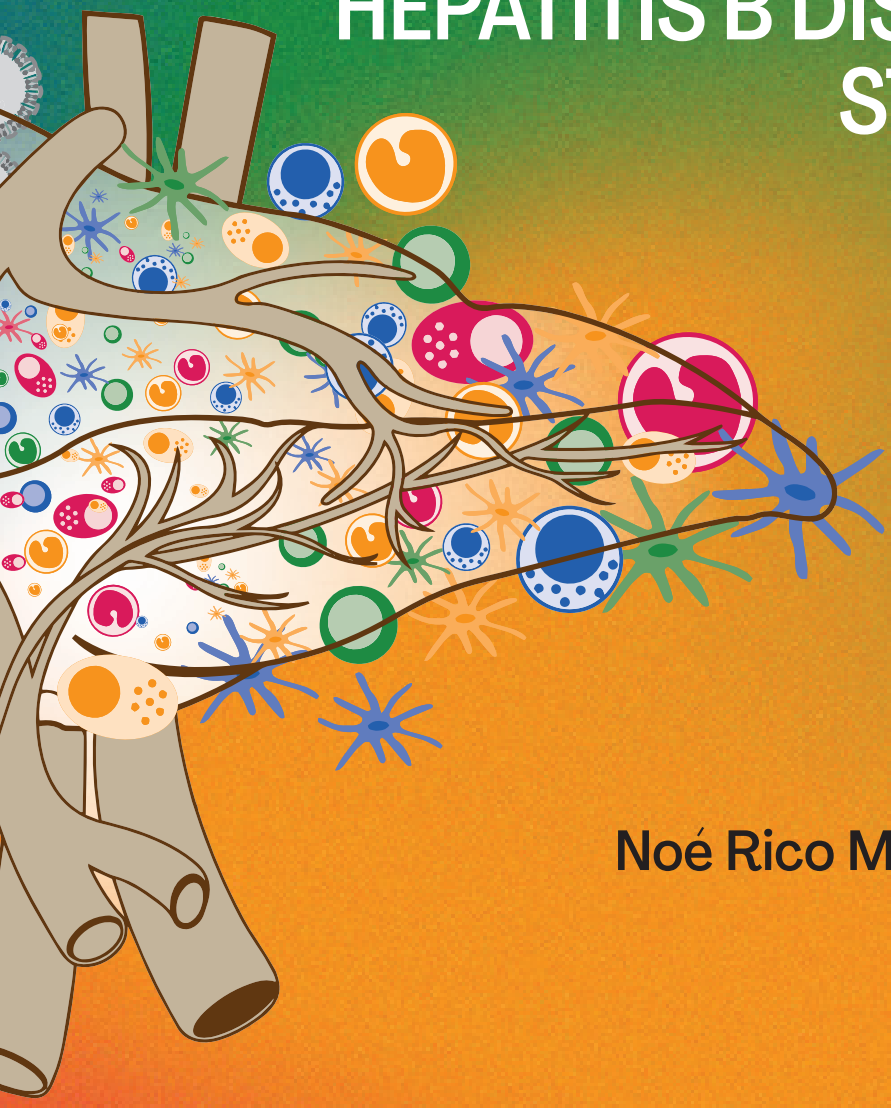
IMMUNE AND TRANSCRIPTOMIC PROFILING OF DISTINCT HEPATITIS B DISEASE STATES



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Immune and Transcriptomic Profiling of Distinct Hepatitis B Disease States

Immuun- en transcriptomische profilering van verschillende ziektetoestanden van hepatitis B

Thesis

to obtain the degree of Doctor from the
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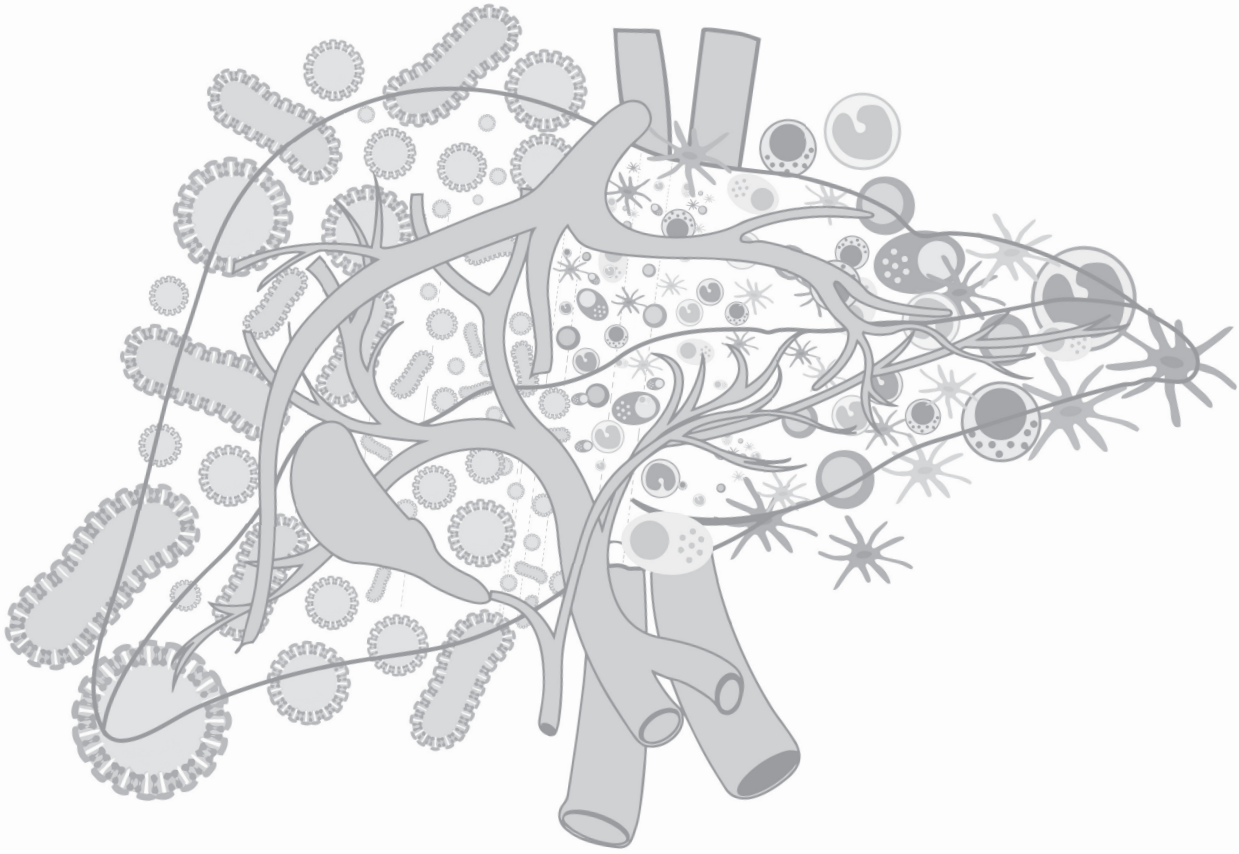

CHAPTER 1

Introduction and Outlines of this Thesis

INTRODUCTION AND OUTLINES OF THIS THESIS

Originally termed *Australian antigen,* the presence of the hepatitis B virus (HBV) was first discovered by Dr. Baruch Blumberg in 1965 and later identified to cause acute and chronic disease in the liver due to the specific viral tropism for that organ [1,2]. Viral transmission is reported to occur through contact with virally contaminated blood and other body fluids as well as during child-birth (perinatal) [3]. Through these routes, an estimated 2 billion individuals have been in contact with the virus at some point in their lifetime as evidenced by antibodies against HBV viral proteins. Whether the infection outcome is acute or chronic is largely dependent on the age the individual exposed. Whilst 95% of infected adults are able to self-contain it, infection during child-birth and childhood results in chronic infection in over 90% of the cases [4]. Despite the existence of an effective vaccine, the World Health Organization most recent estimates indicates close to 260 million people to be chronically infected based on serology status and approximately 900,000 related deaths per year as a result of the long-term consequences of chronic HBV infection: end-stage liver disease and hepatocellular carcinoma (HCC) [5].

Acute HBV infections can be asymptomatic. If symptoms are observed then the most common manifestations are jaundice, extreme fatigue, nausea/vomiting and abdominal pain that may last several weeks [6]. Moreover, a small fraction of patients can develop fulminant hepatitis which can progress into liver failure and result in death [7]. In contrast, chronic infections are generally characterized by a lack of symptoms over the first years of infection which can often last decades [8]. Nonetheless, the host-virus interplay in the infected liver over the infection course may lead to a slow but progressive replacement of the liver parenchyma by fibrotic tissue in a small subset of patients which over time may further develop into liver cirrhosis, and the formation of HCC [9].

From HBV entry to viral persistence

HBV is an enveloped partially double-stranded DNA virus of 3.2 kilobase (kb) in length that belongs to the *Hepadnaviridae* family encoding four open reading frames (ORFs) producing seven functional viral proteins known as HBV polymerase (HBVpol), HBV core (HBc), HBV envelope (HBeAg), HBV X (HBx) and the small, middle and large HBV surface antigen (HBsAg, S, M and L, respectively) [6,10]. Of these proteins, HBeAg and HBsAg can be easily detected in chronic HBV patients' serum where HBsAg is the most abundant HBV protein present in circulation . The different HBsAg forms are present in the HBV outer membrane but carry different functions. The small HBsAg form plays a role in virion secretion whereas the large HBsAg has two main roles. On one hand interacts with the HBV nucleocapsid to produce mature viral particles and on the other interacts with the sodium taurocholate co-transporting polypeptide (NTCP) receptor expressed in hepatocytes explaining HBV tropism for the liver [11,12]. Upon entry, the viral

nucleocapsid is released into the cytoplasm and transported into the nucleus through nuclear pore complexes [12]. Once there, the HBV genome is released and undergoes a series of modifications by host factors prior the formation of the stable covalently closed circular DNA (cccDNA) which can occasionally integrate into the host's genome [13–15]. The stable cccDNA is used for viral replication and acts as a transcriptional template for HBV protein synthesis [12,16]. During the formation of new HBV virions and prior to HBsAg envelopment and cell-release, newly formed nucleocapsids containing the HBV genome can re-circulate into the nucleus maintaining the cccDNA and potentially increasing the number of HBV integrations [12]. Together with the release of infectious viral particles, infected hepatocytes can secrete non-infectious subviral particles (SVPs) [10,12]. Its morphology, spherical and long filamentous, depends on the ratio of the different HBsAg forms [16]. Moreover, SVPs outnumber the number of infectious viral particles with several magnitudes - 1,000–100,000-fold- which may be exploited by HBV as a decoy strategy to avoid antibody neutralization [10,17].

In contrast to infections with the hepatitis C virus (HCV), chronic infections with the HBV virus are characterized by the presence of different phases in which viral replication and liver damage fluctuates [9,18]. Four distinct clinical phases are generally discriminated in patients and have been described on the basis of serum alanine aminotransferase (ALT) levels, HBV DNA levels and the presence of absence of the viral antigen HBeAg in serum [19]. These phases reflect the natural history of chronic HBV and are termed HBeAg-positive chronic infection (i.e, Immuno Tolerant; IT), HBeAg-positive chronic hepatitis (i.e, Immuno Active; IA), HBeAg-negative chronic infection (i.e, Inactive Carrier; IC) and HBeAg-negative chronic hepatitis (i.e, ENEG) (Figure 1) [19].

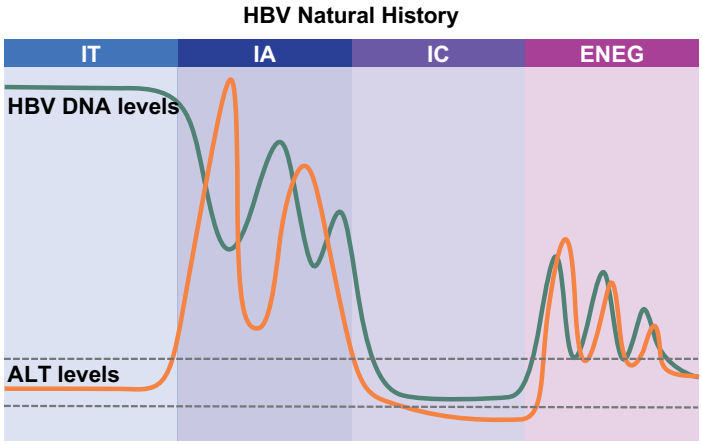

Figure 1. Chronic HBV natural history.
Fluctuations in serum HBV DNA and ALT levels in the distinct clinical phases during chronic HBV infections

Immune responses and exhaustion in chronic HBV

A striking feature of chronic HBV infection, and in fact also of other chronic viral infections such as HCV, human immunodeficiency virus (HIV) and chronic lymphocytic choriomeningitis virus (LCMV) infections, is that viral replication is continuous for a prolonged period of time and viral titers are high. This continuous triggering with high amounts of protein induces an impairment of the T cell compartment, referred to as "T cell exhaustion'' [20,21]. This functional impairment occurs gradually, and can eventually lead to the abolishment of cytotoxic and proliferative capacity and the inability to produce substantial levels of the T cell derived cytokines, IL-2, IFN-γ and TNF, thereby hampering the antiviral capacity of virus-specific T cells [22,23]. Phenotypically exhausted T cells are characterized by enhanced expression of inhibitory receptors, such as PD-1, CTLA-4, Tim-3, LAG-3 and CD160 [23,24]. Blockade of these receptors has been shown to lead to partial restoration of the activity of exhausted cells, therefore indicating the importance of these cell surface markers in inhibiting T cell activity [23,25]. However, this also illustrates the therapeutic capabilities of these markers as novel immunotherapeutic strategies to treat cancers and possibly viral infections as currently used in the clinic [26,27]. However, it is important to note that T cell exhaustion is not homogenous, and the specific inhibitory receptor and their expression levels differ between CD4+ and CD8+ T cells and between individual patients [28].

T cell exhaustion has been described extensively for HBV-specific CD8+ T cells and a better understanding of the specific triggers that induce T cell exhaustion in HBV could be very important to explain persistence of the virus [29]. Compared to the other chronic infections mentioned (HIV, HCV and LCMV), chronic HBV infections are a special case since the levels of circulating viral proteins are extremely high, which makes HBsAg a likely candidate that induces or maintains the state of exhaustion [30]. If this is indeed the case then one would expect that comparison of the reactivity of T cells from chronic HBV patients would demonstrate more frequent responses directed against HBcAg and polymerase, and less frequent responses against HBsAg. Indeed, by assessing IFN-γ production by ELISPOT after stimulation of PBMC with peptides from different viral antigens (polymerase, core and surface), CD8+ T cells from patients generally exhibited the lowest responses towards HBsAg, whereas most reactivity was found towards HBcAg and polymerase [31]. However, it is important to note that all HBV-specific CD8+ T cell responses are generally low, and although HBcAg- and polymerase-specific T cells are detected in blood of chronic HBV patients, these T cells are also functionally impaired and have an exhausted phenotype [32,33]. Reduced functionality of other immune cells, such as B cells and NK cells, has also been described in PBMC obtained from blood of chronic HBV patients. For HBV-specific B cells this is most pronounced for cells with a specificity for HBsAg and less so for HBcAg [34,35]. However, these studies are still in its infancy and more information is needed on the functionality of B cells during the chronic phase of infection. Also NK cells have been reported to be functionally impaired in chronic HBV patients with reduced IFN-y production upon stimulation, while cytotoxic capabilities are not or less affected as compared to cells from healthy individuals [36–38].

Current and future anti-HBV therapies

Several treatment strategies exist for chronic HBV, namely the use of interferons (IFN-a, pegIFN-a) and nucleos(t)ide analogues (NUC) [19,39]. NUCs are naturally resembling nucleotides that act on the HBV polymerase by blocking HBV DNA elongation [40,41]. Current NUC therapies are highly effective at controlling active disease by reducing serum HBV DNA and ALT levels and regressing liver fibrosis progression, but lifelong administration of the drugs is needed, since viral replication is suppressed, but not eliminated [41]. NUC treatment has a very limited direct effect on viral antigen load and secretion, mainly HBsAg, and intrahepatic HBV reservoirs (cccDNA and integrated HBV) [42]. To increase cure rates, two classes of novel therapeutics are under development [42–44]. The first class aims at interfering HBV by modulating the production or secretion of viral antigens or by reducing/eliminating intrahepatic HBV reservoirs. CRISPR/Cas technologies represent an attractive avenue as it could serve to eradicate cccDNA and integrated HBV reservoirs preventing viral reactivation under immune-stress conditions [45]. Furthermore, the activation of lymphotoxin-b receptor via IFN-a or other agonists could result in cccDNA degradation via APOBECs (APOBEC3A and APOBEC3B) activity [46]. However, these are at early pre-clinical stages and carry obstacles such as delivery, specificity for infected hepatocytes and possible off-target effects [47,48]. A more feasible strategy in the short term consists in HBsAg targeting. The rationale consists in lowering circulating HBsAg levels to partially restore the impaired immune response possibly leading to immune-mediated control of viral replication, also known as functional cure [30]. Preliminary results from the first clinical trials testing the effectiveness of different compounds reducing HBsAg are promising as initial data points to safe tolerability profiles and partial HBsAg reduction [49]. For instance, preliminary data obtained from HBV infected chimpanzees treated with the ARC-520 siRNA -developed by Arrowhead pharmaceutical- showed a greater reduction in HBeAg-positive than HBeAg-negative chimps (mean -1.91 Log_{10} vs -0.55 Log_{10}, respectively)[50]. The observed difference is speculated to steam from the different source of HBsAg (cccDNA or host's DNA integrated in HBeAg-positive and negative, respectively)[50]. Similarly, the phase II clinical trial Heparc-2001 showed an overall greater HBsAg reduction in NUC-naïve patients compared to NUC-experienced patients. Furthermore, in NUC-naïve patients positive for HBeAg achieved greater antigen load reduction than those HBeAg-negative [50]. Nonetheless, very scarce immunological data is available from these studies that could complement and enforce decision-making. An alternative class of therapeutics consist of immune-modulators. These include a wide array of strategies that range from checkpoint inhibitors blockade (i.e., anti-PD-1) expressed in exhausted HBV-specific T and B cells, therapeutic vaccination, engineered T cells or TLR-such as TLR-7 and TLR-8 agonist to name a few [51–53].

Advances in HBV research: Towards the liver

Studies conducted on in-vitro and animal model systems continue to be of paramount importance in HBV research where key steps in HBV's viral cycle are subjected to be identified to be susceptible to new therapeutic strategies [54–56]. Furthermore, seminal work conducted on mouse and chimpanzee animal models have served to shed light into HBV pathogenesis and to gather a better understanding in effective anti-HBV immune responses capable of clearing HBV [57,58]. However, these systems do not always mirror the complex and intricate virological and immunological processes acting during chronic HBV infection in humans. In general, peripheral blood mononuclear cells (PBMCs) are the predominant study material in HBV research conducted in humans since sampling procedures are safer and easier than in the liver, which is the site of HBV replication. Although some of the observations made in the liver of chronic HBV patients are reflected, albeit weaker, in peripheral blood, these two compartments present important immunological differences [27,59–62].

Due to its physiological role, the liver is constantly exposed to harmless gut-derived products entering the portal vein [63,64]. As a result, the organ has evolved to be a tolerogenic environment conferred by parenchymal (hepatocytes) and non-parenchymal cells (antigen presentation and regulatory T cells) as well as mediated by soluble cytokines such as IL-10 and TGF-b to prevent excessive immune activation to harmless products while maintaining an effective activation when faced to bacterial or viral pathogens [65]. In addition to the local cytokine milieu and physiological conditions -hypoxia, nutrients and slowed blood flow-, egress from the periphery and tissue residency maintenance exert phenotypical changes on the local and recruited immune cell populations. Furthermore, the immune cell composition in the liver is substantially different than its blood counterpart [59,60]. Therefore, to get a detailed understanding of the immunobiology during chronic HBV infection, it is essential to also include the liver in the studies as it is expected that leukocyte populations sampled in blood and liver may be different. Despite the fact that nowadays liver biopsies are rarely performed, advances in multiplex immunofluorescence -allowing for a greater number of target protein to be visualized altogether- and cost-reduction in gene expression such as RNA sequencing will allow to maximize the knowledge extracted from archived formalin fixed paraffin embedded (FFPE) liver biopsies . Furthermore, advances in liver sampling techniques such as fine needle aspirates (FNA), which have less complications and give less discomfort to patients than regular core needle biopsies, in conjugation with multiparameter flow cytometry and unbiased methods such as bulk and single-cell RNA sequencing and high dimensional analysis be extremely important to advance the field [66,67].

Aims of this thesis:

An effective immune response has proven capable to control HBV infection in adults. However, in chronic HBV patients the immune response is clearly insufficient to eliminate the virus, albeit overtime a fraction of patients can control of viral replication and show signs of limited liver damage. To better understand the dynamics of the immune response during the various clinical HBV phases more detailed studies are needed, especially in the liver. This immune response is likely affected by the levels of HBsAg, which have been postulated to be a major contributing factor leading to an impaired or exhausted functionality of T cells, B cells and NK cells. However, much is still unclear about the role and consequence of HBsAg in modulating the anti-HBV immune response, in blood and in the liver, and conflicting data has been published. An additional area that requires much research and that is still poorly understand include the mechanisms that govern immune control in patients with chronic HBV. This immune control can be either spontaneous during the inactive carrier phase of the infection or treatment induced during antiviral treatment with NUC. Little is known about the state of the immune response during these two different states of immune control in the liver and blood. Detailed information on the above mentioned parameters, i.e. clinical phases, HBsAg and immune control processes, are instrumental for the design of new therapeutic approaches aimed at immune reconstitution for effective anti-HBV control. Therefore, the main aim of this thesis is to better comprehend the host's immune response in chronic HBV to generate knowledge that may serve as the foundation to reach effective tools for immune control.

Outline of this thesis:

The work presented in this thesis addresses three central questions:

1. How does the natural progression of chronic HBV, associated with fluctuations in viral load and liver inflammation affect liver gene expression profiles?
2. Does the mode of viral suppression (spontaneous or NUC-induced) reflect distinct immunological states in major peripheral leukocyte populations in chronic HBV patients?
3. Does the level of serum and intrahepatic HBsAg modulate peripheral innate immune responses and intrahepatic gene expression profiles in non-viremic patients?

First, in **chapter 2**, we set the foundation of this thesis by studying liver biopsies collected from the various clinical phases in chronic HBV and compare them to livers of true uninfected healthy controls to evaluate the composition and degree of leukocyte infiltrate together with the intrahepatic host gene expression in relation to the degree of inflammation and HBV antigen burden. In **chapter 3**, we characterize MAIT cells, an innate immune effector cell with antiviral properties, in the context of chronic HBV and assess their frequency in liver versus blood.

Additionally, we describe their frequency and phenotype in blood across the different clinical phases in chronic HBV and healthy controls and evaluate their functional capacity before therapy and on-NUC therapy. In **chapter 4**, we further expand our peripheral leukocyte populations of interest and study the transcriptome of sorted CD4+ and CD8+ T cells, CD19+ B cells, BDCA1+ dendritic cells, CD14+ monocytes and CD56+ NK cells in non-viremic patient (Inactive Carrier and on-NUC) to determine to what extent these cell populations are modulated by serum HBsAg levels. In **chapter 5**, we focus on the intrahepatic transcriptome of Inactive Carrier patients and compare this to healthy livers so to identify an immune control gene expression profile. Additionally, we compare the transcriptome of patients with distinct serum and liver HBsAg levels to further identify immune-modulating mechanisms associated with HBsAg. Lastly, in **chapter 6**, we contextualized the findings presented in this thesis and discuss its implications in the new era to come of novel anti-HBV therapeutics.

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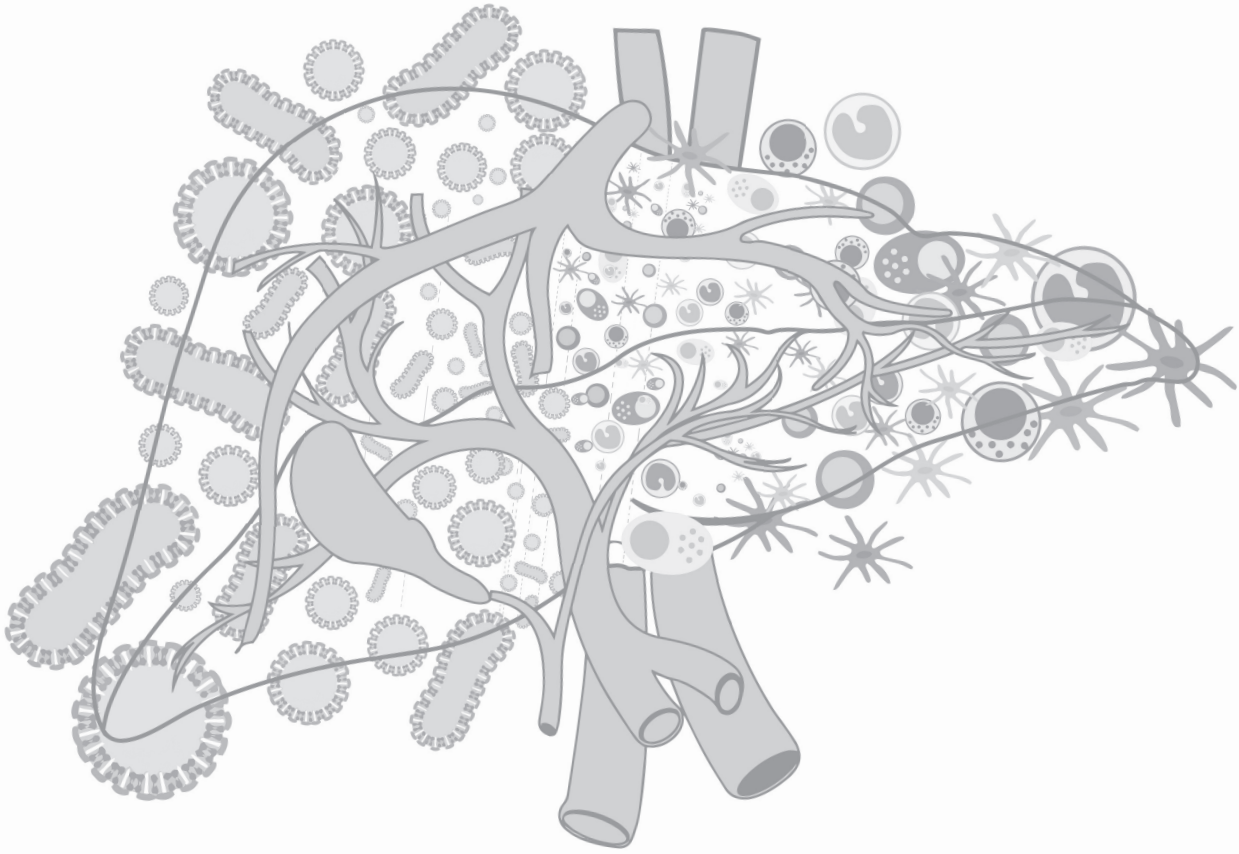

CHAPTER 2

Multi-parametric analysis of human livers reveals intrahepatic inflammation variation across chronic hepatitis B infection phases

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LAY SUMMARY

Immunological studies on chronic HBV remains largely restricted to assessment of peripheral responses due to the limited access to the site of infection, the liver. In this study, we comprehensively analyzed livers from a well-defined cohort of chronic HBV patients and uninfected controls with state-of-the-art techniques, and evaluated the differences in gene expression profiles and inflammation characteristics across distinct disease phases of chronic HBV patients.

ABSTRACT

Background and Aims: Chronic HBV is clinically defined in 4 phases by a combination of serum HBV DNA levels, HBeAg status and ALT: immunotolerant (IT), immune-active (IA), inactive carrier (IC) and HBeAg-negative hepatitis (ENEG). Immune and virological differences between phases as detected in blood have proven useful but are insufficient to appreciate the interrelation between the immune system and the virus since immune dynamics in blood and liver differ. Furthermore, the inflammatory response in the liver and parenchymal cells cannot be fully captured in blood.

Methods: Immunological composition and transcriptional profiles of FFPE core needle liver-biopsies in chronic HBV phases vs healthy were evaluated by multiplex immunofluorescence and RNA-Seq (n = 37 and 78, respectively).

Results: Irrespective of the phase-specific serological profiles, increased immune-gene expression and frequency was observed in chronic HBV compared to healthy livers. Greater transcriptomic de-regulation was seen in IA and ENEG (172 vs 243 DEGs) than in IT and IC (13 vs 35 DEGs) livers. ISG, immune-activation and exhaustion genes (*ICOS*, *CTLA4*, *PDCD1*) together with chemokine genes (*CXCL10*, *CXCL9)* were significantly induced in IA and ENEG livers. Moreover, distinct immune profiles associated with ALT elevation, and a more accentuated immune-exhaustion profile (*CTLA4*, *TOX*, *SLAMF6*, *FOXP3*) found in ENEG and set it apart from IA phase *(LGALS9*, *PDCD1*). Interestingly, all HBV phases showed downregulation of metabolic pathways vs healthy livers (fatty and bile acid metabolism). Finally, we found increased leukocyte infiltrate correlated with serum ALT, but not with HBV DNA or viral proteins.

Conclusion: Our comprehensive multi-parametric analysis of human livers revealed distinct inflammatory profiles and pronounced differences in intrahepatic gene profiles across all chronic HBV phases in comparison to healthy liver.

INTRODUCTION

Chronic hepatitis B is a heterogeneous disease which progresses through distinct disease phases that differ in the levels of viral replication and the degree of liver damage inflicted by immune responses against hepatocytes infected with the hepatitis B virus (HBV)[1]. Four phases have been defined on the basis of the variable serum levels of HBV DNA, HBeAg and alanine aminotransferase (ALT): immunotolerant (IT), immune-active (IA), inactive carrier (IC) and HBeAg-negative hepatitis (ENEG)[2]. The distinction between phases is clinically relevant as it determines the indication for antiviral treatment, aimed at reducing viral replication and liver damage in the IA and ENEG phases[3].

HBV is a non-cytopathic virus, meaning that the liver damage observed during the chronic phase of the disease is a direct consequence of the immune response against the virus[3]. Histopathological evaluation of the liver of infected patients demonstrated that mild leukocyte infiltration is observed in the IT and IC phases, whereas extensive infiltrates are observed in IA and ENEG patients, predominantly in the portal tract area[4]. These distinct characteristics were used by European Association for the Study of the Liver (EASL) to propose a revised nomenclature for HBeAg-positive and HBeAg-negative chronic HBV: chronic infection versus chronic hepatitis, which is thought to better describe the phases[3].

In recent years, several studies have examined the immunological and virological differences between the natural phases of chronic HBV infection. Most of these studies have examined the peripheral compartment where minor differences were reported on the frequencies and phenotype of circulating NK-cells, T-cells and B-cells when comparing blood samples from patients at different clinical phases[5–7]. However, since HBV replication takes place in the liver, assessment of the intrahepatic immune response will provide a more accurate evaluation of the local antiviral and inflammatory processes in the HBV infected liver[8]. Moreover, it is well described that the number, phenotype and function of immune cells, differs between liver and blood[9–11]. To date, no studies have systematically evaluated differences in chronic HBV livers at the distinct clinical phases by means of immunological techniques, such as flow cytometry, and only few studies have reported differences at the gene expression level of livers at distinct clinical phases using core needle biopsies collected for diagnostic purposes. Two studies compared the intrahepatic gene expression by microarray in chronic HBV patients, with respect to the first phase in chronic disease (IT phase) as a control and showed increased immune gene expression during chronic hepatitis phases, albeit relatively modest in number compared to chronic infection phases[12,13]. A third study by Lebossé *et al*. used the Nanostring technology highlighted a general downregulation of innate immune genes in chronic HBV when compared to non-HBV infected patients[14]. However, these studies did not include true uninfected healthy controls, and lack information on immune cell infiltrates in the infected livers.

To better understand the intrahepatic processes underlying viral replication and immune control, it will be critical to characterize the intrahepatic immune

milieu in the HBV-infected livers across different phases. To provide a global molecular profile of each phase, we combined state-of-the-art RNA sequencing (RNA-seq) and whole tissue multiplex immunofluorescence microscopy on core needle liver biopsies from a unique cohort of uninfected healthy controls and chronic HBV patients across the different clinical phases of the disease. This approach allowed us to assess the intrahepatic host-virus interplay by elucidating the level and make up of leukocyte infiltrates and host gene expression in relation to the degree of inflammation and HBV antigen burden in the liver. Contrary to previous observations, we observed a prevalent downregulation of metabolic genes and pathways across all chronic HBV phases and a general upregulation of innate and adaptive immune genes and pathways in chronic HBV, particularly in chronic-hepatitis phases. Moreover, we detected increased gene expression of immune-exhaustion genes in chronic hepatitis phases. Lastly, leukocyte infiltration was predominantly restricted to portal and peri-portal areas and only associated with the inflammation level (ALT) but not with the positivity for intrahepatic HBV antigens (HBcAg and HBsAg) or replication activity (serum HBV DNA).

MATERIAL AND METHODS

Patients and liver samples

Formalin fixed paraffin embedded (FFPE) core needle liver biopsies from 69 chronic HBV patients and 9 healthy controls were used for RNASeq (Table 1), and biopsies from a matched subset of chronic HBV patients (n=30) and healthy controls (n=7) were used for multiplex immunohistochemistry (Table S1). The livers of healthy individuals were collected to determine their eligibility as altruistic liver donors. All other liver biopsies were collected as part of routine clinical care at the Erasmus MC and archived. Patients were classified as IT, IA, IC or ENEG based on their serum HBV DNA, ALT levels and HBeAg status. An ALT of 30 IU/mL was considered normal and chronic hepatitis phases, IA and ENEG, were determined using a threshold of 1.5xULN ALT and/or 20,000 IU/mL the HBeAg status (positive and negative, respectively). Patients received neither antiviral treatment prior to biopsy, nor had any co-existing primary liver disease nor were co-infected with HCV, HEV, HDV or HIV. Histological evaluation of METAVIR staging was performed as described previously by a single liver pathologist in a uniform manner[15]. Due to the retrospective nature of this study, written informed consent was not obtained from each patient. Instead, the ethical review board of the Erasmus MC approved this study as it was in accordance with the COREON guidelines, which describes the use of coded-anonymous residual human tissue for scientific research (www.coreon.org).

Table 1: Patient characteristics of liver biopsies included in the RNASeq analysis

	Healthy control	*IT (Chronic infection HBeAg+)*	*IA (Chronic hepatitis HBeAg+)*	*IC (Chronic infection HBeAg-)*	*ENEG (Chronic hepatitis HBeAg-)*
N samples	9	15	15	23	16
Age (Median, IQR)	52 (28-57)	24 (18-32)	24 (18-27)	35 (29-45)	35 (24-45)
Male, n (%)	4 (44%)	4 (26%)	5 (33%)	10 (43%)	13 (81%)
ALT‡ (IU/mL) (Median, IQR)	24 (22-45)	29 (21–50)	68 (46-164)	27 (21–32)	101 (61–222)
Log_{10}HBV DNA (IU/mL) (Median, IQR)	NA	8.6 (8.1–8.8)	8.6 (7.4–8.7)	2.2 (<1–2.9)	6.0 (4.0–8.3)
HBeAg-positive, n (%)	NA	15 (100%)	15 (100%)	0 (0%)	0 (0%)
Anti-HBeAg Antibody-positive, n (%)	NA	0 (0%)	0 (0%)	23 (100%)	16 (100%)
HBV Genotype, n (%)					
A	NA	1 (7%)	1 (6%)	4 (17%)	2 (12%)
B		4 (27%)	3 (18%)	3 (13%)	2 (12%)
C		8 (53%)	7 (46%)	2 (9%)	2 (12%)
D		2 (13%)	3 (18%)	10 (43%)	7 (44%)
E		0 (0%)	1 (6%)	1 (4%)	2 (12%)
ND		0 (0%)	0 (0%)	3 (13%)	1 (6%)
Fibrosis ≤ F2, n (%) (METAVIR)	9 (100%)	14 (93%)	15 (100%)	23 (100%)	13 (81%)

ALT: Alanine aminotransferase
ENEG: (HBeAg)-negative hepatitis
HBeAg: Hepatitis B E antigen
HBV: Hepatitis B virus
IA: Immuno Active
IC: Inactive Carrier
IT: Immuno Tolerant
IU/mL:International Units/milliliter
NA: Not applicable
ND: Not determine

FFPE liver RNA extraction and transcriptome sequencing

Eight 10 μm-thick core needle liver biopsies were sectioned, deparaffinized, and total RNA was isolated using the RNeasy FFPE Kit (Qiagen, Germany) following the manufacturer's instructions. Prior to library preparation, rRNA was depleted using the Illumina Ribo-Zero rRNA Depletion kit (Illumina, USA) following the manufacturer's instructions, and fragmented into approximately 300 basepairs (bp). cDNA libraries for each sample were prepared using Illumina TrueSeq Total Gold. Libraries were sequenced at EA Genomics (Q^2 solutions, USA) using 100 bp paired-end reads on Illumina HiSeq platform aimed at 50 million reads per sample. Samples with less than 10 million protein-coding reads were excluded from the analysis. Sequencing fastq files will be publicly available at *https://www.ncbi.nlm.nih.gov/geo/* and upon reasonable request.

Ribo-depletion RNA-seq on liver biopsies from healthy individuals and chronic HBV patients

Raw paired-end reads were aligned to human reference genome hg38 downloaded from the USCS Genome Bioinformatics site using the STAR (v.2.7.3a) [16]. Picard tools were employed to detect at least 10 million reads in protein coding mRNA regions for downstream analysis[17]. Quantification of gene expression was calculated using featureCounts (v.1.4.6-p1), converted to RPKM using the R package edgeR[18]. Subsequently, scores were calculated for gene sets from MSigDB v7 using the R GSVA package[19,20]. Gene counts per sample were used to perform differential gene expression analysis between groups using the DESeq2 package (v.1.30.1)[21]. Weakly expressed genes across samples were removed from downstream analysis when fifty or less counts were detected in four samples per experimental group. Genes were considered as differentially expressed the adjusted $p<0.05$ and Fold Change $|>1.5|$. Remaining genes were normalized using the estimateSizeFactors function. Hallmark and gene ontology biological processes (BP) pathway analysis were conducted with Gene set enrichment analysis (GSEA) java software (v.4.1.0)[22] using the default settings "signal2noise" for ranking genes. To estimate immune cell proportions, we supplied RPKM values to the EPIC algorithm in the immunedeconv R package (v2.0.4)[23,24]. Single sample gene set scores for visualization were calculated using GSVA[20]. Reads were also aligned to a combined HBV and hg38 reference sequencing using BWA. Reads with at least 25 bp alignment to the HBV genome were included to calculate HBV burden. Reads with segments mapping to both HBV and hg38 were considered chimeric reads suggesting HBV integration.

Multiplex immunofluorescence assay and image analysis

To investigate the liver immune microenvironment and viral antigen expression, FFPE liver biopy sections, collected from patients at distinct HBV clinical phases (Table S1), were subjected to a novel multiplex immunofluorescence

(mIF) technology, InSituPlex (Ultivue Inc., USA)[25–27]. The custom panel used 3 iterative imaging cycles to detect 12 analytes using a cocktail of primary antibodies conjugated to unique DNA-barcodes (Figure S1). These antibody conjugates were used to identify the protein targets using fluorescently labeled probes, complementary to the barcodes. Between imaging rounds, a gentle dehybridization step was used to remove fluorescent probes from the previous round followed by incubation of the next set of fluorescent probes. Stained slides were scanned at 20X on the AxioScan SpectraX whole slide scanner (Zeiss, Germany) followed by a hematoxylin and eosin (H&E) stain. Images were co-registered with the UltiStacker software (Ultivue, USA) using DAPI as references from the different rounds. H&E images were digitally annotated guided by a board-certified pathologist, to define the liver tissue region. H&E and mIF channels were assessed at individual marker level for suitability of analysis including staining specificity, tissue integrity and presence of artefacts. Whole slide digital image analysis was performed using Visiopharm software (v.21.2.0.9368, Oracle Bio, UK) to generate cell phenotype data. Single cells were identified using DAPI for nuclear detection and NaKATPase was additionally used for hepatocyte formation. Customized cellular analysis algorithms were developed to detect and phenotype individual cells by setting the threshold to ensure only true signal was being detected by the algorithm.

Statistics

Statistical analyses of clinical and virological parameters were performed with GraphPad Prism (v9) software. Baseline variables between cohorts were compared with the use of chi-square tests for categorical variables, Mann-Whitney test or (Unpaired)T-test for continuous variables, unless otherwise indicated. All reported P values are two-sided. Significant differences were considered in all cases when $p<0.05$. Spearman/Pearson correlations were considered significant when $p<0.05$.

RESULTS

Immune genes and inflammatory pathways are enriched in HBV livers with chronic inflammation (IA and ENEG), whereas metabolic pathways are suppressed

Detailed knowledge of the immune and anti-viral responses that act during the distinct HBV clinical phases in chronically infected patients is essential to better understand the processes that underlie the fluctuations in viral replication and liver damage.

To study this, we performed RNA-seq on liver biopsies from 69 chronic HBV patients at the different phases (15 IT, 15 IA, 23 IC and 16 ENEG) and compared them to biopsies obtained from 9 individuals who volunteered as altruistic liver donors (Table 1). In agreement with EASL guidelines, phase-specific serological profiles were observed across chronic HBV based on HBV DNA, ALT and HBeAg.

Serum HBV DNA levels in HBeAg-positive phases (IT and IA) were the highest (mean HBV DNA>8Log10) with limited inter-patient heterogeneity within each phase (SD<1Log10). In contrast, ENEG phase displayed lower HBV DNA levels than HBeAg-positive phases (mean HBV DNA 6 Log10) but with greater heterogeneity (SD>2Log10). In contrast, serum HBV load in almost half of the IC patients (10/23) was undetectable, and in all but one patient displaying HBV DNA levels (<4Log10) (Figure S2). Moreover, peripheral biochemical ALT levels were within the normal range in IT and IC phases (mean<35 IU/mL) whereas IA and ENEG phases displayed elevated serum ALT levels (mean 126 and 208 IU/mL, respectively, clearly showing signs of ongoing liver inflammation (Figure S2).

Comparison of the liver transcriptome across the distinct HBV clinical phases to healthy livers highlighted a large disparity in the number of differentially expressed genes (DEG; adjusted p<0.05 and Fold Change |>1.5|). Although the patients in the IT and IC phase exhibit high variability in HBV DNA and HBeAg, their intrahepatic gene expression profile was comparable with a relatively low number of DEGs, 13 and 35 DEG, respectively (Figure 1A and S3, Table S2). In contrast, livers from IA and ENEG patients displayed 172 and 243 DEGs, respectively, with almost 70% of them being upregulated in chronic HBV compared to healthy livers (Figure 1A and S3, Table S2). Interestingly, this profile was comparable in all IA and ENEG patients (Figure 1B), despite the fact that serum ALT levels were highly variable (Figure S2). As shown in Figure 1B, livers from all four phases of chronic HBV demonstrated a common downregulation of genes involved in metabolic processes (*ADH4*, *ADH5*, *LIPC* and *FOLH1*) and complement system [classical pathway (*C1R* and *C1S*) and MAC attack complex (*C6*, *C8A* and *C8B*)]. Of note, all phases in chronic HBV, except IT, displayed decreased expression of albumin (*ALB*) (Table S2).

Besides a downregulation of metabolic genes, we also observed an increased immune gene expression in livers from chronic HBV as compared to healthy livers; this effect was most pronounced in IA and ENEG phases (Figure 1B). In the IT phase only *STAT1* and immunoglobulin-related gene *IGHG1* were found to be significantly increased, whereas only *GBP5* was found to be induced in IC livers when compared to healthy livers (Figure 1B Table S2). In contrast, among the upregulated DEGs found in the IA and ENEG phases, we identified multiple overlapping genes linked to both the innate and adaptive arms of the immune system (Figure 1B). Several HLA genes (*HLA-A/B/C*), antigen presentation genes (*CD74*, *CIITA* and *TAP*), interferon stimulated genes (ISGs; *STAT1*, *ISG20*), interferon-regulatory factors (IRFs; *IRF1* and *IRF8*) and genes with known antiviral function such as *IKBKE* and *SAMHD1* were detected. Furthermore, we observed an upregulation of cytokine-related genes involved in inflammatory processes, such as *CXCL9* and *CXCL10* together with *IL-32* and interleukin receptor *IL10RA*. Multiple genes related to different leukocyte populations, such as neutrophils (*CD53* and *PREX1*), NK-cells (*CLDC2D* and *CD96*), T-cells (*ZAP70*, *CD8*, *CD2*) and B-cells (*IGHG1*, *IGHG2*, *SIGLEC1*) showed increased gene expression in inflammation phases, with the strongest signal in the ENEG group, in comparison to healthy livers (Figure 1B). Most striking was the detection of genes encoding for molecules pivotal for T-cell activation

upon TCR engagement, such as *ICOS*, *PDK1*, *ITK* and *THEMIS*. Furthermore, we also observed a strong upregulation of genes encoding for immune exhaustion markers expressed on exhausted T-cells, such as *CTLA4*, *LGALS9*, *PDCD1* encoding for PD-1, *TNFRS19* encoding for 4-IBB, *SLAM-6* and NK-cell associated *SLAMF7* among others. Also increased expression of the genes encoding the transcription factors *TOX*, expressed in exhausted T-cells, and *FOXP3*, expressed in regulatory T cells, was observed.

Next, we assessed whether the findings at the individual gene level were supported by Hallmark gene set enrichment analysis (GSEA). Indeed, GSEA identified a general upregulation of inflammatory (inflammatory response, IL-6 JAK/STAT3 signaling) and IFN-associated (IFN-a/g) gene sets in chronic HBV as compared to healthy livers, although it was only significant in IA and ENEG phases (false discovery rate; FDR<0.05; Figure 1C). Moreover, in line with the findings at the individual gene level, metabolic gene sets related to adipogenesis, fatty and bile acid and xenobiotic metabolism were all significantly downregulated in all phases of chronic HBV compared to healthy livers (Figure 1C). In summary, these results demonstrate that at the transcriptional level, livers of chronic HBV patients display a downregulation of numerous metabolic genes in all four clinical HBV phases. Moreover, livers from the IA and ENEG phase exhibit a relatively strong increase in differentially expressed immune genes and inflammatory pathways as compared to healthy livers, while this increase is minimal in IT and IC livers.

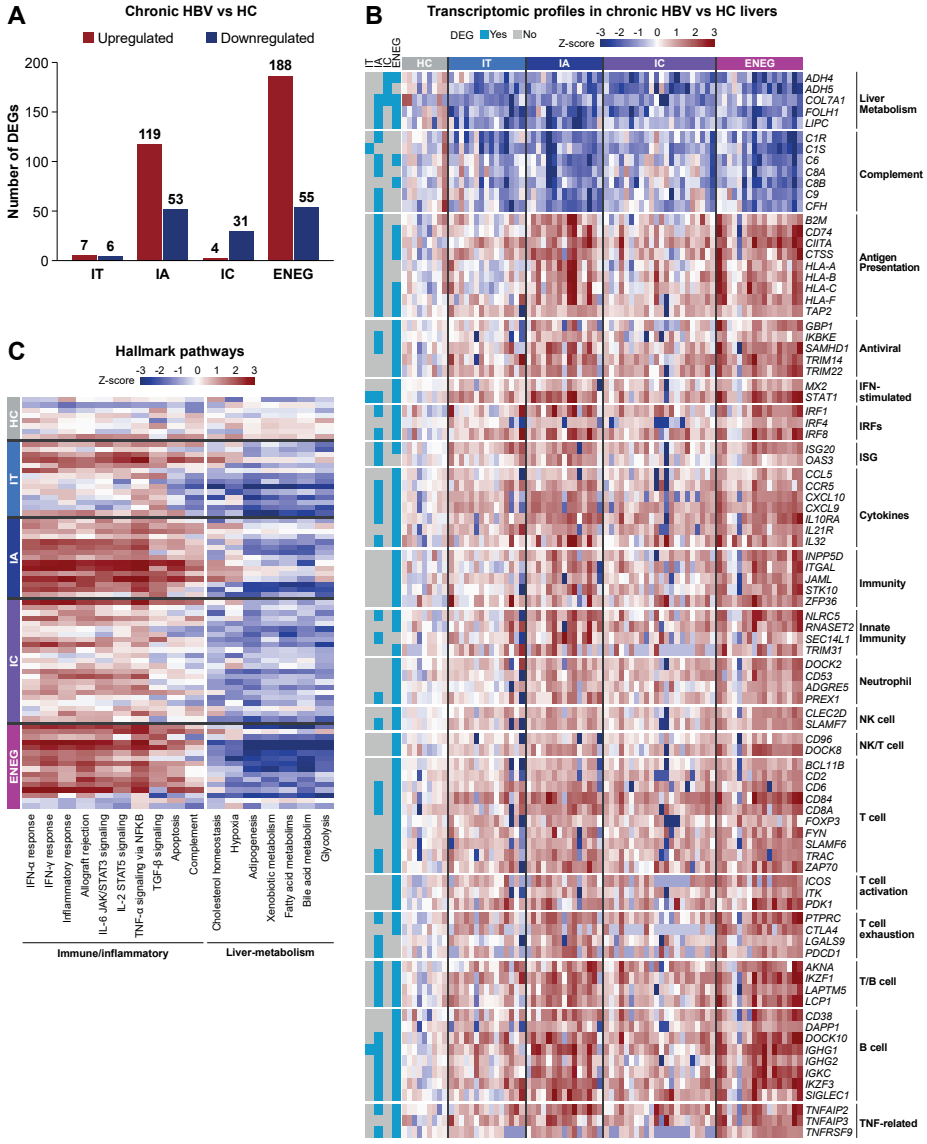

Figure 1. Increased immune gene expression and inflammatory pathways in chronic HBV livers.
(A) Bar plot representing the number of Differentially expressed genes (DEG, adjusted p<0.05 AND Fold Change |>1.5|) in each chronic HBV phases compared to the HC group. Red and blue bars represent upregulated and downregulated genes, respectively. (B) Heatmap depicting z-scores from metabolic, innate and adaptive immune genes in HC and chronic HBV patients. Scaling was conducted per gene (row) using the mean gene expression in the HC group. DEGs in chronic HBV (IT, IA, IC and ENEG) phases compared to HC are indicated highlighted in blue (left side). (C) Heatmap depicting z-scores of selected Hallmark pathways categorized as Immune/inflammatory and liver-metabolism across HC and chronic HBV livers. Scaling was conducted per pathway (column) using the mean pathway score in the HC.
Positive and negative z-scores in panel B and C heatmaps are colored in gradients of red and blue, respectively.

Livers from IA and ENEG phases display considerable transcriptional overlap yet distinct patterns correlate with ALT

To further characterize intrahepatic transcriptomic profiles in the different phases of chronic HBV, we performed GSEA on Biological Processes (BP) from Gene Ontology pathways, which contains an extensive list of additional immune and non-immune gene sets. In line with the Hallmark GSEA, a core group of metabolic BPs (organic acid catabolism, detoxification, fatty acid metabolism and cellular respiration) was found to be significantly downregulated (FDR<0.05) in all chronic HBV phases, except the IA phase (FDR » 0.06), when compared to healthy livers (Figure 2A). In contrast, no BPs were upregulated in the IT and IC group compared to the healthy livers. As depicted in Figure 2A and 2B, several of the downregulated BPs were found to overlap between the IT and IC phase (Figure 2A-B, Table S3 and S4 for IT and IC vs HC, respectively. Overlapping BPs are highlighted in yellow).

In contrast to the IT and IC livers, BPs identified in livers from IA and ENEG patients were almost all upregulated and exhibited a significant increase in those related to leukocyte activation (NK-cell, T-cell, B-cell), neutrophil chemotaxis and immune-activation terms compared to the healthy group (Figure 2A, Table S5 and S6 for IA and ENEG vs HC, respectively). Likewise, a high number of BP terms overlapped (n=202, 63%) between the IA and ENEG phase (Table S5 and S6 highlighted in yellow).

Although the direct GSEA comparison between IA and ENEG samples yielded no differences in BPs (not shown), the observed subtle differences in enriched BPs in IA and ENEG compared to the healthy group made us consider whether both phases could be distinguished by their individual gene expression profile on the basis of the upregulated DEGs. In line with the general overlap seen in immune activation Hallmark gene sets and BPs terms, we found almost 40% overlap (n=83) of DEG between phases (Figure 2C, Table S7). A good representation of these common genes was found in those encoding for the inflammatory chemokines IP-10 (*CXCL10*) and MIG (*CXCL9*) together with immune-activation (*RELT*, *STAT1* and *IRF1*) and T-cell related genes (*CD8A, CD84*), all indicative of the ongoing inflammation during these active phases. On the other hand, 36 (16%) and 105 (47%) of the DEG were exclusively increased in IA and ENEG when compared to the healthy group, respectively. Interestingly, livers of patients in the ENEG phase displayed a higher abundance of genes participating in T-cell activation (*ICOS*, *BTN3A1*, *BTN3A2*) or T-cell immune-exhaustion (*CTLA4*, *TOX*, *FOXP3*, *CD38* and *SLAMF6*) than those found in livers from IA patients (*PDCD1* and *LGALS9*) (Figure 2C, Table S7).

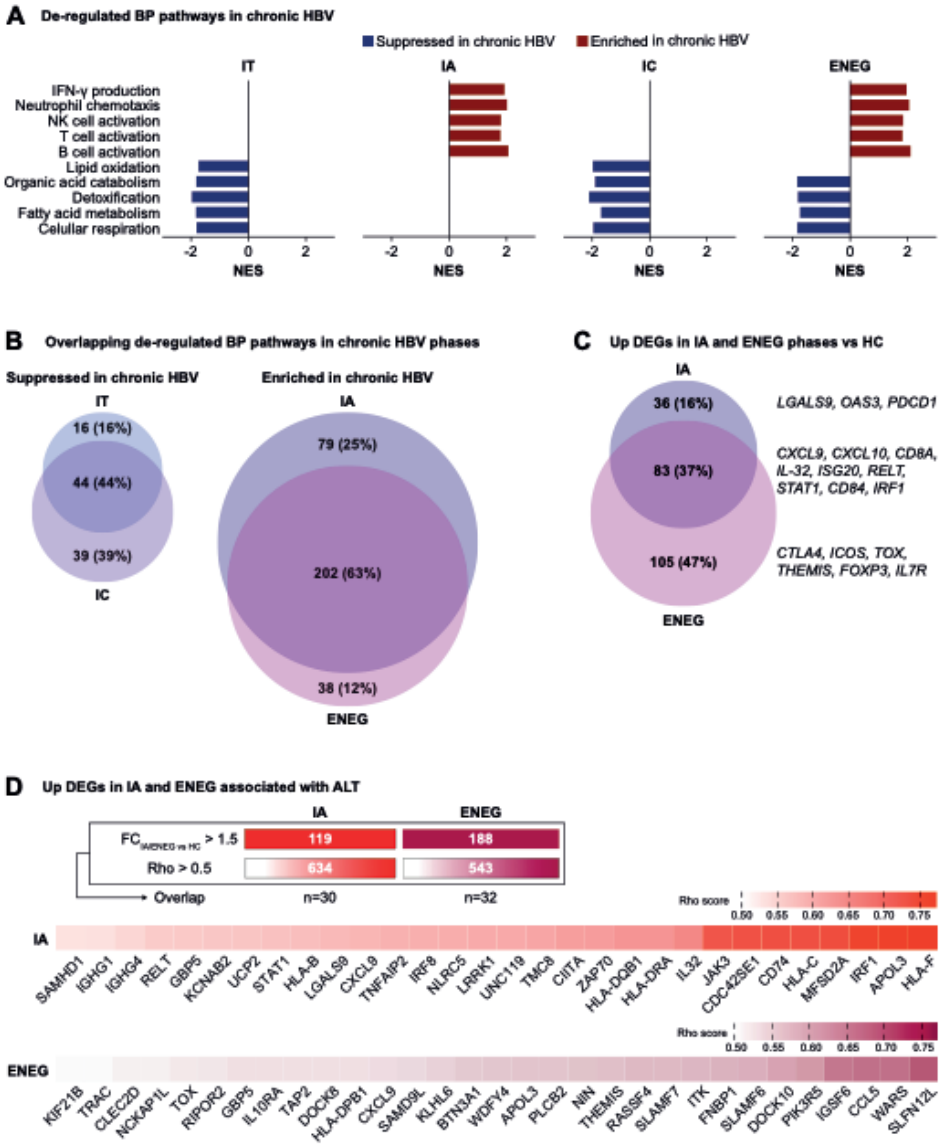

Figure 2. Distinct intrahepatic transcriptomic profiles associated with ALT elevation, and more accentuated immune exhaustion gene profile found in ENEG as compared to the IA livers. (A) Bar plots displaying normalized enrichment scores (NES) of ten significantly de-regulated (adjusted p<0.05) Gene ontology Biological Process (BP) pathways in chronic HBV phases compared to HC. Red and blue bars represent pathways enriched and suppressed in chronic HBV phases, respectively. (B) Venn-diagrams with number and percentage of (non-) overlapping BP pathways downregulated in IT and IC or induced in IA and ENEG when compared to HC. (C) Venn-diagram indicating the number of (non-)overlapping DEG (Fold Change>1.5 and adjusted p<0.05) upregulated in IA and/or ENEG compared to HC. (D) Overlap of DEGs upregulated in livers at the IA or ENEG phase vs HC livers, and significant and positively associated (p<0.05 and Spearman Rho>0.5) with ALT values within IA or ENEG. Color-gradient bars indicate increased Rho scores.

Having observed the minor but significant differences in transcriptomics in IA and ENEG livers with respect to healthy, we assessed whether specific gene expression profiles of IA and ENEG livers were associated with serum ALT levels. Indeed, we identified 30 and 32 candidate genes in the IA and ENEG group, respectively associated with ALT (Rho>0.5), where only *CXCL9*, *GBP5* and *APOL3* correlated in both phases (Figure 2D). Interestingly, ALT correlations with genes related to T-cell related activation and exhaustion were more often found in livers from ENEG patients (*BTN3A1*, *ITK*, *TRAC*, *TOX*, *SLAMF7*, *SLAMF6*) than IA patients (*ZAP70* and *LGALS9*). Taken together, these results highlight two major distinct profiles in chronic HBV based on the inflammation status, yet subtle and significant differences discriminate IA and ENEG chronic hepatitis phases with an even greater immune exhausted gene expression phenotype in ENEG.

Leukocyte infiltration is increased across chronic HBV natural history and correlates with serum ALT levels but not HBV DNA

Gene expression analysis at the individual gene level and GSEA analysis on BP pathways showed increased expression of leukocyte-related genes and leukocyte activation gene sets in chronic HBV phases, particularly the IA and ENEG phases, which are well known to exhibit more pronounced liver inflammation than the IT and IC phases. To further determine if these findings translated at the protein level, we executed a mIF assay by which to detect multiple analytes on a single FFPE slide, enabling us to phenotype the lymphocyte subsets. We stained a cohort of 37 liver biopsies, a subset of the original cohort, across the natural history in chronic HBV – characterized by <F1 and ≤5% steatosis- and compared them to the healthy liver tissues (at least 7 biopsies per phase, Figure 3A, Table S1). In liver biopsies from healthy controls, T-cells (mean 122 cells/mm^2) and B- cells (mean 9 cells/mm^2) immune subsets were primarily found around portal areas while CD68 Kupffer cells and macrophages were present across the liver parenchyma (Fig. 3A). However, liver biopsies across all chronic HBV phases showed increased evidence of immune cell activity. The IT and IC groups showed a modest increase in immune cell densities: T-cells (mean 182 and 185 T-cells/mm^2), B-cells (mean 20 and 14 cells/ mm^2) and macrophages (mean 202 and 264 cells/ mm^2). Consistent with previous observations, liver biopsies from patients belonging to the IA and ENEG groups showed pronounced infiltration predominantly around portal and periportal areas [T-cells (mean 383 and 458 T-cells/mm^2), B-cells (mean 33 and 61 cells/mm^2)] while macrophages (mean 193 and 396 cells/mm^2) were found both, around portal structures, as well as dispersed across the parenchyma (Fig. 3A). Analysis of the staining profiles in chronic HBV livers demonstrated that compared to healthy livers, the number of total CD3$^+$ T-cells showed an increase in all chronic HBV phases, albeit that the increase was not statistically significant in IT patients (Figure 3B). In contrast, the number of CD8$^+$ T-cells was significantly increased in HBeAg-negative patients (IC and ENEG) whereas CD4$^+$ T-cells showed an increased trend

in phases with active inflammation (IA and ENEG, p=0.08 and p<0.05, respectively). These increased trends observed in the $CD4^+$ and $CD8^+$ T-cell subsets were further accentuated when expressed as a percentage of the total CD3 T-cells. Interestingly, the frequency of CD4 and CD8 T-cells in the vast majority of healthy livers was less than 25% resulting in a larger proportion of double-negative CD4 and CD8 T-cells, possible NKT and/or MAIT cells, than in chronic HBV livers (Figure 3C). $CD20^+$ B-cells showed a comparable trend as $CD4^+$ T-cells, albeit in lower numbers, as the ENEG phase showed the greatest increase. Moreover, the number of macrophages and those positive for PD-L1, linked to induce immune-exhaustion/impairment in chronic viral infections[28], remained comparable in livers of healthy individuals and across chronic HBV phases (Figure 3B and S4). Similarly, the number of CD3 T-cells positive for PD-1 remained constant across chronic HBV phases and to a comparable level observed in healthy control livers (Figure S4). Moreover, the in-silico estimated fractions, by EPIC, of the different T-cell subsets (CD4 and CD8), B-cells and Monocytes highlighted a similar pattern of increased leukocyte infiltrate in chronic HBV, further elevated in IA and ENEG, as observed by mIF (Figure 3D). Not surprisingly we observed significant positive associations (rho ≥ 0.5) between the number of detected leukocytes per mm^2 and the transcriptome-based estimated fractions (EPIC) (Figure S5).

Interestingly, the higher degree of heterogeneity in leukocyte infiltrate [number of $CD4^+$ and $CD8^+$ T-cells], which was seen in chronic inflammation phases (IA and ENEG) partially coincided with higher serum ALT levels. Indeed, correlation analysis highlighted the significant positive association between leukocyte infiltration (B-cells, T-cells and CD4 and CD8 T-cell subsets) and serum ALT levels (Figure 3E). In contrast, none of the infiltrating leukocyte populations correlated with serum HBV DNA (Figure 3E).

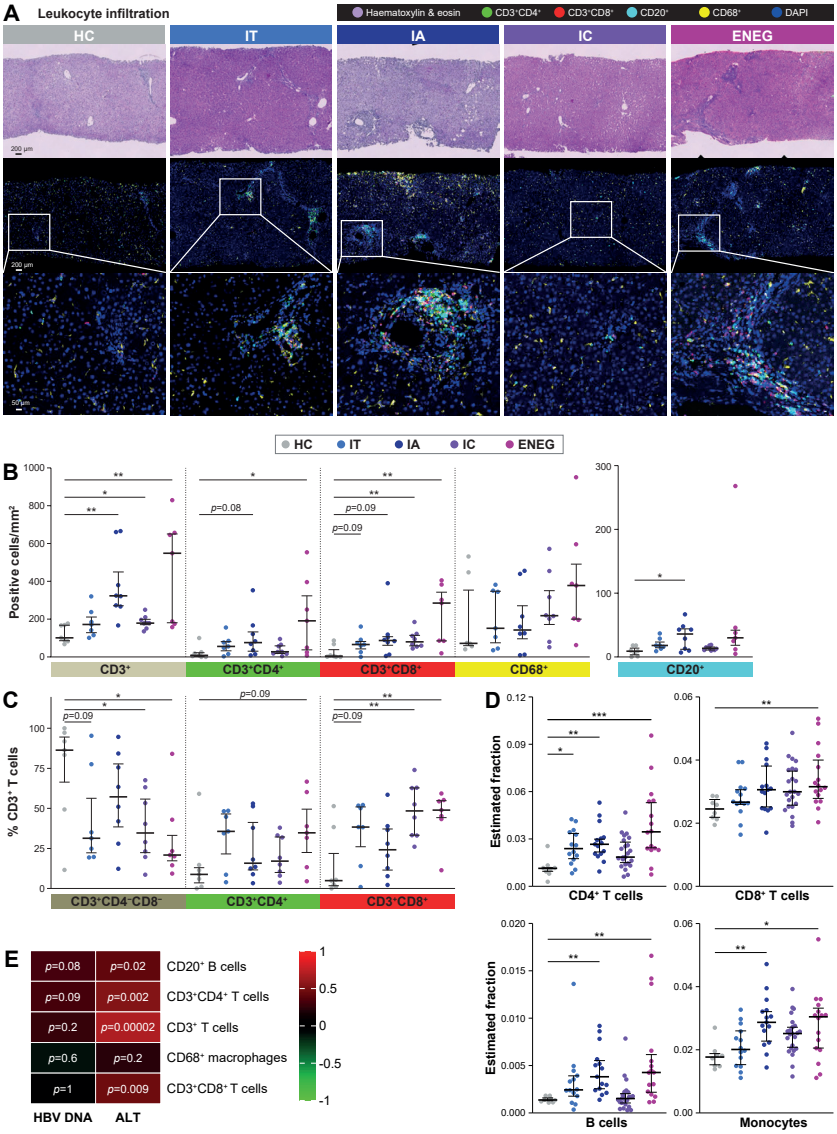

Figure 3. Leukocyte infiltration is increased across chronic HBV natural history and correlates with serum ALT. (A) Leukocyte infiltration by multiplex immunofluorescence staining from HC and chronic HBV phases displaying T-cells ($CD3^+CD4^+$ and $CD3^+CD8^+$, green and red, respectively), macrophages ($CD68^+$; yellow) and B-cells ($CD20^+$; teal). (B) Density of cells per mm^2 positive for CD3, CD4, CD8 T-cells, CD20 B-cells and CD68 macrophages. (C) Percentage of $CD4^-CD8^-$ T-cells, CD4 and CD8 T-cell subsets and in chronic HBV and healthy livers. (D) In silico EPIC estimated fractions of bulk leukocyte populations: CD4 and CD8 T-cells, B-cells and macrophages/monocytes. (E) Spearman correlations between peripheral biomarkers (HBV DNA and ALT) and leukocyte infiltrate [number of $CD3^+$ cells, T-cell subsets ($CD4^+$ and $CD8^+$), $CD20^+$ B-cells and $CD68^+$ macrophages per mm^2] in chronic HBV livers. Correlation Rho coefficients are color-coded as a transitional gradient from red (Rho>1) to green (Rho<0). P-values for each correlation pair is displayed within each quadrant.

Panel B-D display median and IQR bars. *p<0.05, **p<0.01, ***p<0.001; Unpaired T-test compared to healthy samples for panels B, C and D. Inter-quartile range (IQR) is shown.

Intrahepatic viral antigens are heterogeneously expressed across and within CHB natural history phases and do not correlate with leukocyte infiltrate

Previous results have highlighted liver inflammation as the main driver in the highly differential transcriptomic and leukocyte infiltrate profiles seen in chronic HBV livers. However, whether these inflammatory processes are correlated with the intrahepatic expression of the viral proteins HBsAg and HBcAg remains unclear. To that end, we first performed systematic analysis of the viral staining pattern in HBV biopsies in the context of the different chronic HBV phases. We analyzed viral antigen proteins in the liver as part of the mIF assay by detecting for HBsAg and HBcAg and further evaluated the parameters that correlate with the observed liver inflammation.

Infected hepatocytes were detected by positivity for HBsAg and/or HBcAg using Na/K-ATPase, panCK and CD299 as segmentation markers to selectively guide hepatocyte classification at the single cell level (Figure S6). Heterogeneous expression of surface and/ or cytoplasmic HBsAg was detected in infected liver biopsies across all stages of HBV (Figure 4A). Upon quantification using digital image analysis tools on the stained images, a gradual decline in the percentage of infected hepatocytes from IT, IA, IC to ENEG phases was seen (Figure 4B). The percentage of hepatocytes stained positive for HBsAg tended to decline over the course of chronic HBV (mean IT: 57%, IA: 30%, IC: 32% and ENEG: 27%), but was highly heterogeneous within and across groups (Figure 4B). As expected, HBcAg positive hepatocytes were identified only in IT and IA patients (mean IT: 70%, IA: 51%; Figure 4B). To note, HBcAg detection was predominantly localized in the cytoplasm of infected hepatocytes and rarely in the nucleus (nuclear HBcAg in infected cells <25% in 7/7 IT; 6/8 IA; 6/7 ENEG; 7/7 IC patients) (Figure S6). In contrast, HBsAg was heterogeneously detected in the membrane and cytoplasm of infected cells (Figure S6). Moreover, as presented in Figure 4C, the transcriptome-based detected integration of HBV DNA in the host genome (chimeric reads) is relatively constant over the course of chronic infection, while the total non-integrated HBV reads are highest in IT and IA, suggesting that this distinct pattern in HBcAg protein expression is predominantly driven by non-integrated forms of HBV.

When we performed correlation analysis to determine whether the degree of liver inflammation correlated with the intrahepatic expression of HBcAg and HBsAg, no correlation was found between any of the leukocyte populations and the HBV viral proteins HBcAg and HBsAg (Figure 4D). However, we found a strong correlation between HBcAg and HBV DNA ($p<0.001$; $r^2=0.864$) but interestingly, no correlation with HBsAg levels (Figure 4D). Together, these results demonstrate that the degree of liver damage but not HBV virological markers is associated with the degree of leukocyte infiltration in chronic HBV livers.

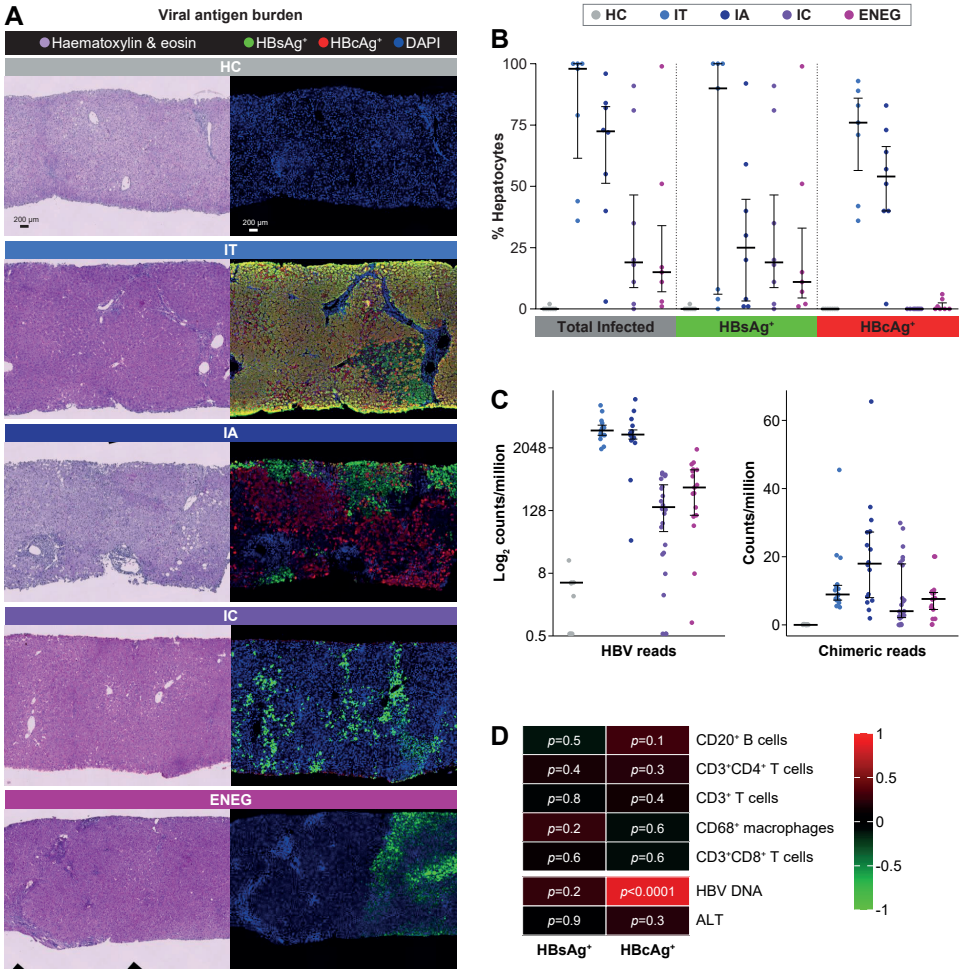

Figure 4. Intrahepatic viral antigens are heterogeneously expressed in chronic HBV livers and do not correlate with leukocyte infiltrate.

(A) HBcAg (red) and HBsAg (green) immunofluorescence staining overlays representative of liver biopsies from healthy individuals and chronic HBV phases. (B) Percentage of hepatocytes positive for HBcAg and/or HBsAg in chronic HBV livers. (C) Number of HBV and host-integrated (chimeric) reads (expressed as counts per million) in chronic HBV phases. (D) Spearman correlation between HBV viral burden (percentage of hepatocytes positive for HBcAg or HBsAg) and the leukocyte infiltrate [number of $CD3^+$ cells, T-cell subsets ($CD4^+$ and $CD8^+$), $CD20^+$ B-cells and $CD68^+$ macrophages per mm^2] in chronic HBV livers.

Correlation Rho coefficients are color-coded as a transitional gradient from red (Rho>1) to green (Rho<0). Panel B-C display median and IQR bars. P-values for each correlation pair is displayed within each quadrant. Inter-quartile range (IQR) is shown.

DISCUSSION

The present study examines in detail the intrahepatic interplay between HBV and the host-immune response in all four phases of chronic HBV. Through mIF and liver transcriptome analysis (RNA-seq) we show that livers of patients in the IT and IC phase show modest levels of CD3 T-cell infiltration and an altered immune gene expression profile when compared to healthy livers. These changes found in IT and IC livers with respect to their healthy counterparts were more pronounced IA and ENEG livers, where serum ALT, but not serum HBV DNA or intrahepatic HBV proteins (HBsAg and HBcAg) correlated with leukocyte inflammation. Besides innate and adaptive immune genes, an upregulation of genes encoding for immune-exhaustion markers was also observed in IA and ENEG livers, with the highest increase in ENEG. All chronic HBV phases showed a common suppression of metabolic genes sets compared to healthy. Moreover, all chronic HBV phases, in particular, IA and ENEG phases showed reduced expression of complement genes.

In contrast to the current view in which HBV can increase metabolic signaling pathways such as lipogenesis via viral proteins such as HBx[31] we demonstrate suppression of liver metabolic gene sets such as fatty/lipid and bile acid metabolism or response to toxic substrate in all chronic HBV phases. A possible explanation for this observation is that the current understanding is largely supported by in vitro studies with HBV-infected hepatoma cell lines, which may not reflect the intrinsic complexity of chronic HBV disease in human livers. Furthermore, a recent study evaluating the transcriptomic changes over time in distinct liver-injury mice models showed metabolic genes and pathway downregulation coinciding in time with inflammatory pathways inducement. This was later confirmed in various etiologies in human livers, including HBV infection[32]. Consistent with this study, we observed the strongest metabolic downregulation in those phases accompanied by increased inflammation. Moreover, metabolomics studies have shown a de-regulated serum lipid profile with strong downregulation of specific lipid metabolites in chronic HBV patients compared to controls[33,34]. However, it cannot be excluded that a more extensive infiltrate in the liver may alter the transcriptomic input from parenchymal cells leading to the observed reduced expression of metabolic genes as compared to healthy livers in our analysis. Single-cell RNA-Seq of liver parenchymal cells and/or spatial transcriptomics may provide more detailed information needed to resolve this issue.

Besides the downregulation of metabolic gene sets in chronic HBV, we observed a general upregulation of diverse innate and adaptive immune genes in chronic HBV, albeit only statistically significant in livers from IA and ENEG phases when compared to healthy livers. These results are in stark contrast with the Nanostring-evaluated transcriptomic characterization of chronic HBV livers conducted by Lebossé et al., where a large number of innate immune genes (i.e., ISGs, TLR-related) were downregulated in livers of chronic HBV patients compared to non-viral infected controls[14]. Of note, whereas we included core needle liver biopsies from altruistic liver donors as healthy uninfected controls, Lebossé's study

included liver material from a mix of six patients with gallbladder stones, one ovarian cystadenoma and two non-metastatic colon cancer who underwent liver surgery as controls, all with arguable ongoing inflammatory processes[14].

Interestingly, the transcriptomic profile we observed in HBV phases was highly homogeneous across patients despite fluctuating levels of serum biomarkers such as HBV DNA and ALT. Hence the fluctuating serological profile characteristic of these phases is not mirrored by changes in the intrahepatic transcriptome. However, this observation might be at least partially explained by the moment in which the liver biopsy was taken in relation to when the ongoing inflammation in the liver started. Moreover, inflammatory chemokines encoding genes such as *CXCL10* and *CXCL9* as well as several innate and adaptive immune genes were found to be upregulated in IA and ENEG phases suggesting that these phases are immunologically more active. Further strengthening this view, we found T-cell activation genes such as *ICOS*, *ITK* and PDK1to be upregulated. However, a number immune-exhaustion encoding genes were also found to be upregulated in livers in the IA and ENEG phases. Besides genes encoding for markers previously observed to be expressed in HBV-specific T-cells (*PDCD1*, *CTLA4*, *LGALS9*) *TOX* was also observed, paralleling a recently identified transcription factor preferentially found in phenotypically and functionally exhausted HBV-specific CD8+ T-cells[35]. Furthermore, newly identified genes were observed encoding for other T-cell related immune-exhaustion or inhibitory markers, such as *SLAMF7*, *SLAMF6*, *TNFRSF9, TNFAIP2* and *TNFAIP3*[36–40]. A larger proportion of these genes were found to be upregulated in ENEG livers which indicates that minor but significant differences may be present in the T-cell responses between IA and ENEG phases.

For the first time, we now demonstrate an increased number of T-cells in livers at chronic HBV phases which were further increased in phases with elevated ALT (IA and ENEG) when compared to healthy livers, contrary to the comparable frequencies of leukocyte subsets in blood across chronic HBV phases[5–7]. In contrast, we detected lower frequencies of CD4CD8 double-negative T-cells in all livers from chronic HBV phases compared to healthy. Whether this population represents NKT and/or MAIT cells, which can comprise up to 15% of the intrahepatic leukocytes[41,42], and what is their functional status warrants further investigation. However, other chronic viral infections such as HIV and HCV have shown lower peripheral and intrahepatic MAIT frequencies with a more exhausted phenotype compared to healthy controls[43,44]. Moreover, some livers from patients classified in the IA and ENEG phase displayed immune infiltrate levels comparable to uninfected controls despite the homogeneity in their transcriptome. Interestingly, leukocyte infiltration positively associated with serum ALT levels, but not with serum HBV DNA and intrahepatic HBcAg and HBsAg proteins expression. This disconnect between intrahepatic immune profiles and viral markers strengthens our previous observation in which intrahepatic HBsAg expression minimally altered the transcriptome in the IC livers[45]. Furthermore, the positive association seen for the leukocyte infiltrate and serum ALT levels coincides with the paradigm in which immune-mediated liver damage induces ALT release by dying hepatocytes[1].

However, further studies are warranted to better understand whether the limited but detectable T-cell infiltrate present in IT and IC phases affects intrahepatic responses and initiates cytopathic damage. In addition, the underlying mechanisms that trigger the inflammatory response during an ongoing HBV infection in the absence of antiviral treatment leading to the observed leukocyte recruitment and elevated ALT levels need more attention. Further studies are needed to confirm these findings, for instance by sampling the liver via fine needle aspirates, and analysis by multi-color flowcytometry and scRNA-Seq can be conducted[11].

In summary, the comprehensive characterization of a unique cohort of livers of healthy individuals and chronic HBV patients revealed a previously unreported increase in leukocyte infiltrate and immune gene expression in chronic HBV phases, and downregulation of metabolic gene sets when compared to healthy controls. These increased immune-gene profiles were significantly accentuated in IA and ENEG livers which showed a common upregulated profile of innate (ISGs, antiviral) and adaptive immune (T-/B-cell) genes, while immune-exhaustion and inhibitory genes were most pronounced in ENEG livers. Given the increased interest of HBV studies in liver over blood, our comprehensive analysis from a large group of well-defined liver biopsies serves as starting point for future more detailed studies also at the single cell level. Our results show the heterogeneity of intrahepatic processes in the different phases, which needs to be taken into account in ongoing efforts to improve treatment strategies for chronic HBV.

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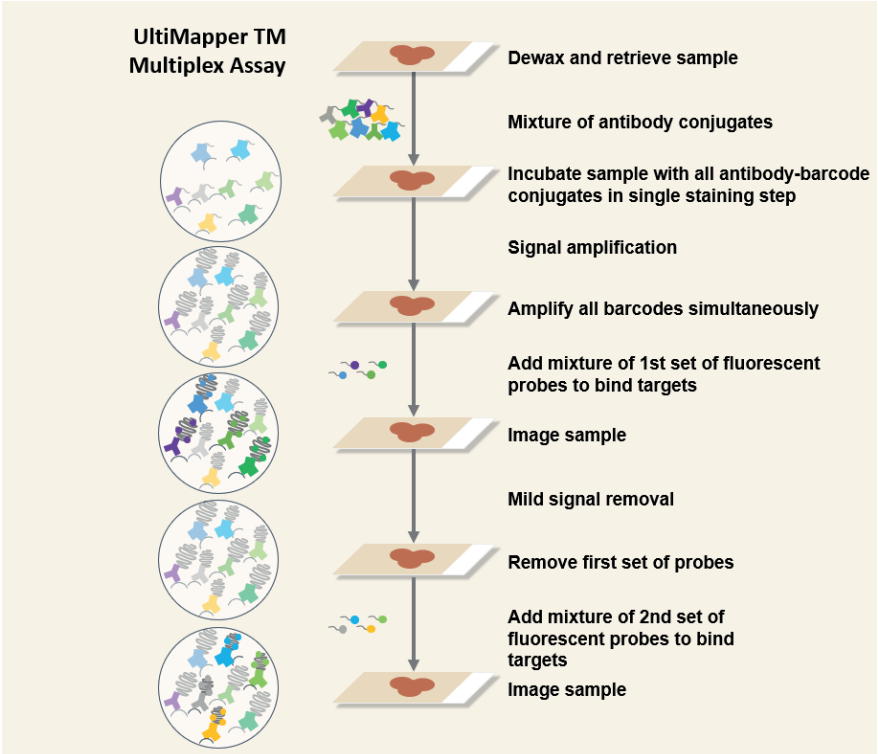
SUPPLEMENTARY MATERIAL

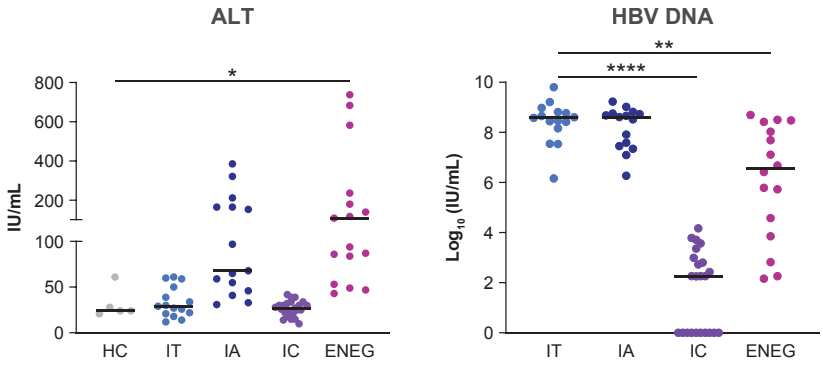
Determination of peripheral clinical and virological parameters

Biochemical and virological characteristics were determined on serum or plasma samples collected at the time of biopsy. ALT and AST levels were measured on an automated analyzer. Serum HBeAg and anti-HBe antibodies were determined quantitatively using the Abbott IMX system (Abbot, USA). HBV DNA levels and HBV genotype were determined using the COBAS AmpliPrep-COBAS TaqMan HBVv2test (Roche, USA) and INNO-LiPA HBV Genotyping assay (Fujirebio, Belgium), respectively. Serum HBsAg quantification was measured by chemiluminescent immunoassay on an automated Liaison analyzer (Diasorin, Italy). ALT, AST and HBV DNA levels are expressed in units/milliliter (IU/mL).

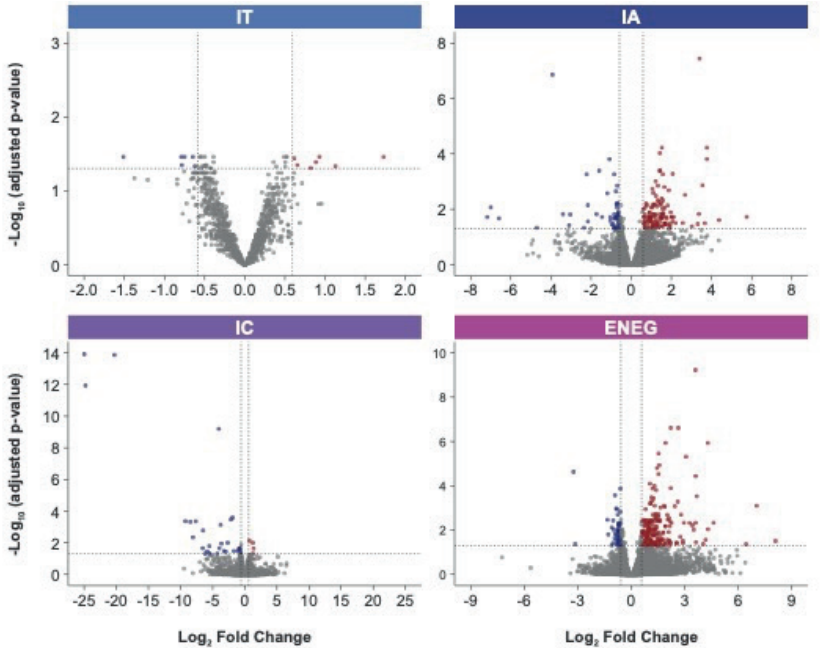


Round/ Filter	FITC	TRITC	Cy5	Cy7
1	HBcAg 366-2 (Gilead Sciences)	CD299 EPR11211 (Abcam)	Na^+K^+ ATPase EP1845Y (Abcam)	HBsAg XTL17 (Gilead Sciences)
2	PanCK AE1/AE3 (Abcam)	PD-1 NAT105 (Abcam)	CD4 SP35 (Abcam)	CD3 BC33 (Abcam)
3	CD8 C8/144B (Abcam)	CD68 KP1 (Abcam)	PD-L1 73-10 (Abcam)	CD20 L26 (Abcam)

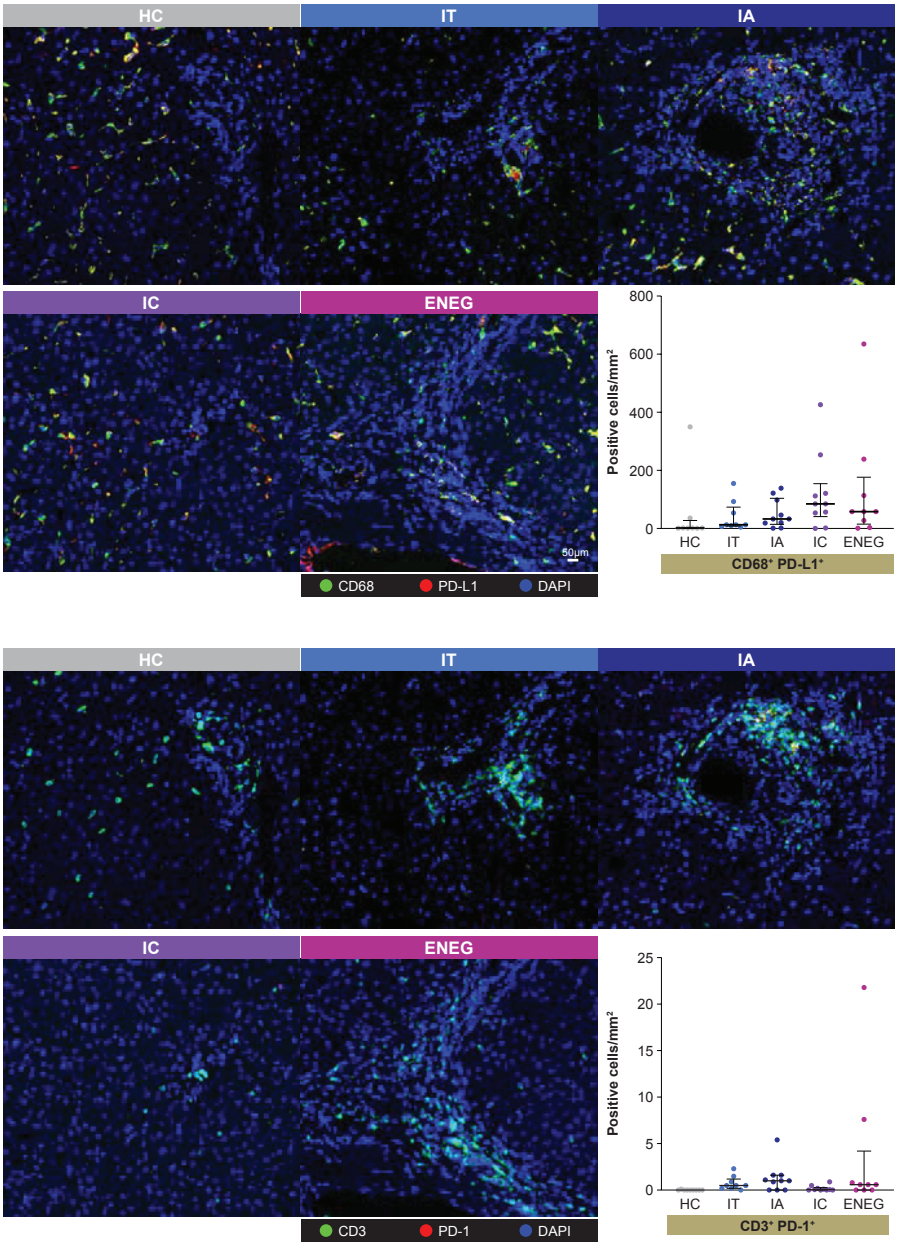
Supplementary Figure 1: Multiplex Immunofluorescence procedural overview.
Schematic representation of the key steps conducted to performed multiplex immunofluorescence on liver biopsies from uninfected healthy control and chronic HBV patients. All biopsies were subjected to 3 rounds of staining, imaging and dye-inactivation. The markers used for this analysis include HBcAg, HBsAg, CD3, CD4, CD8, CD20, CD68, PD-1, PD-L1, CD299, PanCK and NaKATPase.



Supplementary Figure 2: Serological ALT and HBV DNA levels in chronic HBV phases and altruistic healthy donors.
Left and right panels show serum ALT (IU/mL) and HBV DNA (Log10 IU/mL) in the four phases of chronic HBV and healthy (left panel only). Ordinary One-way ANOVA with Dunnett's post-hoc correction for multiple comparisons taking HC or IT as reference group (ALT and HBV DNA, respectively). *p< 0.05, **p< 0.005, ***p<0.0001

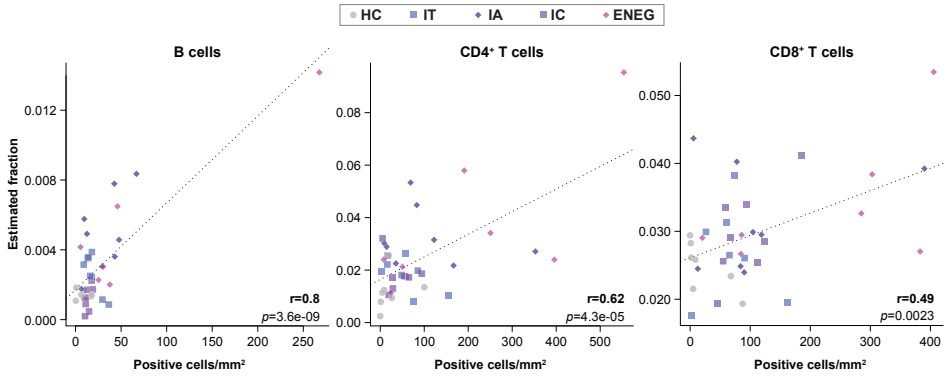


Supplementary Figure 3: Volcano plots of differentially expressed genes in chronic HBV vs healthy livers.
Negative Log10(adjusted p-value) (Y-axis) and fold-change (X-axis) values for each gene in chronic HBV phases vs HC.

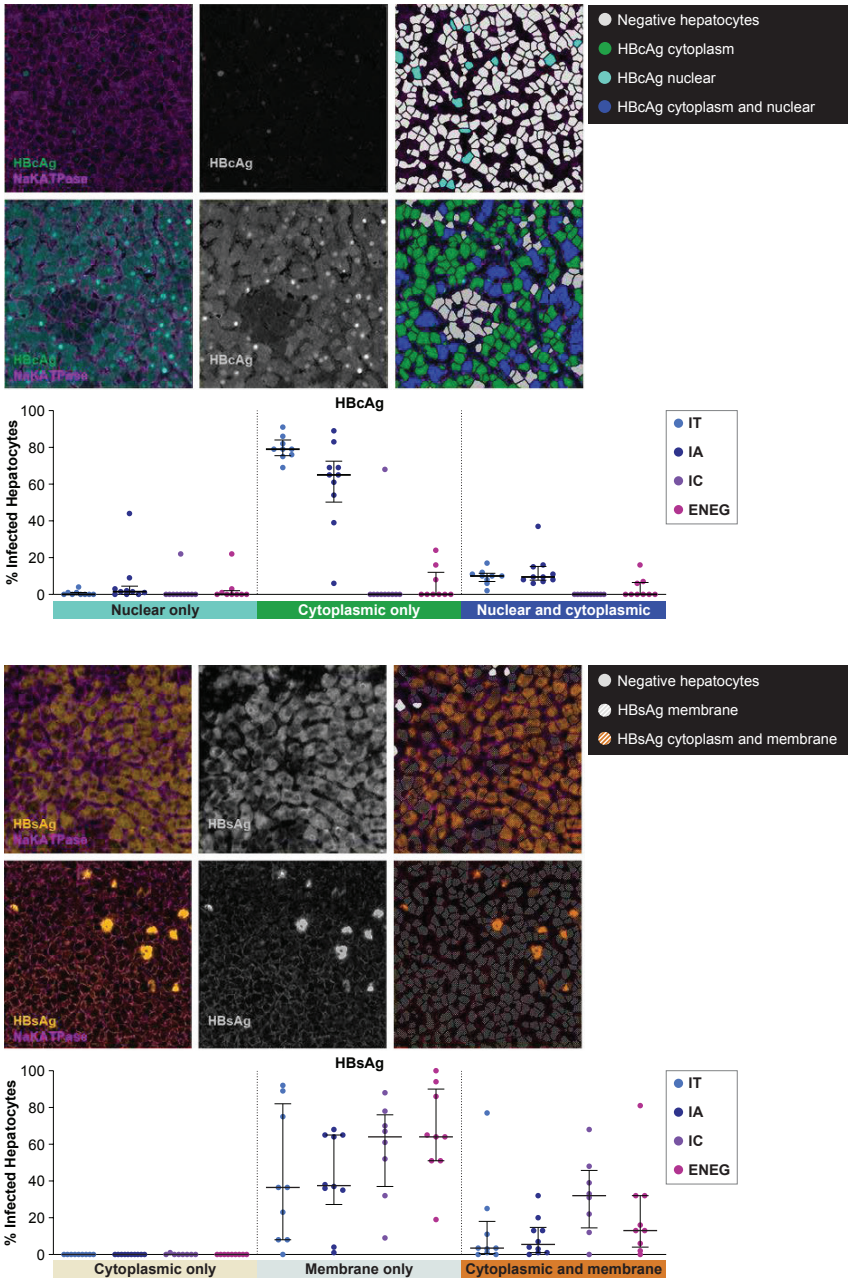


Supplementary Figure 4: Expression of check point inhibitors PD-1 and PD-L1 in liver biopsies across chronic HBV natural history.

Top and bottom panel: Expression of PD-L1 on CD68 macrophages and PD-1 on CD3 T-cells across the natural history of chronic HBV



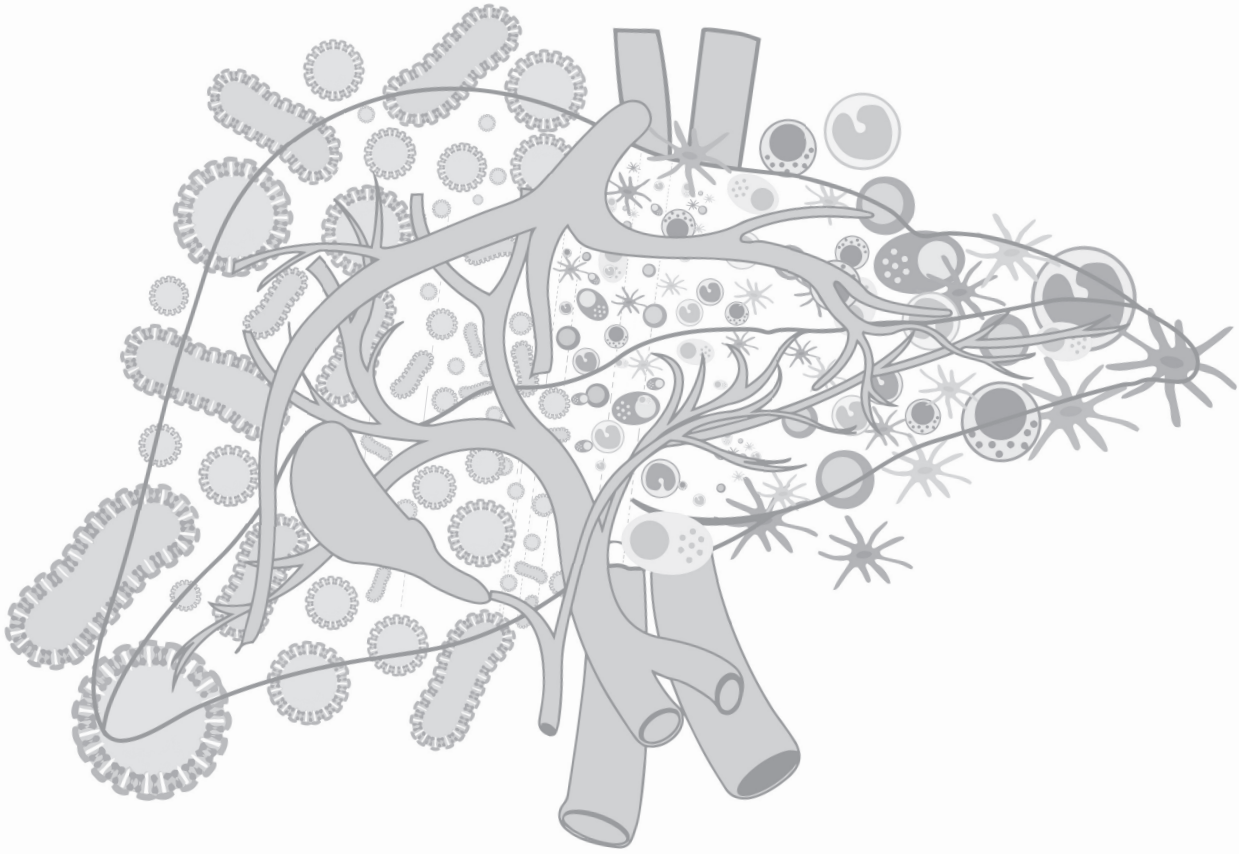
Supplementary Figure 5: Correlation between leukocytes detected by multiplex immunofluorescence and EPIC (in-silico) leukocyte estimated fractions.
Scatter plots illustrating the relationship between the number of T-cell subsets ($CD4^+$ and $CD8^+$) and $CD20^+$ B-cells per mm^2 in chronic HBV liver biopsies (X-axis) and transcriptome-based EPIC leukocyte estimated fractions (Y-axis).



Supplementary Figure 6: Subcellular localization of viral antigens across chronic HBV natural history. Viral antigens subcellular localization was assessed by first identifying individual hepatocytes via NaKATPase stain followed by signal from either HBcAg (nuclear identified by co- localization with nuclei (DAPI) and/ or cytoplasm) or HBsAg (membranous identified by co- localization with NaKATPase and/or cytoplasm). Top and bottom panel represents subcellular localization of nuclear and/or cytoplasmic HBcAg and membrane and/or cytoplasmic HBsAg over total infected (HBsAg+ and/or HBcAg+) hepatocytes in chronic HBV livers.

SUPPLEMENTARY TABLES

To access supplementary tables visit: https://www.journal-of-hepatology.eu/article/S0168-8278(22)00123-4/fulltext#supplementaryMaterial



CHAPTER 3

Mucosal-Associated Invariant T Cells Are More Activated in Chronic Hepatitis B, but Not Depleted in Blood: Reversal by Antiviral Therapy

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ABSTRACT

Background: Mucosal-associated invariant T (MAIT) cells might play a role in control of viral replication during chronic hepatitis B (cHBV) infection, but little is known of their number, phenotype, or function in cHBV patients.

Material and Methods: We performed flow cytometry on CD3+Vα7.2+CD161+ MAIT cells in blood of 55 cHBV patients. Nine patients were sampled before and on entecavir treatment. Six patients on therapy underwent a liver biopsy for flow cytometric analysis. Measurements included MAIT cell frequency, phenotype, and cytokine-producing capacity.

Results: The MAIT cells were not deleted in blood or liver of cHBV patients compared with healthy controls, but they had higher percentages of CD38+ MAIT cells in blood, which declined on entecavir treatment. Peripheral MAIT cells of patients in the HBeAg-negative phase were least activated. Cytokine-producing MAIT cells were as frequent, but granzyme B-producing MAIT cells were more frequent upon stimulation with Escherichia coli compared with healthy controls.

Conclusions: We demonstrate that, in sharp contrast to hepatitis C virus and human immunodeficiency virus patients, MAIT cells isolated from HBV patients are not deleted but are more activated, which can be normalized by nucleoside analog therapy. These observations may aid in deciphering the role of MAIT cells in immune responses to HBV.

INTRODUCTION

Chronic hepatitis B virus (cHBV) infection is a non-cytopathic infection of the host hepatocytes affecting over 240 million people worldwide. Progression to chronicity, control of viral replication, and the degree of liver injury are determined by host immune responses [1]. For early control of viral replication as well as the generation of adaptive immune responses to HBV, local production of the antiviral cytokine interferon gamma (IFNɣ) is key [2]. In response to stimulation with IFNα, interleukin (IL)-12 or IL-18 released by plasmacytoid dendritic cells, or monocytes, IFNɣ _is produced by innate immune cell populations, such as natural killer (NK) cells and NKT cells. When exposed to the same cytokines, mucosal-associated invariant T (MAIT) cells have recently been found to be a major intrahepatic IFNɣ-producing innate effector cell [3]. Mucosal-associated invariant T cells are T cells that express a semi-invariant T-cell receptor specific to bacterial vitamin B2 metabolites presented by major histocompatibility complex-related protein 1 (MR1). It has been proposed that, independently of MR1 binding, MAIT cells can produce IFNɣ, tumor necrosis factor (TNF), and IL-17 [4] and promote antiviral immune responses. This effect would be most prominent in MAIT cell-enriched tissues such as the intestinal mucosa and the liver, where CD161+Vα7.2+ MAIT cells comprise 40% of the T cells (in liver perfusates) compared with 5%–10% in the peripheral blood [5, 6]. It is interestingly that, during infections with hepatitis C virus (HCV) and human immunodeficiency virus (HIV), MAIT cell frequencies in blood are low [7, 8]. In vivo peripheral MAIT cells have an activated phenotype, as reflected by higher expression of the activation maker CD38, but are less functional [8]. More importantly, in HCV and HIV, MAIT cell deletion and functional impairment was shown not to be reversible with antiviral therapy [7– 9]. Taken into account the innate-like phenotype of the MAIT cell, and its broad range of antiviral effector functions and its tissue distribution, MAIT cells may be involved in immunity to HBV, yet no information is available on the possible effect of HBV on the MAIT cells compartment in cHBV patients. Therefore, we formulated the following research questions: (1) does the number, phenotype, and function of MAIT cells in cHBV patients differ from healthy controls; (2) does the clinical phase of HBV disease affect the MAIT cell compartment; (3) does liver inflammation, viral replication, and treatment impact the parameters?

MATERIALS AND METHODS

Patient Selection and Characteristics

Heparinized blood was collected from 55 patients with cHBV and 17 healthy controls. Of all included subjects, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll separation (Ficoll-Paque plus; Amersham) and stored at –150°C until used for the various assays. The 55 cHBV patients were part of 3 different cohorts (**Table 1**). Patients from cohort 1a (n = 18) were sampled cross-sectionally and compared with an uninfected control group matched by gender (11

of 17 male healthy control vs 13 of 18 male HBV), age (average age was 41 in healthy control vs 43 in HBV), and ethnicity (10 of 17 healthy control vs 11 of 18 HBV infected subjects were of Asian descent). Nine of 18 cohort 1a patients were sampled a second time (cohort 1b), when they were on 3 years of nucleoside analog therapy (0.5 or 1 mg entecavir daily). Cohort 2 consisted of 31 patients with cHBV who were cross-sectionally sampled for comparison of different HBV clinical phases. Based on serum HBV deoxyribonucleic acid (DNA), alanine aminotransferase (ALT) levels, and hepatitis B e-antigen (HBeAg) presence at the time of sampling (see **Supplementary Figure 1**), patients were categorized into 4 clinical HBV phases according to international guidelines [1, 10]. Immune tolerant (IT) or HBeAg-positive infection patients had detectable serum HBeAg and repetitive normal ALT values (<40 U/L) for at least 1 year. The HBeAg-positive immune active ([IA] HBeAg-positive hepatitis) and HBeAg-negative ([ENEG] HBeAg-negative hepatitis) patients had repetitive or intermittent abnormal serum ALT (>40 U/L) values and HBV DNA levels >2000 IU/mL. Inactive carrier ([IC] HBeAg-negative infection) patients were HBeAg-negative and had both repetitive normal ALT values (<40 IU/L) and HBV DNA levels below 20000 IU/mL for at least 1 year. Serum ALT was measured on an automated analyzer, qualitative serum hepatitis B surface antigen (HBsAg) and HBeAg levels were measured on an Architect Abbott analyzer, and serum HBV DNA levels were measured using the COBAS AmpliPrep-COBAS TaqMan HBVv2test (CAP-CTM; Roche Molecular Systems). Cohort 3 consisted of 6 cHBV patients who underwent a core liver biopsy (18 Gauge), as part of a clinical trial (NCT02166047), while on successful nucleoside/nucleotide analog therapy (entecavir or tenofovir) at the time of sampling, resulting in undetectable HBV DNA and low ALT values.

Ethical Statement

The study was performed in concordance with the Declaration of Helsinki as adopted by the 64th WMA General Assembly, Fortaleza, Brazil, October 2013. The institutional ethical review board of the Erasmus Medical Center approved the protocols, and written informed consent was obtained from all individuals.

Table 1 Patient's Characteristics

Cohort	1a: cHBV	1b: cHBV+ETV	2: HBV Phase	3: Blood-Liver
Design	Cross-sectional	Longitudinal	Cross-sectional	Cross-sectional
Diagnosis	cHBV	cHBV	cHBV	cHBV
Sample size	n = 18	n = 9/18	n = 31	n = 6
Sampling timepoints	t = 0	t = 0, t = 3	t = 0	t = 0
Age* (years)	43 (25–59)	45 (25–59)	37 (18–49)	49 (29–63)
Gender (M/F)	M: 13, F: 5	M: 9, F: 0	M: 22, F: 9	M: 4, F: 2
Ethnicity				
Asian	11	5	21	4
African	2	2	6	1
Other	5	2	4	1
Genotype	A: 3, B: 4, C: 4, D: 3, E: 2, ND: 2	A: 2, B: 1, C: 2, D: 1, E: 2, ND: 1	A: 4, B: 8, C: 8, D: 2, E: 1, ND: 8	A: 2, B: 1, C: 1, E: 1, ND: 1
HBeAg status	HBeAg+: 4	HBeAg+: 2	HBeAg+: 13	HBeAg+: 1
HBV DNA (IU/mL)	4.1×10^7 ($2.9 \times 102–3.7 \times 10^8$)	2.5×10^7 ($2.9 \times 102–2.2 \times 10^8$)	2.0×10^8 ($2.0 \times 101–1.1 \times 10^9$)	<20
on ETV		<20		
ALT (U/L)	58 (14–366)	81 (23–366)	56 (8–229)	18 (10–32)
on ETV		42 (27–114)		
HBsAg (IU/mL)	1.2×10^4 ($2.1 \times 101–6.1 \times 10^4$)	5.4×10^3 ($2.1 \times 101–2.7 \times 10^4$)	2.1×10^7 ($1.0 \times 10−1–9.3 \times 10^4$)	2.5×10^3 ($1.6 \times 102–5.7 \times 10^3$)
on ETV		2.5×10^3 ($4.2 \times 101–1.6 \times 10^4$)		
Fibrosis grade	F0–F1: 7, F1–F2: 8, F3: 1, F4: 2	F0–F1: 3, F1–F2: 4, F4: 2	F0–F1: 24, F1–F2: 7	F0–F1: 6
Clinical phase*	IT: 2, IA: 3, IC: 4, ENEG: 9	On treatment	IT: 7, IA: 6, IC: 8, ENEG: 10	On treatment

Abbreviations: ALT, alanine aminotransferase; cHBV, chronic hepatitis B virus; DNA, deoxyribonucleic acid; ETV, entecavir; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; ND, not determined.
*At start of treatment. All values are depicted as mean (minimum-maximum).

Expression of Cell Surface Activation Markers on CD161+Vα7.2+CD3+ Mucosal-Associated Invariant T Cells

To determine the frequency and phenotype of MAIT cells, multicolor flow cytometry was performed (as described previously [7]) on PBMCs with anti-CD3-Amcyan (SK7; BD Biosciences), anti-CD161-eFluor450 (HP-3G10; eBiosciences), anti-TCR Vα7.2-PE (3C10; BioLegend), anti-CD56- APC (N901; Beckman Coulter), anti-CD38-APC-efluor780 (HIT2; BD Biosciences), anti-CD25-APC (2A3; eBiosciences), anti-CD69-PeCy7 (TP1.55.3; BioLegend), anti-HLA-DR-APC-eFluor780 (LN3; eBiosciences), anti-CD57-FITC (TB-01; eBiosciences), anti-TIM-3-APC (F38-2E2; eBiosciences), anti-2B4-FITC (DM244; eBiosciences), anti-PD-1-Percp-eFluor710

(eBioJ105; eBiosciences), and CTLA-4-PeCy7 (14D3; BD Biosciences). Anti-CD3-PeCy7 (UCHT1; eBiosciences) and anti-CD38- PerCp-eFluor710 (HB7; eBiosciences) were used in cohort 2. The gates for the activation and exhaustion markers were set on internal controls, ie, the CD3-positive lymphocytes negative for the marker of interest. The PBMCs from cohorts 1 and 2 were assessed using the FACS Canto II Flow Cytometer (BD Biosciences). Of the cohort 3 patients, 18-gauge liver biopsies were collected in ice cold phosphate-buffered saline + 1% fetal calf serum and reduced to cell suspensions in a 48-well, flat bottom plate using a 1-mL syringe. The intrahepatic cells were stained in tandem with paired PBMCs using anti-CD3-PeCy7 (UCHT1), anti-CD56-APC (N901), anti-TCR Vα7.2-PE (3C10), anti-CD161-eFluor450 (HP-3G10), and Live/Dead-aqua (Miltenyi) and assessed using a FACS Aria II Flow Cytometer. If less than 75 viable CD161+Vα7.2+CD3+ MAIT cells were detected during flow cytometry, the sample was excluded. All data were analyzed using FlowJo version 10.1 (Tree Star Inc).

Stimulation and Intracellular Cytokine Analysis of CD161+Vα7.2+CD3+ Mucosal-Associated Invariant T Cells

Per well, 1 million PBMCs in a volume of 500 μL were stimulated with IL-12 (0.25 ng/mL; Miltenyi), IL-18 (10 ng/mL; R&D Systems), and CD28 monoclonal antibody (2 μg/mL; eBiosciences) with or without Escherichia coli (25 colony-forming units/leukocyte of E coli ATCC 25922 or E coli K12, fixed for 5 minutes with 1% formaldehyde) overnight for surface marker expression and intracellular cytokine production. Stimulation with 12-O-tetradecanoyl-phorbol-13-acetate ([PMA] 50 ng/mL; Sigma) and ionomycin (500 ng/mL; Sigma) was used as a positive control. Depending on the stimulus used, after 6 hours (E coli and anti-CD28 2 μg/mL) and 21 hours (IL-12/IL-18 and PMA/ ionomycin) of stimulation, brefeldin A (10 μg/mL; Sigma) was added to the cultures. Stimulated PBMCs were harvested after 24 hours of stimulation. The PBMCs were stained with anti-CD3- Amcyan (SK7), anti-CD161-eFluor450 (HP-3G10), and anti-TCR Vα7.2-PE (3C10). Next, cells were fixed with 2% formaldehyde, permeabilized with 0.5% saponin, and stained with anti-IFNɣ-FITC (25723.11; BD Biosciences), anti-TNF-APC (Mab11; eBiosciences), anti-granzyme-B-FITC (GB11; BD Biosciences), and anti-perforin-PerCP-Cy5.5 (dG9; eBiosciences). Cytokine-producing MAIT cells were assessed by flow cytometry (FACS Canto II).

Statistical Analysis

Data are expressed as means ± standard error of the mean, unless indicated otherwise. The data were analyzed with Prism software, version 5.0 (GraphPad Software) using the Mann-Whitney *U* test to compare the variables between independent groups and Wilcoxon signed-rank test for paired data groups. In all analyses, a 2-tailed $P < .05$ (95% confidence interval) was considered statistically significant.

RESULTS

Baseline Characteristics of Three Cohorts of Patients Infected With Chronic Hepatitis B virus

To characterize the MAIT cell compartment of HBV patients in various clinical phases and during different treatment modalities, we included 3 different cohorts (Table 1). Although different ethnicities were included, most of the patients were of Asian ancestry (65%), as reflected by the high frequencies of HBV genotypes B and C. The majority of patients (71%) was male. Representative of the Dutch HBV-infected population, patients in the asymptomatic IT phase had a relatively high percentage of female subjects (due to past HBsAg screening at time of suspected pregnancy). Virologic parameters differed between cohorts, owing to the selection criteria. At baseline of cohort 1b, the average HBV DNA was 2.5 × 10^7 IU/mL (2.9 × 10^2–2.2 × 10^8IU/mL), and all patients responded to nucleoside analog treatment (response was defined as undetectable HBV DNA viral loads 3 months after start of therapy). As presented in Supplementary Figure 1, cHBV patients were selected on the basis of the level of HBV DNA, ALT, and HBeAg in serum, as described previously [11, 12]. In line with literature, HBeAg-positive patients (IT and IA patients, cohort 2) had higher HBsAg values compared with ENEG patients (averaging 5.7 × 10^8 and 7.5 × 10^5 IU/mL, respectively) [13, 14].

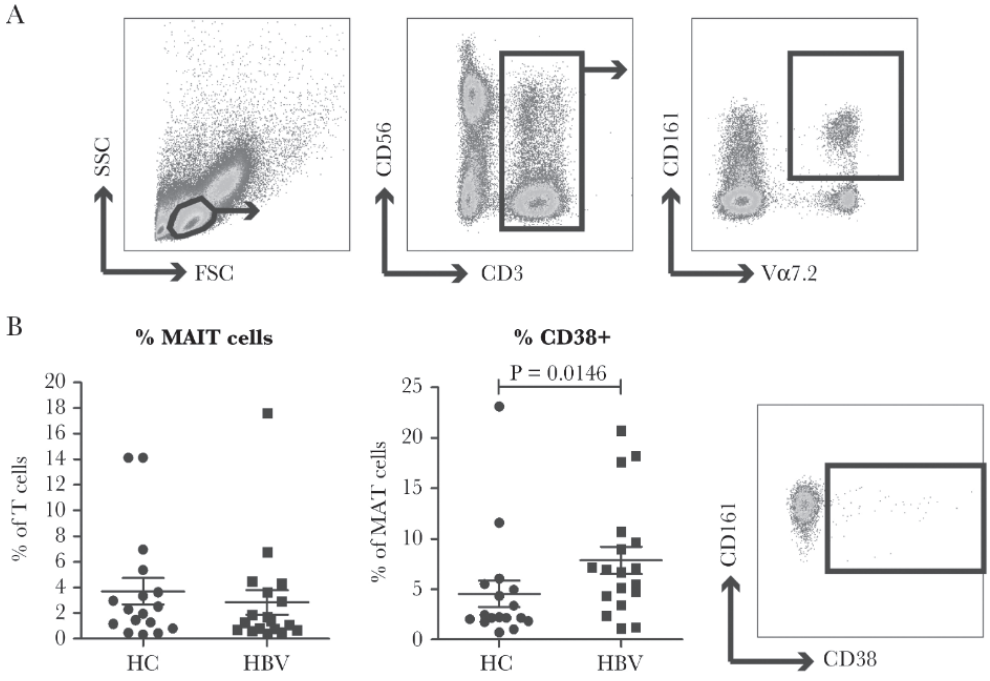

Figure 1. Peripheral CD38+ mucosal-associated invariant T (MAIT) cells are more frequent in chronic hepatitis B (cHBV) patients. (A) Representative flow cytometry plots showing the gating strategy for peripheral MAIT cells from a patient chronically infected with cHBV. (B) The percentage of peripheral CD3+Vα7.2+CD161+

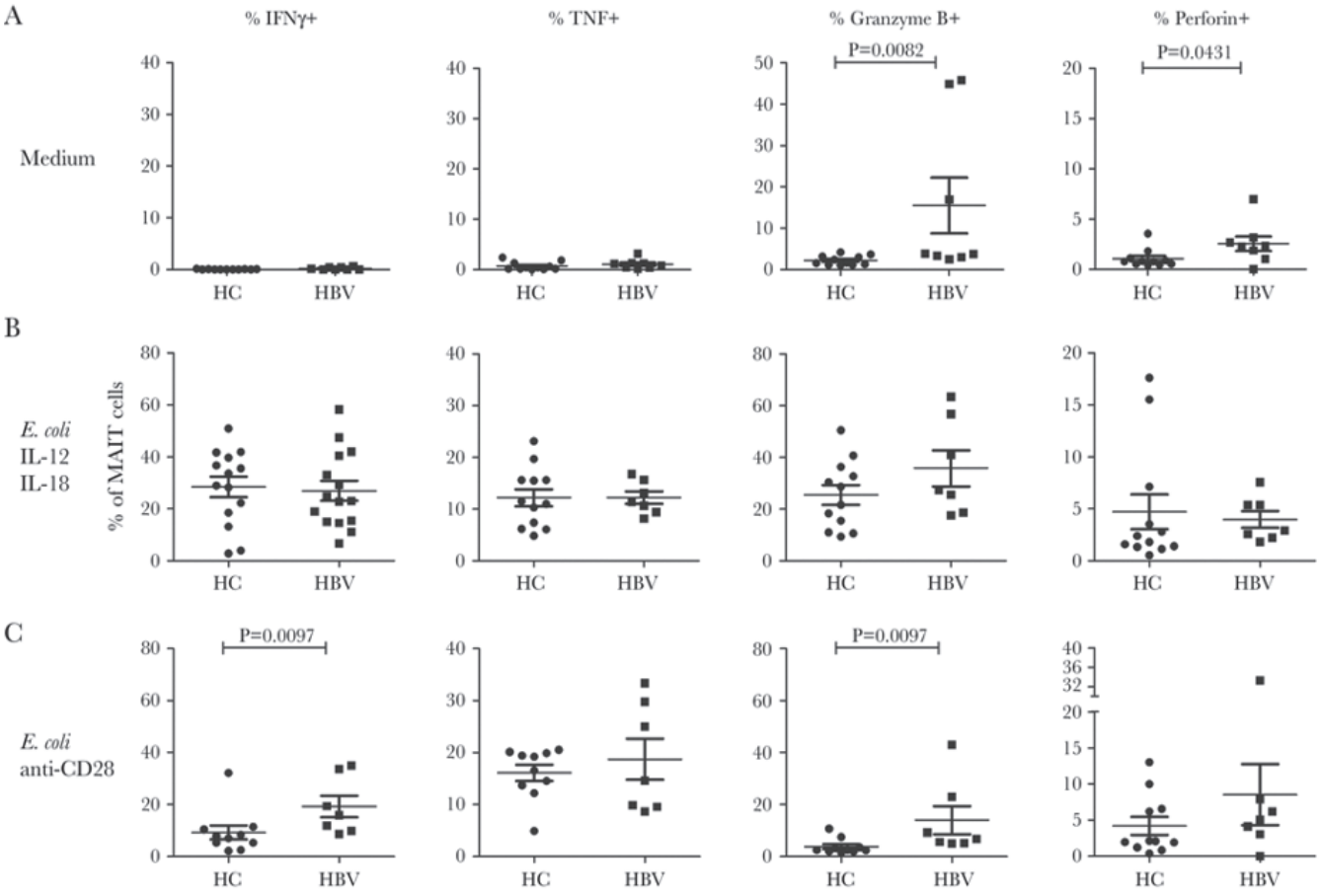

Figure 2. Mucosal-associated invariant T (MAIT) cells of chronic hepatitis B (HBV) patients are not functionally impaired. The percentage of Vα7.2+CD161+ positive for interferon (IFN)γ (15 HBV patients), tumor necrosis factor (TNF), granzyme B, and perforin (8 HBV patients) compared with healthy controls (HC). Intracellular cytokines were stained after 24 hours of incubation with medium (A), interleukin (IL)-12/IL-18/Escherichia coli (B) or anti-CD28/E. coli (C).

MAIT cells of total T cells in 18 patients with cHBV and matched healthy controls (HC). (Second Panel) The percentage of CD3+Vα7.2+CD161+CD38+ MAIT cells in HC and cHBV patients and the representative gating after the gating in (A).

Peripheral Mucosal-Associated Invariant T Cells From Chronic Hepatitis B Virus Patients Have an Activated Phenotype, but Are Not Deleted or Functionally Impaired

To explore the possible role of MAIT cells during immune responses in cHBV infection, we assessed their phenotype and frequency and compared it to a matched healthy control group. In contrast to HCV and HIV patients [6, 7], cHBV patients did not have significantly different MAIT cell frequencies in blood within the T-cell population compared with healthy controls (averaging 2.9% and 3.7%, respectively) (**Figure 1**). It is interesting to note that a larger fraction of MAIT cells in blood of cHBV patients expressed the activation marker CD38 on their surface in comparison to healthy controls (7.9% vs 4.6%, P = .0146). Upon further exploration of MAIT activation and/or exhaustion, we did not observe significant differences in MAIT cell percentages positive for CD25, HLA-DR, CD69, TIM-3, 2B4, CTLA-4, or PD-1 compared with controls (**Supplementary Figure 2**, **Supplementary Figure 3**, and summarized in **Supplementary Table 1**). The mean fluorescence intensity of CD57 on MAIT cells was significantly higher in the HBV group, mainly due to 2 HBV-infected subjects with exceptionally high CD57 expression. Upon functional assessment of MAIT cells stimulated with IL-12/IL-18/E coli, no differences were observed in the frequency of IFNɣ-producing MAIT cells in blood from HBV patients compared with healthy controls (26.9% vs 28.4%, respectively) (**Figure 2B**) and was significantly higher in the HBV group upon anti-CD28/E coli stimulation (19.2% vs 9.2%, respectively; P = .0097) (**Figure 2C**). For the evaluation of the frequency of TNF, granzyme B, and perforin producing MAIT cells, the PBMCs of 10 HBV-infected subjects and 12 healthy controls were stimulated. The potential of MAIT cells to produce TNF and perforin did not differ, but the percentage of granzyme B producing MAIT cells was higher in the HBV-infected group compared with healthy controls (upon E coli: 11.6% vs 3.3% and anti-CD28/E coli: 14.0% vs 3.7%). In the unstimulated condition, a high percentage of MAIT cells was positive for granzyme B in 3 HBV-infected patients, indicative of an activated phenotype (**Figure 2A**). The cytokine-producing potential (IFNɣ, TNF) and positivity for granzyme B and perforin of MAIT cells was not significantly different upon PMA/ionomycin stimulation (**Supplementary Figure 4**). Escherichia coli-only stimulation did not or only mildly increased the frequency of granzyme B-positive cells; induction of granzyme B in MAIT cells seems to require a stronger stimulus such as IL-12/IL-18 or PMA/ionomycin (shown in **Supplementary Figure 4**). Three healthy controls and 3 HBV samples were excluded because less than 75 MAIT cells were detected (resulting in unreliable percentages of IFNɣ-positive MAIT cells).

Chronic Hepatitis B Virus Patients in the Hepatitis B e-Antigen-Negative Clinical Phase Have Lower Frequencies of Cluster of Differentiation 38-Positive Mucosal-Associated Invariant T Cells Compared With the Inactive Carrier Phase

To investigate whether the frequency or activation of MAIT cells was related to the degree of liver inflammation or viral replication in cHBV patients, we assessed the frequency and CD38- positive percentages of MAIT cells in blood of patients at the various clinical phases. As shown in **Figure 3**, MAIT frequencies did not vary significantly in patients in the IT, IA, IC, and ENEG phase (average 1.7%, 0.8%, 1.4%, and 2.1% of total CD3+ T cells, respectively), although a trend was observed for lower MAIT cell numbers in the IA phase. These phases correspond to patients with (1) HBeAg-positive infection or hepatitis and (2) HBeAg-negative infection or hepatitis, respectively, as described in recent EASL guidelines [10]. The frequency of CD3+ T cells within the lymphocyte population was not different in any clinical phase (data not shown). Evaluation of CD38 expression on MAIT cells demonstrated that in the IT, IA, and IC phase, cHBV patients have comparable frequencies of CD38-positive MAIT cells, with the exception of MAIT cells of ENEG patients, who had reduced percentages compared with the precursory IC phase (12.2% vs 24.2%, P = .0031). No significant correlations were observed between the percentage of CD38-positive MAIT cells and patient characteristics such as age, ALT, HBV viral load, serum IL-12p40, or IL-18 levels (data not shown).

Antiviral Therapy for Chronic Hepatitis B Virus Reduces Mucosal-Associated Invariant T-Cell Activation to Levels of Healthy Controls

Using a longitudinal study design, we then investigated the effect of nucleoside analog treatment on MAIT frequencies and activation in 9 male patients with cHBV using paired samples. At baseline, 2 patients were in the IA phase, 1 in the IC phase, and 6 in the ENEG phase. As shown in **Figure 4A**, the frequency of peripheral MAIT cells was not influenced in patients on antiviral therapy for approximately 3 years, but the percentage of CD38+ MAIT cells (11.2%) declined to healthy control levels (5.4%). In line with this finding, the capacity of MAIT cells to produce IFNγ _after stimulation with IL-12/IL-18 and IL-12/IL-18/E coli was higher in 4 of 6 patients before treatment than on treatment (**Figure 4B**), but this was a nonsignificant trend (upon IL-12/IL- 18: 12.6% vs 8.3%, respectively; and upon IL-12/IL-18/E coli: 20.6% vs 16.2%, respectively). Interferonγ _production was not tested in 3 samples due to the unavailability of sufficient cells at both timepoints.

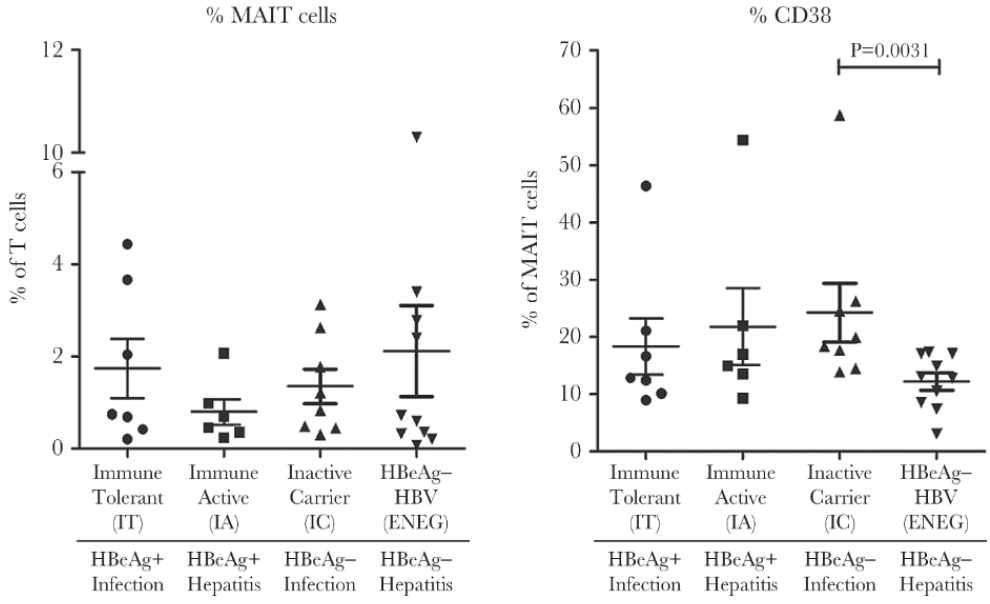

Figure 3. Chronic hepatitis B virus (cHBV) patients in the hepatitis B e-antigen (HBeAg)-negative clinical phase have fewer CD38+ mucosal-associated invariant T (MAIT) cells compared with the inactive carriers. The percentage of CD3+Vα7.2+CD161+ of total T cells (left panel) and CD3+Vα7.2+CD161+CD38+ of total MAIT cells in the peripheral blood of 31 cHBV patients divided in its 4 clinical phases: immune tolerant (IT), immune active (IA), inactive carrier (IC), and HBeAg-negative phase (ENEG). These phases represent patients with HBeAg-positive infection or hepatitis and HBeAg-negative infection or hepatitis, respectively, as described in the most recent EASL guidelines. The percentage of CD38+ MAIT cells in cHBV patients at the ENEG phase was significantly lower than at the IC phase (P = .0031).

Intrahepatic Mucosal-Associated Invariant T Cells Are Not Deleted in the Liver of Chronic Hepatitis B Virus Patients on Nucleoside Analog Treatment

We had the unique opportunity to examine liver and blood samples of 6 HBV patients on continuous nucleoside analog treatment with either entecavir or tenofovir. We observed that the MAIT cell frequencies of total T cells among individual patients varied considerably in both the intrahepatic (range, 6.4%–29.2%) and peripheral (range, 0.8%–4.8%) compartment but are highly enriched in the liver (**Figure 5**).

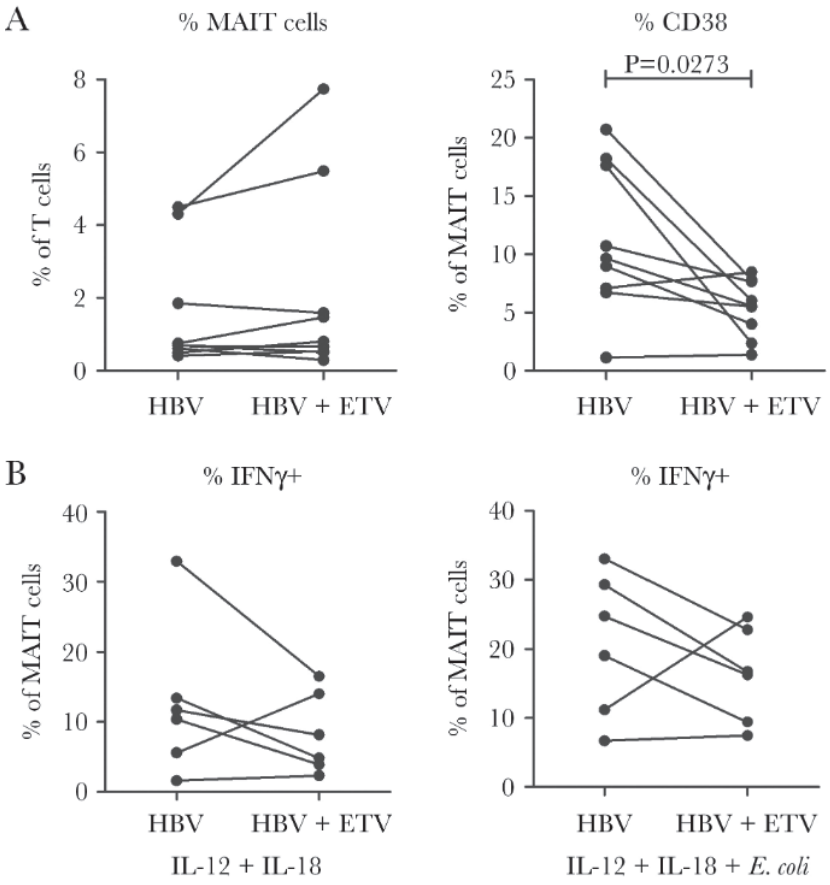

Figure 4. Antiviral therapy for chronic hepatitis B virus (cHBV) reduces mucosal-associated invariant T (MAIT) cell activation to levels of healthy controls, but it does not impact interferon (IFN)ɣ _production by MAIT cells significantly. (A) The percentage of CD3+Vα7.2+CD161+ of total T cells in 9 cHBV patients who donated blood before and during 3 years of entecavir (ETV) therapy resulting in a sustained virological response. The percentage of CD38+ MAIT cells declined significantly (P = .0273) during 3 years treatment. (B) MAIT cell IFNɣ _production stimulation with interleukin (IL)-12/IL-18 (left panel) or IL-12/IL-18/Escherichia coli (right panel) before and on entecavir treatment.

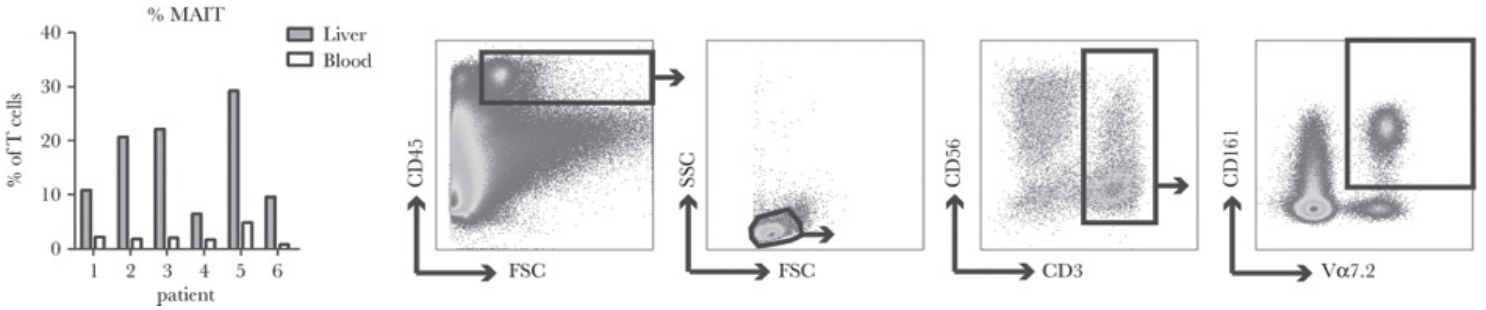

Figure 5. Intrahepatic mucosal-associated invariant T (MAIT) cells are present in high frequencies in the liver compared with blood of chronic hepatitis B virus (cHBV) patients on antiviral therapy. CD3+Vα7.2+CD161+ MAIT cells of total T cells of 6 cHBV patients on continuous nucleoside analog therapy in blood and liver. The depicted gating was preceded by gating on CD235a-negative, live cells and by gating on single cells only using SSC-W/SSC-A and FSC-W/FSC-A plots. FSC-A, forward scatter area; FSC-W, forward scatter width; SSC-A, side scatter area; SSC-W, side scatter width.

DISCUSSION

To the best of our knowledge, we present the first characterization of MAIT cells in cHBV patients, during its highly variable natural history and during antiviral treatment. We show that MAIT cells are not deleted in blood of cHBV-infected patients and have a more activated phenotype that is reversible with nucleoside analog treatment. In the ENEG phase of cHBV, MAIT cells are least activated. In addition, in the majority of patients, antiviral treatment negatively impacts IFNɣ _production by MAIT cells. These observations improve our understanding of the role MAIT cells during immune responses to HBV.

One of our major findings is that in cHBV patients, MAIT cell frequencies are not diminished in the peripheral blood, which is in sharp contrast to various other chronic viral and inflammatory diseases [7, 15, 16]. In HCV infection, low percentages of peripheral MAIT cells in blood have been found by us and others [7, 8]. Migration towards mucosal surfaces in HIV and possibly towards the liver in HCV could explain these low peripheral frequencies. Mucosal-associated invariant T cells have been reported to home to inflamed tissues and may undergo activation-induced apoptosis when exposed to high antigen loads [16, 17]. We have previously shown that in cHBV patients with low levels of viral replication and low degrees of liver inflammation (ICs), serum levels of several proinflammatory cytokines such as IFNɣ-inducible protein-10, IL-18, and IL-12p40 are low [18]. Based on the low peripheral IL-18 and IL-12p40 titers we found in this projects, we expected low percentages of CD38-positive MAIT cells in this phase. Contrary to our expectations, we observed that relatively high numbers of MAIT cells derived from ICs were CD38-positive, in particular in comparison to ENEG patients. It is possible that activated MAIT cells exit the blood and home to the liver during the immunologically more active ENEG phase. Contrary to HIV and HCV infection, the activated MAIT cell phenotype in cHBV patients was reversible with antiviral treatment. Because HBsAg levels in serum are barely affected by nucleoside analog therapy, HBsAg is unlikely to influence the release of MAIT stimulatory signals in cHBV patients. In line with the normalization of MAIT phenotype, we observed a decline of the frequency of IFNɣ-producing MAIT cells after viral suppression in the majority of treated patients, possibly due to changes in the stimulatory milieu after cessation of viral replication [19]. Indeed, after stimulation with E coli or medium only, the percentage of granzyme B+ MAIT cells derived from HBV-infected patients was higher than matched healthy controls, possibly as a result of their increased activation status. None of the stimulations of MAIT cells (E coli, PMA/ionomycin, IL-12/IL-18/E coli) showed a significant difference of the frequency of IFNɣ-positive MAIT cells between healthy controls and HBV patients, except for E coli/anti-CD28. The latter stimulus resulted in higher frequencies of IFNɣ-positive MAIT cells in HBV-infected patients. We did not examine whether MAIT cells from HBV patients have higher expression of CD28 on their surface and are therefore more responsive. In addition, the biological significance of this finding is not clear, because it was not observed for the other stimuli. However, this finding clearly demonstrates that, in contrast to other chronic viral infections, IFNɣ _production is not impaired compared with

healthy controls. The biological relevance of the finding requires more detailed studies to determine whether MAIT cell-derived IFNɣ _may contribute to antiviral responses to HBV infection and affect fibrogenesis. The liver biopsies analyzed by flow cytometry revealed that MAIT cells remain highly enriched in the liver during nucleoside analog therapy and in the absence of liver inflammation. These biopsies indicate that although MAIT cells are known to home to inflamed tissues, ongoing local inflammation may not be necessary for their continuing presence in the liver of HBV patients. It should be noted here that a small fraction of the intrahepatic (or tissue resident) CD161+Vα7.2+CD3+ MAIT cells may be tetramer negative and therefore are not genuine MAIT cells, as has previously been suggested by Reantragoon et al [20], and future studies should include MR1 tetramer stainings, preferably combined with the evaluation of activation and exhaustion markers.

CONCLUSION

In cHBV patients, contrary to chronic HCV or HIV patients, peripheral MAIT cells are not deleted in blood. Significantly more MAIT cells from HBV patients are CD38-positive compared with healthy controls. It is interesting to note that antiviral therapy normalizes the frequency of CD38-positive MAIT cells to healthy control levels, but this does not affect the relative numbers of IFNɣ-producing cells. In patients on continuous nucleoside analog treatment, we show that MAIT cells were consistently enriched in the liver, compared with the peripheral blood. These observations may aid in deciphering the role of MAIT cells in immune responses to HBV.

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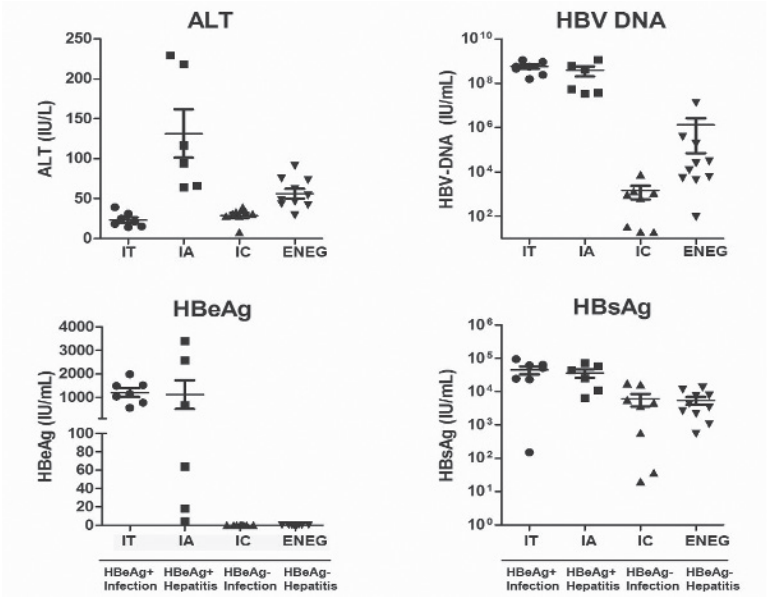
SUPPLEMENTARY DATA

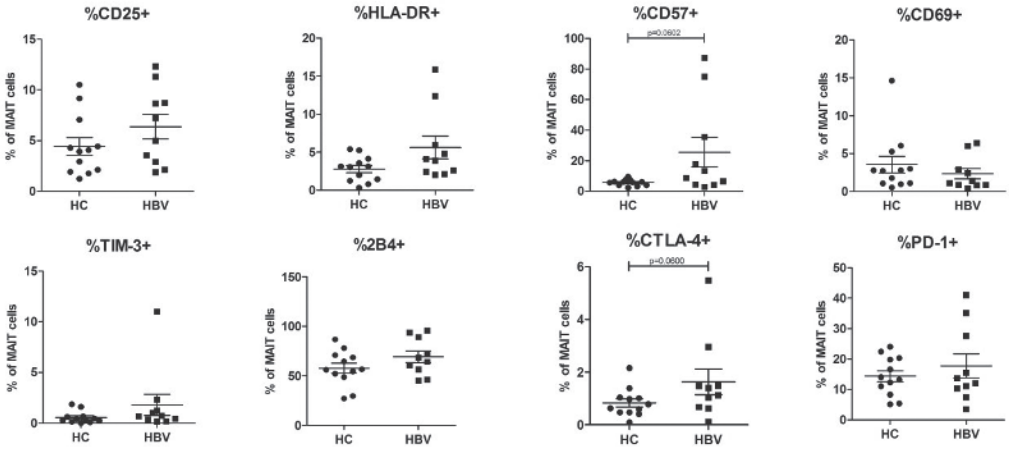
Supplementary Table 1. Percentages of MAIT cells positive for activation and exhaustion markers other than CD38 are not higher in HBV infected patients compared to healthy controls.

		HC (n=12)		HBV (n=10)	
CD25	%MAIT+	4.4	(1.2-10.5)	6.4	(1.9-12.3)
	MFI	65.0	(47.0-94.7)	93.1	(46.5-171.0)
CD57	%MAIT+	5.6	(2.3-9.5)	25.6	(2.9-87.3)
	MFI	37.4	(22.0-65.0)	**701.4***	(34.7-4300.0)
CD69	%MAIT+	3.5	(0.5-14.6)	2.3	(0.4-6.4)
	MFI	48.4	(27.5-140.0)	42.4	(23.4-61.4)
HLA-DR	%MAIT+	2.8	(0.3-5.4)	5.6	(2.0-15.9)
	MFI	21.4	(6.9-38.8)	47.1	(9.5-162.0)
2B4	%MAIT+	57.8	(27.0-86.7)	69.3	(45.3-95.9)
	MFI	544.5	(276.0-1115.0)	732.2	(285.0-1872.0)
CTLA-4	%MAIT+	0.8	(0.1-2.2)	1.6	(0.1-5.5)
	MFI	22.7	(14.6-29.2)	26.0	(15.7-53.1)
PD-1	%MAIT+	14.4	(5.1-24.0)	17.8	(3.5-41.0)
	MFI	62.7	(30.7-104.0)	71.3	(21.9-157.0)
TIM-3	%MAIT+	0.6	(0.0-1.9)	1.8	(0.2-11.0)
	MFI	18.2	(14.8-22.0)	20.4	(12.1-29.3)

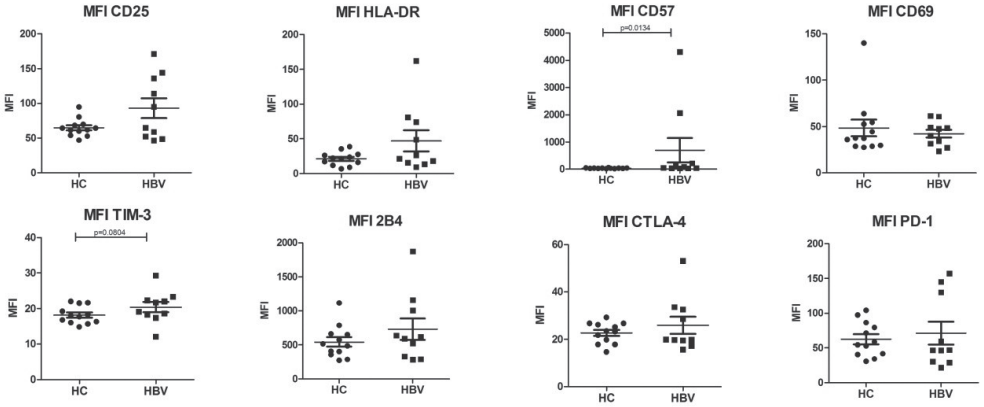
MFI, (Geometric) Mean Fluorescence Intensity. All values are depicted as mean (minimum-maximum). HLA-DR, Human Leukocyte Antigen - antigen D Related; CTLA-4, Cytotoxic T-Lymphocyte-Associated protein 4; PD-1, Programmed cell Death protein 1; TIM-3, T-cell immunoglobulin and mucin-domain containing-3. * $p<0.05$.



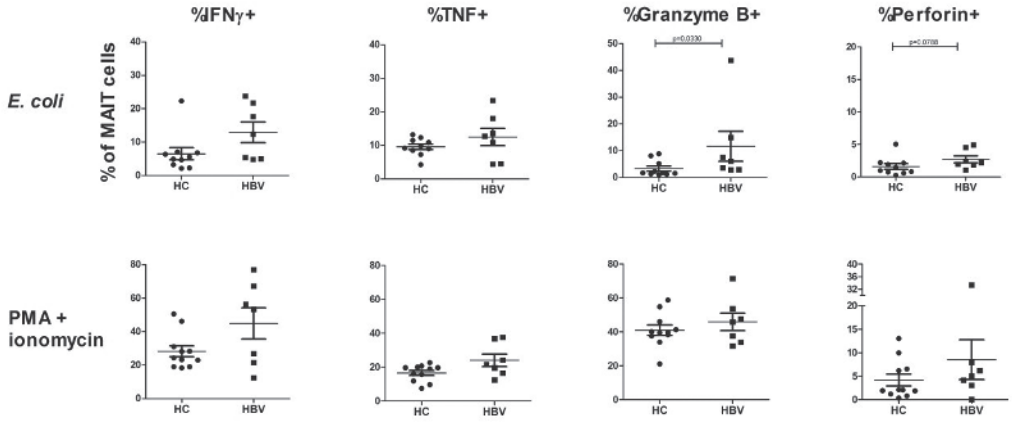
Supplementary Figure 1: cHBV is characterized by 4 phases with different degrees of liver inflammation, HBV replication and HBeAg expression Representation of the patient characteristics of Cohort 2. Based on serum HBV DNA, ALT levels, and HBeAg presence at the time of sampling, patients were categorized into 4 clinical HBV phases according to international guidelines: the immune tolerant, immune active, inactive carrier and HBeAg-negative HBV (European Association For The Study Of The Liver, 2012). The new nomenclature as described in the most recent EASL guidelines of 2017 are also mentioned in the figure: HBeAg-positive infection or hepatitis, and HBeAgnegative infection or hepatitis, respectively. HBsAg levels are presented to illustrate lower values among IC and ENEG patients, characteristic for the natural history of cHBV.



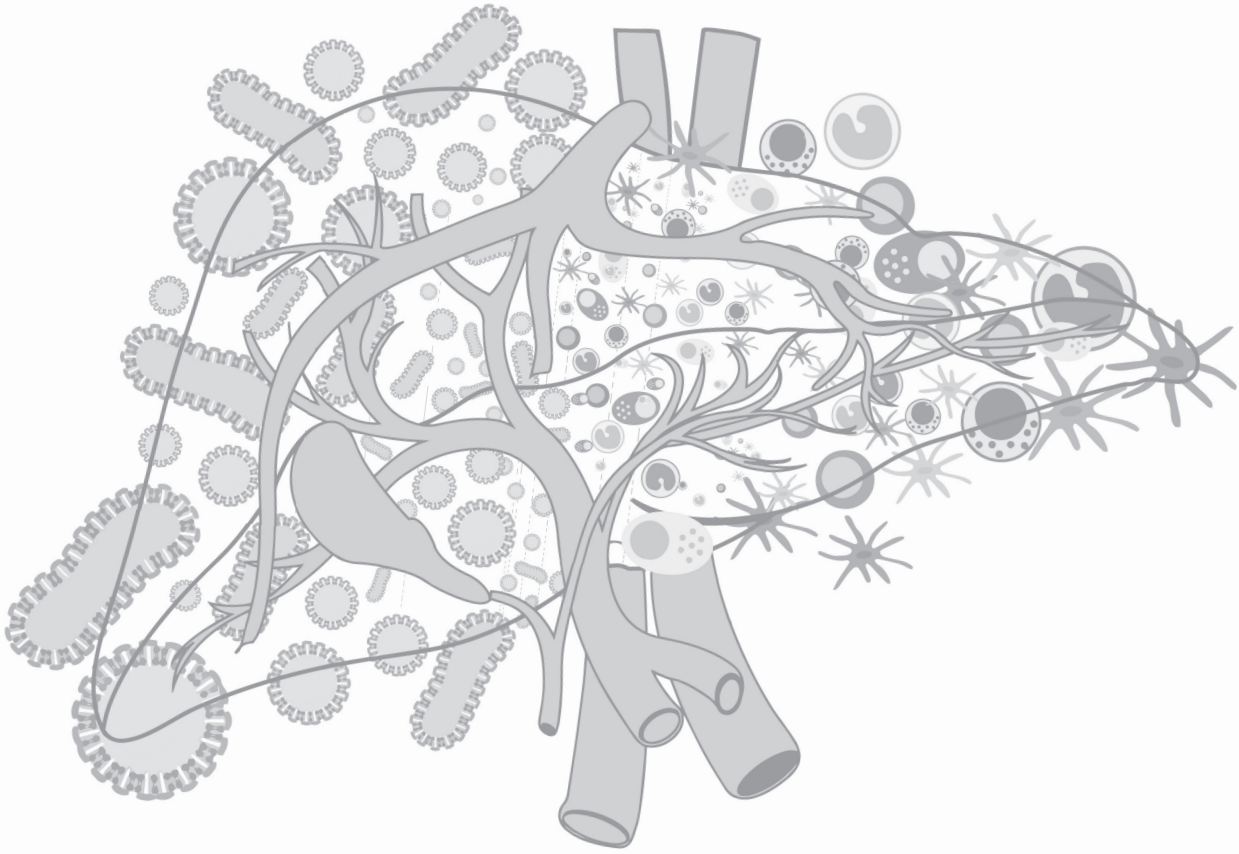
Supplementary Figure 2: Percentages of MAIT cells positive for activation and exhaustion markers other than CD38 are not higher in HBV infected patients compared to healthy controls.
Presented are the percentages of CD3+Vα7.2+CD161+ MAIT cells positive for the activation (CD25, HLA-DR, CD57, CD69) and exhaustion markers (TIM-3, 2B4, CTLA-4, PD-1) of 10 cHBV patients and 12 matched healthy controls of cohort 1. HLA-DR, Human Leukocyte Antigen - antigen D Related; CTLA-4, Cytotoxic T-LymphocyteAssociated protein 4; PD-1, Programmed cell Death protein 1; TIM-3, T-cell immunoglobulin and mucin-domain containing-3.



Supplementary Figure 3: Expression levels of activation and exhaustion markers by MAIT cells is not significantly higher in HBV infected patient compared to healthy controls
Presented are the Mean Fluorescence Intensity (MFI) of CD3+Vα7.2+CD161+ MAIT cells for the measured activation (CD25, HLA-DR, CD57, CD69) and exhaustion markers (TIM-3, 2B4, CTLA-4, PD-1) of 10 cHBV patients and 12 matched healthy controls of cohort 1. HLA-DR, Human Leukocyte Antigen - antigen D Related; CTLA-4, Cytotoxic T-LymphocyteAssociated protein 4; PD-1, Programmed cell Death protein 1; TIM-3, T-cell immunoglobulin and mucin-domain containing-3.



Supplementary Figure 4: MAIT cells from HBV infected patients are not functionally impaired
Intracellular cytokine staining of IFNɣ, TNF, granzyme B and perforin after 24 hours stimulation with *E. coli* (first row) or PMA/ionomycin (second row). Cytokine production was similar in both groups, but the percentage granzyme B-positive MAIT cells was higher in the HBV infected group upon *E. coli* stimulation.



CHAPTER 4

Differential gene expression, irrespective of circulating Hepatitis B Surface Antigen levels, between Inactive Carrier and Nucleos(t)ide Analogue-Treated Hepatitis B Virus patients

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ABSTRACT

Long-term viremia control in chronic HBV patients oc- curs either spontaneously in inactive carrier (IC) patients or therapy-induced by nucleos(t) ide analogues (NUC). To better understand the characteristics of viremia control, we evaluated gene expression in purified leukocyte subsets from IC versus NUC-treated patients, and evaluated the putative modulatory effects of hepatitis B surface antigen (HBsAg). We observed that gene expression in NUC-treated patients differed mark- edly from IC patients, especially in dendritic cells, monocytes, and CD8+ T cells, while serum HBsAg levels had little effect. Nevertheless, based on our findings it cannot be excluded that HBsAg may act locally in the infected liver or preferentially af- fects HBV-specific cells.

Keywords. blood leukocytes; chronic HBV; transcriptome; inactive carriers; antiviral; HBsAg.

INTRODUCTION

Chronic infection with hepatitis B virus (HBV) is a major global health burden. The World Health Organization estimates that 257 million individuals are living with chronic HBV, which can lead to liver fibrosis, cirrhosis, and ultimately hepatocellular carcinoma[1]. Chronic HBV patients can be categorized into 4 clinical phases, which vary in inflammatory activity and are highly var- iable in serum levels of alanine transferase (ALT) and viral rep- lication (HBV-DNA). Patients in the inactive carrier (IC) phase (also known as chronic HBeAg-negative HBV infection) are characterized by the ability to naturally control viral replication, and exhibit low HBV-DNA and normal ALT levels throughout an indefinite period of time, likely via immune-mediated control[2, 3]. In contrast to IC patients, patients with active disease (elevated ALT and HBV-DNA) usually receive antiviral therapy consisting of nucleos(t)ide analogues (NUC)[3]. These medica- tions are not curative but are highly effective in reducing HBV- DNA and normalizing ALT levels. However, even when viral replication is controlled and serum ALT levels are normalized during the IC phase and during NUC treatment, active trans- lation of viral antigens, such as hepatitis B surface antigen (HBsAg), continues. High serum HBsAg has been postulated to be immunomodulatory, which is in line with phenomena seen in other chronic viral infections where continuous high antigen exposure induces a state of exhaustion, particularly in T cells [4]. A clear understanding of whether high concentrations of HBsAg in serum are responsible for the lack of an effective im- mune response to HBV in patients is still lacking.

In this study, we therefore examined the frequencies and transcriptome of blood-sorted monocytes, dendritic cells (DC), B cells, CD4+ and CD8+ T cells, natural killer (NK), and NKT cells of chronic HBV patients who controlled viral replication either spontaneously or by effective NUC treatment. In addi- tion, the putative modulatory effect of HBsAg was evaluated by comparing HBsAg-high versus HBsAg-low IC patients.

METHODS

Patient Cohort

Cryopreserved peripheral blood mononuclear cells (PBMCs) from chronic HBV patients were selected from the Erasmus MC biobank. All patients tested negative for hepatitis D virus (HDV), hepatitis C virus (HCV), and human immu- nodeficiency virus (HIV), and had minimal liver fibrosis (METAVIR ≤ F2). As a case-control comparison, 12 treatment- naive IC patients present in the HBsAg-high (median, 11 513 IU/mL; range, 5707–24 538 IU/mL) and 8 HBsAg-low (median, 99 IU/mL; range, 1–189 IU/mL) were included (Supplementary Table 1). The categorization of patients as HBsAg-high or HBsAg-low was based on the top and 40% serum HBsAg dis- tribution (high, ≥ 1783 IU/mL; low, ≤ 989 IU/mL) of over 800 samples available in the Erasmus MC biobank. For testing of IC versus NUC patients, 24 treatment-naive IC

patients were tested against 16 NUC-treated patients with normalized ALT levels at least 12 months prior to sample collection who were on treatment for a median of 3.5 years (interquartile range, 3–5 years; Supplementary Table 1). The study and its protocols were approved by the institutional ethical review board. All pa- tients provided informed written consent**.**

Flow Cytometry and Cell-Sorting Analysis

PBMCs were sorted for NK cells (CD3 CD56), NKT cells (CD3+CD56+), CD4+ and CD8+ T cells (CD3+CD4+ and CD3+CD8+), monocytes (CD14+ CD19–), B cells (CD19+CD14–), and DC (BDCA1+CD19–CD14–) using a fluorescence-activated cell sorting (FACS)-ARIA-II (Supplementary Figure 1). Five hundred sorted cells of each population were collected in Lyse- and-Go (ThermoFisher, discontinued) and stored at –80°C. Lysates were processed using the GeneChip HT Pico Kit (ThermoFischer).

Microarray Analysis

Fragmented biotin-labelled was hybridized on a human Clariom GO Screen Assay (ThermoFischer) and scanning was done using GeneTitan. Gene expression values were normalized by robust multiarray average normalization on the microarray probe-level data [5], and downstream analysis was done in R version 3.4.2. Unsupervised analysis using spectral maps was performed and showed no batch effects. To validate that the sorting pro- cedure yielded pure populations, we evaluated the expression levels of key marker genes for each leukocyte population such as CD19 for B cells, NKG7 for NK cells, and CD4 and CD8 for T-cell subsets (Supplementary Figure 2). A supervised analysis was performed using the *limma* package [6] for com- parisons between patient groups within cell populations and corrected *P* values for multiple testing across genes ≤ .05 were considered significant. The affected pathways were analyzed using MLP (mean log P analysis) and GO Biological Process [7]. The considered cutoffs for MLP were lower (5) and upper (100) threshold for gene set size where 7100 pathways from the Biological Process source were used.

Statistical Analysis

Group comparison of clinical parameters was performed using 2-way unpaired *t* testing, unless indicated otherwise. Group differences in cell frequencies were analyzed by Mann-Whitney testing using GraphPad Prism.

RESULTS

Diversity in Cell Frequencies and Gene Expression Profiles of Peripheral Sorted Blood Leukocytes Irrespective of Circulating HBsAg Levels

To determine the effect of ongoing HBsAg exposure, gene ex- pression profiling was performed on FACS-sorted peripheral leukocytes obtained from HBsAg-high and HBsAg-low IC patients (Figure 1A). IC patients with distinct HBsAg levels displayed no significant differences in leukocyte subset fre- quencies (Supplementary Figure 3). All IC patients were HBeAg negative, had low HBV-DNA, and normalized ALT levels (Supplementary Table 1). As shown in Figure 1B, comparison of the 2 different groups yielded only few or no differentially expressed genes (DEG), using adjusted *P* value ≤ .05 and 1.5 log fold-change (logFC) as cutoff, for sorted B cells (*GAS6-AS1*), T-cell subsets (CD4± and CD8± (*SLC33A1*, *OBSCN*), NK cells and NKT cells, and DC (*MOCOS*, *BPIFAF3*, *ZN665*, and *MAS4A4E*) (Figure 1D). In monocytes, the vast majority of DEG (22/35) were upregulated in the HBsAg-low group, although some immune-related genes (eg, *C6*, *ADA*, and *DEFB134*) were upregulated in the HBsAg-high group (Figure 1B and 1C).

HBV Viremia Control in the IC Phase Versus During NUC Therapy Exhibits Altered Frequencies and Gene Expression Profiles in Blood Leukocytes

The identification of the underlying mechanisms that deter- mine viral control are essential for the creation of new treat- ment strategies. Therefore, we compared blood leukocyte composition and transcriptome in 24 IC and 16 NUC-treated patients. Although the groups exhibited comparable liver en- zymes (ALT and aspartate transaminase) and virological char- acteristics (HBV-DNA and HBsAg; Supplementary Table 1), higher frequencies of DC (median, 0.48% vs 0.25%, respec- tively; *P* value = .03) and CD8+ T cells (median, 31% vs 22%, re- spectively; *P* value = .001) were found in NUC-treated patients compared to IC patients, whereas the percentage of CD4+ T cells was higher in IC than NUC-treated patients (median 68% vs 51%; *P* value = .002; Supplementary Figure 4). These find- ings suggest an altered balance between the CD4+ and CD8+ T-cell populations in the IC versus NUC-treated groups.

Next, gene expression profiling on FACS-sorted blood leuko- cytes from IC and NUC-treated patients was conducted. Using adjusted *P* values ≤ .05 and 1.5 logFC as cutoff, we observed that DC showed the highest number of DEG (805), followed by CD8+ T cells (189), monocytes (42), and B cells (13). In con- trast, low numbers of DEG to no DEG were detected in CD4+ T cells, NK cells, and NKT cells (3, 1, and 0 DEG, respectively; Figure 2A).

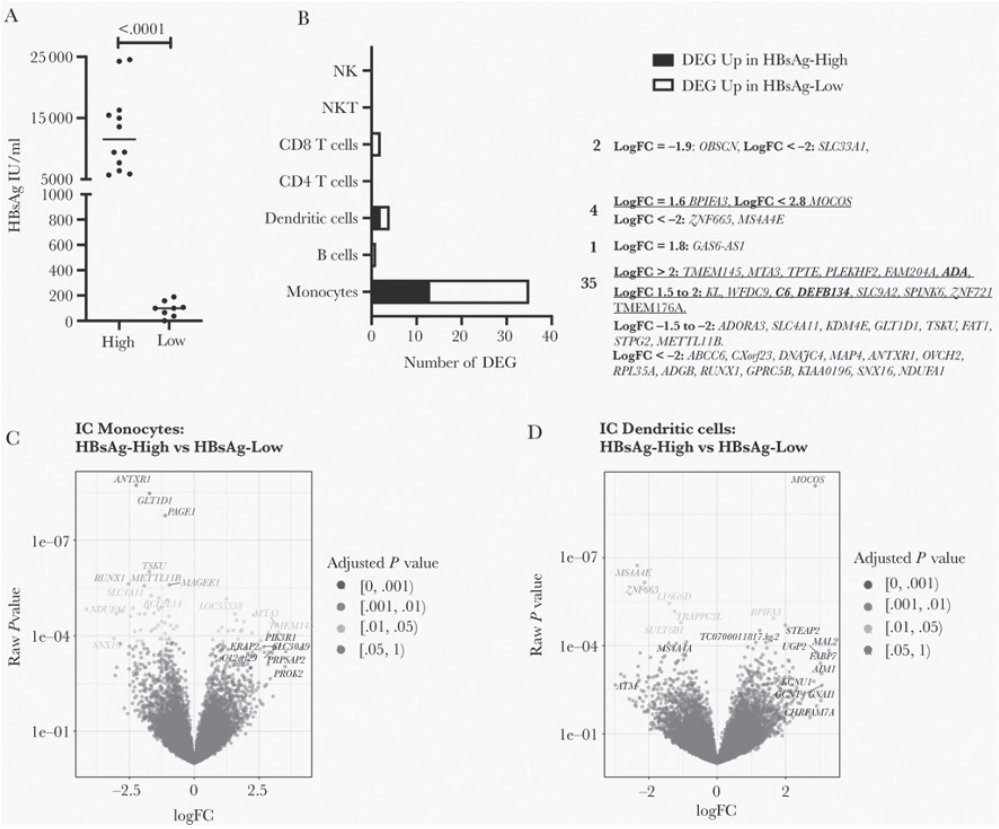

Figure 1. A, Serum HBsAg distribution in IC patients with high and low HBsAg levels (high mean 12 807 IU/mL, minimum 5707 IU/mL, maximum 24 538 IU/mL; low mean 94 IU/mL, minimum 1 IU/mL, maximum 189 IU/mL). Unpaired 2-way t test was conducted. B, Number of DEG (adjusted P value ≤ .05 and 1.5 logFC) in sorted blood leukocytes from IC patients with distinct HBsAg levels. Black bars and underlined genes indicate DEG upregulated in the HBsAg-high group whereas white bars and nonunderlined genes indicate DEG upregulated in the HBsAg-low group. Genes are ordered by logFC increase. C and D, Volcano plot of DEG in monocytes and dendritic cells from IC patients with HBsAg-high versus HBsAg-low. Abbreviations: DEG, differentially expressed genes; HBsAg, hepatitis B surface antigen; IC, inactive carrier; logFC, log fold change; NKT, natural killer T cell.

Among the list of DEGs, DC from NUC-treated patients exhibited a higher expression of HLA-related genes (ie, *HLA-DQB1*, *HLA-C* or *HLA-DMB*), Interferon Stimulated Gene (ISG) (*NKAP*, *IFITM3*, *IRF2BP2*, *ISG15*, *IRAK3*, and *MAPK1*), caspases (*CASP1*, *CASP4*, and *CARD16*), chemokines and chemokines receptors (*CXCL16* and *CXCR4*), and also *TLR8* (lowest adjusted *P* value 7.2e-10; Figure 2B). On the other hand, expression of type I inter- feron (IFN) genes (IFN-ε and IFN-α13) was higher in IC patients. All DEG identified for the CD8+ T cells showed higher expression in NUC-treated patients. Among these genes, we detected some related to cytotoxicity (ie, *KLRD1, KLRG1*, *GZMH*, and *GZMB*), immune-cell trafficking (*S1PR1* and *CX3CR1*), and the transcription factor *IKZF5* from the IKAROS family. Moreover, monocytes

exhibited an increased expression of Toll-like receptor (TLR)-related genes *LY6E* and *STK4* in NUC-treated patients compared to IC. Comparable to CD8+ T cells, most of the DEG in B cells were increased in NUC-treated patients. Among the genes with the highest fold-change, immunoglobulins were the pri- mary component. The DEG list for all leukocyte populations is shown in Supplementary Table 2. Finally, we conducted pathway analysis to identify the major biological processes driving the difference in gene expression profiles between IC and NUC-treated patients. As shown in Figure 2C, in DCs this was largely driven by signal recognition particle (SRP)- dependent protein targeting to membrane and endoplasmic reticulum pathways, followed by type I and II signaling, T-cell costimulation, and regulation of production of various interleukins (see also Supplementary Table 3). Moreover, CD8+ T cells were primarily driven by ubiquitin-related pathways (not shown) and monocytes by gap-junction as- sembly (not shown).

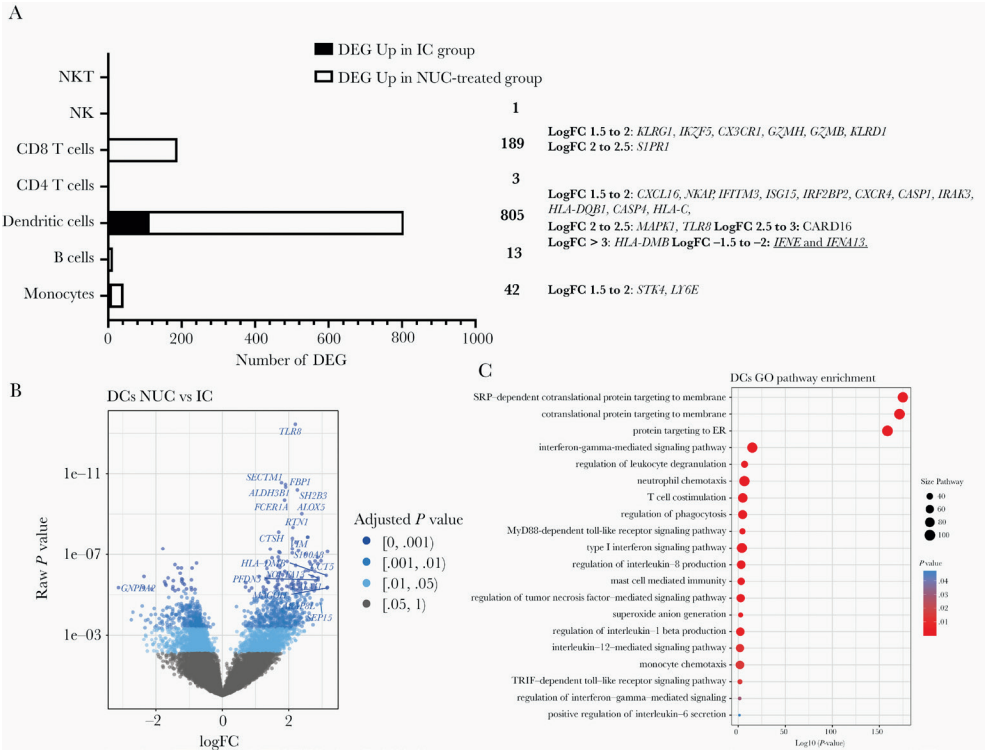

Figure 2. A, Number of DEG (adjusted P value ≤ .05 and 1.5 logFC) in sorted blood leukocytes in IC vs NUC-treated patients. Black bars and underlined genes indicate DEG upregulated in the IC group whereas white bars and nonunderlined genes indicate DEG upregulated in the NUC-treated group. Genes are ordered by logFC increase. B, Volcano plot of DEG in DC in IC vs NUC-treated patients. C, Altered immune-related gene signaling from pathway analysis in DC from IC vs NUC-treated patients. Abbreviations: DEG, differentially expressed genes; IC, inactive carrier; logFC, log fold change; NKT, natural killer T cell; NUC, nucleos(t)ide analogue.

DISCUSSION

In this study, we observed that the gene expression profiles of patients with low/undetectable HBV-DNA and normalized ALT levels receiving NUC therapy differed markedly from IC patients. However, distinct serum HBsAg levels only minimally affected gene expression profiles of blood leukocyte subsets in chronic HBV patients.

Our findings show that the transcriptome, particularly of DC, CD8+ T cells, and, to a lesser extent, monocytes are markedly different between IC and NUC-treated patients. This was unexpected because, although the mode of viral control differs, both groups of patients exhibit prolonged low/ undetectable HBV-DNA and normalized ALT serum levels. The distinct gene expression profiles between IC and NUC- treated patients in DC and CD8+ T cells suggest that distinct regulatory processes are active. Indeed, we observed that the ISG expression levels in DC and cytotoxicity-related genes in CD8+ T cells are increased in NUC-treated patients as com- pared to IC patients. In contrast to DC, CD8+ T cells, and monocytes, differential gene expression was not observed for B, NK, NKT, and CD4+ T cells between IC and NUC-treated HBV patients, and it does not lead to altered functioning of these cell types in the examined patient groups. Thus, it is tempting to speculate that although NUC-treated patients might experience a partial improvement in their immune re- sponse against HBV [8], it fails to reconstitute to an immune viral control state as seen in IC patients. The continuous presence of high levels of HBsAg are gen- erally considered to be an important factor impacting im- mune cell activity, with HBV-specific CD8+ T cells being examined most frequently with respect to the induction of T-cell exhaustion [4, 9, 10], thereby impeding the establishment of a long-lasting and effective immune response capable of eliminating or controlling the infection [11, 12]. To our surprise, transcriptomic characterization of highly pure FACS-sorted blood leukocytes collected from patients with highly distinct HBsAg serum levels exhibited only minimal modulation of gene expression levels. DEG were only identified for monocytes between the HBsAg-high and HBsAg-low groups but not, or in very low numbers, for CD4+ and CD8+ T cells, NK, NKT, B cells, and DC (Supplementary Figure 5B). Importantly, the inclusion of a smaller cohort of NUC-treated patients (Supplementary Table 4; n = 15) with contrasting serum HBsAg level recapitulated the cell fre- quencies data and transcriptomic findings seen in the IC co- hort with DEG identified solely in antigen-presenting cells (Supplementary Figure 5). Interestingly, a previous study on sorted CD4+ and CD8+ T cells from IC patients found the gene expression of TLR-signaling (*MYD88*), cytotoxicity (*GZMA*, *GZMK*), and nuclear factor-κB signaling (*BST2*) to be upregulated in patients with less than 1.5 × 103 versus more than 15 × 104 IU/mL HBsAg [13]. These discrepancies might be explained by differences in patient se- lection, methodology, or cofounding factors—although limited information is available in the study. Moreover, assessment of the major blood leukocyte population revealed comparable fre- quencies irrespective of the HBsAg antigenemia level, as previ- ously reported [14]. In light of these findings,

studies evaluating the immunological effects—in the context of effective immune control—of HBsAg blockers should preferentially also address the intrahepatic compartment and HBV-specific responses.

In conclusion, our approach, using ex vivo evaluation of the transcriptome of sorted highly pure blood leukocytes, does not provide evidence that lower versus higher levels of peripheral HBsAg alters gene expression of immunomodulatory proteins with suppressive activity on the total polyclonal leukocyte population. On the basis of our findings alone, it cannot be excluded that the HBsAg levels observed in this study might still be biolog- ically excessive and thus capable of negatively modulating the immune response at the same level or even higher HBsAg levels. Moreover, it cannot be discounted that HBsAg may preferen- tially act by modulating the activity of HBV-specific cells and that more profound effects are delivered locally in the infected liver. In addition, NUC-treated patients may fail to reconstitute the peripheral immune response comparable to IC patients, which might explain HBV relapses during antiviral withdrawal.

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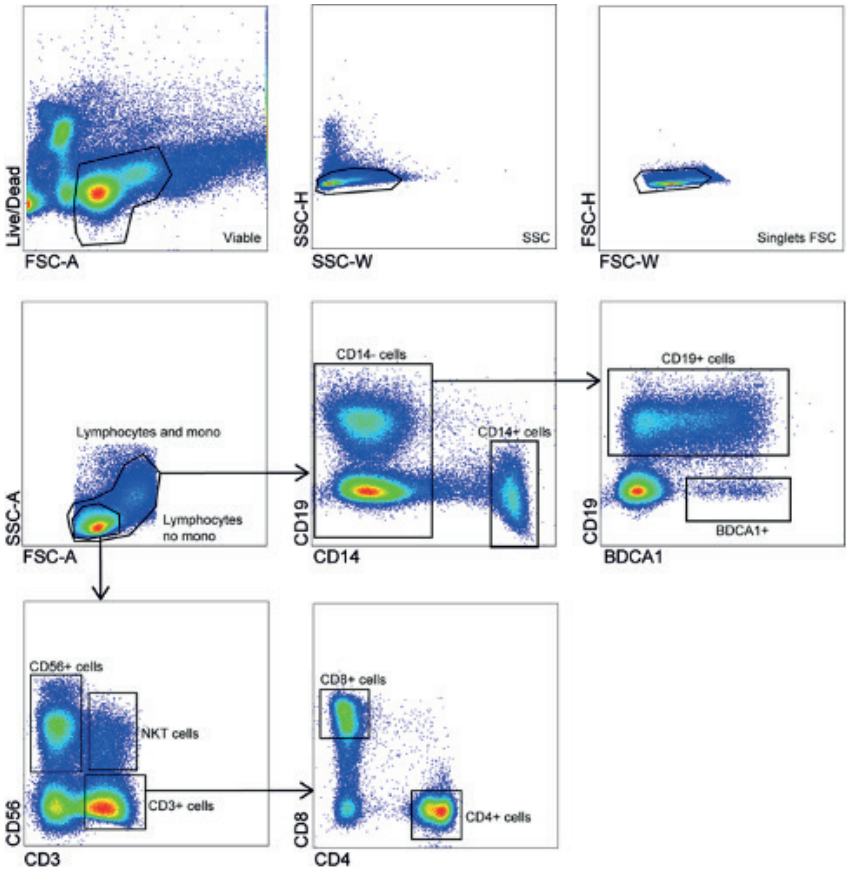
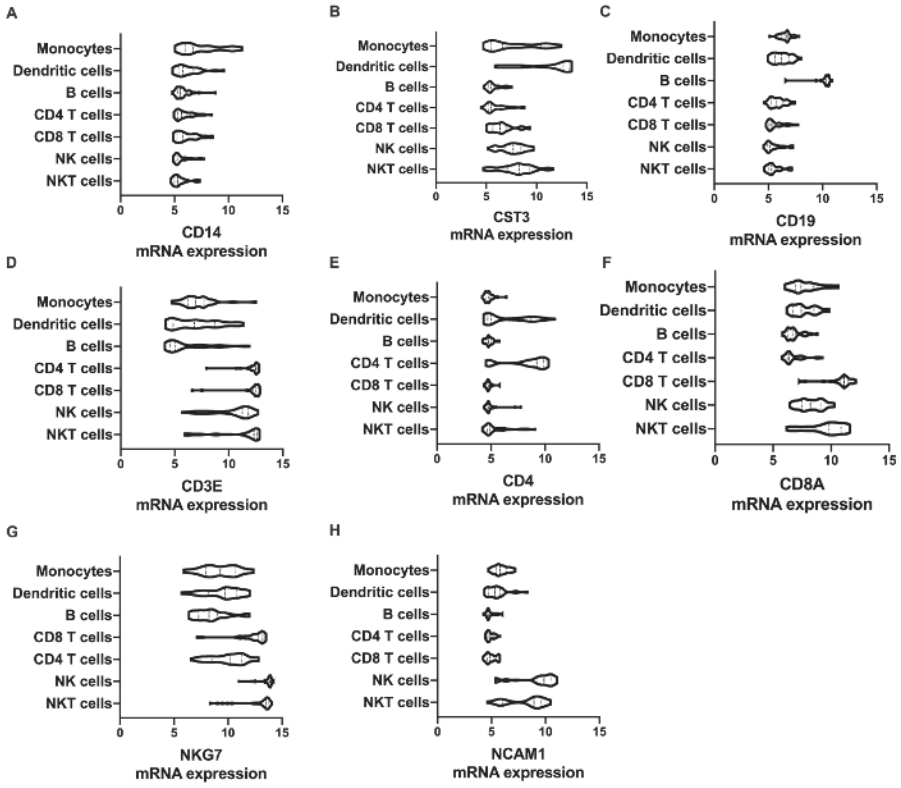
SUPPLEMENTARY MATERIAL

To access supplementary tables visit: https://academic.oup.com/jid/advance-article/doi/10.1093/infdis/jiaa614/5917489#supplementary-data

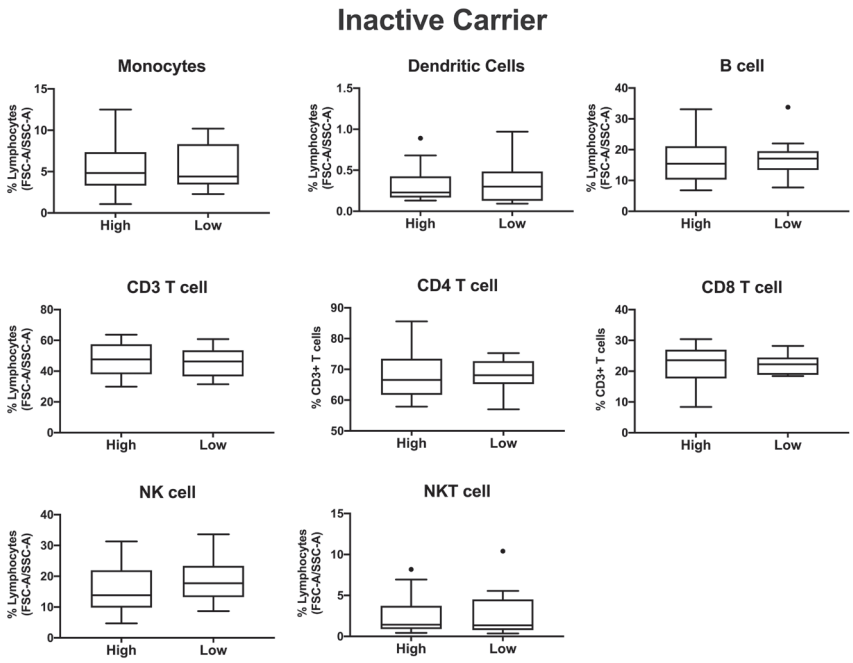
Supplementary Figures



Supplementary Figure 1. Gating strategy.
Cells were gated on Aqua-negative to exclude dead cells followed by SSC-H/SSC-W and FSC-H/FSC-W for doublets removal. Following, lymphoid and monocytic populations were gated (FSC-A/SSC-A). From the lympho-gate, total Natural Killers (NK), Natural Killer T (NKT) and T cell subsets based on CD3 and CD56 expression ($CD3^-$ $CD56^+$ NK, $CD3^+$ $CD56^-$ T cells, $CD3^+$ $CD56^+$ NKT, respectively) were sorted. Also, $CD4^+$ and $CD8^+$ were sorted from $CD3^+$ T cells. Moreover, monocytes ($CD14^+$), B cells ($CD19^+CD14^-$) and dendritic cells ($BDCA1^+CD19^-CD14^-$) were progressively gated from the FSC-A/FSC-W.

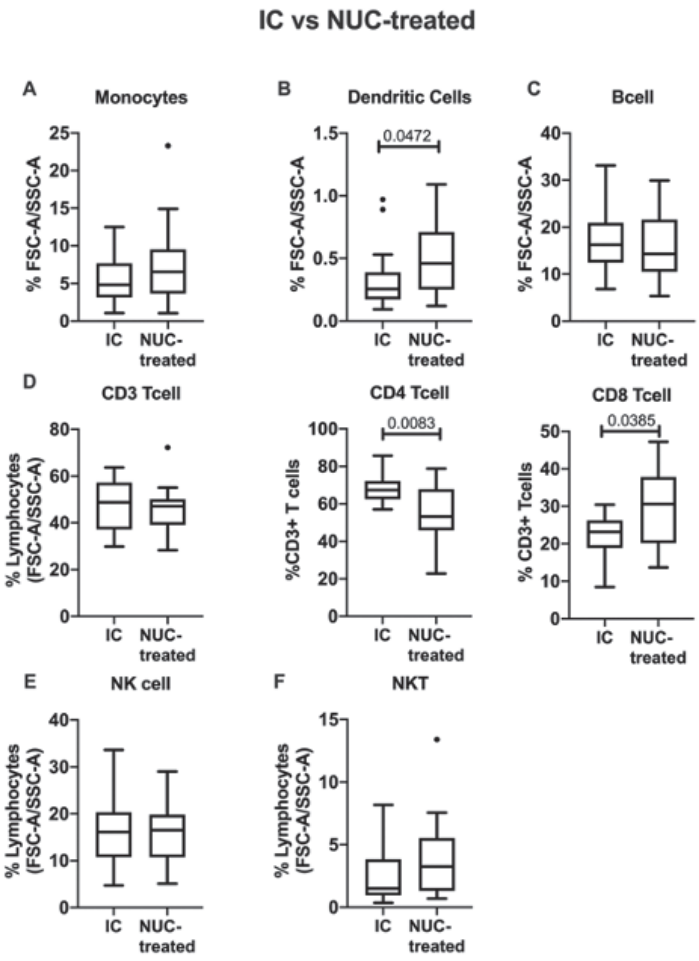


Supplementary Figure 2. Gene expression values of key markers in sorted blood leukocyte populations**.** Raw gene expression values for selected key marker genes in sorted blood leukocytes. A to H. mRNA expression values for *CD14* (monocytes), *CST3* (dendritic cells), *CD19* (B cells), *CD3E* (CD3$^+$ T cells), *CD4* (CD4$^+$ T cells), *CD8A* (CD8$^+$ T cells), *NKG7* (NK cells), *NCAM1* (NKT cells). Data is represented as violin plots.

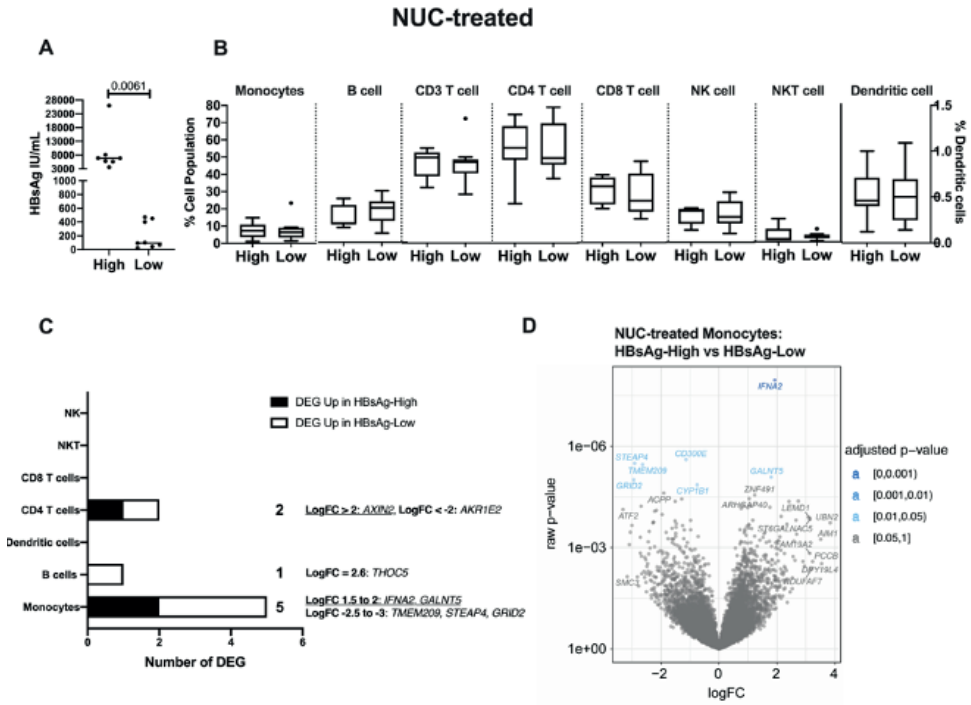


Supplementary Figure 3. Comparable peripheral leukocyte frequencies in inactive carriers with high and low HBsAg.

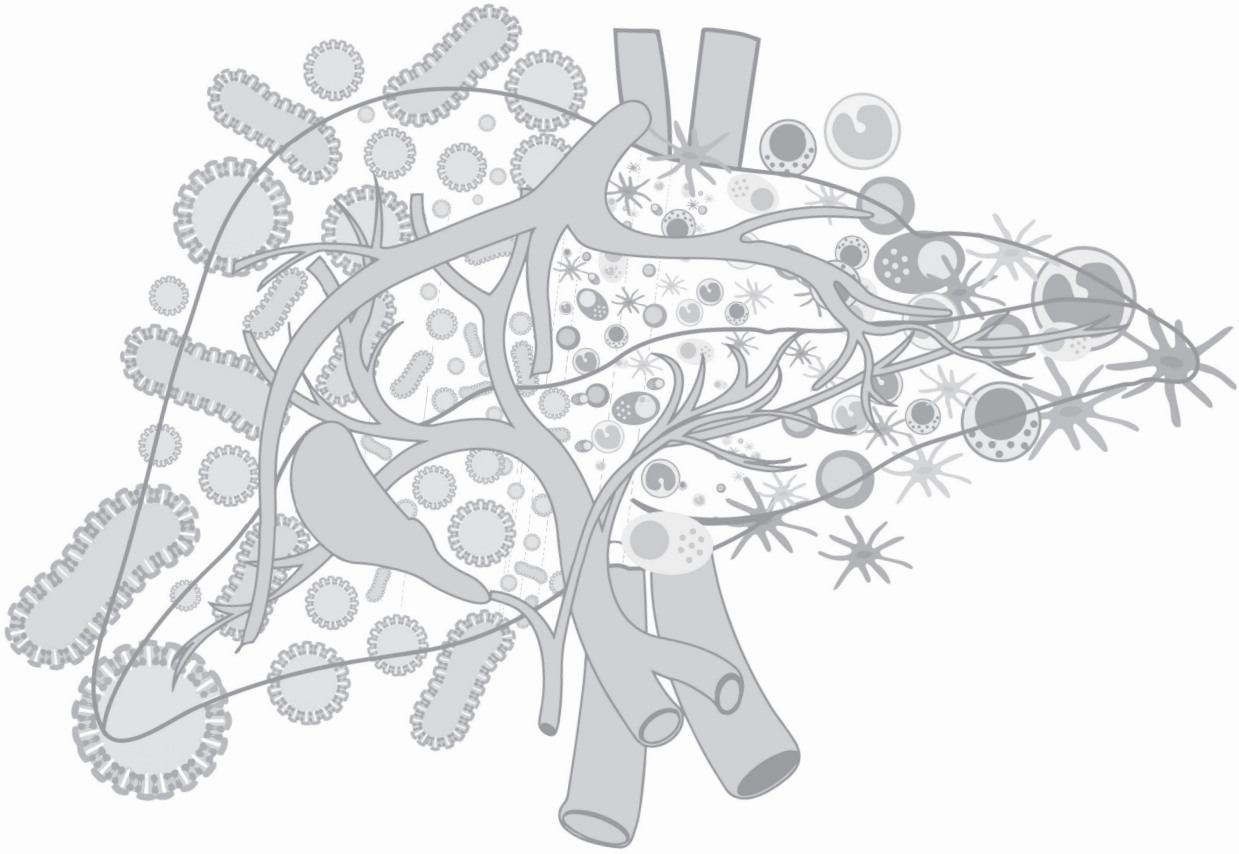
Frequencies of monocytes ($CD14^+$), dendritic cells ($BDCA1^+CD19^-CD14^-$), B cells ($CD19^+CD14^-$), total $CD3^+$ T cells ($CD3^+$), $CD4^+$ T cells ($CD3^+CD4^+CD8$-) and $CD8^+$ T cells ($CD3^+CD8^+CD4^-$), NK cells ($CD56^+CD3^-$), and NKT ($CD3^+CD56^+$) in IC patients with high and low HBsAg. Data are represented as Tukey's boxplots with median, interquartile range (IQR) and outliers. Statistical analysis was performed by Mann-Whitney test. None of the tests was significant (p-value > 0.05).



Supplementary Figure 4. Frequencies of dendritic cells and T cell subsets (CD4 and CD8) differ between Inactive Carrier (IC) vs NUC-treated patients Frequencies of monocytes ($CD14^+$), dendritic cells ($BDCA1^+CD19^-CD14^-$), B cells ($CD19^+CD14^-$), total $CD3^+$ T cells ($CD3^+$), $CD4^+$ T cells ($CD3^+CD4^+CD8$-) and $CD8^+$ T cells ($CD3^+CD8^+CD4^-$), NK cells ($CD56^+CD3^-$), and NKT ($CD3^+CD56^+$). Data are represented as Tukey's boxplots with median, interquartile range (IQR) and outliers. Statistical analysis was performed by Mann-Whitney test.



Supplementary Figure 5. Serum HBsAg inflicts minimal to no changes on frequencies and transcriptome of sorted PBMCs in NUC-treated patients (A) Serum HBsAg distribution in NUC-treated patients in High and Low HBsAg levels. High (mean 8984 IU/mL, min 3600 IU/mL max 26000IU/mL) Low (mean 205 IU/mL, min 23 IU/mL max 470 IU/mL). Unpaired two-way t-test was conducted (B) Frequencies of monocytes ($CD14^+$), dendritic cells ($BDCA1^+CD19^-CD14^-$), B cells ($CD19^+CD14^-$), total $CD3^+$ T cells ($CD3^+$), $CD4^+$ T cells ($CD3^+CD4^+CD8$-) and $CD8^+$ T cells ($CD3^+CD8^+CD4^-$), NK cells ($CD56^+CD3^-$), and NKT ($CD3^+CD56^+$). Data are represented as Tukey's boxplots with median, interquartile range (IQR) and outliers. Statistical analysis was performed by Mann-Whitney test. None of the tests was significant. (C) Number of differentially expressed genes [adjusted p-value ≤ 0.05 and 1.5 log fold change (logFC)] in sorted blood leukocytes from NUC-treated patients. Black bars and underlined genes represent DEG upregulated in the HBsAg-high group whereas white bars and non-underlined genes showcase upregulated in the HBsAg-low group. (D) Volcano plot of DEG in monocytes from NUC-treated patients with HBsAg-high versus HBsAg-low.



CHAPTER 5

Transcriptomic analysis of livers of Inactive Carrier HBV patients with differential HBsAg

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ABSTRACT

Inactive Carrier phases in chronic hepatitis B virus (HBV) infection present minimal liver disease and HBV replication activity suggesting a partial immune-reconstitution although the mechanisms responsible remain elusive. Moreover, HBsAg production -hypothesized to modulate the immune response- is unaltered. Here, we assessed the intrahepatic transcriptome in Inactive Carrier patients vs healthy liver donors, also in the context of diverse HBsAg levels (serum and liver), to better understand the phenomenon of immune control. We found a de-regulated liver transcriptome in Inactive Carrier patients vs healthy controls despite normal liver function. Moreover, diverse HBsAg levels impacted minimally at the liver transcriptome in Inactive Carrier patients although gene correlation analysis revealed leukocyte activation, recruitment and innate responses genes to correlate with liver HBsAg levels. These findings provide more insight into the mechanisms underlying anti-HBV strategies that are currently under development aimed at interfering with HBsAg production or at inducing a state of immune control.

Key words: hepatitis B, HBsAg, intrahepatic transcriptome**.**

INTRODUCTION

Chronic infection with the hepatitis B virus (HBV) remains a global health burden despite the fact that the virus was discovered over 50 years ago and a highly effective prophylactic vaccine exists. According to The World Health Organization, only 9% of the estimated 257 million individuals living with chronic HBV have been diagnosed[1]. Untreated, chronic HBV can lead to liver fibrosis, cirrhosis and ultimately hepatocellular carcinoma. These long-term life threatening complications are thought to be responsible for more than one million deaths annually[2,3]. The virological characteristics in chronic HBV infection are highly diverse and categorize patients into distinct clinical phases on the basis of the presence of the viral protein HBeAg, serum HBV DNA levels and alanine transferase (ALT), a surrogate marker of liver damage and inflammation released by lysed hepatocytes[4]. Particularly interesting is the Inactive Carrier phase, marked by undetectable HBeAg and low HBV DNA and normal ALT serum levels, suggesting that either the virus is inactive or somehow controlled by the immune system. A detailed understanding of the immune status during this phase is still lacking, but may provide important clues for future therapeutic strategies. Characterization of the host immune response with respect to the distinct clinical phases using systems biology approaches has shown changes in host gene expression in phases with active hepatitis vs non-hepatitis during chronic HBV and phases with high versus low viral load[5–7].

Although low serum HBV DNA levels, normal ALT and undetectable HBeAg are a hallmark of the Inactive Carrier phase, the viral protein hepatitis B surface antigen (HBsAg) is abundantly found in the serum of these subjects. It has been hypothesized that high levels of serum HBsAg are responsible for the induction and maintenance of an impaired immune response against HBV, amongst others by inducing T cell exhaustion[8–10]. In fact, various therapeutic approaches aimed at reducing HBsAg production and release are currently tested in trials for clinical efficacy[9]. However, evidence in support of a potent immunomodulatory activity of HBsAg has not been convincingly reported, and studies using blood leukocytes from individuals with variable serum HBsAg levels showed minor differences in gene expression profiles and functional readouts in purified leukocyte subpopulations [11–13]. However, these studies failed to examine the consequence of differential HBsAg expression in the liver, where viral replication and protein synthesis takes place. Moreover, the scarcity and difficulty to sample the livers of healthy individuals using core needle biopsies limits the studies assessing the HBV livers in comparison to healthy livers. To the best of our knowledge, we are the first to profile the transcriptome of liver FFPE biopsies to better understand the putative differences between the immune status of 1) Inactive Carrier patients versus healthy individuals, and 2) Inactive Carrier patients with variable serum and liver HBsAg levels.

MATERIAL AND METHODS

Patient and FFPE liver samples

Core needle liver-biopsies from 21 chronic HBV patients and 7 healthy controls -taken to determine their eligibility as altruistic liver donors- were collected as part of routine clinical care at the Erasmus MC and archived as FFPE tissues. Serum HBV DNA was undetectable in 10/21 HBV patients. The remaining 11 had all below 2,000 IU/mL except for 3 patients (2,290, 3,690 and 5,160 IU/mL). All were HBeAg-negative, had within normal range ALT (< 35 IU/mL), no signs of hepatitis or antiviral treatment prior to biopsy and no co-existing primary liver disease or co-infection with HCV, HEV, HDV or HIV which classified them in the Inactive Carrier state of chronic HBV. Histological evaluation (METAVIR staging system and hepatic activity index [HAI]) was performed as described previously by a single liver pathologist in a uniform manner (Table 1)[14]. Determination of intrahepatic and peripheral virological and clinical parameters is described in Supplementary Material and Methods. This study was conducted in accordance with the Declaration of Helsinki guidelines and the principles of Good Clinical Practice. Given the retrospective nature of this study, written informed consent was not obtained from each patient. Instead, the ethical review board of the Erasmus Medical Center approved this study as it was in accordance with the FEDERA guidelines, which regulate further use of coded-anonymous residual human tissue for scientific research.

Table 1 Patients' Characteristics

	Healthy Controls	***Inactive Carrier***	***P-value***
Nr Samples	7	21	-
Gender % Male (n)	57% (4)	47% (10)	ns‡
Ethnicity	6 Caucasian, 1 Other	5 African, 5 Asian, 7 Caucasian, 4 Other	NA
Age Median (IQR)	51 (27 - 55)	36 (28 - 42)	ns†
ALT IU/mL Median (IQR)	28 (23 - 40)	27 (29 -33)	ns†
AST IU/mL Median (IQR)	25 (23-04)	24 (19 - 30)	ns†
Prothrombin (seconds) Median (IQR)[a]	NA	8 (10.3 – 12.1)	NA
Total Bilirubin (↘mol/L) Median (IQR)[a]	NA	9 (8 – 11.4)	NA
LogHBV DNA IU/mL Median (MAX)[b]	NA	2.3 (3.7)	NA
HBeAg (Pos – Neg)	NA	21 Neg	NA
Anti-HbeAg (Pos - Neg	NA	21 Pos	NA
Serum HBsAg IU/mL Median (IQR)	NA	1750 (555 - 9475)	NA
% HBsAg-positive Liver cells (IQR)*	NA	30 (7 – 69)	NA
HBV Genotype	NA	4A; 2B; 2C; 9D; 1E; 3ND	NA
Fibrosis (<F2)	5, 2ND	21	NA
HAI score (≤5)	7 ND	21	NA
NASH (no/yes)	7/0	20/1	ns‡
Steatosis (no/yes)	7/0	17/4	ns‡

NA = Not applicable; '
ND = Not determined; ns = Not significant
18/21 Inactive Carriers
Undetectable HBV DNA in 10/21 Inactive Carriers.
*16 Inactive Carrier patients
† Unpaired Mann-Whitney test
‡ Fishers' exact test

Intrahepatic transcriptome data analysis

RNA extraction from FFPE liver-biopsies and sequencing are described in Supplementary Material and Methods. Raw paired-end reads were aligned to the human reference genome hg38 downloaded from the USCS Genome Bioinformatics site using the STAR (v2.7.3a)[15]. Picard tools were employed to detect samples with ≥10 million reads in protein coding mRNA regions for downstream analysis and were otherwise excluded[16]. Gene set annotation from the Molecular Signatures Database (v7.0) were quantified per sample as following. Gene expression quantification was calculated using featureCounts, converted to log2CPM using the R package edgeR, then scores were calculated using the R package gsva[17,18]. Raw gene counts per sample were used to performed differential gene expression analysis between groups using the DESeq2 package[19]. Lowly expressed genes across samples were removed and normalized using the estimateSizeFactors function. The normalized gene expression matrix obtained from DESeq2 was used to performed spearman correlations with serum and liver HBsAg levels in Inactive Carrier patients. Pathway analysis was conducted with Gene set enrichment analysis (GSEA)[20].

Statistics

Statistical analyses were performed with the GraphPad Prism (v9). Baseline variables between cohorts were compared with the use of chi-square tests for categorical variables, or Mann-Whitney test for continuous variables. All reported P values (p) are two-sided. Significant differences were considered in all cases when $p < 0.05$. Spearman correlations were considered significant when $p < 0.01$.

RESULTS

Metabolic genes and pathways are downregulated in livers of Inactive Carrier patients

To examine whether the minimal viral activity observed in the Inactive Carrier phase still impacts the liver gene expression we performed RNA sequencing on FFPE liver-biopsies from 21 Inactive Carrier patients with normal ALT levels, minimal to undetectable serum HBV DNA, low fibrosis and low necro-inflammatory scores (METAVIR ≤F2 and HAI ≤5, respectively) and compared them to that of 7 healthy individuals (Table 1). Differential gene expression analysis (Fold Change ≥ 1.5; adjusted p-value ≤ 0.05) identified 110 differentially expressed genes (DEG) (Figure 1A, Table S1). Two thirds (n=78) were downregulated in the livers of Inactive Carrier patients, while the remaining genes (n=32) were increased with respect to healthy individuals (Figure 1B). Half of the downregulated genes were enzymatic genes, predominantly involved in lipid metabolism (*HSD11B1*, *DECR1*, *PTDSS1*) and oxidative processes (*GLUD1*, *GSTA1*, *NDUFS2*) (Table S1). Interestingly, we also found liver-

specific genes (*FABP1*, *TF*), hepatocyte markers (*ALB*, *SLC2A2*) and some immune-related genes (*IRF6*, *SOD1*, *CTSL*, *ORM1* and *ORM2*) to be under-expressed in livers of Inactive Carrier patients. In contrast, a third of the upregulated genes found in Inactive Carrier patients were immune-related and predominantly involved in adaptive immune cell activation (*CD84*, *BTN3A1, IL10RA*).

To better understand in which processes these DEG participate and the level of de-regulation of the host response, we conducted biological processes (BP) GSEA-pathway analysis. We only detected downregulated BP signatures (adjusted p-value <0.05) in Inactive Carriers in comparison to healthy individuals. These were predominantly related to liver metabolism (Table S2) and the top-10 were involved in ATP synthesis and response to toxic substrates (Figure 1C). In sum, we observed a de-regulated intrahepatic gene expression profile in the livers of chronic HBV patients with controlled infection in comparison to healthy individuals, the vast majority affecting metabolic genes and pathways with select immune genes significantly upregulated.

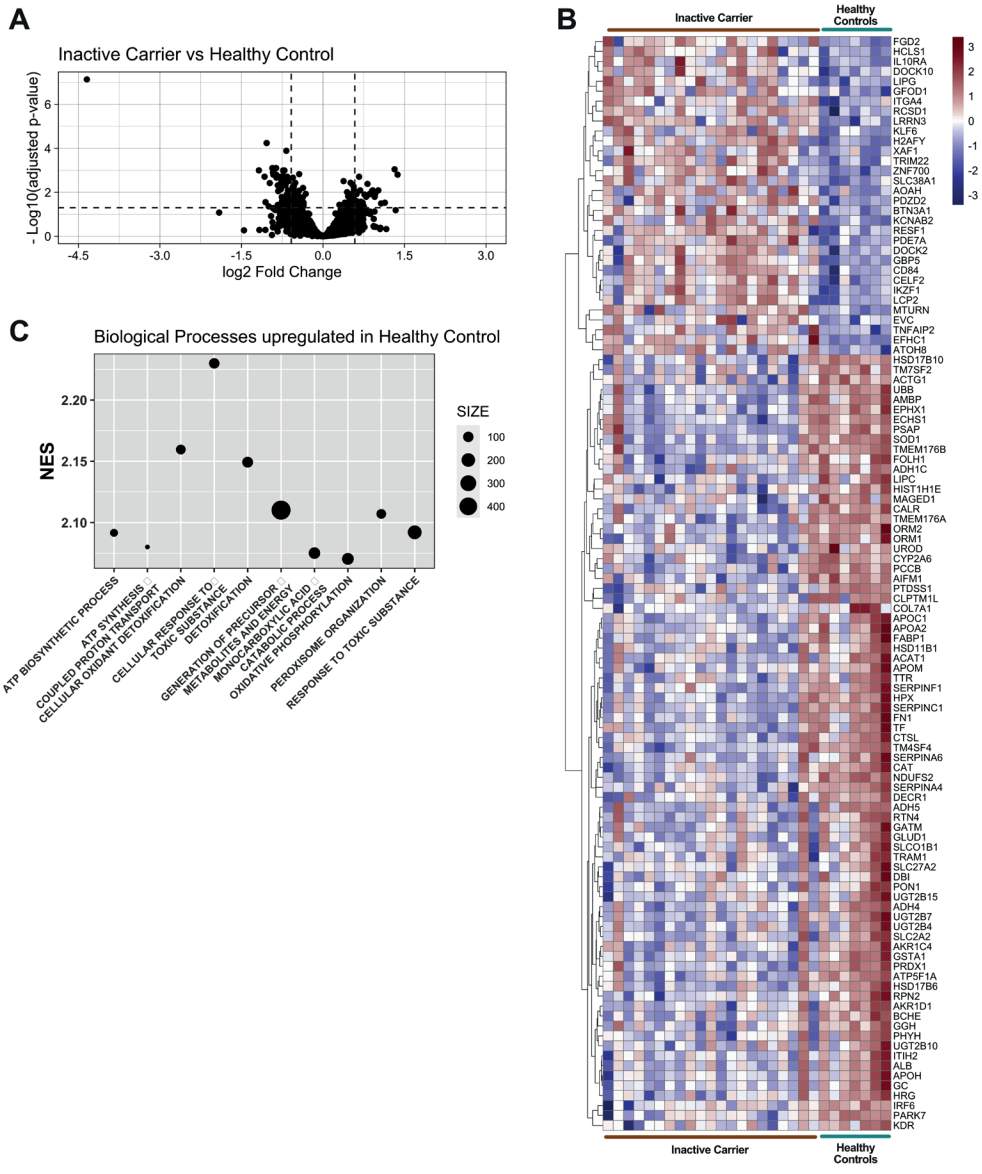

Figure 1: Intrahepatic gene expression comparison in Healthy control (n=7) vs Inactive Carrier patients (n=21). A. Volcano plot of differentially expressed genes (DEG) in 7 Healthy Control vs 21 Inactive Carrier. Vertical and horizontal dashed lines represent 1.5 Fold Change (0.58 log2 Fold Change) and adjusted p-value < 0.05 (-1.3 log10) thresholds, respectively. B. Heatmap of DEG with Fold Change > 1.5 per patient. Color-coded values represent the z-scores of normalized gene counts per samples, scaled per row (gene). C. Top 10 GSEA-Biological Processes (BP) upregulated in Healthy Controls.

Serum HBsAg levels correlate with innate and adaptive liver immune genes

Elevated viral antigen expression has been previously shown to negatively impact immune responses, as shown in LCMV-infected mouse studies[21]. Despite viral replication activity and liver inflammation is low in Inactive Carrier patients, HBsAg is continuously expressed and can easily be detected in serum. Continuous presence of HBsAg has been hypothesized that may negatively impact immune control during all phases of chronic HBV infection[9]. Inactive Carrier patients were selected with a 10-fold difference in serum HBsAg levels (5 Low ≤ 1,000 IU/mL; 5 High ≥ 10,000 IU/mL) (Figure 2A). HBV genotype distribution (not shown) and age (median 39 years vs 36 years in Low and High-HBsAg group, respectively) and were comparable between groups albeit older age and genotype D have been associated with lower serum HBsAg levels[22,23]. Surprisingly, we only detected 4 DEG (*INAVA*, *SNX32* and *RBPMS2* and *GJA5/CX40*) between the Low- and High-HBsAg with all, except *GJA5/CX40,* increased in the High-HBsAg group (Figure 2B). To further examine the possible modulation exerted by HBsAg, we conducted correlation analysis of gene expression with serum HBsAg levels from our full cohort of 21 Inactive Carriers (Spearman, p-value < 0.01 AND Rho |> 0.5|). We found an almost equal number of genes positively (n = 136 Rho > 0.5) and negatively correlating with serum HBsAg (n = 135 Rho < -0.5) (Table S3). Among the extensive list of genes, we detected enzymatic genes (hydrolases, transferases and oxidoreductases) as well as genes involved in transcriptional regulation and cell cycle (Table S3). Furthermore, 24 correlating immune genes were identified among which some were related to B-cell activation (*DAPP1*, *DCLRE1C*, *CD81* and *IGSF8*), T-cell activation (*MAP3K8*, *CD247*), inflammatory response (*IGFBP4*, *MAPKAPK5* and *CASP4*), chemotactic genes (*ACKR4* and *CD99*) and genes involved in antiviral defense (*ISG15* and *IRF9*) and antigen presentation (*ADAM19* and *HLA-DMA*) (Figure 2D and Table S3).

Although transcriptome comparison based on distinct serum HBsAg levels did not reveal HBsAg-induced changes of expression of immune genes, correlation analysis revealed an association between the intrahepatic gene expression levels of few immune genes and serum HBsAg levels which suggests some degree of immune-modulation in livers of Inactive Carrier patients due to HBsAg.

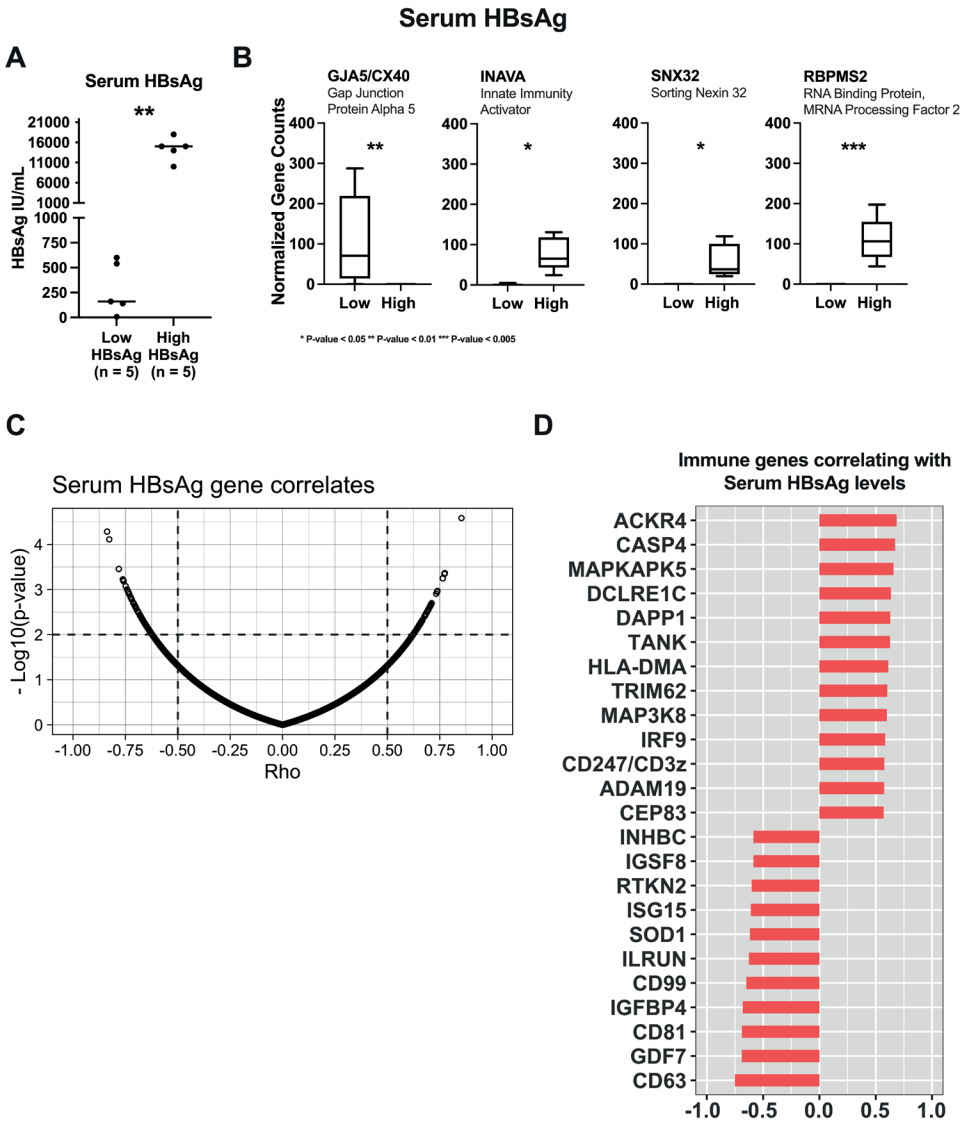

Figure 2. Gene expression differences in Inactive Carrier based on serum HBsAg levels. A. Serum HBsAg distribution in IC patients with High (n=5; >10,000 IU/mL) and Low (n=5; <1,000 IU/mL) HBsAg expression. Mann-Whitney test. B. Differentially expressed genes in Inactive Carriers patients with distinct serum HBsAg levels. C. Spearman correlations with serum HBsAg levels and normalized gene expression values in 21 Inactive Carriers. Vertical and horizontal dashed lines indicate Rho (>0.5) and p-value (<0.01) thresholds, respectively. D. Selected immune-related gene correlates.

Percentage of HBsAg-positive liver cells positively correlates with serum HBsAg expression in Inactive Carrier patients

We generally use serum HBsAg levels as a proxy for HBsAg production in HBV-positive livers. However, little is known on whether serum HBsAg levels are reflective of the intrahepatic HBsAg levels. To study this, we performed immunofluorescence of HBsAg on the FFPE liver-biopsies from 16 RNAseq-matched Inactive Carrier patients. Images of HBsAg staining in the liver are presented in Figure S1. We found that the percentage of HBsAg-positive liver cells significantly correlated with serum HBsAg levels (r = 0.59, p-value = 0.02; Figure 3). However, this monotonic relationship may not be true in all patients (i.e., patient 1, 4 and 12; Figure S1) raising the question of which mechanisms are responsible for the different HBsAg secretion patterns from the liver as well as what are the clinical implications. This positive trend suggests serum HBsAg may be used as surrogate of HBV liver burden.

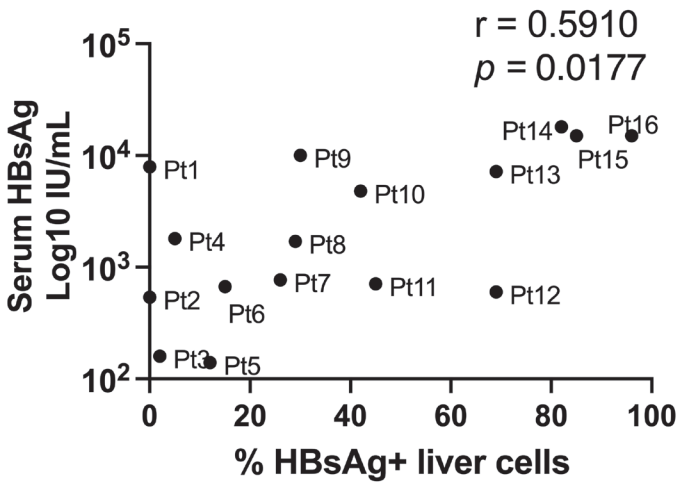

Figure 3. Serum and intrahepatic HBsAg expression correlation. Spearman correlation between serum HBsAg levels and percentage liver HBsAg-positive cells in Inactive Carrier patients (n=16).

Liver HBsAg expression correlates with liver leukocyte activation genes

Although we observed a positive correlation between the HBsAg levels detected in serum and liver, not all patients adhered to this pattern (patient 1, 4 and 12; Figure S1), and therefore we decided to also examine the liver transcriptome with respect to the number of intrahepatic HBsAg-expressing cells. For this, 5 Inactive Carrier patients with more than 60% of cells positive for HBsAg were compared to 6 Inactive Carrier patients who had livers with less than 20% of cells showing

positivity for HBsAg by immunofluorescence (Figure 4A). This analysis resulted in only 5 DEG (*HSF5*, *C16orf74*, *CCER2*, *DCC* and *TAT*). All, except *TAT*, were increased in the Low-HBsAg group (Figure 4B).

Again, we further conducted correlation analysis of gene expression with the percentage of liver HBsAg-positive cells in the cohort of 16 Inactive Carrier patients, whose livers were subjected to HBsAg immunofluorescence. Correlation analysis revealed a total of 270 genes correlating with the percentage of HBsAg positivity in the liver (Table S4). The number of genes that positively (Rho > 0.5) and negatively (Rho < -0.5) correlated with the level of HBsAg in the liver was almost identical (128 genes and 142 genes, respectively: Figure 4C). Among these we found a large number of genes acting as transcription factors/regulators and DNA-binding, cell cycle, enzymatic genes (oxidoreductase, transferase and hydrolase functions) lipid- and protein-transport genes, involved in cytoskeleton arrangement, lipid metabolism, autophagy and apoptotic programs (Table S4). Moreover, 36 immune genes were identified. Most of which were involved in B-cell (*CD320*, *IDO2*, *TNFSF13*, *DNAJB9*, *PLCG2* and *ZAP70*) T-cell (*LGALS3*, *VSIR*, *TSC22D3/GILZ*, *TSC1*, *PLCG2*, *ZAP70*, *BTN2A2*, *ITGAV*) and NK-cell (*SIGLEC7*, *NBEAL2*, *CCD88B*, *SEMA5A*) activation/function. Furthermore, a small fraction of detected genes was related to antigen presentation (*NLRC5*, *YTHDF1*), TGF-b signaling (*TGFBR2*, *VPS39*), leukocyte recruitment (*S1PR3*, *ITGAV*), IFN-lambda 3 stimulated (*PSME2*) and inflammation (*CXCL2*, *LRRC70*, *ARID5A*) (Figure 4D). Interestingly, none of these genes were found among the list of genes correlating with serum HBsAg level where less than 2% (*RPL41*, *SUPV3L1*, *CNPPD1*, *CEP83*, *MON2, USP37*) of the genes found to correlate with liver HBsAg levels also correlated with serum HBsAg levels (not shown). These findings point towards changes in leukocyte activation profiles in the liver of Inactive Carrier patients are associated by the level of HBsAg in the liver.

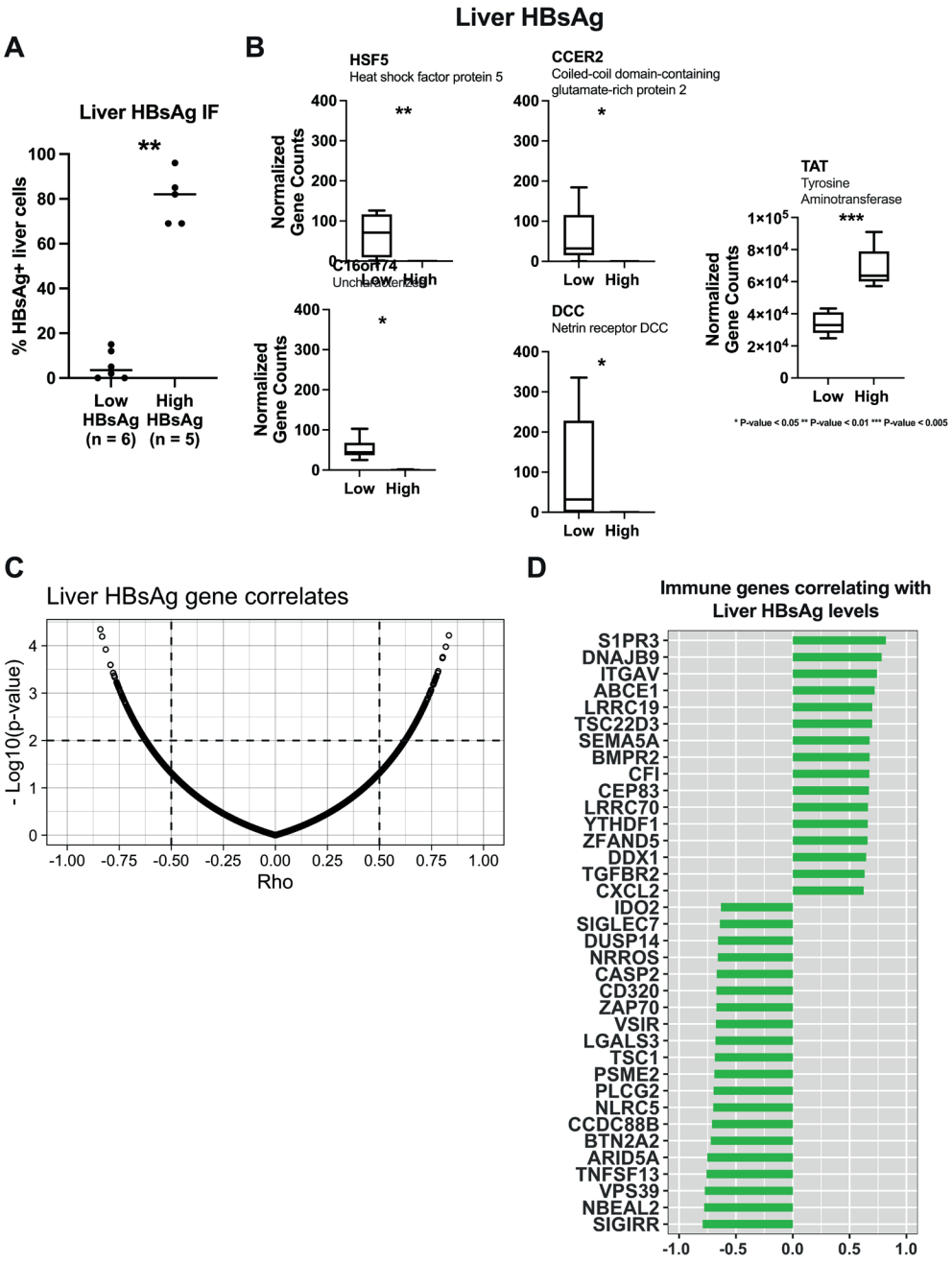

Figure 4. Gene expression differences in Inactive Carrier based on the percentage of intrahepatic HBsAg expression. A. Percentage HBsAg-positive liver cells distribution in Inactive Carrier patients with High (n=5; 60%) and Low (n=6; 20%). Mann Whitney test. B. Differentially expressed genes in Inactive Carrier patients with distinct liver HBsAg expression. C. Spearman correlations with liver HBsAg levels and normalized gene expression values in 16 Inactive Carriers. Vertical and horizontal dashed lines indicate Rho (>0.5) and p-value (<0.01) thresholds, respectively. D. Selected immune-related gene correlates.

DISCUSSION

The present study examines the putative mechanisms underlying immune control during the Inactive Carrier phase of chronic HBV. Through intrahepatic transcriptome analysis in Inactive Carrier patients and healthy liver donors we found a downregulation of metabolic genes and pathways, and a minor increase in immune genes in Inactive Carrier patients compared to healthy individuals. Moreover, whereas gene expression differences in a subset of Inactive Carrier patients based on the HBsAg level in serum or liver were almost not existent, correlation analysis identified a subset of immune genes that correlated with serum or liver HBsAg levels.

To gain insight into the mechanisms that act during the Inactive Carrier phase in chronic HBV patients, we demonstrated that the expression of metabolic genes in the liver of patients is downregulated as compared to livers of healthy individuals. Thus, despite the fact that viral replication and liver damage is minimal in these patients, metabolic dysregulation at the genetic level was observed, including the gene encoding Albumin. Given the key role of hepatocytes in metabolic processes and the abundance of hepatocytes over immune cells, it is likely that the downregulated gene expression is the consequence of either continued translation of viral protein from cccDNA in infected hepatocytes or low HBV replicative activity in hepatocytes. To note, the observed transcriptome differences were unlikely driven by HBV DNA levels as no DEG were identified between Inactive Carrier patients on the basis of HBV DNA (detectable vs undetectable; not shown). Nonetheless, despite the reduced expression of metabolic genes in the liver, prothrombin and total bilirubin levels were within the normal range in the majority of Inactive Carrier patients, suggesting a conserved synthetic liver function as often observed in more advance forms of liver disease. Our findings observed in livers of Inactive Carrier patients differ from earlier studies in which an increase in fatty acid synthesis, gluconeogenesis and aerobic oxidation during this phase of chronic HBV was observed[24,25]. In this respect it is important to note that our study is unique in that we compared core needle liver-biopsies taken from patients and healthy donors which were collected and processed using identical procedures. Most other studies used healthy liver material from surgical procedures or obtained results from in-vitro studies using hepatoma tumor cell-lines, in which the persistence of the HBV infection and the Inactive Carrier phase are difficult to interpret[26–29]. In contrast, a recent study evaluating the transcriptomic changes overtime in distinct liver-injury mice models revealed a downregulation of metabolic genes and pathways during peak upregulation of inflammatory genes and pathways, which were controlled by the same upstream regulators suggesting that these changes participate in liver regeneration upon liver injury[30]. Furthermore, in the same study they also showed that these transcriptomic signatures were present in different liver etiologies in human livers, including HBV infection[30]. Whether these findings can be translated to our dataset, composed of HBV patients with no signs of hepatitis and minimal liver inflammation (HAI < 5) warrants further investigation.

However, it is important to mention that we previously demonstrated that serum metabolomics conducted in chronic HBV showed deregulated lipid metabolite patterns in Inactive Carrier patients[31], which might be the consequence of altered expression of metabolic genes.

Besides an effect on expression levels of metabolic genes, transcriptome comparison showed upregulation of a small number of immune genes when comparing the livers of Inactive Carrier patients with those from healthy individuals. Most of these genes are involved in leukocyte activation (*DOCK2* and *ITGA4*) and T-cell responses (*BTN3A1* and *CD84*). Whether these signatures reflect inflammatory activity during chronic hepatitis or contribute to partial immune control in the livers of Inactive Carrier patients is difficult to ascertain and warrants functional studies. However, enhanced expression of *DOCK2* and *IL10RA* in addition to the previously mentioned genes suggest either infiltration of immune cells, pronounced activation or regulation of intrahepatic immune responses during the inactive phase of chronic HBV infection. Nonetheless, we detected an increased expression of *TRIM22*, a potent HBV replication and transcription suppressor, in infected patients suggesting an active anti-HBV response[32]. Although signs of immune control in our cohort of Inactive Carrier patients are limited, a previous study in which Nanostring analysis was performed on FFPE core needle liver-biopsies from chronic HBV patients observed a general gene expression downregulation of innate antiviral effectors, TLR- and IFN-related genes in HBeAg-negative patients compared to uninfected controls[33]. These discrepancies might be explained by differences in cohort composition between studies, since a third of the HBeAg-negative patients showed signs of active hepatitis and no matching details were provided for the uninfected cohort. Furthermore, HBeAg-negative patients with minimal HBV DNA and normal ALT levels displayed a comparable gene expression in some of the studied ISGs compared to healthy controls supporting the findings in our cohort of Inactive Carrier patients[33].

The putative HBsAg immune-modulatory effect on the immune response in chronic HBV remains under continuous debate as human data fails to recapitulate in-vitro and animal models observations. Here, we show that liver gene expression differences in Inactive Carrier patients with variable serum and liver HBsAg levels are minimal, with less than 5 genes found to be differentially expressed. These results mirror the findings from our previous study where we showed that variations in serum HBsAg levels resulted in minimal gene expression changes in different cell-sorted peripheral leukocytes populations (i.e., monocytes, CD4 and CD8 T-cells, B-cells, NK-cells and DCs)[11]. Likewise, phenotypic characterization and function of peripheral T and NK cells in chronic HBV patients has been shown to be comparable irrespective of serum HBsAg levels[34]. However, in mouse models, knockdown of HBV antigen expression using siRNA increased CD8+ T cell responses against HBV following therapeutic vaccination[35]. In contrast, a different study showed that the frequency and function of HBsAg-specific T cells in HBV transgenic mice were found to be independent of the level of circulating serum HBsAg levels[36]. In line with this the study, Le Bert et al. showed that not circulating HBsAg levels,

but rather the duration of HBV infection associated with the frequency of HBsAg-specific T cells in chronic HBV patients[34]. These findings could not be extrapolated to our study, since we found that the intrahepatic transcriptome variance was not associated with patients' age (not shown), albeit that HBsAg burden in serum and particularly in the liver negatively associated with patients' age (Figure S2). Likewise, infection with HBV genotype D, which was predominant in our cohort and has been shown to associate with lower serum HBsAg levels[23], showed no transcriptomic differences when compared to samples from patients with non-D genotypes (not shown).

Additional correlation analysis on a continuous scale of serum and liver HBsAg levels showed a fraction of the correlating genes to be immune-related. For instance, the antiviral effector *ISG15* was found to negatively correlate with serum HBsAg, an observation in line with Lebossé´s study who showed that HBeAg-negative patients with lower HBsAg levels displayed increased *ISG15* expression[33]. Although we found a set of immune genes related to innate and adaptive immune responses to correlate with serum HBsAg, it is unclear whether these changes have consequences for the functionality of the immune response.

Interestingly, the positive correlation in HBsAg level in the liver with gene expression of innate immune genes (*CFI*, *DDX1* and *LRRC19*), chemotactic genes (*CXCL2*) and genes involved in leukocyte recruitment (*S1PR3* and *ITGAV*) may reflect signs of HBsAg-induced immune-activation. Similarly, the negative correlations observed between the low liver HBsAg levels and the increased expression of T- and B-cell activated genes may point towards a limited HBsAg-induced immune-exhaustion and enhanced immune control and/or a higher proportion of leukocytes in the liver of these patients.

In summary, profiling of liver transcriptomes in healthy individuals versus Inactive Carrier patients with variable HBsAg levels revealed limited immune activation signals in livers of Inactive Carrier patients and a previously unreported downregulated metabolic profile. Furthermore, our results underline the limited but detectable signs of immunomodulation in the livers of Inactive Carrier patients, and mildly more active T-cell and B-cell gene signatures in patients with low liver HBsAg levels. Our findings are highly relevant in the current search for novel anti-HBV strategies that aim at re-invigorating the antiviral response by interfering with HBsAg-production or at inducing a state of immune control.

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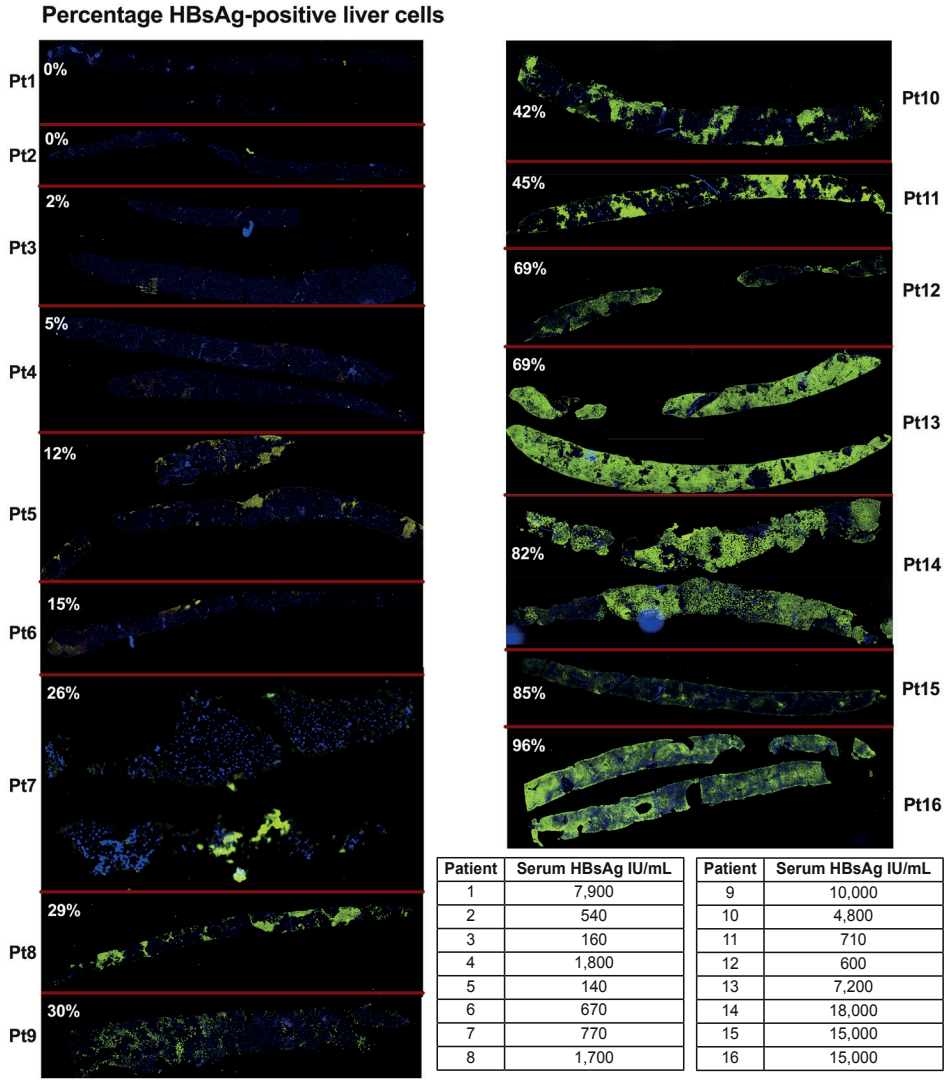
SUPPLEMENTARY MATERIAL

To access supplementary tables visit https://academic.oup.com/jid/advance-article/doi/10.1093/infdis/jiab381/6324056#supplementary-data

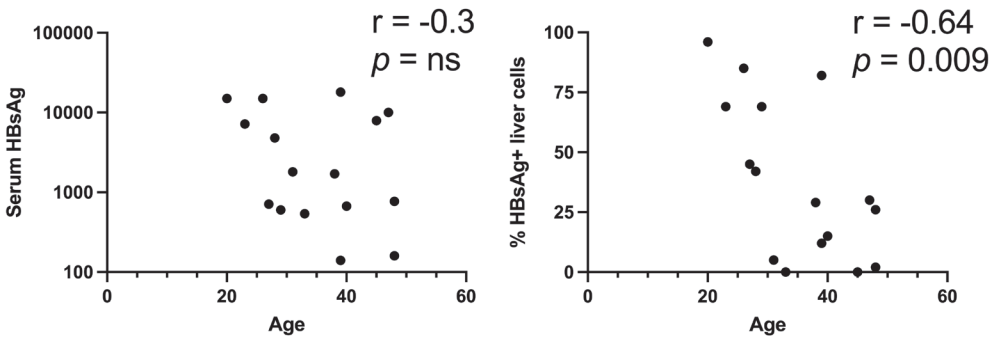
SUPPLEMENTARY FIGURES



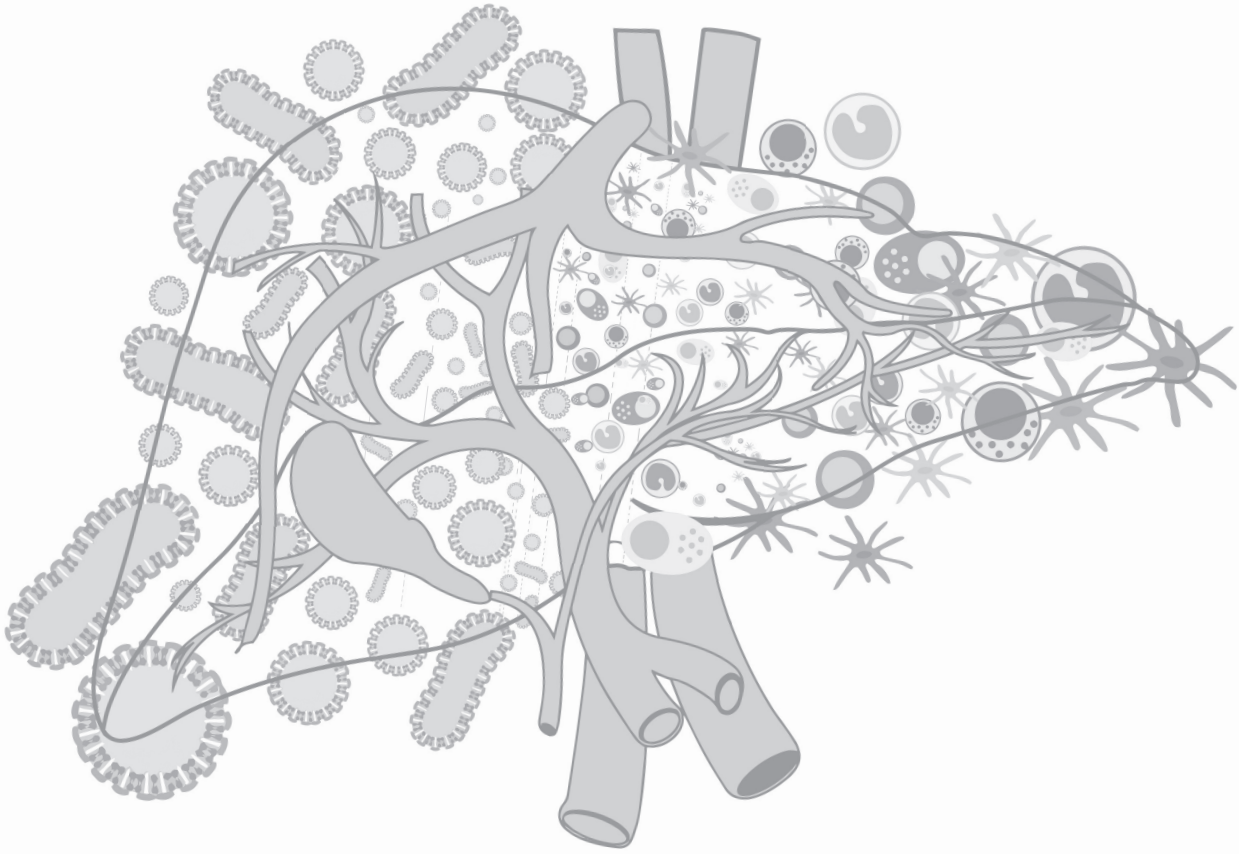
Patient	Serum HBsAg IU/mL
1	7,900
2	540
3	160
4	1,800
5	140
6	670
7	770
8	1,700

Patient	Serum HBsAg IU/mL
9	10,000
10	4,800
11	710
12	600
13	7,200
14	18,000
15	15,000
16	15,000

Supplementary Figure 1. Intrahepatic HBsAg expression levels
Immunofluorescence detection and percentage of HBsAg-positive (green) liver cells (DAPI+) in Inactive Carrier FFPE tissue sections (n=16) calculated with digital analysis software QuPath.



Supplementary Figure 2. Spearman correlations between Serum HBsAg or percentage of HBsAg-positive liver cells and age in Inactive Carriers (n=16).



CHAPTER 6

Discussion

DISCUSSION

Chronic HBV infection is a highly heterogeneous disease with four distinct clinical phases characterized by fluctuating shifts in viral load and liver damage. Left unchecked, the active disease states can progress to develop cirrhosis and ultimately liver cancer. However, the increase roll-out of effective antiviral therapies (nucleos(t)ides analogues; NUCs) capable of controlling active disease and achieve regression in the grade of liver damage (fibrosis) has pivoted the end goals in chronic HBV management towards curative strategies. Hence, much of the research has focused on shedding light onto which mechanisms are responsible for the observed immune-exhaustion and exploring and designing new strategies, through novel therapeutics, to re-invigorate the anti-HBV immune response to reach a state of functional immune-mediated control termed functional cure. However, a deeper understanding on the immunological states and degree of dysfunction throughout the clinical phases is lacking. Likewise, it remains unclear whether natural and NUC-induced viremia control present similar immune responses and can be further boosted by new therapeutic strategies to reach functional cure. Therefore, the overall aims of this thesis were set to characterize the immunological profiles in distinct clinical phases and to evaluate the immunological changes undergone during NUC-therapy. Furthermore, we set out to explore the immune states during viremia control and the degree of immune-exhaustion exerted by viral antigen load (HBsAg). It is important to highlight that contrary to the animal models and in-vitro systems, the work present in this thesis captures the complexity of chronic HBV infection in humans.

Advancements in research approaches permit more easily study intrahepatic immune responses

To address these unsolved questions related to disease progression and immune control more refined assays focusing on the intrahepatic immune responses is essential. Recent advances in sampling techniques and immune-assays can now provide the possibility to study the liver in more detail. The introduction of fine needle aspirate biopsies (FNAB) employs finer needles permitting to sample immune populations residing on the infected liver[33]. This technique minimizes the risk of potential complications associated with larger needles (used for diagnostic liver biopsies). Furthermore, the material obtained from FNABs is collected as a cell suspension which allows for a limitless battery of research assays available to the researcher. In addition, newly developed research techniques such as fluorescently-labelled antigens and single-cell sequencing (scRNA-seq) now offers the ability to study previously unidentified immune cells and explore in further detail the role of neglected leukocytes such as HBV-specific B-cells[19,25,34]. Altogether, the combination of these two factors has opened up a new era in HBV research where studying the liver is no longer a limitation and the information obtained is much more comprehensive. FFPE liver biopsies are

limited as traditional liver biopsies are used taken in clinical practice as a result of accurate disease staging given by non-invasive techniques such as fibroscan[35]. Thus, these technical advances in liver sampling make more feasible to conduct follow-up studies to examine which events are the initial trigger promoting liver damage, impossible to address cross-sectional studies. Prospective recruiting of Immune Tolerant patients and/or patients susceptible to experience viral relapses (upon NUC-discontinuation) should be designed using unbiased approaches as just described. Additionally, paired sampling of blood and liver tissue in this setting might help identify biomarkers indicative of liver damage.

Study of intrahepatic immune responses is pivotal to understand clinical disease states

Chronic HBV infection is characterized by distinct phases with shifts in viral load and liver disease (hepatitis). Based on the serological levels of HBV DNA, ALT and detection of viral antigen HBeAg, patients are clinically categorized in four, often sequential, phases known as Immune Tolerant (IT), Immune Active (IA), Inactive Carrier (IC) or E-antigen negative (ENEG)[1]. Hepatitis is an immune-mediated event present in IA and ENEG phases, whereas it is weaker or virtually absent in IT and IC phases[1]. In acutely infected primates intrahepatic NK-cells and cytotoxic CD8+ T-cells are the main drivers in liver damage[2,3]. However, it is not fully characterized by which mechanism liver damage occurs in chronic HBV infection as peripheral NK-cells have shown minimal phenotypical and functional alterations irrespective of ongoing/absent liver disease and CD8+ T-cells are generally exhausted[4-7]. This observation does not rule out their pathological role as peripheral immune responses are less accentuated than their liver counterparts and do not fully reflect the intrahepatic compartment as the leukocyte composition differs between the two[8-12]. Moreover, partial immune control can be reached in chronic HBV infection. This state is reflected in the IC phase where reduced serum HBV DNA levels, often undetectable, and no signs of active liver disease (within normal ALT levels) can last for decades without alterations[1,13]. The observed increase in peripheral HBV-specific T-cell responses in these patients, compared to active disease states (IA and ENEG) or with elevated HBV DNA and no liver damage (IT), may partially lie behind immune control[14].

To decipher the different immune states underlying the four clinical states in chronic HBV infection and to further understand immune control, we comprehensively evaluated the intrahepatic compartment in all phases of chronic HBV and compared them to altruistic healthy donors (Chapter 2). Albeit that leukocyte infiltrates were observed in all chronic HBV livers, the active hepatitis phases (IA and ENEG) showed the greatest influx. This infiltrate was characterized by not only T-cells, but also by B-cells to a lesser extent. The study of B-cells has gained recent interest in the community due to its dual role in HBV infection. On one hand, antibody responses mediate protection against HBV infection and prevent HBV re-activation, as observed in chronic HBV patients upon immune-

suppressive therapies[15,16]. On the other hand, antibody-dependent cytotoxicity via anti-HBcore antigen (HBcAg) antibodies has been described to be a mechanism promoting liver damage in acute liver failure[17,18]. Recently, peripheral studies have highlighted that HBV-specific antibody responses are mainly found in IA and ENEG phases and directed against HBcAg. These responses are presumed to be in response to antigen release from dying hepatocytes[19]. Interestingly, the livers of ENEG patients included in our study minimally expressed HBcAg. Whether trace amounts of antigen are sufficient to generate a response or whether other mechanisms lie behind B-cell influx and activation should be further explored. However, our data suggests it to be independent of viral antigen expression as we detected higher HBcAg protein expression in IT than ENEG livers and B-cell influx was markedly lower in liver of the IT than ENEG phase. Furthermore, we observed a lack of correlation between the two. Interestingly, serum ALT levels positively correlated with B-cell influx. Thus, it is tempting to speculate that these observations are the result of bystander activation due to the ongoing inflammation present during active liver disease. However, should that be the case, then it remains elusive why antibodies against other viral antigens, such as HBsAg, are hardly detected. In addition, recent studies comparing the transcriptomic state between HBcAg-specific and HBsAg-specific B-cells showed a predominant IgG+ memory phenotype in the former population[20], which raises the question on what drives these distinctive profiles. Moreover, we showed that gene expression of immune-exhaustion markers was solely observed in livers with active disease. Whether this phenotype, likely to be observed in T- and B-cells, is transient and is a mechanism related in response to ALT increases to mitigate liver damage similarly to what has been shown in infants and neonatal mice models requires further evaluation[21]. However, spontaneous HBeAg-positive seroconversion and progression to Inactive Carries states[22] together with the dynamic expression of immune-exhaustion markers as shown for PD-1 in the context of other chronic viral infections (i.e.,Hepatitis C virus)[23] suggests to be an event dependent on ALT raises. Indeed, we found a large number of immune-exhaustion genes to correlate with ALT levels in active disease phases. Similarly, the leukocyte infiltrate associated during active disease may arise in response to the observed increase of chemoattractant proteins, such as *CXCL9* and *CXCL10,* during active liver disease[24]. Moreover, although our experimental approach did not allow to distinguish the HBV-specificity of the recruited leukocyte infiltrate, it is likely that a fraction might correspond to specific responses as these are enlarged in the liver compared to blood. However, the functional state of these cells remains to be explored. The minimal penetration of the infiltrate in the liver parenchyma (most of the leukocytes were within periportal areas) suggests that cytotoxic clearance of infected hepatocytes may be a less frequent event than previously hypothesized, and it should be further explored which functions these cells exert in the infected livers. To our surprise, the intrahepatic gene expression and leukocyte infiltrate in IC patients were almost identical to IT patients although they markedly differ in the levels of viral replication[1]. This begs to ask how comparable intrahepatic profiles

lead to two diametrically opposed clinical phenotypes. A plausible explanation might be the methodology employed in the study, bulk RNA-sequencing, which cannot capture the cellular origin of the detected transcripts[25]. Furthermore, it is not possible to identify subtle differences linked to HBV-specific or other unidentified cell populations participating in immune control as the majority of the detected transcriptome is derived from parenchymal cells[25].

NUC-viremia control does not reconstitute anti-HBV immune responses to allow immune control

Besides IC patients, treatment with nucleos(t)ide analogues (NUC) induces a state where serum viral load becomes undetectable and ALT returns to normal levels. However, NUC-therapy discontinuation has highlighted the limited functional recovery that occurs in patients as viral rebound occurs in the vast majority of patients requiring re-treatment[26,27]. This suggests that underlying differences exist in the immune state of NUC-treated patients compared to IC patients responsible for the inability to control HBV upon NUC withdrawal. This observation may be partially explained by work summarized by Boeijen et al. where changes induced by NUC-therapy in multiple leukocyte populations from non-specific NK cells and T regulatory cells to HBV-specific CD4+ and CD8+ T-cells were reviewed[28]. Interestingly, NUC-induced changes in the HBV-specific compartment are often transient [i.e., in-vitro proliferation, cytotoxic and non-cytotoxic potential (TNF-a or IFN-g producing cells)] returning to pre-treatment levels by week 12-16 on-treatment. Hence, to further increase our knowledge on the potential different immune states in natural and NUC-induced viremia control, we evaluated the peripheral compartment of IC and NUC-treated patients (Chapter 4). We observed marked differences in the frequency and gene expression of dendritic cells and CD8+ T-cells, which were largely increased in NUC-treated patients despite the fact that treatment initiation dated at least 3 years from assay conduction. Interestingly, expression of interferon genes (*IFNA13* and *IFNE*) was increased in IC patients, which may partially contribute to viremia control without exacerbating liver damage by acting non-cytopathically on infected hepatocytes[29,30]. In line with the reviewed changes induced by NUC-therapy on the immune response, much of the observed induced gene expression in NUC-treated patients resembled what we observed in livers of patients eligible for NUC-therapy (IA and ENEG) with greater expression of HLA, ISG and chemokine receptors genes (see Chapter 2). It should be noted that most of the reported transient changes were primarily observed in in-vitro HBV-specific T-cells whereas we focused on global ex-vivo T-cell populations where HBV-specific cells are only a small fraction. Thus, whether transient changes took place in our NUC-treated cohort could not be addressed in our study. However, should transient recovery take place it ought to be explored which mechanisms underlie the point in which undetectable viral load and ALT normalization is reached (week 12-16)[31]. Nonetheless, changes observed on dendritic cells (DCs) and the increase in cytotoxic genes in CD8+

T-cells suggest that the transcriptome of these patients remains active, despite the absence of clinical signs of liver disease or viremia. How this cytotoxic-prone immune-activation profile fits with the observed regression of fibrosis under NUC-therapy[32] is unclear. It is tempting to speculate that the detected transcriptome phenotype might be the result of a stronger HBV transcriptional activity which is unaffected by NUC-therapy.

HBsAg suppression in chronic HBV might not result in immune-reconstitution/functional cure

It appears evident that new treatment strategies will require tailoring to different chronic HBV patients to achieve a functional cure state. With that end, several novel therapeutics are in development and can be broadly categorized as immune-boosters aiming at immune reconstitution or viral suppressors which target different steps in the HBV life cycle such as blockade of viral translation via small interfering RNAs.

The importance of Hepatitis B surface antigen (HBsAg) in HBV infection has long been established and is of paramount importance as it participates in viral entry via the NTCP receptor expressed in hepatocytes[36]. Not surprisingly, vaccines against HBV are developed to elicit a potent and functional anti-HBsAg antibody response neutralizing infective viral particles[37,38]. These responses are not only observed in healthy vaccinated individuals but also in acute self-limited HBV infected patients whom not only display functional B-cell responses directed against HBsAg but also multi-specific T-cell responses proving that the immune system is capable to mount effective and long-lived anti-HBV responses[34,39]. However, a hallmark in chronic HBV patients –infected at child birth – is the scarcity and exhaustion of HBV-specific immune responses[6,40]. Several mechanisms have been shown to be active in chronic HBV that can alter anti-HBV immune responses: expansion of suppressive monocyte-derived suppressor cells (MDSC) [41], increased levels of IL-10 and arginase limiting T-cells function[42,43], depletion of HBV-specific T-cells via NK-mediated killing[44] and reduced NK-mediated IFN-g production[45,46] among others. However, a common mechanism in chronic viral settings such as LCMV infections and cancer is antigen burden where elevated antigen load induce T-cell immune-suppression[47,48]. In chronic HBV, HBsAg is postulated as the main culprit given the kinetics in HBsAg production – several orders of magnitude higher than infective viral particles[49] - over a large time-span with limited reduction over the course of the infection.

Interestingly, clinical studies on natural viremia-control patients have shown quantitative serum HBsAg (qHBsAg) to be a predictive biomarker for HBsAg seroclearance[50,51]. Similarly, greater seroclearance rates in NUC-treated cohorts have also been observed in patients with low baseline qHBsAg[52,53].

Immunologically, the putative immune-suppressive role of HBsAg on the immune response has been explored in mouse models. Xiaowen et al. described a mechanism in which impairment of T follicular helper (Tfh) cells via regulatory

T cells was responsible for non-functional anti-HBsAg antibody responses[54]. Interestingly, blockade of the inhibitory marker CTLA-4 could increase the frequency of Tfh cells in an hydrodynamically-injected HBV mouse model resulting in more potent anti-HBs antibody responses and an increase of peripheral Tfh cells frequencies in chronic HBV patients[54]. Moreover, Michler et al. showed how artificial removal of HBsAg via small interfering RNA (siRNA) was a pre-requisite for developing poly-functional T-cell responses upon therapeutic vaccination in the same HBV mouse model[55]. Based on this concept, new therapeutics targeting steps of the HBV life cycle including disruption of HBsAg production are being tested[56]. To date, limited data is available on immune-suppressive mechanisms exerted by HBsAg that may take place in chronic HBV in humans, and that may be responsible for maintenance of the chronic state, even in non-viremic patients, either upon NUC treatment or during the IC phase.

Therefore, in chapter 4 and 5 we explored in detail the effects that distinct HBsAg concentrations may carry in chronic HBV infection in humans at the peripheral and intrahepatic level. In chapter 4, we comprehensively analyzed the frequency and transcriptome – via microarray- of FACS-sorted leukocyte populations (monocytes, DC, NK-cells, CD4 and CD8 T-cells subsets and B-cells) in peripheral blood of non-viremic chronic HBV patients (antivirally/NUC-induced and natural; Inactive Carrier) with large differences in their serum HBsAg levels. We demonstrated that frequencies of the different leukocyte populations in both cohorts of non-viremic chronic HBV patients were maintained irrespective of serum HBsAg levels as shown by others[57]. We showed that transcriptome modulation, albeit very limited, was only observed in monocytes and DCs, populations shown to be stimulated by HBsAg in-vitro [58,59]. Thus, gene expression profiles of purified NK cells, CD4 T cells and CD8 T-cells isolated from blood of NUC-treated individuals were not different from IC patients. It is important to acknowledge that this observation was at the transcriptomic level, and that we did not collect functional data in our study. In addition, a step-wise classification of patients based on HBsAg thresholds and inclusion of an extra control group such as chronic HBV patients who experienced HBsAg seroclearance (S-loss) could further support our findings. We expect that HBsAg-induced changes are more pronounced in liver than blood, but no studies have addressed this before. Therefore, in chapter 5 we profiled the intrahepatic transcriptome of natural viremia-controlled chronic HBV patients with distinct serum and liver HBsAg levels (10 and 2-fold, respectively). To our surprise, comparable to the peripheral transcriptome, intrahepatic gene expression differences were minor, irrespective of whether the assessment was against serum HBsAg levels or the degree of HBsAg positivity in the liver. Moreover, albeit a previous assessment (Chapter 2) of the intrahepatic immune infiltrate showed a lack of association with intrahepatic viral antigens expression (HBsAg and HBcAg), this dataset suggests that stronger immune responses might be occurring in patients with lower HBsAg levels as seen by the transcriptomic upregulation of T- and B-cell activated genes suggesting a stronger immune-control state. Whether this transcriptomic activation relates to HBV-responses or not requires further

research. Nonetheless, the nature of these findings does not necessarily reflect a causative effect exerted by HBsAg load. Chronic HBV patients have shown an expansion of functionally and phenotypically exhausted HBsAg-specific atypical memory B-cells (AtM) in liver and liver[60], albeit antibody responses could be partially restored in peripheral AtM B-cells by PD-1 blockade[34,60]. It is important to mention that dysfunctional HBV-specific B-cell responses in blood are limited to HBsAg, but not HBcAg suggesting that immune-dysfunction is mainly restricted to HBsAg-specific responses. However, even in the absence of detectable HBsAg in sera of seroconverted (S-loss) patients anti-HBsAg antibodies may not be detectable for months[61]. Hence, what mechanism lies behind inducing an effective antibody response to a specific viral antigen (HBcAg) but not another (HBsAg) warrants further research. Similarly, detectable HBV-specific T-cell responses against HBsAg are less frequent than HBcAg and have recently been shown to decline over the course of the infection, contrary to T-cell responses directed against other HBV viral antigens[62]. Furthermore, whereas no changes have been reported on the frequency of HBsAg-specific T-cells irrespective of serum HBsAg levels, HBcAg-specific T-cells comprise a large fraction of the HBV-specific T-cell pool in chronic HBV patients with low serum HBsAg and those who seroconverted (S-loss)[57,63]. In these patients, the HBV-specific T- and B-cell responses seem to be different to the poly-functional responses seen in acute HBV patients which may suggest that functional cure in chronic HBV may not replicate immune responses seen in self-acute HBV. Altogether, current data does not support the idea that HBsAg is a major immune-modulator which may explain the limited sustained seroclearance at the end of the treatment period albeit all siRNAs phase II clinical trials have shown transient reductions in serum HBsAg[64,65]. Nevertheless, immune control associated with HBsAg seroconversion remains understudied and not well understood. With the introduction of new therapeutics (viral targeting and immune-boosters) currently in clinical trial stages it is imperative to maximize our understanding of the immune dynamics in HBV, especially considering the limited number of inclusions and low rates of S-loss. Hence, prospective study designs employing new state of the art research assays, as earlier described, ought to be considered. Lastly, more strict inclusion criteria are needed to limit the impact of confounding factors on the reported responses there is a clear need of conducting collaborative studies to increase sample sized while maintaining a good representation of the HBV patient population not specific to concrete geographical areas.

Take home messages:

Before new therapeutics can be effectively brought into the clinic, several important questions remain unanswered in chronic HBV disease. For instance, we lack vital information on intrahepatic immune responses and how these reflect the different clinical states of chronic HBV. Furthermore, what constitutes immune control and whether viral antigen loads impair immune responses are yet to be

explored. Thus, this thesis has focused on shedding light on these key questions.

We presented for the first time a comprehensive characterization of the intrahepatic immune state across the natural phases of HBV as well as uninfected healthy controls were we observed an increased leukocyte influx across chronic HBV, particularly in active disease states with ongoing liver inflammation (hepatitis) whereas non-hepatitis phases showed a less pronounced influx. Similarly, active disease states highlighted increased expression of exhausted transcriptomic signatures, albeit not identical in HBeAg-positive and HBeAg-negative disease, that included newly identified genes from HBV-specific T-cells. Interestingly, these phenotypes were unrelated to HBV DNA or viral antigens (HBcAg and HBsAg) levels. In contrast, ALT level (the degree of inflammation) was the most convincing determinant as it positively associated with the degree of influx and the immune-exhaustion genes. Moreover, contrary to the general assumption that HBsAg modulates the immune response, our studies revealed that HBsAg did not exert changes at the peripheral level on the transcriptome of the major blood leukocytes' or in the liver of carefully selected cohort of infected patients with limited to undetectable viremia and liver damage. Nevertheless, we identified T-cell and B-cell activation signatures to associate with limited HBsAg expression in the liver which may explain help understand the increased functional cure rates in patients with low HBsAg levels. Lastly, we observed a more active transcriptomic profile in peripheral immune cells of antivirally-treated patients vs spontaneous viremia controllers which was reminiscent of that of livers from active disease patients. This finding may help explain viral rebound rates upon antiviral discontinuation.

Altogether, these studies provide novel clues relevant for new forms of therapy focused on attaining functional cure and lie a foundation on the heterogeneous clinical disease states characteristic of chronic HBV. These findings and new ones born out of advances in research assays in combination with novel liver sampling techniques independent of the limited liver biopsies biobank material will further pave the way to deepen our knowledge on the distinct aspects of chronic HBV from disease progression to immune control.

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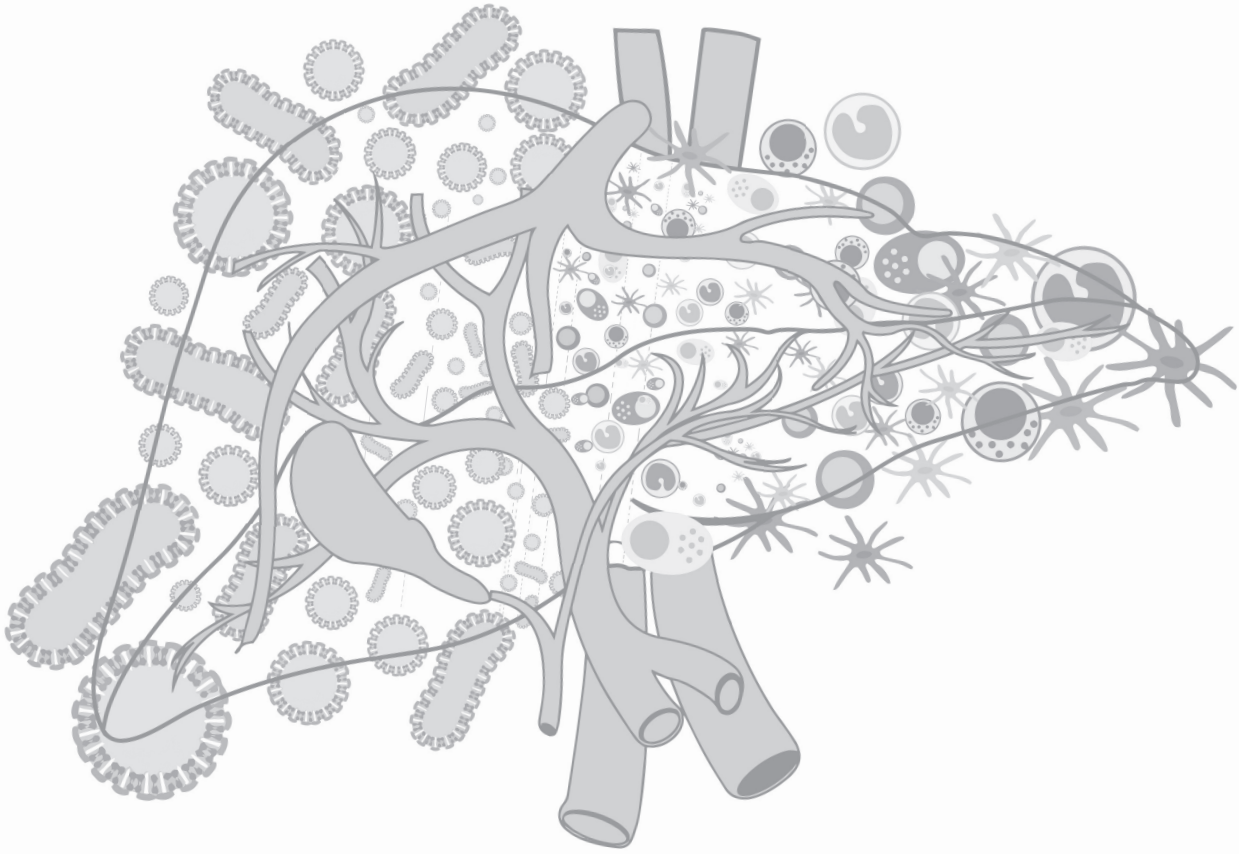

CHAPTER 7

Summary

Summary

Implementation of vaccination programs against the hepatitis B virus (HBV) has proven effective in decreasing HBV prevalence. However, a large number of individuals (270 million worldwide) remain chronically infected due to infection at childbirth. Disease progression occurs over a long period of time and is characterized by distinct clinical phases. Ultimately, chronic infection can lead to liver cancer, where HBV infection is the most common risk factor. Moreover, antiviral therapy (with NUCs) is effective in controlling disease progression. However, these are life-long therapeutics and associate with elevated socio-economic burden. Given that the immune response has been shown to be able to resolve acute HBV infections in virtually all infected adults, the current goal in chronic HBV management has shifted towards reaching a state in which the immune response is capable to suppress the virus, defined as functional cure.

It is of paramount importance to first understand how the distinct clinical phases translate immunologically, particularly in the liver, as distinct approaches may be required to induce functional cure. However, knowledge in this area is limited as access to liver material is difficult. In this thesis, we are the first to comprehensively characterize HBV infected livers across the different stages in chronic HBV infection and compare them to altruistic healthy donors from which tissue availability is even scarcer. This thesis shows large differences in the intrahepatic compartment between clinical phases and highlights greater immune-activation/exhaustion during hepatitis phases. More importantly, we describe previously unseen distinct gene expression signatures associated with ALT increase on basis of HBeAg status. To our surprise, HBV activity (serum viral load and antigen expression in the liver) has limited impact on HBV activity of the intrahepatic immune response. Moreover, partial immune control, characterized by long-term viral suppression (antiviral-induced or spontaneous) can be achieved in a small subset of patients. However, upon antiviral therapy discontinuation viral rebound occurs in a large fraction of chronic HBV patients in most studies. This thesis identifies changes at the peripheral level in the frequency and transcriptome of peripheral leukocytes in NUC-treated patients which are reminiscent of phases with active inflammation suggesting that initial normalization upon antiviral therapy may be temporal. These observations may have important consequences for the implementation of future treatment strategies as they might need to be personalized based on the patients' characteristics.

Nonetheless, rates of functional cure are marginal in patients who control their viremia. Viral antigen load, HBsAg, is hypothesized to be the predominant causative factor for immune-exhaustion in chronic HBV. This thesis explores whether viral antigen levels modulate the degree of immune-exhaustion at the peripheral and intrahepatic level and shows no direct evidence of immune-transcriptomic modulation exerted by serum or liver HBsAg levels arguing that artificial removal of HBsAg per se may not result in increased functional rates. Thus, boosting the impaired immune response in combination with HBV viral cycle disruption may be necessary.

Chapter 1 serves as an introduction that describes basic features of the HBV viral cycle that serve to explain the intrinsic complexities of viral eradication and cure. Furthermore, it summarizes the current knowledge of the immune response during chronic infection and brushes upon the current and future directions in treatment strategies to improve clinical outcomes (i.e., functional cure).

Chapter 2 is a comprehensive characterization of the intrahepatic compartment during the natural history of chronic HBV infection using new state of the art assays (Journal of Hepatology, 2021, submitted) where we combine RNA-sequencing with multiplex immunofluorescence in matched liver biopsies. We highlight that immune-gene expression and leukocyte infiltrate is greater in chronic HBV infected livers than in healthy livers. We also show induced immune-exhaustion gene expression profiles in livers of chronic hepatitis patients and distinct gene signatures to associate with ALT levels in IA and ENEG patients where a subset of genes encode for immune-exhaustion markers. Lastly, we indicate that the level of leukocyte infiltrate seen in chronic HBV livers associates with serum ALT, but not with serum HBV DNA levels or intrahepatic viral protein expression.

Chapter 3 focuses on the study in chronic HBV of MAIT cells, a leukocyte population known to possess antiviral properties and largely present in liver under homeostatic conditions. Contrary to other chronic viral infections such as HCV and HIV that had shown a depletion of this non-conventional T cell population, we show that MAIT cells are not depleted in blood or liver of chronic HBV patients (Journal Infectious Diseases, 2017). In addition, we also describe a normalization in their activation status, similar to healthy controls, upon antiviral therapy.

Chapter 4 evaluates the effect of serum HBsAg and distinct modes of long-term viremia control (antiviral and natural) on the transcriptome of sorted peripheral immune cells (Journal Infectious Diseases, 2019). Serum HBsAg levels exerts little changes in frequency and transcriptome of the immune cells studied, whereas gene expression in antiviral-treated chronic HBV patients shows marked differences in the gene expression of blood dendritic cells, monocytes and CD8+ T cells which may partially explain viral re-bound upon antiviral discontinuation.

Chapter 5 focuses on the intrahepatic compartment of natural viremia-controlled chronic HBV patients to identify immune-signatures associated with partial immune control and to what extent HBsAg modulates the intrahepatic compartment (Journal Infectious Diseases, 2020). We are the first to show a de-regulated liver transcriptome in chronic HBV patients despite normal liver function and identify gene signatures related to leukocyte activation and recruitment to correlate with HBsAg levels.

Samenvatting (NL)

De implementatie van vaccinatieprogramma's tegen het hepatitis B-virus (HBV) is effectief gebleken bij het verminderen van de HBV-prevalentie. Een groot aantal individuen (270 miljoen wereldwijd) blijft echter chronisch geïnfecteerd door infectie bij de bevalling. Ziekteprogressie vindt plaats over een lange periode en wordt gekenmerkt door verschillende klinische fasen. Uiteindelijk kan chronische infectie leiden tot leverkanker, waarbij HBV-infectie de meest voorkomende risicofactor is. Bovendien is antivirale therapie (met NUC's) effectief bij het beheersen van ziekteprogressie. Dit zijn echter levenslange therapieën en worden geassocieerd met een verhoogde sociaaleconomische last. Aangezien is aangetoond dat de immuunrespons acute HBV-infecties bij vrijwel alle geïnfecteerde volwassenen kan oplossen, is het huidige doel bij de behandeling van chronische HBV verschoven naar het bereiken van een toestand waarin de immuunrespons in staat is het virus te onderdrukken, gedefinieerd als functioneel genezing.

Het is van het grootste belang om eerst te begrijpen hoe de verschillende klinische fasen zich immunologisch vertalen, met name in de lever, aangezien verschillende benaderingen nodig kunnen zijn om functionele genezing te induceren. De kennis op dit gebied is echter beperkt omdat toegang tot levermateriaal moeilijk is. In dit proefschrift zijn we de eersten die HBV-geïnfecteerde levers uitgebreid karakteriseren in de verschillende stadia van chronische HBV-infectie en deze vergelijken met altruïstische gezonde donoren waarvan de beschikbaarheid van weefsel nog schaarser is. Dit proefschrift laat grote verschillen zien in het intrahepatische compartiment tussen klinische fasen en benadrukt een grotere immuunactivatie/uitputting tijdens hepatitis-fasen. Wat nog belangrijker is, we beschrijven voorheen ongeziene verschillende genexpressiesignaturen geassocieerd met ALT-toename op basis van HBeAg-status. Tot onze verbazing heeft HBV-activiteit (serum viral load en antigeenexpressie in de lever) een beperkte invloed op de HBV-activiteit van de intrahepatische immuunrespons. Bovendien kan bij een kleine subgroep van patiënten een gedeeltelijke immuuncontrole worden bereikt, die wordt gekenmerkt door langdurige virale onderdrukking (antiviraal geïnduceerd of spontaan). Bij het staken van de antivirale therapie treedt in de meeste onderzoeken echter een virale rebound op bij een groot deel van de chronische HBV-patiënten. Dit proefschrift identificeert veranderingen op perifeer niveau in de frequentie en het transcriptoom van perifere leukocyten bij met NUC behandelde patiënten die doen denken aan fasen met actieve ontsteking, wat suggereert dat initiële normalisatie na antivirale therapie tijdelijk kan zijn. Deze observaties kunnen belangrijke gevolgen hebben voor de implementatie van toekomstige behandelstrategieën, aangezien deze mogelijk gepersonaliseerd moeten worden op basis van de kenmerken van de patiënt.

Desalniettemin zijn de percentages van functionele genezing marginaal bij patiënten die hun viremie onder controle hebben. Virale antigeenbelasting, HBsAg, wordt verondersteld de belangrijkste oorzakelijke factor te zijn voor

immuunuitputting bij chronische HBV. Dit proefschrift onderzoekt of virale antigeenniveaus de mate van immuunuitputting moduleren op het perifere en intrahepatische niveau en toont geen direct bewijs van immuun-transcriptomische modulatie uitgeoefend door serum- of lever-HBsAg-niveaus, met het argument dat kunstmatige verwijdering van HBsAg op zich niet leidt tot verhoogde functionele tarieven. Het kan dus nodig zijn om de verstoorde immuunrespons te versterken in combinatie met een verstoring van de HBV-virale cyclus.

Hoofdstuk 1 dient als een inleiding die de basiskenmerken van de HBV-virale cyclus beschrijft die dienen om de intrinsieke complexiteit van virale uitroeiing en genezing te verklaren. Bovendien vat het de huidige kennis van de immuunrespons tijdens chronische infectie samen en geeft het inzicht in de huidige en toekomstige richtingen in behandelingsstrategieën om klinische resultaten te verbeteren (d.w.z. functionele genezing).

Hoofdstuk 2 is een uitgebreide karakterisering van het intrahepatische compartiment tijdens het natuurlijke beloop van chronische HBV-infectie met behulp van nieuwe state-of-the-art assays (Journal of Hepatology, 2021, ingediend) waarbij we RNA-sequencing combineren met multiplex immunofluorescentie in gematchte leverbiopten. We benadrukken dat immuungenexpressie en leukocytinfiltraat groter zijn in chronische HBV-geïnfecteerde levers dan in gezonde levers. We tonen ook geïnduceerde genexpressieprofielen voor immuunuitputting in levers van chronische hepatitispatiënten en verschillende gensignaturen om te associëren met ALT-niveaus bij IA- en ENEG-patiënten waar een subset van genen codeert voor markers voor immuunuitputting. Ten slotte geven we aan dat het niveau van leukocytinfiltraat dat wordt gezien in chronische HBV-levers associeert met serum-ALT, maar niet met serum-HBV-DNA-niveaus of intrahepatische virale eiwitexpressie.

Hoofdstuk 3 richt zich op de studie in chronische HBV van MAIT-cellen, een leukocytenpopulatie waarvan bekend is dat ze antivirale eigenschappen bezit en grotendeels aanwezig is in de lever onder homeostatische omstandigheden. In tegenstelling tot andere chronische virale infecties zoals HCV en HIV die een uitputting van deze niet-conventionele T-celpopulatie hadden laten zien, laten we zien dat MAIT-cellen niet zijn uitgeput in het bloed of de lever van chronische HBV-patiënten (Journal Infectious Diseases, 2017). Daarnaast beschrijven we ook een normalisatie in hun activeringsstatus, vergelijkbaar met gezonde controles, na antivirale therapie.

Hoofdstuk 4 evalueert het effect van serum HBsAg en verschillende manieren van viremiecontrole op lange termijn (antiviraal en natuurlijk) op het transcriptoom van gesorteerde perifere immuuncellen (Journal Infectious Diseases, 2019). Serum HBsAg-spiegels oefenen weinig veranderingen uit in frequentie en transcriptoom van de bestudeerde immuuncellen, terwijl genexpressie bij met antivirale middelen behandelde chronische HBV-patiënten duidelijke verschillen vertoont in de genexpressie van bloeddendritische cellen, monocyten en CD8+ T-cellen, wat de virale re- gebonden na stopzetting van de antivirale behandeling.

Hoofdstuk 5 richt zich op het intrahepatische compartiment van natuurlijke

viremie-gecontroleerde chronische HBV-patiënten om immuunsignaturen te identificeren die geassocieerd zijn met gedeeltelijke immuuncontrole en in hoeverre HBsAg het intrahepatische compartiment moduleert (Journal Infectious Diseases, 2020). We zijn de eersten die een gedereguleerd levertranscriptoom laten zien bij chronische HBV-patiënten ondanks een normale leverfunctie en identificeren gensignaturen gerelateerd aan leukocytactivering en rekrutering om te correleren met HBsAg-niveaus.

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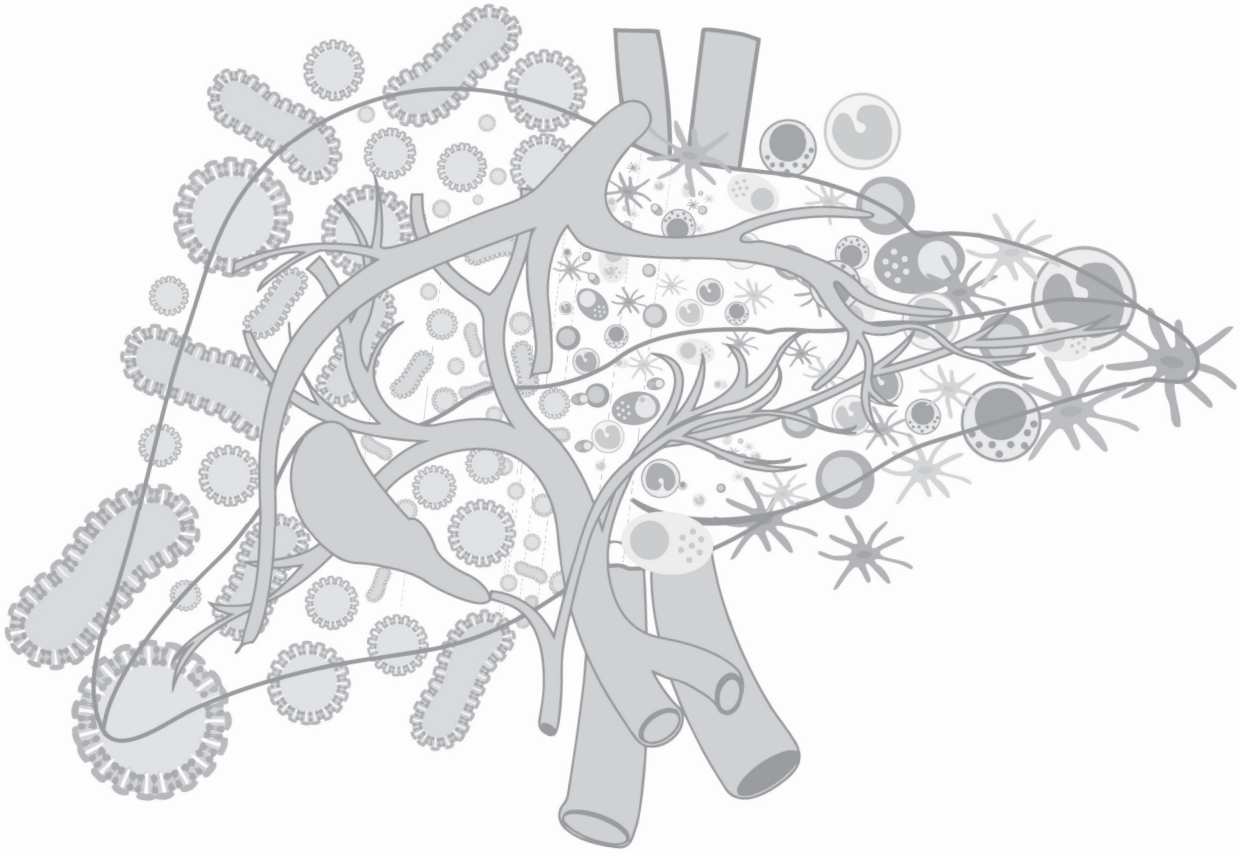

CHAPTER 8

APPENDICES

PhD portfolio

Bibliography

Curriculum Vitae

Acknowledgements (EN, SPA, ITA)

PHD PORTFOLIO

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COURSES

2021 EMC Research Integrity

2021 Molecular Medicine Workshop on Photoshop and Illustrator CC

2021 Molecular Medicine Workshop on career support

2020 UMN course on advance flow cytometry

2020 UMN workshop on Improve presentation skills

2019 Datacamp course on data analysis

2019 UMN course on REDCap

2017 Molmed Medicine Workshop on Introduction to R

2017 Molmed Medicine course on Gene Expression data analysis using R: How to make sense out of you RNA-Seq/microarray data

OTHER CONFERENCES

2022 The international Liver Congress (ILC)

2022 Dutch Liver Retreat (*Oral Presentation*)

2021 The international Liver Congress (ILC)

2021 International HBV meeting

2020 The international Liver Congress (ILC)

2018 Erasmus Liver Day

2018 Nederlandse Vereniging voor Immunologie (NVVI)

2018 European Conference for Immunology(ECI) (Amsterdam)

2017 Nederlandse Vereniging voor Immunologie (NVVI)

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CURRICULUM VITAE

Noé Axel Rico Montanari received a degree in Microbiology from the Universitat Autonoma de Barcelona (UAB) in 2013. During his bachelor internship he studied part of the SOS system in bacteria and its role in bacteria motility and virulence. In 2015, he enrolled in the Infection and Immunity master program at the Erasmus MC, The Netherlands, where he did an 18 months internship in Andre Boonstra's lab. During this period he studied the immunological effects of long-term antiviral therapy in chronic HBV patients. Since 2017, as a PhD-candidate under the supervision of Andre Boonstra and Jose Debes, he has performed translational studies focusing on studying the effect of HBV activity in the immune response in blood and liver using omics approaches.

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