significant.

Figure 4. Dominance of HBV-specific T cells in relation to age

Composition of HBV-specific T cells in individual CHB patients with a detectable T cell response (164/189). (**A**) The percentage of HBV-specific T cells against HBs- (red), HBcore- (purple), HBpol- (green) and HBx-protein (blue) in each patient is shown. (**B**) Pie charts below show the median percentages of T cells specific for each HBV protein in different age-groups.

Figure 5. Duration of infection affects the HBV-specific T cell profile

(**A**) Quantity of HBV-specific T cells (spot forming units; SFU) in acute resolved, AR (n=27; left panel) and chronic resolved, CR (n=11; right panel) patients. Bars represent SFU per 10^5 cells in response to indicated HBV proteins in each patient. Pie charts show the percentage of patients with HBV-specific T cells. (**B**) Composition of HBV-

specific T cells in each patient. Pie charts show the mean percentages of T cells specific for the individual HBV proteins in both patient groups. (**C**) Quantity (SFU; upper panel) and frequency among total HBV-specific T cells (%; lower panel) of HBs-specific T cells in the indicated categories of patients. Red lines indicate median. Statistical significance using acute resolved group as baseline was determined by one-way ANOVA with Dunnett test. *, ** indicate $p<0.05$ and $p<0.01$ respectively.

Table 1: Clinical data of patients selected for the study of global T and NK cells by CyTOF

Characteristics	CHRONIC HBV patients (n = 48)			
	treatment naïve HBeAg+	treatment naïve HBeAg-	TDF HBeAg+	TDF HBeAg-
Number of patients	12	12	12	12
HBsAg level (IU/mL): range (median)	3394 – 365623 (26665)	21 – 32171 (5418)	75 – 38534 (2462)	2 – 17351 (2113)
HBsAg level (log_{10}IU/mL): range (median)	3.5 – 5.6 (4.4)	1.3 – 4.5 (3.7)	1.9 – 4.6 (3.3)	0.4 – 4.2 (3.0)
Age (years): range (median)	24 – 47 (31)	20 – 51 (35)	24 – 60 (41.5)	22 – 57 (50)
Sex (male/female)	9/3	6/6	5/7	9/3
ALT level (IU/L): range (median)	36 – 298 (99.5)	15 – 165 (32.0)	14 – 78 (22.5)	17 – 67 (29.0)
Median HBV-DNA level (log_{10}IU/mL)	8.0	4.9	< 1.3	< 1.3
HBV genotype: A/B/C/D/E/unknown	2/2/3/2/2/1	2/1/2/1/2/3	2/4/0/1/0/5	3/0/0/1/1/7

Table 2: Clinical data of patients selected for the study of HBV-specific T cells

Characteristics	Patient groups							
	CHRONIC (n = 189)						ACUTE RESOLVED	CHRONIC RESOLVED
Age (years): range (median)	**3 – 14 (11.0)**	**15 – 24 (21.5)**	**25 – 34 (28.0)**	**35 – 44 (39.5)**	**45 – 54 (49.4)**	**55 – 75 (60.0)**		
Number of patients	18	42	58	26	32	13	27	11
Median age (years)	11	22	28	40	49	60	42	47
HBsAg level (IU/ml): range (median)	471 – 244,560 (28,249)	75 – 1,678,804 (23,146)	4 – 257,781 (14,049)	21 – 365,623 (6,396)	2 – 17,351 (1,873)	7 – 9,501 (570)	0	0
HBsAg level (log_{10}IU/ml): range (median)	2.6 – 5.4 (4.5)	1.9 – 6.2 (4.4)	0.6 – 5.4 (4.1)	1.3 – 5.6 (3.8)	0.4 – 4.2 (3.3)	0.8 – 4.0 (2.8)	0	0
HBeAg+ chronic infection	9	18	7	0	0	0	NA	NA
HBeAg+ chronic hepatitis	2	12	16	4	5	0	NA	NA
HBeAg- chronic infection	7	9	13	5	1	13	NA	NA
HBeAg- chronic hepatitis	0	3	22	17	26	0	NA	NA
ALT level (IU/L): range (median)	14 – 93 (31.5)	14 – 157 (36.0)	11 – 539 (36.5)	14 – 565 (30.0)	14 – 666 (36.5)	13 – 1067 (28.0)	20	20
Median HBV DNA level (log_{10}IU/ml)	5.73	8.19	5.15	3.04	3.28	3.05	< 1.3	< 1.3
HBV genotype: A/B/C/D/E/unknown	0/1/1/15/0/1	1/4/4/19/2/12	8/4/10/20/4/12	1/5/2/10/4/4	3/10/3/6/4/6	0/6/3/3/0/1	NA	NA
No treatment / NA therapy	18/0	41/1	53/5	21/5	17/15	3/10	NA	NA
Sex (male/female)	5/13	21/21	31/27	21/5	24/8	6/7	unknown	10/1