HUMAN IMMUNE RESPONSES DURING HEPATITIS B VIRAL LOAD FLUCTUATIONS

Lauke L. Boeijen

Human Immune Responses during Hepatitis B Viral Load Fluctuations

Immuunreacties van de mens bij fluctuerende virale hepatitis B titers

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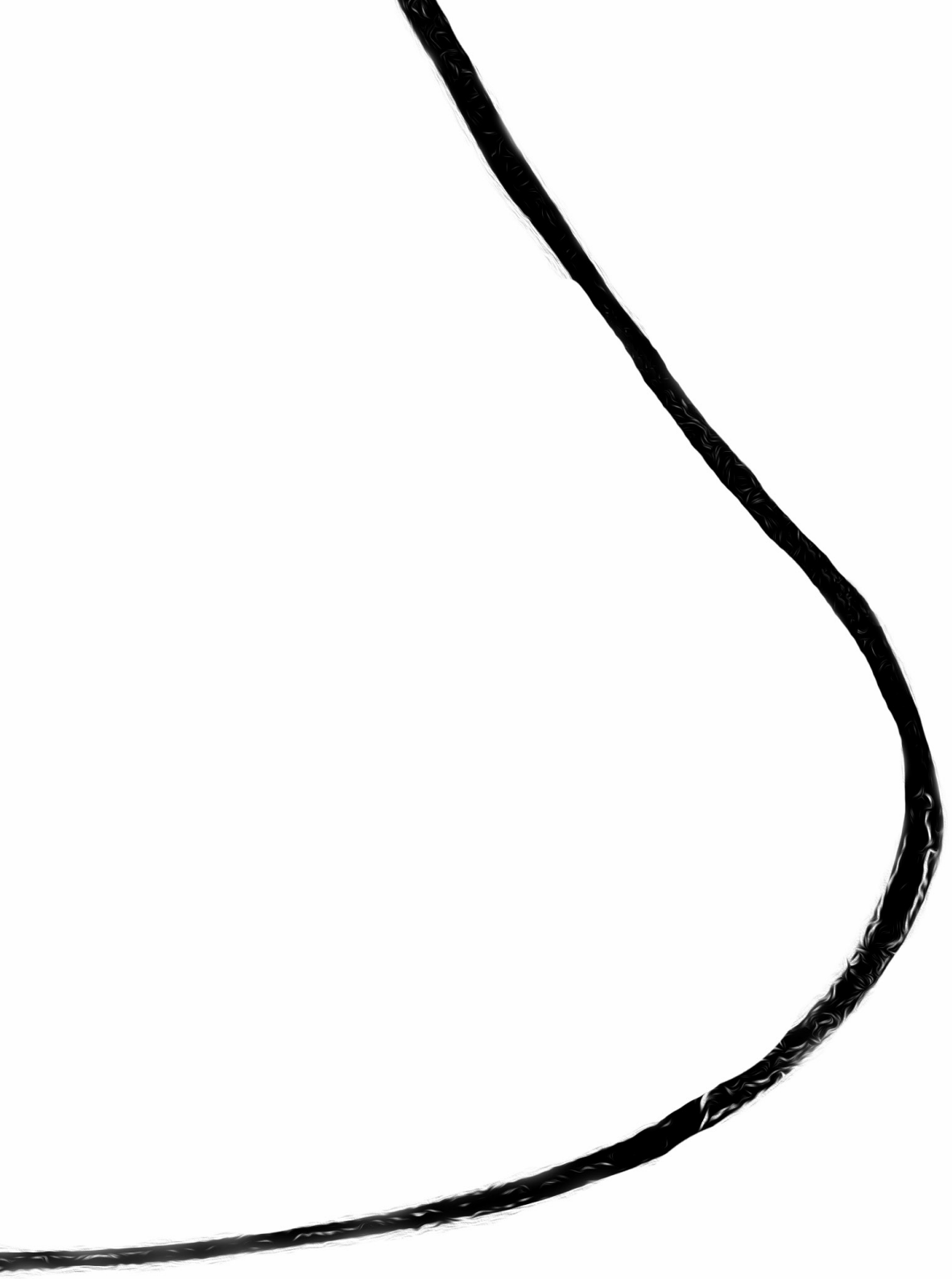

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General Introduction

Hepatitis B Virus (HBV) is a virus with a specific tropism for the human liver**.** The virus is transmitted through contact with virally contaminated blood, by sexual contact, and perinatally. Through these routes, an estimated 2 billion individuals have been exposed to HBV at some point in their life, as evidenced by antibodies to viral antigens in serum[1]. The great majority of exposed adults clear the virus within weeks: only approximately 5% of acutely infected people develop a chronic infection[2]. In contrast, approximately 90% of exposed infants do not clear the virus and become chronically infected. Worldwide, an estimated 250 million people are infected with chronic HBV, which can be diagnosed by detection of HBsAg (a protein in the viral envelope) in the serum. A vaccine based on the recombinant HBsAg protein is available and prevents chronic infection in almost all vaccinated individuals. Despite this effective vaccine, mortality related to chronic HBV remains high and may even be increasing. Every year, approximately 800,000 people die from the consequences of chronic HBV, end stage liver disease, and hepatocellular carcinoma (HCC)[1]. This exceeds the yearly death toll from diseases like malaria and HIV and although the disease may be more prevalent in developing countries, HBV mortality is certainly not limited to specific regions of the world[3].

THE LIFE CYCLE OF HBV

Hepatitis B virus (HBV) is a partially double stranded relaxed circular DNA virus. It is 3.2 kilobase in length, which makes it one of the shortest and simplest viruses that can infect human beings. The HBV genome has 4 open reading frames encoding for a total of 7 viral proteins: Hepatitis B e antigen (HBeAg, a secreted dimeric protein), HBV core antigen (HBcAg the protein that forms the viral capsid), HBV polymerase (Pol, reverse transcriptase), large, medium, and small surface envelope glycoproteins (PreS1/PreS2/HBsAg), and HBVx antigen (HBx, a regulator of transcription required for the initiation of infection). Infection with HBV (and other members of the hepadnaviridae family) is characterized by incorporation of the template for replication into the host genome as well as establishment of circular covalently closed DNA (cccDNA) in the hepatocyte[4]. From this viral mini-chromosome, and from the integrated viral sequence in host DNA, the viral protein HBsAg can be continuously produced by hepatocytes[5].

CHRONIC HBV, A HIGHLY VARIABLE DISEASE

HBV is a non-cytolytic virus: liver damage is caused by the host immune response to the infection. Symptoms of acute infection can include nausea, abdominal pain and/or jaundice, which may occur 30 to 180 days after transmission[1]. However, acute infection often passes without any symptoms, and the first signs of chronic infection may only become evident after decades of viral

replication. The liver damage is related to the accumulation of collagen in the parenchyma, via a process called liver fibrosis. Over time, this can develop into liver cirrhosis, end stage liver disease and HCC in severe cases. Patient specific factors like male gender, older age, as well as behavioral factors like alcohol use and smoking are associated with a worse prognosis[6], and account for some of the variation observed in the presentation of chronic HBV. The natural history of chronic HBV infection (**Figure 1**) follows four clinical phases, defined by HBeAg positivity or negativity in the serum, differential levels of viral replication (HBV DNA titers), liver inflammation and liver fibrosis progression (**Table 1**).

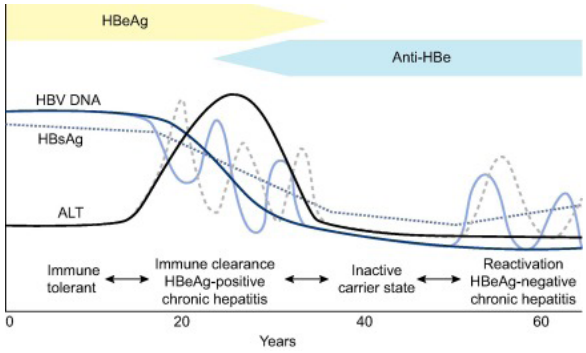


Figure 1. clinical phases of chronic HBV (adapted from Lok et al[7])

Table 1. characteristics of clinical phases of HBV, according to EASL clinical guidelines 2017[8], as well as the dynamics of the clinical markers during high resistance barrier nucleos(t)ide analogs like tenofovir or entecavir

	Natural history of HBV				Treatment	
	HBeAg positive		HBeAg negative			
	Chronic infection	**Chronic hepatitis**	**Chronic infection**	**Chronic hepatitis**	**Functional cure**	**NUC -treated HBV**
HBsAg	High	High/ intermediate	low	intermediate	undetectable	unchanged
HBeAg	Positive	Positive	Negative	Negative	undetectable	barely affected
HBV DNA	>10^7 IU/mL	10^4 – 10^7 IU/mL	<2000 IU/mL	>2000 IU/mL	undetectable	undetectable
ALT	Normal	Elevated	Normal	Elevated	Normal	Normal
Alternative term	Immune tolerant (IT)	Immune active (IA)	Inactive carrier (IC)	HBeAg negative (ENEG)		

TREATMENT FOR HBV

The goal of treatment for HBV is primarily to improve the prognosis of the chronic HBV patient. Complete eradication of HBV from the host is not possible with the current available antivirals. The loss of HBsAg from the serum (and the detection of anti-HBs antibodies) is associated with a great improvement of the long-term outcome. HBsAg loss is also called a functional cure, as reactivation of HBV is still possible during periods of immune suppression, due to residual cccDNA in the liver. Nonetheless, HBsAg loss remains the most favorable treatment outcome for patients with chronic HBV. Pegylated-interferon injections were the first choice for treatment for chronic HBV for many years, and resulted in a loss of HBsAg in 7-17% percent of the treated individuals [9, 10]. However, as pegylated-interferon injections are not well tolerated by patients, nucleotide or nucleoside analogs (NUCs) are currently more frequently used. NUCs are structurally similar to nucleotides and are incorporated into newly created DNA strands by viral DNA polymerase, and act as chain terminators. NUCs can effectively suppress replication of HBV and thereby reduce the incidence of deadly HBV associated complications, like end stage liver disease and HCC. However, NUCs only result in HBsAg loss in a small portion of patients (0-3%) and have to be administered continuously, often lifelong[11-13]. It is therefore of great importance that, in combination with the intensification of vaccination worldwide, a durable cure is developed, to lower the considerable yearly HBV related death toll.

INNATE IMMUNE RESPONSES: NK CELLS AND MAIT CELLS

The host immune response is considered key for clearance of HBV infection from the liver. Many immune populations have been characterized over the years in order to better understand the pathogenesis of the disease. Natural Killer (NK) cells can recognize and lyse virally infected cells, and may play an important role during immune responses to HBV. NK cells were first described in 1975 by Kiessling, Klein and Wigzell[14] following earlier observations on non-B and non-T cell lymphocytes with clear cytotoxic effects[15]. The balance of NK cell stimulatory and inhibitory signals impacts their cytolytic activity and their production of inflammatory cytokines like IFN-γ. The most frequently used marker for the identification of NK cells is CD56 or neural cell adhesion molecule (NCAM), which plays a functional role in cell to cell adhesion. NK cells with low expression of CD56, $CD56^{dim}$ NK cells, have a potent cytotoxic activity, while the $CD56^{bright}$ NK cell are considered to be a subset characterized by the production of inflammatory cytokines like IFN-γ and TNF. Natural cytotoxicity receptors like NKp46 or NKp30 can bind proteins expressed in extracellular matrix, of which some are associated with tumor cells or viral infection[16]. Ligation of CD16 with the Fc portion of antibodies bound to a target cell can also contribute to its lysis by the NK cell. $CD56^{dim}$ NK cells in particular are characterized by

high CD16 expression. The inhibitory Killer-cell immunoglobulin-like receptors (KIRs) regulate cytolysis by NK cells by interaction with Human Leukocyte Antigen (HLA) class I molecules. Cells that do not express cognate HLA molecules or cells with downregulated HLA expression, which can be caused by viral infections like HBV[17] can decrease inhibitory NK cell signaling via this route, leading to their activation and possibly lysis of the infected cell. In addition to cell to cell contact, soluble cytokines like IL-2, IL-12, IL18, and interferon-α can also activate NK cells.

Mucosal-Associated Invariant T cells (MAIT) cells, are CD3+ T cells with an innate effector like function that express an invariant T cell receptor and can be activated by similar cytokines as NK cells. The invariant TCR recognizes small vitamin B metabolites (produced by gram negative bacteria like *E. coli*) presented by MR1 (a non-classical MHC molecule). The TCR of MAIT cells is composed of an invariant TCR α chain and a limited set of β chains. Interestingly, these cells are enriched in the bowel and liver of human subjects[18]. Regardless of their specificity for bacterial antigen[19], MAIT cells have also been investigated in the context of antiviral immune responses, and their frequency, and possibly their capacity to produce inflammatory cytokines, is decreased during chronic viral infection[20, 21]. However, not much is known of their frequency and function during chronic HBV.

IMMUNE RESPONSES DURING CHRONIC HBV IN HUMANS AND ANIMAL MODELS

Clinical events and the host immune response during HBV infection are intricately linked. By combining the information from clinical observations and experimental models, our understanding of key immunological events that define the pathogenesis of HBV has improved steadily over the years. Results from in vitro models[22, 23] and in vivo models[24, 25] suggest that the host immune system is the cause of liver damage during HBV infection and not the viral infection itself. Furthermore, immunological data suggests that both the innate[26] and the acquired branches[27] of the immune system can have profound effects on the outcome of infection. Several studies, on animal and human immune cells, have had considerable impact on the way we think about immune responses to HBV. The potential of innate immune cells to clear HBV infection has been demonstrated in mouse models. NKT and NK cell migration to the infected liver and their activation upon treatment with α-galactoceramide was associated with clearance of the virus[26]. In human subjects with acute HBV infection, the innate immune cells were not affected despite an early spike of HBV DNA and liver inflammation. However, after viral clearance the NK cells were more frequent and displayed an activated phenotype. HBV is considered a ‘stealth virus’, as it was shown that HBV was not detected by the host immune system in the early stage of infections[28, 29]. Apart from early innate responses to HBV, it is

clear that HBV virus specific T cells strongly correlate with the clinical outcome of HBV. Mouse experiments have shown that class I-restricted CD8+ T cells, also called cytotoxic T lymphocytes, can remove HBV from the liver by IFN-gamma production without lysis of the hepatocytes[30]. In the chimpanzee model for HBV infection, the CD8+ T cells, but not CD4+ T cells were essential for the clearance of HBV[27]. In line with the animal model data, in human patients the specific CD8+ T cells were activated during effective antiviral immune responses[31]. Importantly, in the blood of human patients, clearance of HBV during acute infection is associated with activation of both virus specific CD4+ T cell and CD8+ T cell mediated response. While some studies show that NK cells can contribute to the control of viral replication by supporting T cell responses [32], it has also been shown that NK cells can suppress the adaptive arm of the immune system by deletion of HBV-specific CD8+ T cells[33].

THE LIVER ENVIRONMENT

Although considerable progress has been made on the pathogenesis of HBV, many of these observations have not been confirmed in human patients or in cells derived from infected liver, but only in blood. This is not surprising, as blood is much easier and safer to acquire from chronically infected patients parallel to routine blood tests at the outpatient clinic. From a single heparinized blood tube, isolated peripheral blood mononuclear cells or PBMC, 10-15 million live PBMC can be readily isolated and used for flow cytometric analysis, functional assays or for fluorescence assisted cell sorting (FACS) and subsequent RNA isolation. In humans and in animal models, the phenotype and function of liver derived immune cells may be very different from blood[34-37]. Egress from the peripheral blood by immune cells require phenotypical changes that facilitate this migration, for example expression of the chemokine receptors on the surface of T cells, rendering the tissue resident cell phenotypically different by definition. Moreover, the environment of the liver is very different from the peripheral blood, with its slow blood flow, low blood pressure, different concentrations of metabolites, massive exposure to foreign antigen via the portal vein, and a high frequency of immune cells that produce predominantly immune regulatory cytokines like regulatory T cells. These inhibitory signals are likely essential to prevent excessive immune activation in the organ, in balance with activation when confronted with pathogens, viral or otherwise. This view of the liver as an organ with strict regulation of its immunological signals is in sharp contrast to previous beliefs. Before, the liver was considered to be a non-immunological organ, with main functions predominantly related to metabolism, storage of glucose and detoxification[38]. Another potential factor underlying the fundamental difference between tissue resident and peripheral leukocytes is the concentration of viral products, like HBsAg. According to some reports, differentially mutated HBV can even replicate within the same individual, specific to the blood or liver compartment[39]. For all of these

reasons, it can be expected that cells of the same lymphocyte subpopulation isolated from liver and from blood will differ greatly, with regard to phenotype, function, and gene expression profile, and that host-virus interaction should ideally be studied at both sites.

AIM OF THIS THESIS

The aim this thesis is to increase the knowledge of human NK cells and MAIT cell mediated immune responses in the blood and liver, during the various phases of chronic HBV infection, as well as during antiviral treatment. In addition, it is our goal to expand the range and applications of biomedical techniques to analyze liver derived immune cells, to acquire maximal information from the small amount of tissue available for research.

OUTLINE OF THIS THESIS

The work presented in this thesis addresses three central questions:

1. How does the progression of the various clinical phases that characterize the natural history of HBV, associated with huge shifts of viral load and liver inflammation, affect the phenotype and transcriptome of innate immune cells like NK cells and MAIT cells?
2. How does suppression of the viral load by nucleotide or nucleoside analog treatment affect the number and phenotype of innate immune cells in liver and blood?
3. Can we improve the information yield from liver biopsy material to maximize the output of studies investigating liver resident immune cells?

First, in **chapter 2**, we review some of the factors that underlie the currently incomplete information on the pathogenesis of HBV infection, and establish the current knowledge of immune cells that may impact the course of the disease. In **chapter 3**, we describe the subtle interactions between viremia and host antiviral immune response, by focusing on the peripheral blood NK cell transcriptome during all four clinical phases of HBV, characterized by fluctuating viral loads and ALT levels. We offset these results to healthy control derived NK cells, as well as NK cells from chronic HCV and chronic HIV infected individuals. In **chapter 4,** we show that it is feasible to perform cell sorting combined with gene expression profiling on fresh core 18 Gauge core liver biopsies from HBV infected chronic patients on ongoing NUC treatment. We isolated NK cells and bulk T cells from the blood and liver from the same patient, and were able to repeat this procedure on another liver biopsy after experimental TLR7 agonist (GS-9620) treatment. In **chapter 5,** we characterize MAIT cells using PBMC from HBV infected patients in the various phases that characterize the natural history of HBV, including measurements done

on NUC treated patient material from blood and liver. In **chapter 6** we summarize the current knowledge on the immunological effects of nucleotide analog treatment for chronic HBV. A general discussion on all the findings, and an evaluation of the used techniques in this thesis are described in **chapter 7**. In addition, we will propose the methods of choice for future work, in order to fill the gaps that still exist in our knowledge of HBV in the coming years in this chapter.

REFERENCES

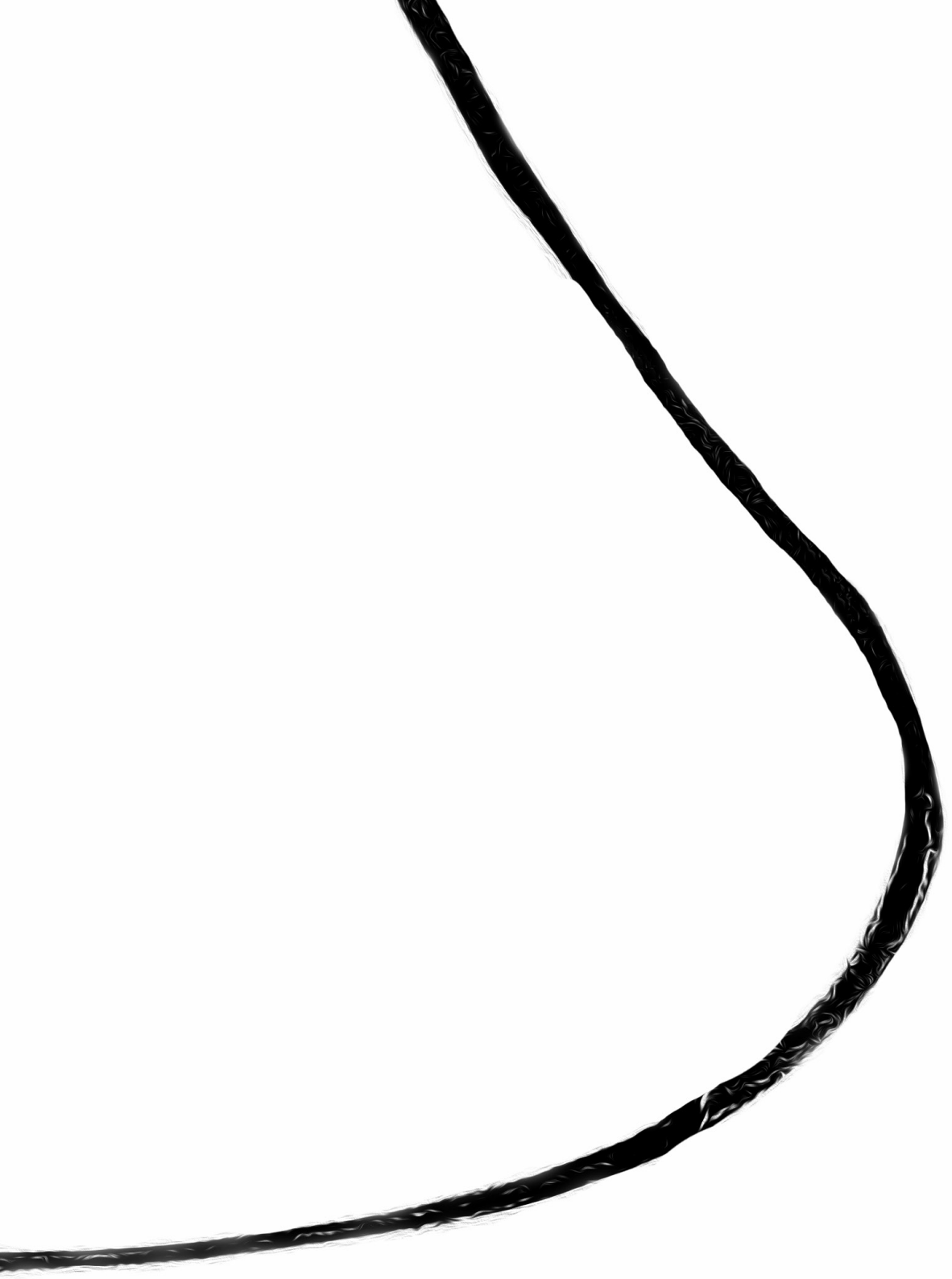
1. World Health Organization. Global Hepatitis Report, 2017 (WHO, 2017).
2. Hyams, K.C., Risks of chronicity following acute hepatitis B virus infection: a review. Clin Infect Dis, 1995. 20(4): p. 992-1000.
3. Hofman, R., et al., [Mortality due to chronic viral hepatitis B and C infections in the Netherlands]. Ned Tijdschr Geneeskd, 2016. 160: p. D511.
4. Tu, T., et al., HBV DNA Integration: Molecular Mechanisms and Clinical Implications. Viruses, 2017. 9(4).
5. Allweiss, L. and M. Dandri, The Role of cccDNA in HBV Maintenance. Viruses, 2017. 9(6).
6. Petrick, J.L., et al., Tobacco, alcohol use and risk of hepatocellular carcinoma and intrahepatic cholangiocarcinoma: The Liver Cancer Pooling Project. Br J Cancer, 2018. 118(7): p. 1005-1012.
7. Lok, A.S., et al., Hepatitis B cure: From discovery to regulatory approval. J Hepatol, 2017. 67(4): p. 847-861.
8. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol, 2017. 67(2): p. 370-398.
9. van Zonneveld, M., et al., Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. Hepatology, 2004. 39(3): p. 804-10.
10. Buster, E.H., et al., Factors that predict response of patients with hepatitis B e antigen-positive chronic hepatitis B to peginterferon-alfa. Gastroenterology, 2009. 137(6): p. 2002-9.
11. Zoutendijk, R., et al., Serum HBsAg decline during long-term potent nucleos(t)ide analogue therapy for chronic hepatitis B and prediction of HBsAg loss. J Infect Dis, 2011. 204(3): p. 415-8.
12. Dienstag, J.L., et al., Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med, 1999. 341(17): p. 1256-63.
13. Lai, C.L., et al., Telbivudine versus lamivudine in patients with chronic hepatitis B. N Engl J Med, 2007. 357(25): p. 2576-88.
14. Kiessling, R., E. Klein, and H. Wigzell, “Natural” killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur J Immunol, 1975. 5(2): p. 112-7.
15. Greenberg, A.H., et al., Antibody-dependent cell-mediated cytotoxicity due to a “null” lymphoid cell. Nat New Biol, 1973. 242(117): p. 111-3.
16. Pazina, T., et al., Regulation of the Functions of Natural Cytotoxicity Receptors by Interactions with Diverse Ligands and Alterations in Splice Variant Expression. Front Immunol, 2017. 8: p. 369.
17. Michalak, T.I., P.D. Hodgson, and N.D. Churchill, Posttranscriptional inhibition of class I major histocompatibility complex presentation on hepatocytes and lymphoid cells in chronic woodchuck hepatitis virus infection. J Virol, 2000. 74(10): p. 4483-94.
18. Kurioka, A., et al., MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets. Mucosal Immunol, 2015. 8(2): p. 429-40.
19. Treiner, E., et al., Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1.

Nature, 2003. 422(6928): p. 164-9.
20. van Wilgenburg, B., et al., MAIT cells are activated during human viral infections. Nat Commun, 2016. 7: p. 11653.
21. Spaan, M., et al., Frequencies of Circulating MAIT Cells Are Diminished in Chronic HCV, HIV and HCV/HIV Co-Infection and Do Not Recover during Therapy. PLoS One, 2016. 11(7): p. e0159243.
22. Schulze-Bergkamen, H., et al., Primary human hepatocytes--a valuable tool for investigation of apoptosis and hepatitis B virus infection. J Hepatol, 2003. 38(6): p. 736-44.
23. Quasdorff, M., et al., A concerted action of HNF4alpha and HNF1alpha links hepatitis B virus replication to hepatocyte differentiation. Cell Microbiol, 2008. 10(7): p. 1478-90.
24. Allweiss, L. and M. Dandri, Experimental in vitro and in vivo models for the study of human hepatitis B virus infection. J Hepatol, 2016. 64(1 Suppl): p. S17-s31.
25. Yang, D., et al., A mouse model for HBV immunotolerance and immunotherapy. Cell Mol Immunol, 2014. 11(1): p. 71-8.
26. Kakimi, K., et al., Natural killer T cell activation inhibits hepatitis B virus replication in vivo. J Exp Med, 2000. 192(7): p. 921-30.
27. Thimme, R., et al., CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J Virol, 2003. 77(1): p. 68-76.
28. Wieland, S., et al., Genomic analysis of the host response to hepatitis B virus infection. Proc Natl Acad Sci U S A, 2004. 101(17): p. 6669-74.
29. Niu, C., et al., The Smc5/6 Complex Restricts HBV when Localized to ND10 without Inducing an Innate Immune Response and Is Counteracted by the HBV X Protein Shortly after Infection. PLoS One, 2017. 12(1): p. e0169648.
30. Chisari, F.V., Hepatitis B virus transgenic mice: models of viral immunobiology and pathogenesis. Curr Top Microbiol Immunol, 1996. 206: p. 149-73.
31. Maini, M.K., et al., Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. Gastroenterology, 1999. 117(6): p. 1386-96.
32. Andrews, D.M., et al., Innate immunity defines the capacity of antiviral T cells to limit persistent infection. J Exp Med, 2010. 207(6): p. 1333-43.
33. Peppa, D., et al., Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion. J Exp Med, 2013. 210(1): p. 99-114.
34. Spaan, M., et al., Longitudinal analysis of peripheral and intrahepatic NK cells in chronic HCV patients during antiviral therapy. Antiviral Res, 2015. 123: p. 86-92.
35. Claassen, M.A., et al., Abundant numbers of regulatory T cells localize to the liver of chronic hepatitis C infected patients and limit the extent of fibrosis. J Hepatol, 2010. 52(3): p. 315-21.
36. Tjwa, E.T., et al., Similar frequencies, phenotype and activation status of intrahepatic NK cells in chronic HBV patients after long-term treatment with tenofovir disoproxil fumarate (TDF). Antiviral Res, 2016. 132: p. 70-5.
37. Norris, S., et al., Resident human hepatic lymphocytes are phenotypically different from circulating

lymphocytes. J Hepatol, 1998. 28(1): p. 84-90.

38. Robinson, M.W., C. Harmon, and C. O'Farrelly, Liver immunology and its role in inflammation and homeostasis. Cell Mol Immunol, 2016. 13(3): p. 267-76.

39. Datta, S., et al., Genetic characterization of hepatitis B virus in peripheral blood leukocytes: evidence for selection and compartmentalization of viral variants with the immune escape G145R mutation. J Virol, 2009. 83(19): p. 9983-92.



2

Hepatitis B virus infection and the immune response: The big questions

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The natural history of HBV infection is exceptionally complex, and, not surprisingly, so are the host immune responses. For almost every clinical or virological change during the course of HBV infection, there are proven or suspected correlates in host immunity, some of which might represent the underlying cause of the observed alterations in disease, while others will be consequences of the change, for example in viral load. Recent advances in experimental and analytical capabilities now allow researchers to address seemingly basic but still unsolved questions surrounding HBV immunity. We have formulated several questions regarding host–virus interactions whose answers we deem most relevant for a better understanding of HBV immunopathogenesis and for the development of novel therapeutic strategies (**Figure 1**). After addressing these issues, we will highlight some of the progress that has already been made in the field of chronic HBV.

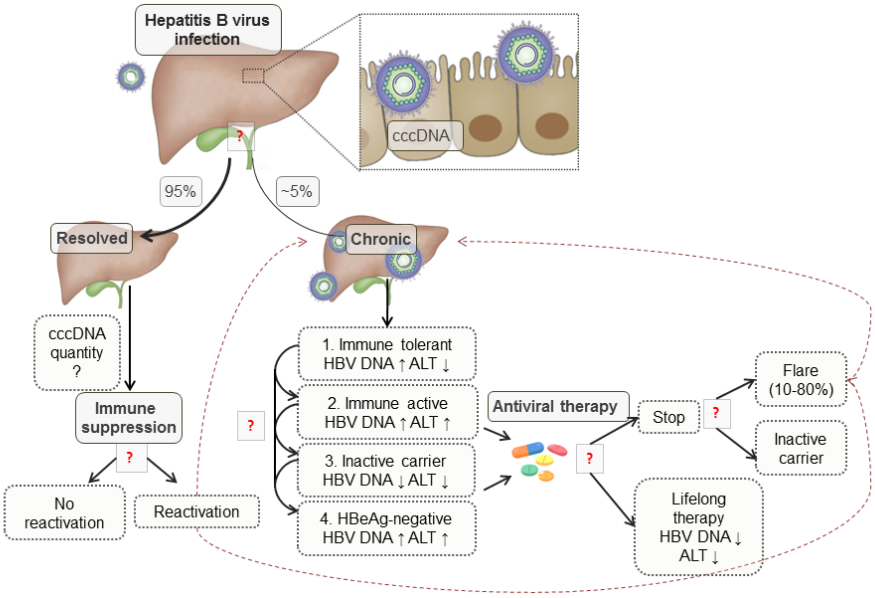

Figure 1. Clinical events during the natural history and treatment that remain unanswered. Currently, it is insufficiently clear which immune factors cause persistence, determine the degree of inflammation and viral replication during chronic infection, and which functionally cured patients reactivate upon immune suppressive therapy, or why. Patients with liver inflammation are usually treated with nucleoside/nucleotide analogs, but due to unknown immunological events, some relapse and have renewed liver inflammation, while others do not.

First is the question of what determines the different outcome in acute HBV infection, as the virus is spontaneously controlled in some subjects, while becoming chronic in others. Here we have to consider two completely distinct scenarios: adult HBV infection typically leads to HBV control, whereas natal infection results in chronicity in almost all cases, with declining but still very significant chronicity rates if infection occurs before the age of 5. However, not much is known of the immunological processes, resulting in these striking differences. We know much more about acute and chronic infection "after the fact", i.e. in adult patients who have established chronic infection or successful HBV control. Studies during the early phase of infection, when the outcome is not yet determined, are much more difficult to undertake. The best opportunity for further insights will be studies in young children who present with early infection. Immunological studies in this population are feasible, but until recently had to be very limited in scope, in part because only small amounts of blood can be drawn for analysis. New technologies now enable analysis of low volume samples with high resolution and without the requirement of research facilities close to the clinic.

Immune responses of chronically infected adults have been performed for decades, but, as we will describe in this review, often have conflicting results. These variations are partly caused by methodological differences, but the variation among patient characteristics also has significant impact [1]. In addition to establishing the range and variation of immune cell parameters during frequent within the clinical phases of chronic infection, we also need to know if, and to what degree, antiviral therapy is able to reconstitute those parts of the HBV immune response previously insufficient for functional control. At least based on the different antibody patterns observed in treated patients, it seems obvious that restoration of immunity is not uniform, and certainly in most subjects not sufficient for viral control in the absence of therapy. But what exactly is restored, whether immune restoration is just a consequence of diminished viral replication during antiviral treatment, or whether restored immunity is independent or even the cause of viral suppression, needs to be evaluated in much more detail. In this context it is also important to define whether circulating viral proteins, such as HBs antigen (HBsAg), are indeed the key immunosuppressive agents that in vitro studies have suggested [2].

Another important question is what level of HBV control can actually be achieved through the host immune response. It is clear that even after HBsAg clearance from the blood, covalently closed circular DNA (cccDNA) can remain in hepatocytes indeterminately, evident from the well-documented cases of HBV reactivation in anti-HBsAg positive subjects undergoing immune-ablative therapies. Whether this is the dominant or even the only scenario in "resolved" HBV infection, or whether some patients are indeed able to completely clear HBV DNA based on an even more effective immune response, will require larger studies analyzing liver tissue for cccDNA in these populations.

Finally, greater insights need to be generated into what actually happens in the liver as the site of infection, since both the composition of immune populations as well as their functional and phenotypic profiles are different from what is observed in the blood. New technologies now allow analysis of rare immune populations in the liver, down to cells on the single cell level. Similarly, we should utilize the improved tools for the integrative analysis of cellular processes and immune functions in order to understand the immune response to HBV more holistically. Many components of the immune response, both innate and adaptive, have to act in concert in different scenarios of viral control and viral persistence. Below we will broadly summarize the current knowledge of both the innate and adaptive arms of the immune responses to HBV infection. While the studies described below do not yet provide definitive answers, they are the foundation for future investigations into the issues raised above.

Innate immune responses to HBV

HBV is transmitted upon contact with blood or body fluids of an infected person. A minute amount of HBV virions in the bloodstream is sufficient for infection of hepatocytes [3]. The HBV virion enters the hepatocyte via the sodium taurocholate co-transporting polypeptide (NTCP), a bile receptor located on the basolateral membrane, contributing to its specificity to human or chimpanzee hepatocytes [4]. After the envelope protein mediates fusion of the viral and endosomal membranes, the capsid enters the cytoplasm and the viral DNA is released into the nucleus through nuclear pores. Upon import into the nucleus, HBV can integrate into the host genome or be present as non-integrated cccDNA. The cccDNA molecule will serve as a template for replication leading to infection of more hepatocytes, and can persist even after HBsAg loss [5]. As circulating blood passes the liver, HBV can easily spread to other hepatocytes.

The detection of HBV by infected hepatocytes

The sensing of HBV and triggering of intracellular antiviral mechanisms can occur on the cell membrane by Toll-like receptors (TLR), in the endosome by TLR7 or TLR9, and in the cytoplasm by sensors, such as intracellular retinoic acid inducible gene I (RIG-I) and melanoma differentiation gene 5 (MDA5) upon ligation with viral proteins or nucleic acids. Although some controversy exists, it has been reported that TLR2, MDA-5 and RIG-I are involved in sensing of HBV. For the cytoplasmic sensor RIG-I, it was demonstrated that HBV pre-genomic RNA triggered its activation, resulting in the release of interferon (IFN)-λ but not type-I IFN by HBV infected primary human hepatocytes and hepatoma cell lines [6]. Release of IFN by hepatocytes and possibly other cells induces expression of hundreds of IFN-stimulated genes (ISGs) with potent antiviral activity. However, the HBV-induced IFN responses are weak [7, 8], which is reflected by the usual lack of clinical symptoms during the acute HBV infection. Also, early data from animal models showed that HBV does not induce the release of type-I IFN [9, 10]. The absence of symptoms and the modest IFN induction by HBV led to adaptation of the term ‘stealth virus’. However, IFN

responses and ISG induction are present, albeit marginal compared to other chronic viruses [7, 8]. The use of cccDNA as a transcriptional template in the nucleus likely contributes to HBV's capacity to limit detection in hepatocytes. Adding to this, viral proteins, like HBV polymerase and HBx protein, directly inhibit the cellular machinery that detects replication intermediates. It is currently unknown which pattern recognition receptor or signaling pathway is essential for early viral control in vivo, and perhaps more relevant to the majority of patients, to HBV persistence in humans.

As the human liver is the site of HBV replication and contains high viral protein concentrations, the most appropriate approach for addressing basic questions concerning HBV detection is to evaluate liver material. Unfortunately, such studies are rare. Lebosse et al. [11] analyzed RNA extracted from liver biopsies of chronic HBV patients and showed low intrahepatic IFNα expression relative to healthy controls, which was unaffected by viral replication. Previously, we analyzed liver biopsies from patients in specific clinical phases of chronic HBV. By comparing the transcriptome of liver and blood samples from patients in distinct clinical phases of HBV we found that, ISG are transcribed even in patients presumed to be 'immune tolerant' to HBV [12]. Transcription of catalytic polypeptide-like 3B, a protein related to cccDNA degradation [13], was increased in the immune tolerant phase, which suggests that the capacity to limit the establishment of high amounts of cccDNA may be different between phases, but not absent in any phase. These two studies using human liver tissue raise significant doubt about the labels 'stealth virus' and 'immune tolerant', as HBV is indeed sensed by the host immune system and antiviral responses are initiated, even as they are not sufficient to halt viral replication and spread in persons with chronic infection. Whether these differences of innate intrahepatic responses are caused by chronic HBV replication, or reflect immune characteristics that select patients for persistent infection, remains to be clarified using longitudinally acquired samples of human liver.

The interaction of antigen presenting cells and HBV

Intrahepatic leukocyte populations lining the sinusoidal lumen of the portal branches as well as hepatocytes are constantly in contact with huge amounts of bacterial antigen derived from the gut. Tolerance during exposure to high antigen loads is essential for host survival, and the relative tolerogenic milieu of the liver is well described [14]. Illustrative of the complex balance of the liver immune system is the fact that, despite its tolerant nature, HBV is cleared after transmission in the vast majority of infected individuals. For the development of treatment strategies, it is important to evaluate the leukocyte populations involved in this process. Liver residing antigen presenting cells, like Kupffer cells and dendritic cells (DC), could potentially modulate host immune responses to a phenotype enabling chronic viral infection, but limited information exists on the interaction between these cells and HBV (**Figure 2**). DC are crucial for their ability to efficiently present antigen to naïve CD4 T cells, or to CD8 T cells via cross-priming.

At present, the impact of persistent HBV infection on the DC compartment is not fully clear. In vitro studies have demonstrated that the presence of viral antigen may limit DC functionality [15]. Both plasmacytoid and myeloid DC can present antigens to T cells, and can, depending on the cytokine milieu, skew T cell effector function towards tolerance or activation. Plasmacytoid DC are specialized in the production of high levels of type I and type III IFN, thereby potentially initiating innate immune responses to HBV after recognition of viral nucleic acids via TLR7 or TLR9. However, based on ex vivo assays, DC function during chronic HBV patients may be hampered. The frequency of both myeloid DC and plasmacytoid DC is not changed in the majority of studies with patient material, but others have reported various functional differences compared to healthy controls [16, 17, 18, 19]. Possibly via downregulation of TLR7 or TLR9, plasmacytoid DC seem to be less capable to produce IFNα, especially when DC are derived from patients with considerable liver inflammation [17, 20].

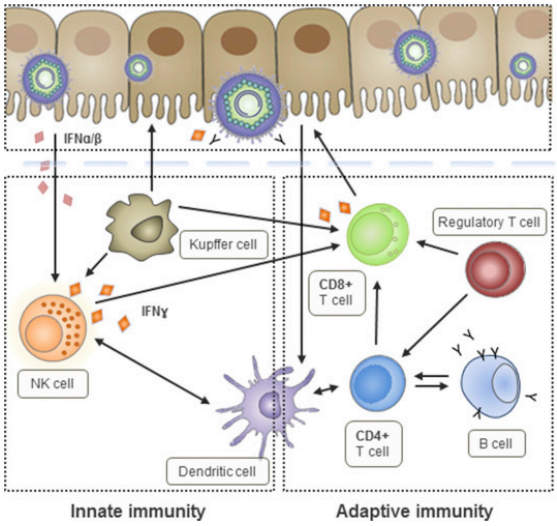

Figure 2. The persistence of HBV infections is determined by the complex interactions of multiple leukocytes.

Kupffer cells are liver resident macrophages, and make up 15–20% of the intrahepatic leukocytes [21]. We have previously shown that Kupffer cells can interact with HBsAg in vivo, which led to increased pro-inflammatory cytokine production compared to healthy controls [22]. However, HBV proteins like HBeAg may interfere with Kupffer cell activation by downregulation of TLR expression as shown in vitro [23]. In line with this finding, patient-derived peripheral monocytes were also found to have lower TLR2 expression compared to healthy controls [24]. Kupffer cells can play different roles in the presence of HBV antigens, as was illustrated in a rat model, where HBV causes Kupffer cells to produce more of the pro-fibrogenic and tolerogenic cytokine

TGF-β1, as opposed to the pro-inflammatory cytokines IL-6, IL-1 and TNF [25]. To prevent continuous activation and excessive intrahepatic inflammation, Kupffer cells become refractory to subsequent endotoxin challenge, which may contribute to the tolerogenic liver environment. Furthermore, Kupffer cells can interact and influence other immune cells, either directly via cell–cell contact or indirectly via the activity of cytokines. Indirect activation of natural killer (NK) cells can take place via Kupffer cell-derived IL-12 and IL-18 [22, 26].

NK cell function and phenotype in chronic HBV

NK cells can recognize and lyse infected cells, and may therefore be an important effector cell that plays a role during persistence of HBV. Indeed, the frequency and function of NK cells in peripheral blood of chronic patients has been studied extensively, and it seems clear that the balance of NK cell stimulatory and inhibitory signals significantly impacts their activity, which in turn correlates with patient outcomes during the natural history of chronic HBV. One of the major effector functions of NK cells, the production of the antiviral and anti-fibrotic cytokine IFNγ, is reported to be unaffected during chronic HBV by some studies [27, 28] but reduced in others [25, 29, 30]. IFNγ release by NK cells mediates the non-cytolytic clearance of HBV by virus-specific CD8 T cells [31, 32]. Another beneficial effect may be that IFNγ induces the production of the antiviral APOBEC proteins A3A and A3B directly affecting the integrity of cccDNA [33]. In a transgenic mouse model, the potential of IFNγ was dramatically demonstrated by injection of the NKT cell activating agent alpha-galactosylceramide, which stopped HBV from replicating, and led to infiltration of NK cells into the liver [34]. In humans, however, alpha-galactosylceramide affected HBV DNA levels in only a subset of human chronic HBV patients, and the treatment was not well tolerated [35]. During chronic HBV infection, the cytotoxic capacity of NK cells seems to remain intact [36], which together with the impaired cytokine production as described by some studies, suggests the existence of a functional dichotomy of NK cells in chronic HBV. However, given the conflicting reports in the literature, it is obvious that more detailed and more controlled studies need to be performed. A recent study performed by our group compared NK cell phenotype and function in blood among HBV patients in different clinical phases. Interestingly, despite big differences in viral load and ALT levels between patients, the NK cell compartment demonstrated only subtle differences between the patients' cohorts [37]. Concerning their activation, a combination of IL-12, IL-15 and IL-18 secreted by other intrahepatic immune cell populations, including activated Kupffer cells, seems to be the most likely mechanism. Direct contact with virally infected cells or HBV DNA in the blood may be a less effective stimulus for NK cells, based on work by Bonoroni et al., who describe no correlation between viral load and peripheral NK activation [38], which is in line with our previous study [37]. Also supporting this finding is the observation that tenofovir-induced viral load reduction did not significantly alter intrahepatic NK cell activation as demonstrated using the analysis of fine needle aspirate liver biopsies, even after six years of viral suppression [39]. The capacity of intrahepatic NK cells to inhibit fibrogenesis

through IFNγ production [40], or by killing stellate cells that are driving collagen syntheses in the liver [41], may also be relevant for clinical outcomes. However, in contrast to the above described beneficial effects of NK cells, their activation may alternatively facilitate HBV persistence, as was recently illustrated by the finding that HBV-specific CD4 T cells expressing death ligands (not seen in healthy controls) can be targets for NK cell-mediated killing [42].

Adaptive immune responses during chronic HBV

Adaptive immune responses by virus-specific CD4 and CD8 T cells, B cells and antibodies are indispensable for HBV control [43]. These responses develop relatively late in HBV infection compared to other viral infections: at ten to twelve weeks post exposure [44]. This late emergence of adaptive immunity is thought to be a consequence of the stealthy nature of early HBV infection, with low viral replication and minimal to no activation of innate immunity. This delayed response is not per se a factor in HBV chronicity, as it is also observed in the vast majority of adult patients who go on to control the virus spontaneously. While we have learned many important details about how adaptive immune responses emerge and evolve from the early phase of infection throughout viral resolution or chronic viremia [43], much remains to be determined in order to fully appreciate the exact mechanisms driving successful and failing adaptive immune responses targeting HBV. This is especially true as our appreciation of the complexity and diversity of different players of the adaptive immune response, mostly defined in animal models, has grown faster than our ability to translate such integrative concepts into human infection [45].

Detection of HBV-specific CD4 T cells

Virus-specific CD4 T lymphocytes are key regulators of both efficient B cell/antibody and CD8 T cell responses and thus are thought to be essential to the control of most viral infections [46]. Unfortunately, virus-specific CD4 T cells are also notoriously difficult to study, as they are rather low in frequency and complex in their phenotypes and function. Overall, our understanding of the HBV-specific CD4 response remains incomplete. The seminal chimpanzee studies defining the importance of CD4 T cells by analyzing the impact of CD4 T cell depletion on the course of infection are not straightforward in their findings, as depletion of CD4 T cells before infection leads to chronic HBV infection, while depletion just before the rise of viremia and liver enzymes did not alter the natural course of infection [46, 47]. These findings support a critical role of CD4 T cells in the control of HBV infection, but also raise questions about the exact role of T cell help and its mode of action. Generally, HBV-specific CD4 T cells appear in the blood seven to ten weeks after infection, in parallel with the emergence of HBV-specific CD8 T cells and antibodies [44, 48]. The CD4 T cells mostly target epitopes in HBV core, though minor responses against surface, polymerase and x protein have also been described [49, 50, 51, 52]. In patients with acute or controlled HBV infection, CD4 responses are more broadly directed and more vigorous,

compared to patients with established chronic viremia. Functionally, HBV-specific CD4 T cells have been shown to predominantly secrete Th1 type cytokines [53], though overall studies assessing the phenotype and function of the cells directly ex vivo are very few and rather limited. In this context it is important to remember that most of these studies were performed in the era when standard proliferation and ELISpot assays where the only means to assessing virus-specific CD4 T cell responses, and as we have learned from other infections such as with HCV, these functional assays might miss significant parts of the response [54]. In addition, HBV-specific CD4 T cells at the site of infection might be quite different than those analyzed in the blood. Indeed, after in vitro expansion from liver biopsies obtained in patients with chronic infection, intrahepatic CD4 T cells have shown a distinct functional profile compared to those derived from blood, with the secretion of IL-4 and IL-5 in addition to Th1 type cytokines [55]. A reassessment of HBV-specific CD4 T cell responses using current methodologies, i.e. HLA class II tetramers, for direct ex vivo phenotyping and flow based cell sorting, followed by omics analyses, seems imperative in order to obtain a more detailed understanding of this central component of the adaptive immune response. These methods enable analysis of single cells, and thus might allow ex vivo studies even in infection at young age, for which we currently have no significant data about the CD4 T cell response.

Detection of HBV-specific CD8 T cells

In contrast to CD4 T cells, CD8 T cell responses have been studied much more widely and in greater detail, including direct ex vivo analyses [56]. They are essential for HBV control, as their depletion invariantly led to chronicity in chimpanzees [46]. HBV-specific CD8 T cells become readily detectable six to eight weeks after infection in adults, and their appearance coincides with a decrease in viral load that is observed even before the onset of liver injury [44], supporting the contribution of non-cytolytic elimination of intracellular virus to viral control. CD8 lymphocytes targeting all HBV proteins have been identified [43] and shown to have cytolytic as well as non-cytolytic effector functions [57]. Non-cytolytic effector functions could be especially relevant, as hepatocytes have been shown to be quite resilient to cell-mediated killing in vitro [58]. While the exact immunological determinants of CD8 mediated HBV control are difficult to define with certainty in humans, the development of a sustained, broad and polyclonal CD8 T cell response that is highly functional is seen as conditio sine qua non for HBV control. To what degree such a CD8 response is dependent on other arms of the immune response, be it CD4 T cell help or a preceding innate response, is not fully clear.

Importantly, there is also a good amount of literature on intrahepatic CD8 T cells targeting HBV, mostly from chronic infection. Detection of HBV-specific CD8 T cells by tetramer staining of cultured intrahepatic lymphocytes revealed that the frequencies of virus-specific CD8 T cells were inversely correlated with the degree of liver injury [59], supporting the hypothesis that

specific responses might be not only important for disease resolution, but also for protection from progressive liver disease. Another pioneering study using fine needle aspirate biopsies to analyze intrahepatic lymphocytes during acute infection provided longitudinal characterization of HBV-specific CD8 T cells directly ex vivo without in vivo expansion. HBV-specific CD8 T cells were highly enriched in the liver during the acute phase of infection and remained detectable after HBsAg seroconversion and full clinical recovery [60]. A similar approach was also employed in chronically infected patients; revealing that virus-specific CD8 T cells were most readily detected during the inactive carrier phase [61]. Altogether, current evidence illustrates differences in phenotype and function between peripheral and intrahepatic lymphocytes, and during different stages of HBV infection. Since one can now analyze cells from liver biopsies or fine needle aspirates with much more powerful analytic tools, this opportunity should be used to deepen our understanding of the CD8 T cell response in acute and chronic infection, and during therapy.

Chronic HBV and functional T cell impairment

Once patients are chronically infected, especially in those patients exposed in early childhood, there is usually an extended phase that has been described as immune tolerant, with high levels of viremia but no or very little liver disease. It has been postulated that during this phase T cell responses are mostly absent and/or non-functional. Recent data indicate that this is not the case, and instead T cells are readily detectable in these patients and seem, if anything, more and not less functional than those in later, so called immune active, stages of disease [12]. This “immune tolerant” phase of HBV might hold more surprises and certainly warrants further investigation. During later stages of chronic infection, the repertoire of detectable HBV-specific T cells is limited and their frequencies are rather low, at least in the blood, and especially so in patients with high viral loads [62]. Preserved HBV-specific CD8 T cells become gradually more functionally impaired, or exhausted, which is thought to be driven by persistent antigen exposure. CD8 T cell exhaustion in chronic HBV infection mirrors that described in other chronic viral infections in mice and humans, with the sustained expression of inhibitory receptors, such as PD-1, TIM-3 and 2B4, reduced proliferative capacity and poor effector functions such as reduced IFNγ and IL-2 secretion [63, 64, 65]. The exhausted state is also associated with distinct expression patterns of transcription factors, compared to functional effector or memory T cells, most notably low expression of T-bet [66] The cells seem also increasingly susceptible to apoptosis through upregulation of Bim and TRAIL-R2 [67, 68], two key regulators of cell death. Whether the functional exhaustion of HBV-specific CD8 T cells is the sole or dominant contributor to CD8 T cell failure, or whether the virus can also escape through the generation of viral escape sequence variants, like in HIV and HCV infection, is currently an open question. HBV as a DNA virus is much more genetically stable compared to HIV and HCV, and early studies supported the idea that HBV displayed little variability that could be linked to immune pressure [69]. However, a recent study revealed viral variation compatible with escape mutations in both the core and envelope

sequence and to a lesser extent in the polymerase sequence of HBV [70, 71]. Future studies in this area should further define the mechanisms of T cell exhaustion and whether exhausted T cells in chronic HBV infection could potentially be reinvigorated through immunotherapeutic interventions. In addition, we need to define the contribution of viral variants to the failure of HBV adaptive immunity.

Functional T-cell restoration under treatment

If T cell exhaustion is principally maintained by persistent antigen exposure, long-term therapy with successful control of viremia, should, to some degree, be capable of restoring T cell function. This would be especially relevant for immunotherapeutic approaches that most likely will be applied in the context of antiviral therapy. This has been studied in some detail, though the complexity of antiviral treatments, but also of the course of chronic HBV infection itself, makes interpretation of the data challenging [72, 73, 74, 75]. Boni et al. compared nucleos(t)ide treated chronic HBV infected patients to those with untreated or resolved infection. PBMC were analyzed either directly ex vivo with class I dextramers or for their proliferative capacity and cytokine production after in vitro expansion. Ex vivo analysis of virus-specific T cells suggested continued impairment even after long-term treatment, though after in vitro expansion some functional properties were partially restored, indicating some improvement of the T cell populations [73]. A similar study design has also been performed in IFNα treated chronic HBV patients and failed to detect improved T cell function, at least in terms of cytokine production [74]. Whether the modest T cell recovery is due to the remaining high levels of HBsAg in serum, despite the control of viral replication, is an important and controversial question. While it is widely assumed that surface antigen has a directly negative effect on T cell function, this hypothesis is mostly based on in vitro studies using high doses of recombinant proteins. One wonders how this effect mediated by circulating proteins could be specific to HBV-specific immune cells as there is no experimental evidence that adaptive immune responses to other pathogens are similarly impaired as those targeting HBV. Clinically chronic HBV patients are also not showing signs of significant immune impairment. Further studies analyzing the impact of circulating HBV antigens on HBV immune responses in vivo are needed, together with a more detailed and comprehensive assessment of the integrated adaptive immune response in well-defined longitudinal cohorts undergoing structured antiviral treatment and treatment interruptions.

Regulatory T cells

Regulatory T (Treg) cells are T cells that can regulate the local immune response via cell–cell contact or via secretion of cytokines, such as TGF-β or IL-10. Treg cells play a central role in immunological tolerance to self- and foreign antigens by suppressing activation, proliferation and effector functions of a wide range of lymphocyte subsets [76]. The best-known are CD25 + FoxP3 + CD4 natural Treg cells that directly inhibit other T cells. In addition, an increasing number of

other regulatory T cell types have been described, including CD8 T cells with inhibitory functions. Most studies in HBV have focused on natural CD4 Treg cells in the peripheral blood, where these cells usually constitute between 3 and 10% of the total CD4 T cell population. Confusingly, while some studies have found increased intrahepatic and peripheral Treg cell levels in chronic HBV compared to healthy individuals and self-limited HBV infection, others did not, similar to results in HCV infection [77, 78, 79, 80, 81, 82]. This is complicated by different methods to define the Treg populations, which have evolved over time from simple staining of CD25 antigen to increasingly more complex and specific combinations of phenotypic markers, including FoxP3, PD-1 and CD127. Given their local mode of action, the presence and functionality of intrahepatic Treg cells should be most consequential, but almost all data is from the blood. It remains to be seen whether their main role is enabling chronic infection or rather protection from active liver disease in the context of long-term viremia.

B cells and antibodies

HBV-specific antibodies are clearly able to provide sterilizing immunity after vaccination. Clinically, different antibody profiles are important for the diagnosis and characterization of acute and chronic HBV infection. Much less is known about the relative contribution of B cells and HBV antibodies to viral control once infection has been established. B cells have been reported to display an activated phenotype and seem functionally intact, even at later stages of infection. We also recently demonstrated that blood gene signatures indicative of B cell responses were highly active during the immune active phase in chronic HBV patients, using a systems biology approach [12]. HBV antibodies target the surface, polymerase, core and x proteins of HBV, and appear ten to twelve weeks after infection. Detection of antibodies against HBsAg is the clinical correlate of protective immunity, but HBs antibodies likely contribute to control of viremia even in chronic infection where they are not detected by the standard antibody assays as they form immune complexes that prevent viral attachment and entry. In contrast, the core antibody (anti-HBc) is detectable in all stages of infection and considered not to mediate viral control, though the passive immunization of anti-HBc/HBe does seem to prolong the incubation period in chimpanzees [83]. Overall a better characterization of how both B cells and antibody responses contribute to viral control during acute and chronic infection is urgently needed, as most likely a concerted effort by T cells and antibodies will offer the highest likelihood of effective HBV control. In this context it should also be noted that treatment with immunomodulatory drugs can lead to reactivation of controlled HBV infection [84]. The classic example is rituximab, a monoclonal antibody targeting CD20-expressing B cells, thus eliminating B cells and suppressing antibody production. It is not clear, however, whether the effect on antibodies is the sole or main cause for HBV reactivation, as rituximab treatment also impacts CD4 T cells and potentially indirectly also CD8 T cell memory [84, 85]. A detailed characterization of virus-specific immune responses during treatment with such agents should

reveal important insights into immunological changes that might lead to diminished HBV control.

SUMMARY

Despite numerous immunological studies performed in HBV infected patients several key questions remain unanswered. For decades, the lack of HBV models facilitating replication of human strains hampered scientific progress, and even findings from clinical studies can be misleading due to the huge variation among study cohorts of patients with chronic infection. Through careful patient selection and the use of modern biomedical techniques, like genomics and proteomics, researchers can now tackle basic questions regarding HBV immunopathogenesis. The aim should be to determine immune parameters associated with persistence, clearance and recurrence of HBV. Also, the mechanisms of recognition of viral antigens in chronic infections by hepatocytes in vivo remain unclear, with a particular need for ex vivo assays. Potential antiviral effector cells like Kupffer cells, natural killer cells and dendritic cell populations may be less functional during chronic infection, possibly leading to infrequent and exhausted HBV-specific T cells in adults. Using modern techniques, the function and phenotype of both peripheral and intrahepatic lymphocyte populations as well as hepatocytes can be determined, which may aid in the rational design of immunotherapeutic strategies.

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REFERENCES

1. G. Fattovich, F. Bortolotti, F. Donato. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J Hepatol, 48 (2008), pp. 335-352
2. J. Cheng, H. Imanishi, H. Morisaki, W. Liu, H. Nakamura, T. Morisaki, et al. Recombinant HBsAg inhibits LPS-induced COX-2 expression and IL-18 production by interfering with the NFkappaB pathway in a human monocytic cell line, THP-1. J Hepatol, 43 (2005), pp. 465-471
3. Y. Komiya, K. Katayama, H. Yugi, M. Mizui, H. Matsukura, T. Tomoguri, et al. Minimum infectious dose of hepatitis B virus in chimpanzees and difference in the dynamics of viremia between genotype A and genotype C. Transfusion, 48 (2008), pp. 286-294
4. H. Yan, G. Zhong, G. Xu, W. He, Z. Jing, Z. Gao, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus Elife, 1 (2012), p. e00049
5. B. Werle-Lapostolle, S. Bowden, S. Locarnini, K. Wursthorn, J. Petersen, G. Lau, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology, 126 (2004), pp. 1750-1758
6. S. Sato, K. Li, T. Kameyama, T. Hayashi, Y. Ishida, S. Murakami, et al. The RNA sensor RIG-I dually functions as an innate sensor and direct antiviral factor for hepatitis B virus. Immunity, 42 (2015), pp. 123-132
7. C. Dunn, D. Peppa, P. Khanna, G. Nebbia, M. Jones, N. Brendish, et al. Temporal analysis of early immune responses in patients with acute hepatitis B virus infection. Gastroenterology, 137 (2009), pp. 1289-1300
8. J.T. Guo, J.A. Sohn, Q. Zhu, C. Seeger Mechanism of the interferon alpha response against hepatitis C virus replicons. Virology, 325 (2004), pp. 71-81
9. C. Seeger, W.S. Mason Hepatitis B virus biology. Microbiol Mol Biol Rev, 64 (2000), pp. 51-68
10. S. Wieland, R. Thimme, R.H. Purcell, F.V. Chisari. Genomic analysis of the host response to hepatitis B virus infection. Proc Natl Acad Sci USA, 101 (2004), pp. 6669-6674
11. F. Lebosse, B. Testoni, J. Fresquet, F. Facchetti, E. Galmozzi, M. Fournier, et al. Intrahepatic innate immune response pathways are downregulated in untreated chronic hepatitis B patients. J Hepatol, 66 (2017), pp. 897-909
12. T. Vanwolleghem, J. Hou, G. van Oord, A.C. Andeweg, A.D. Osterhaus, S.D. Pas, et al. Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells. Hepatology, 62 (2015), pp. 87-100
13. J. Lucifora, Y. Xia, F. Reisinger, K. Zhang, D. Stadler, X. Cheng, et al. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. Science, 343 (2014), pp. 1221-1228
14. I.N. Crispe. Immune tolerance in liver disease. Hepatology, 60 (2014), pp. 2109-2117
15. S. Lan, L. Wu, X. Wang, J. Wu, X. Lin, W. Wu, et al. Impact of HBeAg on the maturation and function of dendritic cells. Int J Infect Dis, 46 (2016), pp. 42-48
16. S. Beckebaum, V.R. Cicinnati, G. Dworacki, J. Muller-Berghaus, D. Stolz, J. Harnaha, et al. Reduction in the circulating pDC1/pDC2 ratio and impaired function of ex vivo-generated DC1 in chronic hepatitis B infection. Clin Immunol, 104 (2002), pp. 138-150

17. R.G. van der Molen, D. Sprengers, R.S. Binda, E.C. de Jong, H.G. Niesters, J.G. Kusters, et al. Functional impairment of myeloid and plasmacytoid dendritic cells of patients with chronic hepatitis B. Hepatology, 40 (2004), pp. 738-746
18. S. Tavakoli, I. Mederacke, S. Herzog-Hauff, D. Glebe, S. Grun, D. Strand, et al. Peripheral blood dendritic cells are phenotypically and functionally intact in chronic hepatitis B virus (HBV) infection. Clin Exp Immunol, 151 (2008), pp. 61-70
19. J. Martinet, T. Dufeu-Duchesne, J. Bruder Costa, S. Larrat, A. Marlu, V. Leroy, et al.,Altered functions of plasmacytoid dendritic cells and reduced cytolytic activity of natural killer cells in patients with chronic HBV infection. Gastroenterology, 143 (2012). 1586–1596.e8
20. A.M. Woltman, M.L. Op den Brouw, P.J. Biesta, C.C. Shi, H.L. Janssen. Hepatitis B virus lacks immune activating capacity, but actively inhibits plasmacytoid dendritic cell function. PLoS One, 6 (2011), p. e15324
21. T. Yamamoto, C. Kaizu, T. Kawasaki, G. Hasegawa, H. Umezu, R. Ohashi, et al. Macrophage colony-stimulating factor is indispensable for repopulation and differentiation of Kupffer cells but not for splenic red pulp macrophages in osteopetrotic (op/op) mice after macrophage depletion Cell Tissue Res, 332 (2008), pp. 245-256
22. A. Boltjes, N. van Montfoort, P.J. Biesta, M.L. Op den Brouw, J. Kwekkeboom, L.J. van der Laan, et al. Kupffer cells interact with hepatitis B surface antigen in vivo and in vitro, leading to proinflammatory cytokine production and natural killer cell function. J Infect Dis, 211 (2015), pp. 1268-1278
23. J. Wu, Z. Meng, M. Jiang, R. Pei, M. Trippler, R. Broering, et al. Hepatitis B virus suppresses toll-like receptor-mediated innate immune responses in murine parenchymal and nonparenchymal liver cells. Hepatology, 49 (2009), pp. 1132-1140
24. K. Visvanathan, N.A. Skinner, A.J. Thompson, S.M. Riordan, V. Sozzi, R. Edwards, et al. Regulation of Toll-like receptor-2 expression in chronic hepatitis B by the precore protein Hepatology, 45 (2007), pp. 102-110
25. H. Li, H.W. Zheng, H. Chen, Z.Z. Xing, H. You, M. Cong, et al. Hepatitis B virus particles preferably induce Kupffer cells to produce TGF-beta1 over pro-inflammatory cytokines. Dig Liver Dis, 44 (2012), pp. 328-333
26. Z. Tu, A. Bozorgzadeh, R.H. Pierce, J. Kurtis, I.N. Crispe, M.S. Orloff TLR-dependent cross talk between human Kupffer cells and NK cells J Exp Med, 205 (2008), pp. 233-244
27. Z. Zhang, S. Zhang, Z. Zou, J. Shi, J. Zhao, R. Fan, et al. Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients. Hepatology, 53 (2011), pp. 73-85
28. J. Zhao, Y. Li, L. Jin, S. Zhang, R. Fan, Y. Sun, et al. Natural killer cells are characterized by the concomitantly increased interferon-gamma and cytotoxicity in acute resolved hepatitis B patients PLoS One, 7 (2012), p. e49135
29. D. Peppa, L. Micco, A. Javaid, P.T. Kennedy, A. Schurich, C. Dunn, et al. Blockade of immunosuppressive cytokines restores NK cell antiviral function in chronic hepatitis B virus infection. PLoS Pathog, 6 (2010), p. e1001227
30. S. Lunemann, D.F. Malone, J. Hengst, K. Port, J. Grabowski, K. Deterding, et al. Compromised function of natural killer cells in acute and chronic viral hepatitis. J Infect Dis, 209 (2014), pp. 1362-1373
31. S. Phillips, S. Chokshi, A. Riva, A. Evans, R. Williams, N.V. Naoumov. CD8(+) T cell control of hepatitis B virus replication: direct comparison between cytolytic and noncytolytic functions. J Immunol, 184 (2010), pp. 287-295
32. M. Zheng, R. Sun, H. Wei, Z. Tian. NK cells help induce anti-hepatitis B virus CD8+ T cell immunity in mice. J Immunol, 196 (2016), pp. 4122-4131
33. K. Kimura, K. Kakimi, S. Wieland, L.G. Guidotti, F.V. Chisari. Interleukin-18 inhibits hepatitis B virus replication in the

livers of transgenic mice. J Virol, 76 (2002), pp. 10702-10707

34. K. Kakimi, L.G. Guidotti, Y. Koezuka, F.V. Chisari. Natural killer T cell activation inhibits hepatitis B virus replication in vivo. J Exp Med, 192 (2000), pp. 921-930
35. A.M. Woltman, M.J. Ter Borg, R.S. Binda, D. Sprengers, B.M. von Blomberg, R.J. Scheper, et al. Alpha-galactosylceramide in chronic hepatitis B infection: results from a randomized placebo-controlled phase I/II trial. Antivir Ther, 14 (2009), pp. 809-818
36. B. Rehermann. Natural killer cells in viral hepatitis. Cell Mol Gastroenterol Hepatol, 1 (2015), pp. 578-588
37. R.A. de Groen, J. Hou, G.W. van Oord, Z.M. Groothuismink, M. van der Heide, R.J. de Knegt, et al. NK cell phenotypic and functional shifts coincide with specific clinical phases in the natural history of chronic HBV infection. Antivir Res, 140 (2017), pp. 18-24
38. P. Bonorino, M. Ramzan, X. Camous, T. Dufeu-Duchesne, M.A. Thelu, N. Sturm, et al. Fine characterization of intrahepatic NK cells expressing natural killer receptors in chronic hepatitis B and C J Hepatol, 51 (2009), pp. 458-467
39. E.T. Tjwa, R. Zoutendijk, G.W. van Oord, L.L. Boeijen, J.G. Reijnders, M.J. van Campenhout, et al. Similar frequencies, phenotype and activation status of intrahepatic NK cells in chronic HBV patients after long-term treatment with tenofovir disoproxil fumarate (TDF). Antivir Res, 132 (2016), pp. 70-75
40. F. Fasbender, A. Widera, J.G. Hengstler, C. Watzl. Natural killer cells and liver fibrosis. Front Immunol, 7 (2016), p. 19
41. S. Radaeva, R. Sun, B. Jaruga, V.T. Nguyen, Z. Tian, B. Gao. Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners. Gastroenterology, 130 (2006), pp. 435-452
42. W.C. Huang, N.J. Easom, X.Z. Tang, U.S. Gill, H. Singh, F. Robertson, et al. T cells infiltrating diseased liver express ligands for the NKG2D stress surveillance system. J Immunol, 198 (2017), pp. 1172-1182
43. A. Bertoletti, C. Ferrari. Adaptive immunity in HBV infection. J Hepatol, 64 (2016), pp. S71-S83
44. G.J. Webster, S. Reignat, M.K. Maini, S.A. Whalley, G.S. Ogg, A. King, et al. Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. Hepatology, 32 (2000), pp. 1117-1124
45. E. Thomas, T.J. Liang. Experimental models of hepatitis B and C – new insights and progress. Nat Rev Gastroenterol Hepatol, 13 (2016), pp. 362-374
46. R. Thimme, S. Wieland, C. Steiger, J. Ghrayeb, K.A. Reimann, R.H. Purcell, et al. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J Virol, 77 (2003), pp. 68-76
47. S. Asabe, S.F. Wieland, P.K. Chattopadhyay, M. Roederer, R.E. Engle, R.H. Purcell, et al. The size of the viral inoculum contributes to the outcome of hepatitis B virus infection. J Virol, 83 (2009), pp. 9652-9662
48. C. Ferrari. HBV and the immune response. Liver Int, 35 (Suppl. 1) (2015), pp. 121-128
49. A. Penna, M. Artini, A. Cavalli, M. Levrero, A. Bertoletti, M. Pilli, et al. Long-lasting memory T cell responses following self-limited acute hepatitis B. J Clin Invest, 98 (1996), pp. 1185-1194
50. E. Mizukoshi, J. Sidney, B. Livingston, M. Ghany, J.H. Hoofnagle, A. Sette, et al. Cellular immune responses to the hepatitis B virus polymerase. J Immunol, 173 (2004), pp. 5863-5871
51. M.C. Jung, M. Stemler, T. Weimer, U. Spengler, J. Dohrmann, R. Hoffmann, et al. Immune response of peripheral blood mononuclear cells to HBx-antigen of hepatitis B virus. Hepatology, 13 (1991), pp. 637-643
52. A. Penna, A. Bertoletti, A. Cavalli, A. Valli, G. Missale, M. Pilli, et al. Fine specificity of the human T cell response to

hepatitis B virus core antigen. Arch Virol Suppl, 4 (1992), pp. 23-28

53. A. Penna, G. Del Prete, A. Cavalli, A. Bertoletti, M.M. D'Elios, R. Sorrentino, et al. Predominant T-helper 1 cytokine profile of hepatitis B virus nucleocapsid-specific T cells in acute self-limited hepatitis B. Hepatology, 25 (1997), pp. 1022-1027
54. M.U. Lokhande, R. Thimme, P. Klenerman, N. Semmo. Methodologies for the analysis of HCV-specific CD4(+) T cells. Front Immunol, 6 (2015), p. 57
55. A. Bertoletti, M.M. D'Elios, C. Boni, M. De Carli, A.L. Zignego, M. Durazzo, et al. Different cytokine profiles of intraphepatic T cells in chronic hepatitis B and hepatitis C virus infections. Gastroenterology, 112 (1997), pp. 193-199
56. K. Nitschke, H. Luxenburger, M.M. Kiraithe, R. Thimme, C. Neumann-Haefelin. CD8+ T-cell responses in hepatitis B and C: the (HLA-) A, B, and C of hepatitis B and C. Dig Dis, 34 (2016), pp. 396-409
57. L.G. Guidotti, T. Ishikawa, M.V. Hobbs, B. Matzke, R. Schreiber, F.V. Chisari. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity, 4 (1996), pp. 25-36
58. M.I. Kafrouni, G.R. Brown, D.L. Thiele. Virally infected hepatocytes are resistant to perforin-dependent CTL effector mechanisms. J Immunol, 167 (2001), pp. 1566-1574
59. M.K. Maini, C. Boni, C.K. Lee, J.R. Larrubia, S. Reignat, G.S. Ogg, et al. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J Exp Med, 191 (2000), pp. 1269-1280
60. D. Sprengers, R.G. van der Molen, J.G. Kusters, R.A. De Man, H.G. Niesters, S.W. Schalm, et al. Analysis of intrahepatic HBV-specific cytotoxic T-cells during and after acute HBV infection in humans. J Hepatol, 45 (2006), pp. 182-189
61. D. Sprengers, R.G. van der Molen, J.G. Kusters, B. Hansen, H.G. Niesters, S.W. Schalm, et al. Different composition of intrahepatic lymphocytes in the immune-tolerance and immune-clearance phase of chronic hepatitis B. J Med Virol, 78 (2006), pp. 561-568
62. G.J. Webster, S. Reignat, D. Brown, G.S. Ogg, L. Jones, S.L. Seneviratne, et al. Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. J Virol, 78 (2004), pp. 5707-5719
63. B. Raziorrouh, W. Schraut, T. Gerlach, D. Nowack, N.H. Gruner, A. Ulsenheimer, et al. The immunoregulatory role of CD244 in chronic hepatitis B infection and its inhibitory potential on virus-specific CD8+ T-cell function. Hepatology, 52 (2010), pp. 1934-1947
64. A. Schurich, P. Khanna, A.R. Lopes, K.J. Han, D. Peppa, L. Micco, et al. Role of the coinhibitory receptor cytotoxic T lymphocyte antigen-4 on apoptosis-Prone CD8 T cells in persistent hepatitis B virus infection. Hepatology, 53 (2011), pp. 1494-1503
65. G. Nebbia, D. Peppa, A. Schurich, P. Khanna, H.D. Singh, Y. Cheng, et al. Upregulation of the Tim-3/galectin-9 pathway of T cell exhaustion in chronic hepatitis B virus infection. PLoS One, 7 (2012), p. e47648
66. P.D. Kurktschiev, B. Raziorrouh, W. Schraut, M. Backmund, M. Wachtler, C.M. Wendtner, et al. Dysfunctional CD8+ T cells in hepatitis B and C are characterized by a lack of antigen-specific T-bet induction. J Exp Med, 211 (2014), pp. 2047-2059
67. A.R. Lopes, P. Kellam, A. Das, C. Dunn, A. Kwan, J. Turner, et al. Bim-mediated deletion of antigen-specific CD8 T cells in patients unable to control HBV infection. J Clin Invest, 118 (2008), pp. 1835-1845
68. D. Peppa, U.S. Gill, G. Reynolds, N.J. Easom, L.J. Pallett, A. Schurich, et al. Up-regulation of a death receptor renders

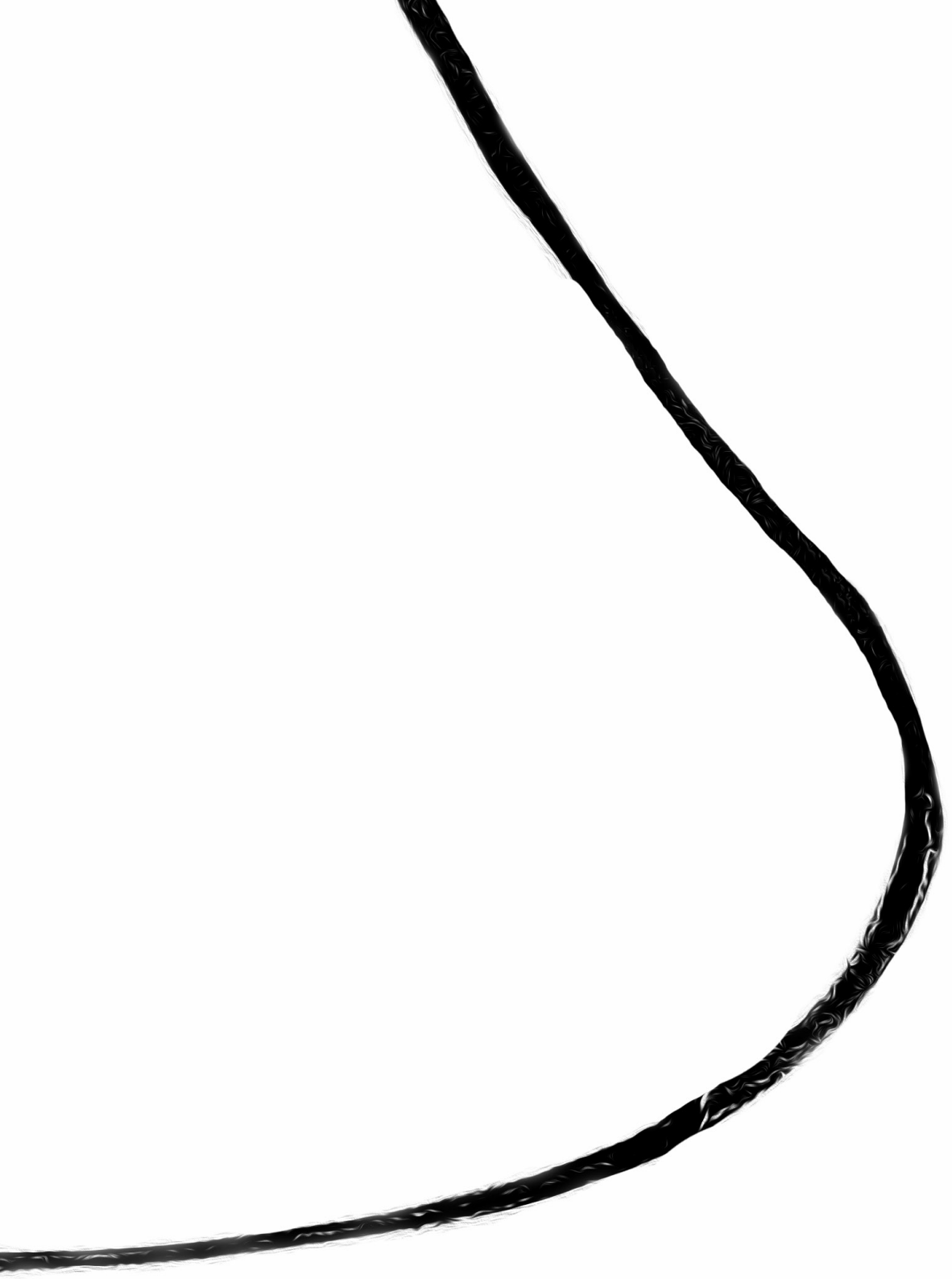
antiviral T cells susceptible to NK cell-mediated deletion. J Exp Med, 210 (2013), pp. 99-114

69. B. Rehermann, C. Pasquinelli, S.M. Mosier, F.V. Chisari. Hepatitis B virus (HBV) sequence variation of cytotoxic T lymphocyte epitopes is not common in patients with chronic HBV infection. J Clin Invest, 96 (1995), pp. 1527-1534
70. H. Kefalakes, B. Budeus, A. Walker, C. Jochum, G. Hilgard, A. Heinold, et al. Adaptation of the hepatitis B virus core protein to CD8(+) T-cell selection pressure. Hepatology, 62 (2015), pp. 47-56
71. C.P. Desmond, S. Gaudieri, I.R. James, K. Pfafferott, A. Chopra, G.K. Lau, et al. Viral adaptation to host immune responses occurs in chronic hepatitis B virus (HBV) infection, and adaptation is greatest in HBV e antigen-negative disease. J Virol, 86 (2012), pp. 1181-1192
72. A. de Niet, F. Stelma, L. Jansen, M.J. Sinnige, E.B. Remmerswaal, R.B. Takkenberg, et al. Restoration of T cell function in chronic hepatitis B patients upon treatment with interferon based combination therapy. J Hepatol, 64 (2016), pp. 539-546
73. C. Boni, D. Laccabue, P. Lampertico, T. Giuberti, M. Vigano, S. Schivazappa, et al. Restored function of HBV-specific T cells after long-term effective therapy with nucleos(t)ide analogues. Gastroenterology, 143 (2012). 963–973.e9
74. A. Penna, D. Laccabue, I. Libri, T. Giuberti, S. Schivazappa, A. Alfieri, et al. Peginterferon-alpha does not improve early peripheral blood HBV-specific T-cell responses in HBeAg-negative chronic hepatitis. J Hepatol, 56 (2012), pp. 1239-1246
75. M.F. Sprinzl, C. Russo, J. Kittner, S. Allgayer, A. Grambihler, B. Bartsch, et al. Hepatitis B virus-specific T-cell responses during IFN administration in a small cohort of chronic hepatitis B patients under nucleos(t)ide analogue treatment. J Viral Hepat, 21 (2014), pp. 633-641
76. T. Manigold, V. Racanelli. T-cell regulation by CD4 regulatory T cells during hepatitis B and C virus infections: facts and controversies. Lancet Infect Dis, 7 (2007), pp. 804-813
77. O. Franzese, P.T. Kennedy, A.J. Gehring, J. Gotto, R. Williams, M.K. Maini, et al. Modulation of the CD8+-T-cell response by CD4+ CD25+ regulatory T cells in patients with hepatitis B virus infection. J Virol, 79 (2005), pp. 3322-3328
78. J.N. Stoop, R.G. van der Molen, C.C. Baan, L.J. van der Laan, E.J. Kuipers, J.G. Kusters, et al. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. Hepatology, 41 (2005), pp. 771-778
79. D. Xu, J. Fu, L. Jin, H. Zhang, C. Zhou, Z. Zou, et al. Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. J Immunol, 177 (2006), pp. 739-747
80. G. Yang, A. Liu, Q. Xie, T.B. Guo, B. Wan, B. Zhou, et al. Association of CD4+CD25+Foxp3+ regulatory T cells with chronic activity and viral clearance in patients with hepatitis B. Int Immunol, 19 (2007), pp. 133-140
81. G. Peng, S. Li, W. Wu, Z. Sun, Y. Chen, Z. Chen. Circulating CD4+ CD25+ regulatory T cells correlate with chronic hepatitis B infection. Immunology, 123 (2008), pp. 57-65
82. J.N. Stoop, M.A. Claassen, A.M. Woltman, R.S. Binda, E.J. Kuipers, H.L. Janssen, et al. Intrahepatic regulatory T cells are phenotypically distinct from their peripheral counterparts in chronic HBV patients. Clin Immunol, 129 (2008), pp. 419-427
83. W. Stephan, A.M. Prince, B. Brotman. Modulation of hepatitis B infection by intravenous application of an immunoglobulin preparation that contains antibodies to hepatitis B e and core antigens but not to hepatitis B surface

antigen. J Virol, 51 (1984), pp. 420-424

84. S. Paul, A. Dickstein, A. Saxena, N. Terrin, K. Viveiros, E.M. Balk, et al. Role of surface antibody in hepatitis B reactivation in patients with resolved infection and hematologic malignancy: a meta-analysis. Hepatology (2017), 10.1002/hep.29082

85. A. Palanichamy, S. Jahn, D. Nickles, M. Derstine, A. Abounasr, S.L. Hauser, et al. Rituximab efficiently depletes increased CD20-expressing T cells in multiple sclerosis patients. J Immunol, 193 (2014), pp. 580-586



3

Persistent Replication of HIV, Hepatitis C Virus (HCV), and HBV Results in Distinct Gene Expression Profiles by Human NK Cells

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ABSTRACT

Natural killer (NK) cells during chronic viral infection have been well studied in the past. We performed an unbiased next-generation RNA-sequencing approach to identify commonalities or differences of the effect of HIV, HCV, and HBV viremia on NK cell transcriptomes. Using cell sorting, we obtained $CD3^-$ $CD56^+$ NK cells from blood of 6 HIV-, 8 HCV-, and 32 HBV-infected patients without treatment. After library preparation and sequencing, we used an in-house analytic pipeline to compare expression levels with matched healthy controls. In NK cells from HIV-, HCV-, and HBV-infected patients, transcriptome analysis identified 272, 53, and 56 differentially expressed genes, respectively (fold change, >1.5; q-value, 0.2). Interferon-stimulated genes were induced in NK cells from HIV/HCV patients, but not during HBV infection. HIV viremia downregulated ribosome assembly genes in NK cells. In HBV-infected patients, viral load and alanine aminotransferase (ALT) variation had little effect on genes related to NK effector function. In conclusion, we compare, for the first time, NK cell transcripts of viremic HIV, HCV, and HBV patients. We clearly demonstrate distinctive NK cell gene signatures in three different populations, suggestive for a different degree of functional alterations of the NK cell compartment compared to healthy individuals.

IMPORTANCE

Three viruses exist that can result in persistently high viral loads in immunocompetent humans: human immunodeficiency virus (HIV), hepatitis C virus, and hepatitis B virus. In the last decades, by using flow cytometry and *in vitro* assays on NK cells from patients with these types of infections, several impairments have been established, particularly during and possibly contributing to HIV viremia. However, the background of NK cell impairments in viremic patients is not well understood. In this study, we describe the NK cell transcriptomes of patients with high viral loads of different etiologies. We clearly demonstrate distinctive NK cell gene signatures with regard to interferon-stimulated gene induction and the expression of genes coding for activation markers or proteins involved in cytotoxic action, as well immunological genes. This study provides important details necessary to uncover the origin of functional and phenotypical differences between viremic patients and healthy subjects and provides many leads that can be confirmed using future *in vitro* manipulation experiments.

INTRODUCTION

Acute viral infection is usually accompanied by early production of infectious virions and rapid eradication of the virus by the host immune system. However, ineffective clearance of the virus can result in latent and even persistent infection within the host. In contrast to viruses such as Epstein-Barr virus, cytomegalovirus (CMV), or herpesviruses that may reinitiate replication after long periods of latency [1], three viruses—human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV)—can persistently replicate, resulting in considerable viral loads in the blood of immunocompetent individuals. Untreated patients infected with these viruses may harbor high viral loads for decades, which can be associated with variable degrees of immune activation or inflammation, but their antiviral host immune responses are insufficient to eradicate the virus [2].

The natural killer (NK) cell is an important antiviral effector cell [3]. NK cells are capable of lysing virus-infected cells following major histocompatibility complex class I downregulation, not necessitating prior licensing. In addition, NK cells can produce inflammatory cytokines, such as gamma interferon (IFN-γ) and tumor necrosis factor (TNF), as well as support and regulate virus-specific immunity [4]. Despite the fact that HIV, HCV, and HBV do not directly infect NK cells (HIV mainly infects CD4+ T cells, and hepatitis viruses infect hepatocytes), chronic exposure to replicating virus may affect NK cell phenotype and function indirectly. These changes may be the consequence of either alterations in the cytokine milieu, exposure to circulating viral components, or abnormal interaction with other leukocytes. HIV patient-derived NK cells have

been shown to produce less IFN-γ following stimulation with cytokines like interleukin-12 (IL-12) and IFN-α [5, 6]. Importantly, the capacity of NK cells to lyse target cells in vitro, such as K562 cells, is lower in HIV patients [7,–9]. This is in contrast to NK cells from chronic HCV- and HBV-infected patients that display an unaltered or mildly augmented cytotoxic potential compared to healthy individuals. Our group has previously shown that NK cell-mediated cytokine production in viral hepatitis patients is not or only slightly impaired, but results on this topic are conflicting since other researchers previously measured lower IFN-γ production by NK cells from hepatitis patients [4, 10,–12]. NK cells from HBV and HCV patients are described to have an activated phenotype (a high percentage of NK cells positive for NKp30, NKp46, and NKG2C) compared to healthy controls (HCs) [4]. It should be noted here that study results often vary in this field [13] and that all studies performed to date on this topic examine a limited number of functions and phenotypical markers of NK cells, mainly by flow cytometry [3, 4, 14, 15]. More unbiased approaches examining gene expression profiles of NK cells from patients with chronic HIV, HCV, and HBV have not been performed. These approaches may identify specific genes and signaling pathways affected in NK cells from patients with ongoing viral replication in vivo that were not recognized using conventional flow cytometry.

Therefore, in the present study, we performed an unbiased RNA sequencing approach on purified NK cells from viremic patients. We show for the first time that HIV and HCV viremia induced numerous interferon-stimulated genes (ISG) in NK cells, but this was not observed in NK cells from chronic HBV patients. In addition, HIV viremia affected more genes and signaling pathways in NK cells than viral hepatitis, including immune-related genes and genes involved in cytotoxic action. This study provides important details necessary to uncover the origin of the functional and phenotypical differences between viremic patients and healthy subjects and clearly demonstrates intrinsic differences in NK cells obtained from patients with three distinct chronic infections.

MATERIALS AND METHODS

Patient selection and characteristics

All patients were recruited from the outpatient clinic of the Erasmus MC Rotterdam. The HBV cohort was of Asian, and the HCV/HIV cohorts were of Caucasian ethnicity (**Table 1**). All infected cohorts were matched by age, gender, and ethnicity to healthy individuals (HBV patients to healthy control group 1 and HIV/HCV to healthy control group 2 [**Table 1**]). Included patients did not have other liver disease, were not pregnant, and were not on anti(retro)viral therapy for 2 years. Liver fibrosis was determined by histology or transient elastography (maximum of F2 Metavir score/7.0 kPa). Patients were categorized into four clinical HBV phases according to

the 2017 EASL Guidelines [16] and in correspondence with our previous work [17, 18] (**Figure 3A**). In clinical practice, the HBV clinical phases have been used to guide the moment to start antiviral suppression with nucleotide analogs (recommended in the IA and ENEG phase). CD4 counts were measured using Canto II flow cytometer (BD). The serum ALT level was measured on an automated analyzer, and serum HBsAg and HBeAg levels were measured on an Abbott Architect analyzer. The serum HIV viral load, the HCV viral load, and the HBV DNA levels were measured using the COBAS AmpliPrep-COBAS TaqMan HIV, HCVv2, and HBVv2 (Roche Molecular Systems), respectively.

Ethics statement

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

Sorting of CD3− CD56+ NK cells

PBMC were isolated from the peripheral blood of 8 chronic HCV patients, 6 HIV patients, 8 HBV patients in each HBV phase, and 20 healthy controls and stored at −150°C. After thawing, the cells were stained using anti-CD3-Alexa-Fluor 700 (OKT-3, Beckman) and anti-CD56-APC-eFluor 780 (CMSSB, Beckman). CD3− CD56+ lymphocytes were purified using a FACSARIA cell sorter (BD) as depicted in **Figure 1A**. The purities of all samples included in the analysis were higher than 95% (**Figure 1B** and and 1C). Sorted NK cells were kept in RNAlater (Qiagen), and RNA was isolated by using a PicoPure RNA isolation kit (Arcturus). Although a trend toward higher percentage of CD56bright NK cells was observed in patients versus healthy individuals, we found no significant differences between the ratios of CD56bright versus CD56dim NK cells when comparing the HIV-infected (10.1% versus 5.3% CD56bright/total NK cells [P = 0.46]), HCV-infected (8.8% versus 5.3% of CD56bright/total NK cells, P = 0.17), and HBV-infected (3.6% versus 1.6% CD56bright/total NK cells, P = 0.41) cohorts to controls.

RNA sequencing of purified NK cells

Total RNA was isolated from purified NK cells using a PicoPure RNA isolation kit (Arcturus), and the RNA concentration was measured using the NanoDrop ND1000 spectrophotometer. The quality and integrity of the RNA samples was analyzed on a Bioanalyzer 2100 (Agilent Technologies). mRNA was isolated from total RNA using the poly-T-oligo-attached magnetic beads. After fragmentation of the mRNA, cDNA synthesis was performed. This was used for ligation with the sequencing adapters and PCR amplification of the resulting product. The sequence libraries were prepared using a TruSeq RNA kit (Illumina) according to the manufacturer's protocol. The quality and yield after sample preparation was measured with a DNA 1000 Lab-on-a-Chip. The size of

the resulting products had a peak around 300 bp on a DNA 1000 chip. The cluster generation, hybridization to a flow cell, amplification, linearization, denaturation, and sequencing were performed on an Illumina HiSeq 2000, HiSeq 2500, or HiSeq 4000 platform at GenomeScan (Leiden, the Netherlands). The sequencing strategy and data quality control was performed as described previously [19] (~92% reads; Phred score, >36).

Alignment of short reads to genome

The generated reads were demultiplexed and trimmed to remove low quality nucleotides as well as reads with low quality score (Phred score, <30) using a trimming tool based on the FASTX tool kit (http://usegalaxy.org). The clean reads in the FastQ files were independently aligned to the human reference genome (UCSC hg19) using Bowtie 2.0, with no mismatch allowed. About 70% (66 to 74%) of the reads were uniquely aligned to the reference genome. The aligned reads in SAM files were sorted in SAMTools by the genomic tracks using the default parameters and then exported into BAM files. Before annotating the aligned reads, eight individual BAM files of each sample were merged and indexed in SAMTools.

Differential gene expression profiling and pathways analysis

The sorted reads were assembled to the transcriptomes and quantified to get gene expression abundances by using human genome track on hg19 as the reference. The abundance of read counts for each selected transcript was quantified by assessing the total number of reads mapped to the transcript using the Cufflinks program. The abundance was normalized per kilobase of transcripts per million mapped reads (FPKM). The expression values of each sample summarized at the gene level (summed FPKM of transcripts sharing the same gene symbol) were first normalized to the overall median of that sample then used to perform differential expression testing between NK cells from two groups (significance was defined as >1.5-fold, q-values of <0.2). DEGs were analyzed using core expression analysis (IPA; Ingenuity Systems). The DEG symbols were mapped against the complete Ingenuity knowledge base (genes only) to identify affected canonical pathways. The P values and log2-fold change of the DEG were used by IPA to predict alterations in the activity of the network or pathway. The significance of the prediction made by IPA is based on an activation z-score (values of >2 suggests increased activity), which infers activation states of the pathway compared to a model that assigns predicted changes (20). We focused on canonical pathways that are significantly deregulated in one viral infection. The significance was defined based on P values calculated using the right-tailed Fisher exact test ($P < 10-4$) and a minimum of 5% deregulated pathway molecules in the disease condition compared to the controls.

Data availability

RNA sequencing data have been deposited in NCBI's Gene Expression Omnibus (GEO) database under accession number GSE125686.

RESULTS

Characteristics of three untreated patient cohorts chronically infected with HIV, HCV and HBV

To identify the effects of chronic viremia on NK cell gene transcripts, we isolated NK cells from cohorts representing the three causes of chronic viremia in humans: HIV-, HCV-, and HBV-infected individuals (**Table 1**). None of the patients were on anti(retro)viral therapy, and all had relatively high serum viral loads. The average viral load of the HIV-infected cohort was 1.1 × 105 copies/ml and the average CD4 count was 374. The average viral loads of the HCV and HBV cohorts were 3.9 × 106 and 2.5 × 108 IU/ml, respectively. We matched the HBV-infected cohort—based on age, gender, and ethnicity—to an Asian healthy control group (**Table 1**, healthy control 1) and matched the HIV- and HCV-infected cohorts to a second, predominantly Caucasian control group (**Table 1**, healthy control 2).

Table 1. Patient characteristics of HBV, HCV and HIV patients and matched healthy control groups

	HC 1	HC 2	HIV	HCV	HBV (all)	HBeAg+ infection (IT)	HBeAg+ hepatitis (IA)	HBeAg- infection (IC)	HBeAg- Hepatitis (ENEG)
Subjects	8	12	6	8	32	8	8	8	8
Gender (M/F)	4/4	12/0	6/0	8/0	19/13	4/4	4/4	4/4	7/1
Age (years)	27 (25-30)	37 (26-51)	42 (21-65)	47 (32-65)	35 (18-55)	29 (18-38)	33 (18-49)	36 (22-55)	40 (29-47)
Ethnicity	Asian	Caucasian	Caucasian	Caucasian	Asian	Asian	Asian	Asian	Asian
Liver Fibrosis	-	-	-	F1-2: 7 F3: 1	F0-F1: 29 F2: 3	F0-F1: 7 F2: 1	F0-F1: 7 F2: 1	F0-F1: 8	F0-F1: 7 F2: 1
ALT (IU/ml)	-	-	51 (21-121)	69 (18-146)	52 (8-229)	25 (15-39)	104 (32-229)	29 (8-39)	50 (29-73)
Viral Load (IU/ml)†	-	-	$1.1x10^5$ ($2.1x10^3$-$2.2x10^5$)	$3.9x10^6$ ($1.0x10^6$-$8.3x10^6$)	$2.5x10^8$ ($2.0x10^1$-$1.2x10^9$)	$5.9x10^8$ ($1.6x10^8$-$1.2x10^9$)	$4.0x10^8$ ($2.2x10^2$-$1.1x10^9$)	$4.8x10^3$ ($2.0x10^1$-$2.8x10^4$)	$1.7x10^6$ ($9.4x10^1$-$1.3x10^7$)
Genotype	-	-	-	HCV-1: 7 HCV-6: 1	A: 5 B: 6 C:9 D: 4 E: 2 ND: 6	B: 3 C: 2 D: 1 ND: 2	A: 1 B: 2 C: 4 D: 1	A: 1 B: 1 C: 1 D: 1 E: 2 ND: 2	A; 3 C: 2 D: 1 ND: 2
HBeAg (IU/ml)	-	-	-	-	560 (0-3389)	1091 (243-1824)	1316 (0.5-3389)	0	0

Continuous variables are displayed as means (minimum to maximum). The Metavir scoring system was used for liver fibrosis grade. Abbreviations: IT, immune tolerant; IA, immune active; IC, inactive carrier; ENEG; hepatitis B envelope antigen-negative hepatitis. ND, not determined. † HIV load is depicted as the mean number of copies/ml.

NK cell ISG are not induced by HBV, but both HIV and HCV infection have parallel patterns of upregulated ISG.

To determine whether the NK cell transcriptional signature in blood was modulated as a consequence of chronic infection, gene expression profiles of sorted NK cells from patients were

compared to their matched controls. In NK cells from HIV patients, 272 differentially expressed genes (DEGs) with ≥1.5-fold-higher expression compared to healthy controls were identified (q-value, 0.2). A total of 241 of the 272 DEGs were downregulated, while 31 were upregulated in HIV NK cells compared to their controls (see **Table S1** in the uploaded supplemental material). The numbers of DEGs were lower in NK cells from HCV and HBV patients (53 and 56, respectively), and the downregulated versus upregulated genes were more balanced compared to their respective controls (30 versus 23 in HCV and 27 versus 29 in HBV, respectively). In NK cells from HIV and HCV patients, 22 genes were differentially expressed in both groups, but there was no overlap with the HBV-infected cohort. In HCV patients, 2 ISGs were among the top 10 upregulated genes compared to 5 during HIV: ISG15, MX1, IRF7, STAT1, and OAS1 (**Figure 1D**). Despite the observation that, during HCV and HIV infection, these individual genes are among the genes with the highest fold change (**Figure 2A**) compared to healthy controls, we did not detect large clusters common to HIV- or HCV-derived NK cells (**Figure 2B**). Apart from the therapeutic application of IFN-α [21,–23], it has been shown that chronic exposure to type I IFN during HIV infection leads to desensitization to its signaling, as well as ongoing immune activation [24, 25]. To investigate the specific ISGs that are induced during viremia in humans, we evaluated the expression levels of 139 ISGs in NK cells (**Table S2** in the uploaded supplemental material and **Figure 2**). In NK cells from the HIV-, HCV-, and HBV-infected cohorts, differential expression of 9, 14, and 1 ISG, respectively, was observed. In sharp contrast, NK cells from HBV-infected patients did not differentially express any ISG, except for IRF4 (log2-fold change of 1.75), encoding a protein that may compete with IRF-5 for binding to MyD88, thereby inhibiting downstream TLR-mediated induction of proinflammatory cytokines, including IFN-γ [26].

NK cells of HIV-infected patients differentially express genes coding for NK activating surface markers and cytotoxicity-related genes and display downregulation of ribosome assembly genes. In NK cells derived from HIV-infected individuals, genes coding for surface markers impacting NK cell activation status and cytotoxicity were differentially expressed (see **Table S1** in the uploaded supplemental material). Genes encoding NK cell-activating receptors, such as CD160 and TNFSFR1 (log2-fold changes of −1.8 and −0.9, respectively) were downregulated. Conversely, six genes coding for proteins whose ligation results in inhibitory signaling were also downregulated in HIV-derived NK cells only: KLRG1, KLRF1, CD244, PILRA, LAIR1, and CD300A/C (log2-fold changes of −0.6, −4.0, −1.5, −1.3, −1.1, and −1.3/−2.7, respectively). We did not observe this degree of modulation of NK cell receptor transcription in HCV or HBV. Importantly, two genes directly involved in the mechanism of cytotoxic action, CTSC [27] (coding cathepsin C, log2-fold change of −1.1) and HCST [28] (coding part of the activating complex KLRK1-HCST, log2-fold change of −3.8) were downregulated in NK cells from HIV-infected individuals only. Interestingly, NK cells derived from HIV-infected individuals also downregulated several genes related to ribosome formation (**Table S1**, **Figure 1D**), including RPL27, RPS7, RPL24, RPS13, and RPL10L.

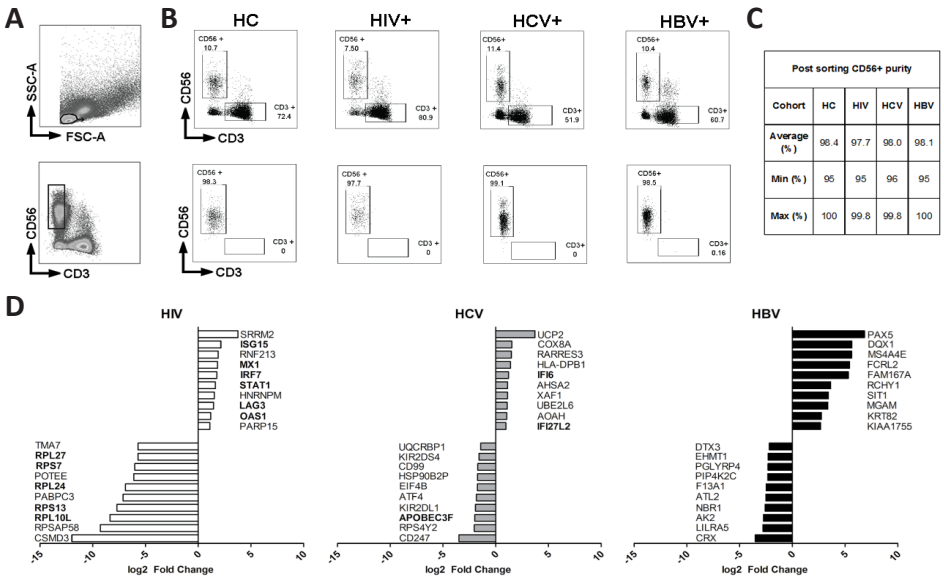

Figure 1. NK cells of HIV-infected patients differentially express numerous genes encoding NK activating surface markers and cytotoxicity-related genes and display a downregulation of ribosome assembly genes. (A) Representative flow cytometry plots showing the gating strategy for peripheral CD56+ CD3− NK cells used for all included subjects. (B) Summary of the post-sort purity percentages of CD56+ CD3− cells in virally infected and healthy subjects. (C) Representative flow cytometry plots showing comparable pre- and post-sort percentages in healthy individuals in comparison with HIV-, HCV-, and HBV-infected patients. (D) Relative expression of the 10 most upregulated genes in HIV-, HCV-, and HBV-infected individuals compared to matched healthy controls (fold change, ≥1.5; q-value, 0.2), including several common ISGs in HIV/HCV infection. NK cells derived from HIV-infected individuals strikingly downregulated several genes related to ribosome biogenesis (RPL27, RPS7, RPL24, RPS13, and RPL10L), and during both HIV and HCV infection, ISGs (IFI6 and IFI27L2) were among the top 10 most upregulated genes.

Fluctuations in serum HBV DNA and alanine aminotransferase (ALT) levels impact the expression by NK cells of immune genes but only minimally affect cytotoxicity or IFN signaling-related genes.

As described above, when analyzing the complete HBV-infected cohort, to our surprise only a single ISG was detected to be differentially expressed (IRF4, **Figure 2A**). However, since chronic HBV infection is a heterogeneous disease characterized by fluctuating ALT levels and viral loads, we investigated the consequence of these variable virological parameters on ISG expression in purified NK cells by stratifying the HBV cohort into four separate groups (as depicted in **Figure 3A**). Compared to healthy controls, the IT (immune tolerant or HBeAg+ infection) phase, the IA (immune active or HBeAg+ hepatitis) phase, the IC (inactive carrier or HBeAg− infection) phase,

and the ENEG (HBeAg− hepatitis) cohorts differentially expressed 37, 47, 64, and 45 genes, respectively (**Figure 3B**; see also **Table S3**). Further examination of each subgroup revealed 5, 11, 8, and 8 immune-related genes differentially expressed in each phase (**Table S3**). Despite this limited variation of NK cell gene transcription, we found that among the DEGs identified, the majority of genes actively transcribed in the IA phase were immune related genes (**Figure 3C**; see **Table S3**). IRF4, the single upregulated ISG in NK cells of the HBV cohort, was upregulated in all phases, except in the ENEG phase. Overall, the analysis of NK cell-derived transcripts of heterogeneous HBV patients show little correlation with fluctuations of clinical parameters (ALT and viral load), which is in concordance with the stable NK cell phenotype as measured by flow cytometry, previously described by our group [17].

Altered activity of specific pathways in NK cells from HCV and HIV patients, but not from HBV patients.

In addition to analysis of the expression of individual genes, additional analysis for the activity of specific networks or pathways was conducted using Ingenuity Pathway Analysis (IPA). Using stringent criteria (P < 10−4; overlap, >5%), nine pathways were identified to be modified in NK cells from HIV patients, four pathways were identified to be modified in chronic HCV patients, and none were identified to be modified in chronic HBV patients compared to NK cells from healthy individuals. The pathway of IFN signaling in NK cells of both HIV and HCV patients was upregulated compared to their respective healthy controls (**Table 2**). Also, the NK cell signaling pathway was modified in NK cells from both chronic HIV and HCV patients, as well as pathways active during DNA repair and stress (nucleotide excision repair, DNA double-strand break repair by NHEJ, and GADD45 signaling). Likely related is the downregulation of the eukaryotic initiation factor 2 (eIF2) pathway in NK cells from the HIV-infected cohort, which includes various ribosomal assembly genes and other eukaryotic initiation factors (pathway P value, 7.58 × 10−8; z-score, −2.4). Upon induction by stress signals, eIF2 has a strong negative impact on cellular mRNA translation [29]. Also, modulation of various pathways related to cell-cell contact or actin-mediated processes (such as remodeling of epithelial adherence junctions, regulation of actin-based motility by Rho, and FcγR-mediated phagocytosis in macrophages and monocytes) was found and was exclusively observed for the NK cells of chronic HIV patients. Several of the genes in these pathways encode proteins involved in the exocytosis of secretory lysozymes, and low abundance of their protein products might directly affect NK cell effector function [29]. Finally, pathways involved in glycolysis and oxidative phosphorylation were altered in the NK cells of HIV patients only compared to NK cells from healthy controls. These altered metabolic pathways may affect NK cell functionality and may be linked to dysfunctional NK cell responses in HIV patients, as has been observed for CD8+ T cells [30]. However, little is known regarding how metabolic processes impact NK cell activity during chronic viral infections [31].

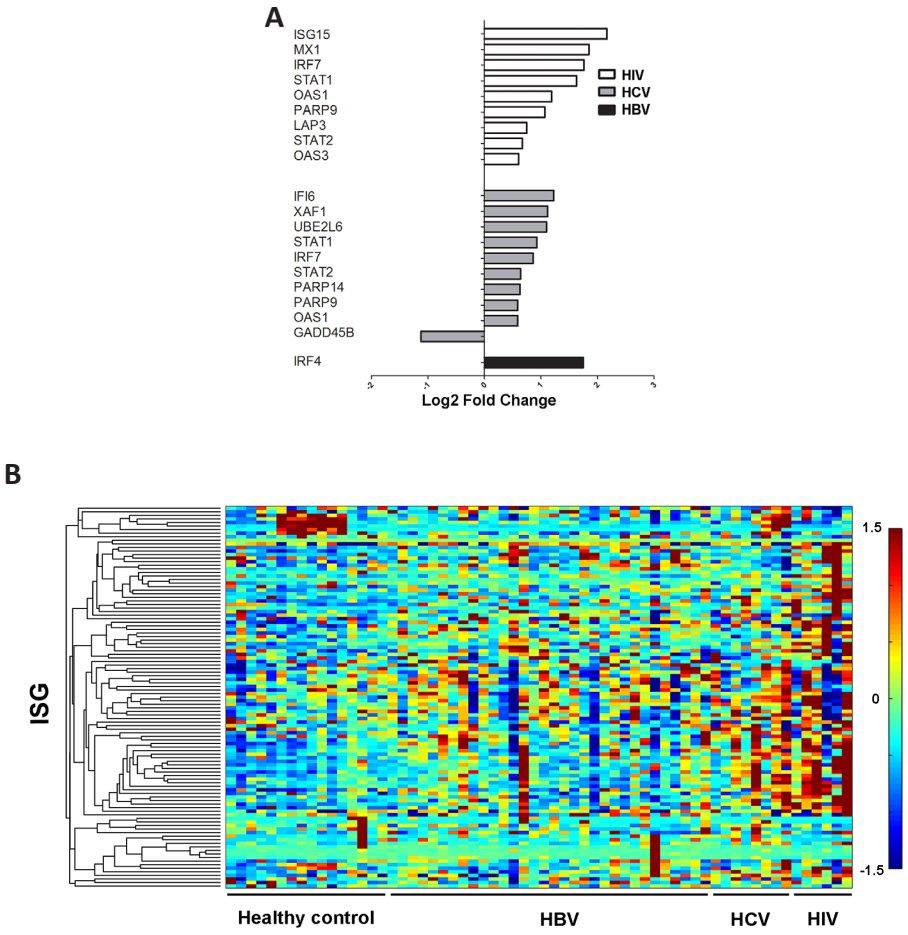

Figure 2. NK cell ISG are not induced by HBV, but both HIV and HCV infection have parallel patterns of upregulated ISGs. (A) Expression relative to matched healthy subjects of several ISGs induced during chronic HCV and HIV infections that is indicative of parallel ISG induction in NK cells. Chronic infection with HCV and HIV induces the expression of several ISGs, but in HBV patients only a single NK cell ISG was upregulated compared to healthy controls. (B) Relative NK cell ISG expression of NK cells from HIV, HBV, and HCV patients and healthy subjects. Each row represents a single ISG. No large clusters of up- or downregulation are common to the viremic subjects, confirming that the expression patterns of individual subjects vary considerably. Patients are presented in the following order: healthy control and HBV, HCV, and HIV infections. The colors represent a range of fold differences from −1.5 (blue) to 1.5 (red).

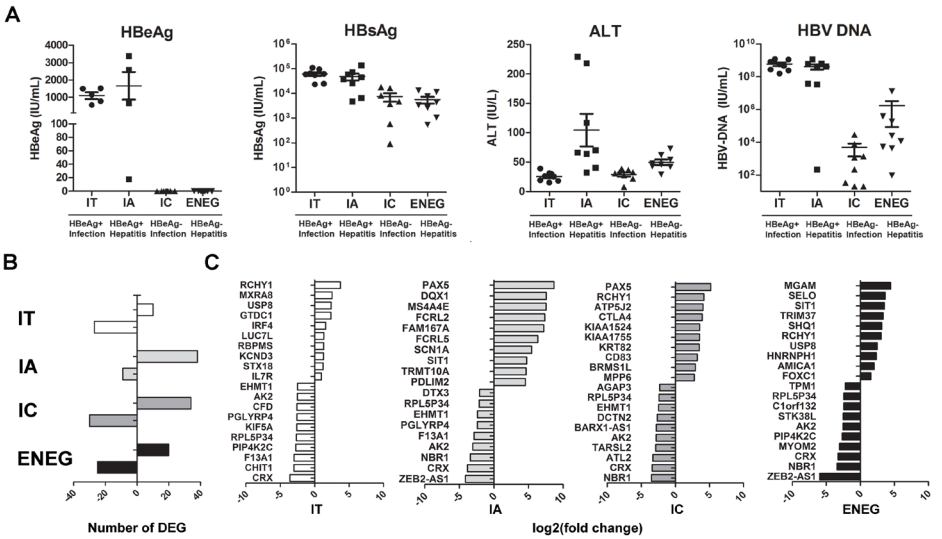

Figure 3. Variation of serum HBV DNA and ALT levels among four HBV cohorts is associated with variation of NK cell immunological genes but has minimal impact on the expression of individual genes related to cytotoxicity or IFN signaling. (A) Graphical representation of the patient characteristics of the HBV-infected cohort. Based on serum HBV DNA, ALT levels, and HBeAg presence at the time of sampling, patients were categorized into four clinical HBV phases according to international guidelines (European Association for the Study of the Liver, 2017). The HBsAg levels are presented to illustrate lower values among IC and ENEG patients which are characteristic for the natural history of HBV. (B) Total number of DEGs in each HBV clinical phase. The numbers of upregulated (rightward) or downregulated (leftward) genes per phase are shown as horizontal bars. (C) The top up- or downregulated genes are depicted per phase compared to healthy individuals. Regardless of serum ALT levels, no ISGs were induced, except for IRF4. IRF4 was highly expressed in all phases, except for the ENEG phase. Among the genes upregulated in the IA phase, a few are immune related, including FCRL2/5, PDLIM2, and SIT1.

DISCUSSION

Using a highly sensitive RNA-sequencing-based approach, we were able to compare, for the first time, the gene expression profiles of NK cells of chronic HIV-, HCV-, and HBV-infected individuals. We clearly demonstrate distinctive gene signatures in the NK cells of the three different patient populations, suggesting different degrees of functional alterations of the NK cell compartment compared to that of healthy individuals. The induction of ISG was pronounced in HIV and HCV patient-derived NK cells compared to their healthy counterparts but was almost completely absent in chronic HBV patients. In addition, the downregulation of ribosome assembly genes, as well as inhibitory receptor-encoding and cytotoxicity-elated genes, was observed in NK cells

from HIV-infected patients, but not or to a lesser extent in NK cells from chronic HBV- and HCV-infected patients. This study provides important details necessary to uncover the origin of the functional and phenotypical differences between viremic patients and healthy subjects and clearly demonstrate intrinsic differences in NK cells obtained from patients with three distinct chronic infections.

NK cells from HBV-infected individuals did not differentially express any ISG compared to healthy individuals, except for IRF4 which is a nonclassical ISG [32], inducible in a type I IFN-independent manner. In 2004, the Chisari group showed that innate responses in the liver are not triggered or are only weakly triggered by HBV, resulting in limited systemic effects during the chronic phase of infection [33]. Supporting this, serum levels of type I and type III IFNs are (undetectably) low during HBV infection [18, 34]. However, in the blood of HIV and HCV patients, we and others have shown that systemic IFN and elevated IP-10 levels can be readily detected, which corresponds to the degree of ISG induction, as shown in **Figure 2A** [35–39]. In line with a stronger IFN response during HCV infection, it has been shown that STAT1 is upregulated [40]. Here, we show by direct ex vivo analysis that STAT1 and STAT2 are indeed upregulated in NK cells during HCV and HIV infection. STAT4, however, which is downstream of the IL-12 receptor, is significantly downregulated in HIV infection, further pointing toward a dominance for an IFN-induced over an IL-12-induced response in NK cells from these chronic infections.

Despite the fact that HIV does not replicate in NK cells, HIV patient-derived NK cells displayed downregulation of genes involved in ribosome assembly. The cause of this downregulation of ribosomal protein transcription remains uncertain. However, similar findings have been reported for primary CD4+ T cells and Jurkat T cell lines in which pre-rRNA processing was perturbed in HIV infection [41, 42]. Several of these ribosomal genes are in the pathway downstream of eIF2. For eIF2 specifically, the phosphorylation of alpha subunit of eIF2 blocks protein translation [43], allowing cells to conserve resources, and subsequently switch to a new gene expression program to prevent stress damage [44]. Interestingly, due to additional interactions of HIV with cellular translational machinery, the synthesis of viral structural proteins is reported to be sustained [45]. The downregulation of ribosome assembly in both CD4+ T cells and NK cells may reflect a common effect of systemic HIV proteins on both lymphocyte populations. Further studies will be necessary to dissect the link between ribosome biogenesis alteration and viral infection, possibly unraveling a novel intricate network of interactions between HIV and host cells.

The analysis of NK cell-derived transcripts of heterogeneous HBV patients showed little correlation with fluctuations of clinical parameters (serum ALT level and HBV DNA). Using FFPE liver biopsy specimens from HBV patients, we have previously shown that liver resident NK cells have an increased transcriptional activity in the phases with increased ALT levels [18], which was

recently confirmed by others [46]. In the peripheral blood compartment, fluctuating serum HBV DNA and ALT levels did not lead to dramatic changes in the expression of NK cell surface markers or production of IFN-γ evaluated by flow cytometry [17]. Our present study, using a completely different, unbiased approach, confirms that only few immune genes are modulated when comparing NK cells from healthy individuals with HBV patients in each of the clinical phases. This indicates that HBV DNA or serum HBsAg does not trigger NK cell activation, which corresponds to previous reports [14, 47]. It is important to decipher in coming studies whether the conflicting findings reported in literature have a technical or biological reason.

Our study has a few limitations. First, we restricted this study to transcriptional analysis and did not include analysis at the protein level. This is important, since the abundance of a transcript does not necessarily reflect protein expression. Similarly, we have not taken the degree of protein phosphorylation into account using these methods, which may impact STAT1/4 function. Second, the study investigated baseline mRNA expression in the peripheral blood only, aiming to capture the effects of ongoing viremia on NK cell transcripts in the blood compartment. However, since leukocytes residing in the liver parenchyma (infected by HCV and HBV) are phenotypically and functionally not identical to their circulating counterpart [13], our findings may not necessarily be generalized to intrahepatic NK cells. Third, although we did not determine killer-cell immunoglobulin-like receptor (KIR) or human leukocyte antigen (HLA) polymorphisms in this study, these polymorphisms, as well as the CMV status of the included subjects, may affect NK cell differentiation, phenotype, and function. We matched the infected cohorts to healthy controls based on ethnicity (as well as gender and age), and we therefore estimate the effect of uneven KIR/HLA distribution and CMV status to be of minor influence. Fourth, we sorted bulk CD56+ lymphocytes and did not separately analyze the functionally distinct CD56bright and CD56dim NK cell subpopulations. However, this approach is sufficient to uncover major effects of viremia on bulk NK cell transcripts, and the frequencies of these subpopulations were similar in all infected cohorts. Despite these limitations, this is the first observational study in which gene expression profiles in sorted NK cells were compared, and it provides leads that may be the first step in uncovering the origin of the functional and phenotypical differences between viremic patients and healthy subjects.

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REFERENCES

1. Traylen CM, Patel HR, Fondaw W, Mahatme S, Williams JF, Walker LR, Dyson OF, Arce S, Akula SM. 2011. Virus reactivation: a panoramic view in human infections. Future Virol 6:451–463.
2. Zuniga EI, Macal M, Lewis GM, Harker JA. 2015. Innate and adaptive immune regulation during chronic viral infections. Annu Rev Virol 2:573–597.
3. Jost S, Altfeld M. 2013. Control of human viral infections by natural killer cells. Annu Rev Immunol 31:163–194.
4. Rehermann B. 2015. Natural killer cells in viral hepatitis. Cell Mol Gastroenterol Hepatol 1:578–588.
5. Alter G, Teigen N, Davis BT, Addo MM, Suscovich TJ, Waring MT, Streeck H, Johnston MN, Staller KD, Zaman MT, Yu XG, Lichterfeld M, Basgoz N, Rosenberg ES, Altfeld M. 2005. Sequential deregulation of NK cell subset distribution and function starting in acute HIV-1 infection. Blood 106:3366–3369.
6. Naluyima P, Eller MA, Laeyendecker O, Quinn TC, Serwadda D, Sewankambo NK, Gray RH, Michael NL, Wabwire-Mangen F, Robb ML, Sandberg JK. 2014. Impaired natural killer cell responses are associated with loss of the highly activated NKG2A+ CD57+ CD56dim subset in HIV-1 subtype D infection in Uganda. AIDS 28:1273–1278. doi:10.1097
7. Vieillard V, Fausther-Bovendo H, Samri A, Debre P, French Asymptomatiques a Long Terme (ALT) ANRS-CO-15 Study Group . 2010. Specific phenotypic and functional features of natural killer cells from HIV-infected long-term nonprogressors and HIV controllers. J Acquir Immune Defic Syndr 53:564–573.
8. Ahmad A, Menezes J. 1996. Defective killing activity against gp120/41-expressing human erythroleukaemic K562 cell line by monocytes and natural killer cells from HIV-infected individuals. AIDS 10:143–149.
9. Ullum H, Gotzsche PC, Victor J, Dickmeiss E, Skinhoj P, Pedersen BK. 1995. Defective natural immunity: an early manifestation of human immunodeficiency virus infection. J Exp Med 182:789–799.
10. Spaan M, Janssen HL, Boonstra A. 2012. Immunology of hepatitis C virus infections. Best Pract Res Clin Gastroenterol 26:391–400.
11. Oliviero B, Varchetta S, Paudice E, Michelone G, Zaramella M, Mavilio D, De Filippi F, Bruno S, Mondelli MU. 2009. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. Gastroenterology 137:1151–1160.
12. Ahlenstiel G, Titerence RH, Koh C, Edlich B, Feld JJ, Rotman Y, Ghany MG, Hoofnagle JH, Liang TJ, Heller T, Rehermann B. 2010. Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner. Gastroenterology 138:325–335.
13. Boeijen LL, Hoogeveen RC, Boonstra A, Lauer GM. 2017. Hepatitis B virus infection and the immune response: the big questions. Best Pract Res Clin Gastroenterol 31:265–272.
14. Zhang QF, Shao JY, Yin WW, Xia Y, Chen L, Wang X, Hu HD, Hu P, Ren H, Zhang DZ. 2016. Altered immune profiles of natural killer cells in chronic hepatitis B patients: a systematic review and meta-analysis. PLoS One 11:e0160171.
15. Lunemann S, Malone DF, Hengst J, Port K, Grabowski J, Deterding K, Markova A, Bremer B, Schlaphoff V, Cornberg M, Manns MP, Sandberg JK, Ljunggren HG, Bjorkstrom NK, Wedemeyer H. 2014. Compromised function of natural killer cells in acute and chronic viral hepatitis. J Infect Dis 209:1362–1373.
16. European Association for the Study of the Liver. 2017. EASL 2017 clinical practice guidelines on the management of hepatitis B virus infection. J Hepatol 67:370–398.

17. de Groen RA, Hou J, van Oord GW, Groothuismink ZM, van der Heide M, de Knegt RJ, Boonstra A. 2017. NK cell phenotypic and functional shifts coincide with specific clinical phases in the natural history of chronic HBV infection. Antiviral Res 140:18–24.
18. Vanwolleghem T, Hou J, van Oord G, Andeweg AC, Osterhaus AD, Pas SD, Janssen HL, Boonstra A. 2015. Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells. Hepatology 62:87–100.
19. Hou J, Groothuismink ZM, Koning L, Roomer R, van IWF, Kreefft K, Liu BS, Janssen HL, de Knegt RJ, Boonstra A. 2014. Analysis of the transcriptome and immune function of monocytes during IFNα-based therapy in chronic HCV revealed induction of TLR7 responsiveness. Antiviral Res 109:116–124.
20. Kramer A, Green J, Pollard J Jr, Tugendreich S. 2014. Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics 30:523–530.
21. Lane HC. 1994. Interferons in HIV and related diseases. AIDS 8(Suppl 3):S19–S23.
22. Tavel JA, Huang CY, Shen J, Metcalf JA, Dewar R, Shah A, Vasudevachari MB, Follmann DA, Herpin B, Davey RT, Polis MA, Kovacs J, Masur H, Lane HC. 2010. Interferon-alpha produces significant decreases in HIV load. J Interferon Cytokine Res 30:461–464.
23. Rehermann B, Bertoletti A. 2015. Immunological aspects of antiviral therapy of chronic hepatitis B virus and hepatitis C virus infections. Hepatology 61:712–721.
24. Chehimi J, Campbell DE, Azzoni L, Bacheller D, Papasavvas E, Jerandi G, Mounzer K, Kostman J, Trinchieri G, Montaner LJ. 2002. Persistent decreases in blood plasmacytoid dendritic cell number and function despite effective highly active antiretroviral therapy and increased blood myeloid dendritic cells in HIV-infected individuals. J Immunol 168:4796–4801.
25. Cha L, Berry CM, Nolan D, Castley A, Fernandez S, French MA. 2014. Interferon-alpha, immune activation, and immune dysfunction in treated HIV infection. Clin Transl Immunol 3:e10.
26. Negishi H, Ohba Y, Yanai H, Takaoka A, Honma K, Yui K, Matsuyama T, Taniguchi T, Honda K. 2005. Negative regulation of Toll-like-receptor signaling by IRF-4. Proc Natl Acad Sci U S A 102:15989–15994.
27. Sanchez-Martinez D, Krzywinska E, Rathore MG, Saumet A, Cornillon A, Lopez-Royuela N, Martinez-Lostao L, Ramirez-Labrada A, Lu ZY, Rossi JF, Fernandez-Orth D, Escorza S, Anel A, Lecellier CH, Pardo J, Villalba M. 2014. All-trans retinoic acid (ATRA) induces miR-23a expression, decreases CTSC expression and granzyme B activity leading to impaired NK cell cytotoxicity. Int J Biochem Cell Biol 49:42–52.
28. Karimi M, Cao TM, Baker JA, Verneris MR, Soares L, Negrin RS. 2005. Silencing human NKG2D, DAP10, and DAP12 reduces cytotoxicity of activated CD8+ T cells and NK cells. J Immunol 175:7819–7828.
29. Wek RC, Jiang HY, Anthony TG. 2006. Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans 34:7–11.
30. Shehata HM, Murphy AJ, Lee MKS, Gardiner CM, Crowe SM, Sanjabi S, Finlay DK, Palmer CS. 2017. Sugar or fat? Metabolic requirements for immunity to viral infections. Front Immunol 8:1311.
31. Gardiner CM, Finlay DK. 2017. What fuels natural killers? Metabolism and NK cell responses. Front Immunol 8:367.
32. Forero A, Moore PS, Sarkar SN. 2013. Role of IRF4 in IFN-stimulated gene induction and maintenance of Kaposi sarcoma-associated herpesvirus latency in primary effusion lymphoma cells. J Immunol 191:1476–1485.

33. Wieland S, Thimme R, Purcell RH, Chisari FV. 2004. Genomic analysis of the host response to hepatitis B virus infection. Proc Natl Acad Sci U S A 101:6669–6674.

34. de Groen RA, McPhee F, Friborg J, Janssen HL, Boonstra A. 2014. Endogenous IFNλ in viral hepatitis patients. J Interferon Cytokine Res 34:552–556.

35. Hajarizadeh B, Lamoury FM, Feld JJ, Amin J, Keoshkerian E, Matthews GV, Hellard M, Dore GJ, Lloyd AR, Grebely J, Applegate TL. 2016. Alanine aminotransferase, HCV RNA levels and proinflammatory and pro-fibrogenic cytokines/chemokines during acute hepatitis C virus infection. Virol J 13:32.

36. Jain MK, Adams-Huet B, Terekhova D, Kushner LE, Bedimo R, Li X, Holodniy M. 2015. Acute and chronic immune biomarker changes during interferon/ribavirin treatment in HIV/HCV-coinfected patients. J Viral Hepat 22:25–36.

37. van den Dries L, Claassen MAA, Groothuismink ZMA, van Gorp E, Boonstra A. 2017. Immune activation in prolonged cART-suppressed HIV patients is comparable to that of healthy controls. Virology 509:133–139.

38. Spaan M, van Oord G, Kreefft K, Hou J, Hansen BE, Janssen HL, de Knegt RJ, Boonstra A. 2016. Immunological analysis during interferon-free therapy for chronic hepatitis C virus infection reveals modulation of the natural killer cell compartment. J Infect Dis 213:216–223.

39. Hardy GA, Sieg S, Rodriguez B, Anthony D, Asaad R, Jiang W, Mudd J, Schacker T, Funderburg NT, Pilch-Cooper HA, Debernardo R, Rabin RL, Lederman MM, Harding CV. 2013. Interferon-alpha is the primary plasma type-I IFN in HIV-1 infection and correlates with immune activation and disease markers. PLoS One 8:e56527.

40. Miyagi T, Takehara T, Nishio K, Shimizu S, Kohga K, Li W, Tatsumi T, Hiramatsu N, Kanto T, Hayashi N. 2010. Altered interferon-alpha-signaling in natural killer cells from patients with chronic hepatitis C virus infection. J Hepatol 53:424–430.

41. Kleinman CL, Doria M, Orecchini E, Giuliani E, Galardi S, De Jay N, Michienzi A. 2014. HIV-1 infection causes a downregulation of genes involved in ribosome biogenesis. PLoS One 9:e113908.

42. Chang ST, Sova P, Peng X, Weiss J, Law GL, Palermo RE, Katze MG. 2011. Next-generation sequencing reveals HIV-1-mediated suppression of T cell activation and RNA processing and regulation of noncoding RNA expression in a CD4+ T cell line. mBio 2:e00134-11.

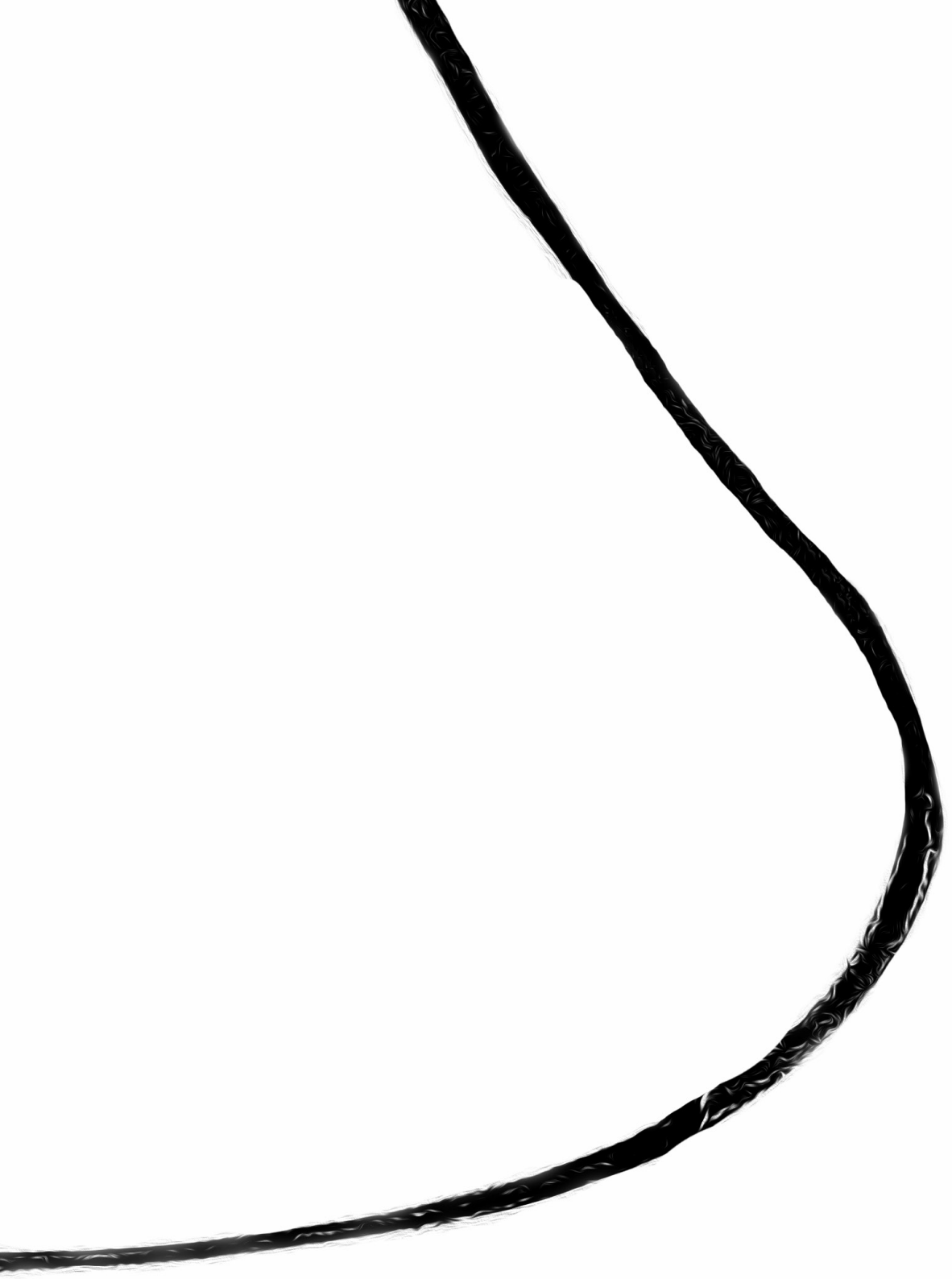
43. Endo-Munoz L, Warby T, Harrich D, McMillan NA. 2005. Phosphorylation of HIV Tat by PKR increases interaction with TAR RNA and enhances transcription. Virol J 2:17.

44. Baird TD, Wek RC. 2012. Eukaryotic initiation factor 2 phosphorylation and translational control in metabolism. Adv Nutr 3:307–321.

45. Guerrero S, Batisse J, Libre C, Bernacchi S, Marquet R, Paillart JC. 2015. HIV-1 replication and the cellular eukaryotic translation apparatus. Viruses 7:199–218.

46. Liu H, Li F, Zhang X, Yu J, Wang J, Jia J, Yu X, Shen Z, Yuan Z, Zhang X, Zhang Z, Zhang X, Lu L, Li H, Lu M, Zhang J. 2018. Differentially expressed intrahepatic genes contribute to control of hepatitis B virus replication in the inactive carrier phase. J Infect Dis 217:1044–1054.

47. Tjwa ET, Zoutendijk R, van Oord GW, Boeijen LL, Reijnders JG, van Campenhout MJ, de Knegt RJ, Janssen HL, Woltman AM, Boonstra A. 2016. Similar frequencies, phenotype and activation status of intrahepatic NK cells in chronic HBV patients after long-term treatment with tenofovir disoproxil fumarate (TDF). Antiviral Res 132:70–75.



4

Gene expression profiling of human tissue-resident immune cells: Comparing blood and liver

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ABSTRACT

In this study, we describe a method to reliably characterize intrahepatic leukocyte populations using flow cytometry and next-generation RNA sequencing on fresh human liver biopsies. Over the last decades, immune responses of viral hepatitis patients, and of other liver diseases, have been incompletely characterized. Most studies include peripheral blood samples only, foregoing the possibility to investigate the site of inflammation directly. Here, we show that with an optimized protocol that combines cell sorting and RNA sequencing, we can perform a side by side comparison of both intrahepatic and peripheral immune cells. Using core liver biopsies from chronic hepatitis B virus patients, we show that the expression levels of IFN-stimulated genes and leukocyte-specific genes are markedly different in the liver compartment as compared to the peripheral blood. These observations emphasize the need to sample the liver directly. The variation of gene expression profiles in these chronic hepatitis B patients was considerable, despite the uniform treatment with nucleotide analogs and absence of liver inflammation in these patients. Finally, we show that this method can provide a detailed characterization of previously undetected liver-specific effects of novel candidate therapeutic compounds.

INTRODUCTION

Our knowledge on immune processes in diseased tissues is still incomplete. The main reason for this is that sampling of many diseased organs is not routinely performed for research purposes, and because blood is the most accessible compartment, the more invasive procedure to obtain tissue is less frequently conducted. This is very clear in case of infections of the liver with hepatitis B virus (HBV) or hepatitis C virus: most studies have examined peripheral blood. Importantly, the few studies that examined the liver demonstrated that the phenotype and function of intrahepatic immune cells differ considerably to that of their peripheral counterparts. [1-5] In recent years, an increasing number of viral hepatitis researchers are describing efforts to fill this gap of knowledge, and are in agreement that a better understanding of liver immunology is crucial for the development of new curative antiviral treatment strategies for chronic hepatitis B.1, [5-7] Unfortunately, progress in the field of liver immunology is hampered by practical and technical difficulties.6 Core liver biopsies are rarely collected for research purposes only, because the associated complication rate is only accepted if the biopsy assists clinical decision making.8 Also, with the expanding use of noninvasive tests of liver disease, such as fibroscan,[9, 10] rest material from liver biopsies is less frequently available. Finally, not all clinical units have a research laboratory able to receive and process liver tissue within hours, which is essential because liver cells are prone to die from apoptosis.[11, 12] Because the number of liver biopsies available is limited, it is essential to make optimal use of all material for research purposes. In the current study, we therefore examined the possibility to combine cell sorting of lymphocytes from liver biopsies by flow cytometry with RNA sequencing to obtain gene expression profiles of liver-resident leukocytes. To do this, we used liver biopsies of 6 patients with chronic hepatitis B who were on continuous nucleotide analog therapy (**Supplementary Table 1**). A number of groups have reported on the evaluation of liver material collected by fine needle aspiration using a [22–25] Gauge needle for flow cytometry or bulk gene expression profiling.[13, 14] In this study, we aimed to combine these approaches and purify T cell and NK cells from liver biopsies, followed by next-generation sequencing of the RNA isolated from these cells, to increase the information gained from biopsies during future immunological studies.

MATERIALS AND METHODS

Collection of liver biopsies and blood samples

We collected core liver biopsies (18 Gauge) of 6 patients chronically infected with HBV and treated with nucleotide analog therapy. Biopsies were collected in 10 ml PBS + 1% FCS in a 15 ml tube, and kept on ice. The liver biopsy tissue was homogenized by carefully mashing the biopsy using the plunger of a 1 ml syringe in a 48 well flat bottom plate. PBS + 1% FCS was added until a 200

µl cell suspension was formed. Erythrocyte lysis was not performed and only fresh material was used (no cryopreservation). All biopsies were taken between 9–11 AM and processed for FACS analysis and sorting within the hour. Rapid processing and analysis was crucial to minimize cell death. Patients were monitored for at least 2 h following biopsy collection and no complications related to the liver biopsies were recorded. Heparinized blood was collected from all patients immediately prior to the biopsy procedure. PBMC were isolated by ficoll separation (Ficoll-Paque™ plus, GE Healthcare Life Sciences, Amersham, United Kingdom), counted and 106 cells stained using the same antibody panel as the liver biopsies.

FACS sorting procedure

Intrahepatic and peripheral leukocytes were sorted using the FACS Aria II (BD, Franklin Lakes, New Jersey, United States) equipped with a 130 µm nozzle. Blood and liver samples were immunophenotyped and various leukocyte populations were subsequently sorted for gene expression analysis by RNA sequencing, using the gating strategy depicted in **Figure 1A** and **B**. We selected CD235a-negative, CD45-positive cells (non-erythrocyte, all leukocytes) cells, followed by further gating on forward scatter (FSC) and sideward scatter (SSC), positivity for CD56 or CD3 (**Figure 1**), and exclusion of dead cells (5–10% in the liver biopsies) and doublets (using FSC-W/SSC-W and FSC-H/SSC-H). For more detailed and FACS plots (representative patient 6) we refer to the **Supplementary Figure 1A** (liver biopsy, before GS-9620 treatment), 1B (liver biopsy, after GS-9620 treatment), 1C (PBMC, before GS-9620 treatment) and 1D (PBMC, after GS-9620 treatment). These flow cytometric data plots also further illustrate that MAIT cell and NK cell percentages differ considerably between the liver and blood compartment in the absence of blood contamination. This sorting strategy resulted in T and NK cells with purities of at least 95%. The following populations were purified (**Figure 1C**): CD45+CD3+ (T cells) and CD45+CD56+ (total NK cells) using CD3:PeCy7 (clone UCHT1, eBioscience, Waltham, Massachusetts, United States), CD8:APC-H7 (clone SK1, BD Biosciences), Live/Dead:AmCyan (Miltenyi Biotec), CD45:PE-eFluor610 (eBioscience, HI30), and CD235a:FITC (clone HIR2, eBioscience).

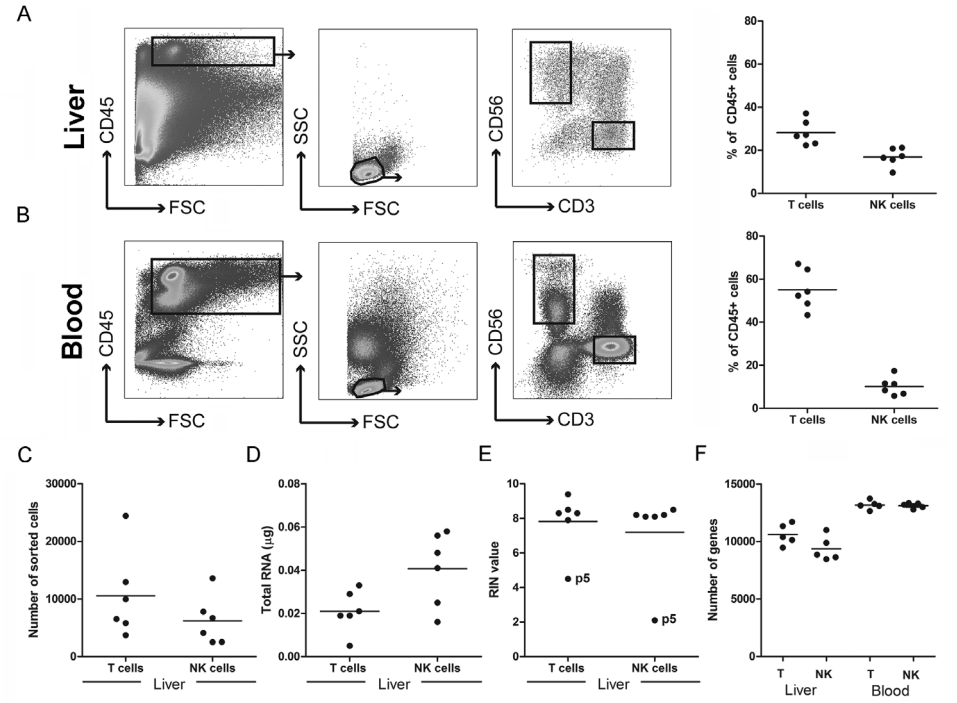

Figure 1. Description of the gating strategy and quality control steps for isolated mRNA derived from liver immune cell populations. (A) FACS plots showing the gating strategies for liver T cells (CD45+CD3+CD56- lymphocytes), and NK cells (CD45+CD3-CD56+ lymphocytes) of 6 chronic HBV patients treated with nucleotide analogs. The panel on the right-hand side shows the T cell and NK cell frequencies of total intrahepatic CD45+ cells. (B) The gating strategy for peripheral blood T cells and NK cells, including the T cell and NK cell frequencies of the total blood CD45+ population (right panel). (C) The absolute number of intrahepatic T and NK cells sorted by flow cytometry. (D) The total amount of RNA obtained from the sorted cells. (E) The RNA integrity number (RIN value) of the RNA samples. (F) The total number of unique genes identified in T cells and NK cells from the 5 liver biopsies passing quality control, in comparison to blood-derived T and NK cells

RNA isolation

RNA was isolated using Arcturus® PicoPure® RNA Isolation Kit according to the manufacturer instructions: Briefly, sorted cells were centrifuged (3000 × g, 9 min), the supernatant was discarded, and the pellet incubated for 30 min with extraction buffer. Ethanol (70%) was pipetted onto the supernatant and the cell extract and ethanol mixture was then pipetted onto the prepared purification column and centrifuged twice (2 min at 100 × g, 30 s at 16,000 × g). DNA was removed using by DNAse treatment. RNA integrity was measured using Eukaryote Total RNA Pico assay (Agilent Technologies Inc, Santa Clara, California, United States). Technical information

on the RNA sequencing of purified leukocyte populations, the alignment of short reads to the genome and, the analysis of expression of genes and pathways can be found in the Extended Methods in the Supplementary Information.

Clinical trial

This study was part of the international trial investigating the safety, tolerability, and efficacy of vesatolimod (GS-9620) in patients with chronic hepatitis B on nucleotide analog therapy (Clinical Trial Number: GS-US-283-1059; NCT 02166047).15 The liver biopsies (18 Gauge) used for the current study were performed at baseline and 24 h after the last eighth dose of vesatolimod. Vesatolimod (GS-9620) is an oral agonist of TLR7. It is currently under investigation for the treatment of both HBeAg-positive and negative chronic HBV. Our center was the only site to perform liver immune cell isolation (in tandem with PBMC isolation), using an 18 Gauge needle, before and after vesatolimod treatment, and on continuous nucleotide analog therapy. Six noncirrhotic virally suppressed chronic hepatitis B patients on nucleotide analog therapy (age: 29–63 years; 4 males and 2 females) were treated once a week for 7 weeks with 1 mg GS-9620 (n = 3), 2 mg GS-9620 (n = 1), 4 mg GS-9620 (n = 1), or placebo (n = 1). Blood was collected from the patients prior (week 0) and at 4, 8, 12, 24, and 48 weeks after start of GS-9620 treatment.

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.

RESULTS AND DISCUSSION

As expected, the frequency of NK cells was higher in the liver as compared to the blood (**Figure 1A** and B), while the opposite was observed for T cells. Surprisingly, a significant percentage of the CD45+ liver immune cells were not identified as T cells or NK cells (**Figure 1A**) and may reflect the higher fraction of CD3+CD56+ NKT cells (16.5% vs. 2.9%) and the relative enrichment of B cells and myeloid cells in the liver. All samples that passed the quality control were analyzed further. In this project, bulk CD3+ T cells were sorted to map general gene expression of T cells in liver and blood, but because the frequencies of CD4+/CD8+ T cells usually differ in liver and blood, sorting T cell subset separately would likely be more informative. The numbers of genes detected were higher in the peripheral blood-derived immune cells, as compared to liver-derived T cells and NK cells (**Figure 1F**), which is likely due to higher input of sorted T and NK cells (average of 1,609,073 vs. 11,242 T cells and 389,820 vs. 7289 NK cells in blood vs. liver, respectively). Ninety-

five percent of the genes detected in liver-resident NK cells and T cells overlapped with their peripheral counterpart (data not shown). The transcriptional profiles of T and NK cells in the liver biopsies, and in the blood, were evaluated for expression of IFN-stimulated genes (ISG; **Figure 2A** and **B**, **Supplementary Table 2**), T cell genes (**Supplementary Table 3**, published online) and NK cell genes (**Figure 2C**, **Supplementary Table 4**, published online). It should be noted here that for a relevant assessment of clinical parameters of the patients included, a larger cohort of patients would be necessary. As previously described at the protein level in both cellular compartments, the immune profiles in blood and liver of chronic hepatitis B patients exhibit considerable variation2, 16: only small gene clusters (<20 genes) were modulated in the same direction in 2 individual patients (**Figure 2**). We observed less variation in the blood compared to the liver, which emphasizes the need for sampling of the liver, preferably using multiple measurements. In sequential biopsies from a single individual, the frequency of liver-resident lymphocyte populations, including T cells and NK cells, did not vary >2% between biopsies, confirming the reproducibility of the protocol used in our study. All RNA samples derived from T cells and NK cells had good RNA integrity scores, with RIN (RNA integrity number) values of 7 or higher (**Figure 1D** and **E**). In the current study, a similar pattern of ISG induction could be observed in T cells and NK cells in liver for each patient, suggesting that these intrahepatic lymphocyte populations are exposed to the same signals (such as type I IFNs), and possibly regulate the transcription of ISG in a similar way. The expression of T cell genes in CD3+ cells in the liver (**Figure 2C**) was different from the blood, as was the expression of NK cell genes in NK cells. In addition to type I IFNs and lymphocyte specific genes, we detected several genes involved in T or NK cell-mediated immune responses (**Table 1**). Of these genes, a few were identified to be highly expressed in the liver T cells (CXCR6, TNF, CD160, CCR5, and IFNG; blood/liver ratio 0.03, 0.04, 0.06, 0.06, and 0.07, respectively) and NK cells (CXCR6, CCL3L3, and TNFSF14; blood/liver ratio 0.015, 0.09, and 0.13, respectively). This observation corresponds to previous reports17 that liver-resident NK cells, but not peripheral blood leukocytes, express CXCR6, as well as high levels of Eomes,18 but low levels of T-bet (blood/liver ratio EOMES 0.2, TBX21 2.3). Interestingly, we detected high expression of genes coding for TNF receptor ligands (**Table 1**), like TNF and FASLG (liver T cells), as well as TNFSF14 (liver NK cells). These genes were among the most liver-enriched genes in these HBV patients, which may be related to earlier reports of their role in initiation of fibrogenesis.19

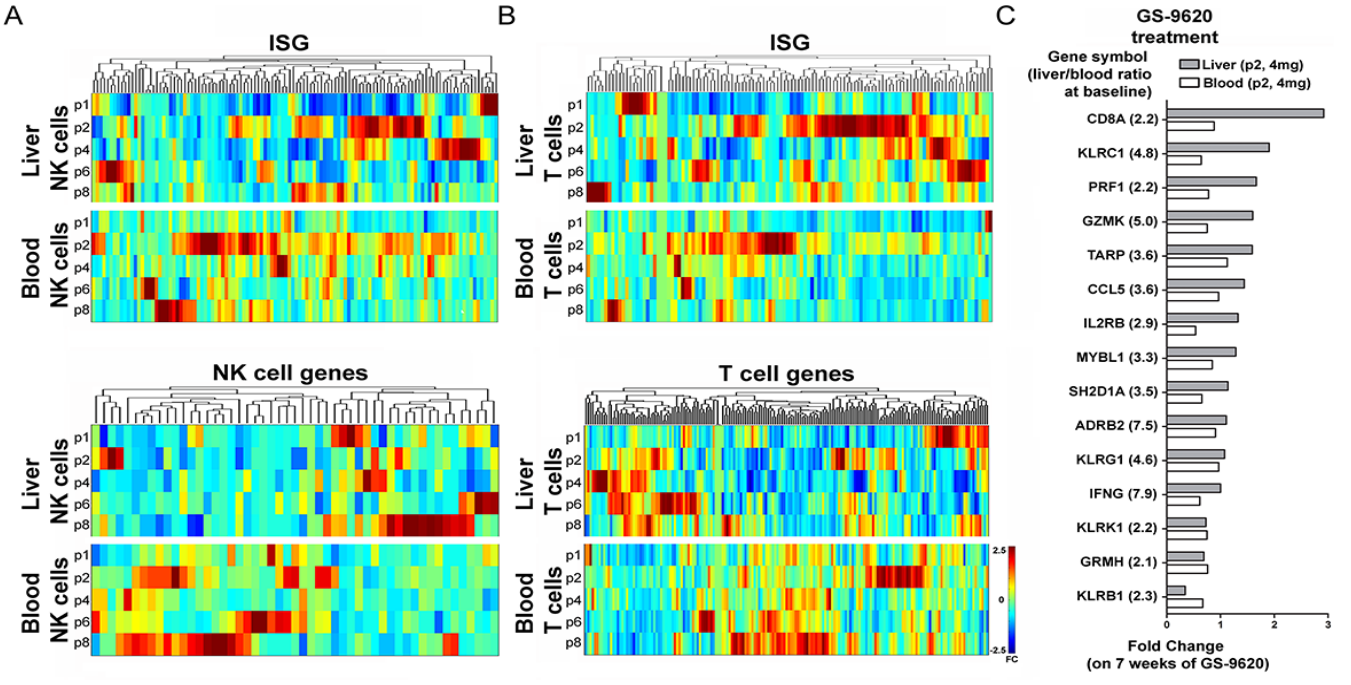

Figure 2. Relative expression of ISG and cell type specific genes in liver and blood of virally suppressed patients with chronic hepatitis B. (A) The relative baseline expression level of ISG (upper panel) and NK cell genes (lower panel) in liver and peripheral blood NK cells of 5 nucleotide analog-treated chronic HBV patients. The colors represent a range of fold difference from −2.5 (blue) to 2.5 (red). (B) The relative baseline expression of ISG in T cells of 5 nucleotide analog-treated chronic HBV patients is shown in the upper panel and of T cell genes in the lower panel. (C) The modulation of ISG expression after 7 weeks of treatment with GS-9620 (Vesatolimod), evaluated longitudinally in T cells isolated from liver biopsies in 1 virally suppressed chronic HBV patient (patient 2, treated with 4 mg Vesatolimod weekly). Vesatolimod (GS-9620) is a TLR7 agonist under investigation for treatment of chronic HBV. The 15 genes whose expressions are most specific to the liver at baseline (before Vesatolimod treatment) are shown, as identified by calculating the liver/blood ratio of expression levels in both compartments. The FC before and after treatment in liver (grey bars) and blood (white bars) is shown for each individual gene, revealing liver-specific treatment effects. FC; fold change

Table 1. The top liver-enriched genes in T cells and NK cells derived from blood and liver during virally suppressed HBV

T cells				NK cells			
Rank	**Gene**	***P*-value**	**Ratio blood/liver**	**Rank**	**Gene**	***P*-value**	**Ratio blood/liver**
1	**CXCR6**	0.0006	0.02869	1	**CXCR6**	0.00001	0.01512
2	**TNF**	0.00001	0.04143	2	SPRY2	0.00006	0.02319
3	**CD160**	0.0007	0.0593	3	EXPH5	0.01919	0.08083
4	**CCR5**	0.00002	0.05955	4	CCL3L3	0.00219	0.08839
5	SLC4A10	0.00801	0.05977	5	LDB2	0.00011	0.09216
6	**IFNG**	0.00024	0.07045	6	SLC4A10	0.00615	0.09528
7	B3GALT2	0.00211	0.07435	7	DET1	0.02772	0.10272
8	**FASLG**	0.00008	0.0766	8	IGIP	0.00241	0.10989
9	MS4A1	0.00014	0.09095	9	MBOAT1	0.00188	0.12441
10	CMTM1	0.00208	0.10766	10	**TNFSF14**	0.00479	0.13107

Finally, using pathway analysis, we aimed to identify the degree of pathway activation in our samples, and compare the blood and liver compartment (**Table 2**). In liver T cells, the NK-cell-mediated cytotoxicity, hepatitis B and Th1 and Th2 differentiation pathway are relatively active, which was expected given the more direct exposure to the virally infected liver tissue and increased T cell differentiation state in most tissues. The oxidative phosphorylation genes were active in T cells and NK cells in the peripheral blood, which may be due to its superior oxygenation compared to the portal blood entering the liver sinusoids. In both T cells and NK cells, cell cycle genes (and DNA Mismatch repair genes) have low expression in the blood suggesting that these cells proliferate slightly faster in the liver. Interestingly, in NK cells in the blood, the pathways related to direct NK cell effector functions, like Lysosome and NK cell mediated cytotoxicity, were more active, possibly reflective of the immune suppressive environment of the liver.

Table 2. Relative activation of signaling pathways in T cells and NK cells from blood and liver † percentage of overlap with the original list of genes in that pathway to estimate the degree of representation of each pathway in every sample. FC; fold change.

Pathway name	**Genes in pathway (total)**	**Blood CD3 FC**	**Liver CD3 FC**	**Blood NK FC**	**Liver NK FC**
Glycolysis / gluconeogenesis	68 (100%)	**2.8** (64%†)	**2.5** (58%)	−2.7 (64%)	−2.2 (51%)
Cell cycle	124 (100%)	0.0 (84%)	**0.9** (77%)	−1.3 (89%)	**1.7** (73%)
Lysosome	123 (100%)	−4.2 (89%)	−4.0 (82%)	**7.3** (88%)	**1.8** (71%)
NK cell-mediated cytotoxicity	133 (100%)	−11.7 (71%)	−3.7 (70%)	**3.6** (77%)	**2.6** (69%)
Oxidative phosphorylation	133 (100%)	**7.2** (68%)	−5.0 (67%)	**4.5** (68%)	−4.3 (62%)
Hepatitis B	144 (100%)	−0.2 (77%)	**1.7** (73%)	**1.6** (79%)	−1.4 (67%)
Th1 and Th2 cell differentiation	92 (100%)	−5.9 (86%)	**1.5** (82%)	**1.6** (85%)	−1.0 (72%)
Mismatch repair	23 (100%)	**2.3** (96%)	−0.5 (88%)	**0.8** (98%)	−1.6 (83%)

In this study, we had the opportunity to obtain a second 18 Gauge core liver biopsy from the same patients at a later time point to map the modulation by immunotherapeutic intervention on the phenotype and gene expression profile of specific lymphocyte populations in addition to baseline gene expression. Analysis of the paired samples showed the modulation of gene expression in T cells from a chronic HBV patient before and after 7 weeks of treatment with 4 mg GS-9620 (Vesatolimod), an oral TLR7 agonist under investigation for the treatment of chronic HBV and may (re-)activate host immune responses to the virus20 (Clinical Trial Number: GS-US-283-1059; NCT 02166047). To detect possible liver-specific effects of the compound, we evaluated the modulation of genes highly enriched in the liver (high liver/blood ratio). In the example depicted in **Figure 2C**, on the gene expression modulation of liver T cells of patient 2, treated with the highest dose of GS-9620 (4 mg), we found CD8A, KLRC1, PRF1, GRMK, and IL2RB (**Figure 2C**) to be up-regulated by GS-9620 treatment in this patient's liver, but not in the blood.

In summary, we describe here a method to investigate intrahepatic leukocyte populations using FACS and next generation RNA sequencing on fresh human liver biopsies directly ex vivo. Fewer liver biopsies are available for research, and more sophisticated methodologies are needed to provide an insight into the pathogenesis of immune-mediated liver disease, including viral hepatitis, non-alcoholic steatohepatitis (NASH)/Non-alcoholic fatty liver disease (NAFLD), and primary sclerosing cholangitis (PSC). The expression of ISG and leukocyte-specific genes are markedly different in the liver compartment as compared to the peripheral blood, emphasizing the need to sample the liver directly. The variation of gene expression profiles in these nucleotide analog-treated chronic hepatitis B patients was considerable, despite undetectable viral loads and the absence of liver inflammation in all included subjects. We show that this method can provide a detailed characterization of the effect of new immune therapeutic compounds on liver-residing leukocyte populations.

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DISCLOSURES

A.B. was advisory board member for Gilead Sciences and has received research support from Gilead Sciences, Fujirebio, BMS, and Roche. L.L.B., G.W.O., J.H., M.H.M., and R.K. have no conflicts of interest. A.G., L.L., and S.F. are employees of Gilead Sciences.

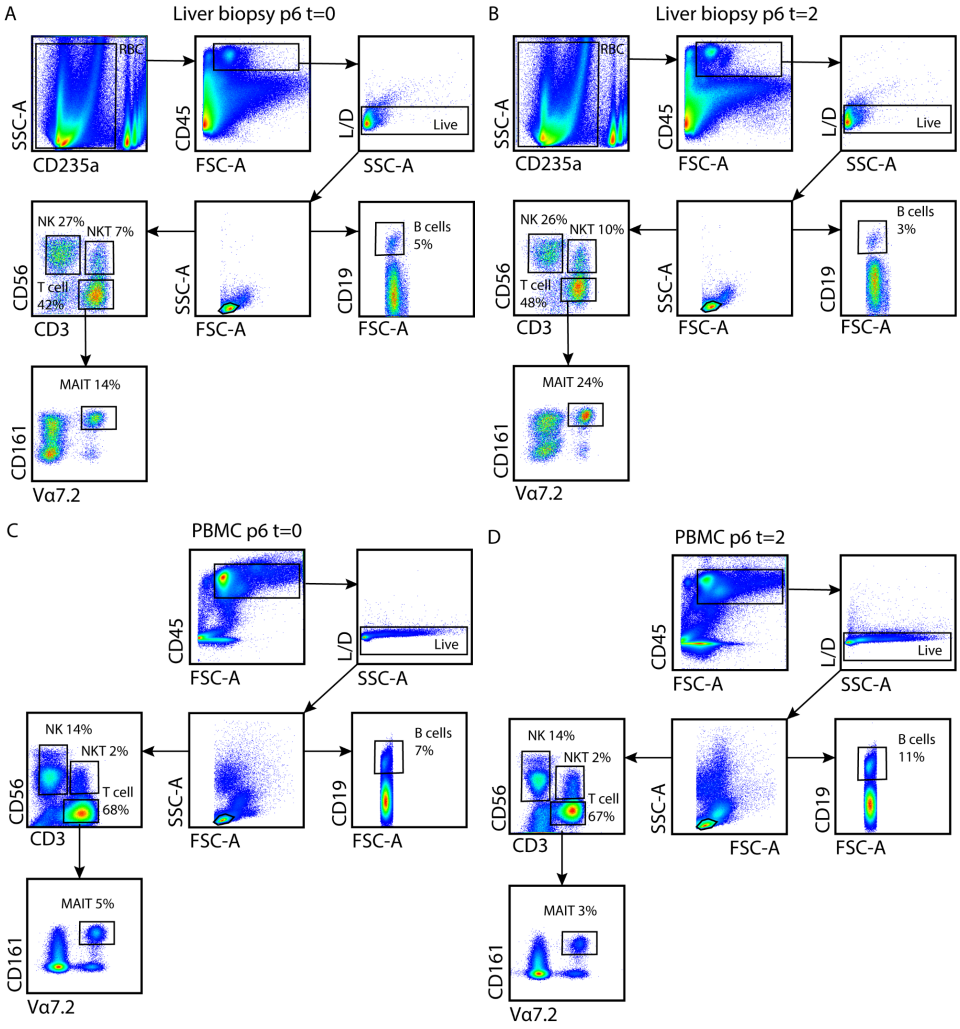
REFERENCES

1. Lebosse F, Testoni B, Fresquet J, et al. Intrahepatic innate immune response pathways are downregulated in untreated chronic hepatitis B. J Hepatol. 2017;66:897–909.
2. Tjwa ET, Zoutendijk R, van Oord GW, et al. Similar frequencies, phenotype and activation status of intrahepatic NK cells in chronic HBV patients after long-term treatment with tenofovir disoproxil fumarate (TDF). Antiviral Res. 2016;132:70–75.
3. Spaan M, Claassen MA, Hou J, Janssen HL, de Knegt RJ, Boonstra A. The intrahepatic T cell compartment does not normalize years after therapy-induced hepatitis C virus eradication. J Infect Dis. 2015;212:386–390.
4. Pallett LJ, Davies J, Colbeck EJ, et al. IL-2(high) tissue-resident T cells in the human liver: sentinels for hepatotropic infection. J Exp Med. 2017;214:1567–1580.
5. Boeijen LL, Hoogeveen RC, Boonstra A, Lauer GM. Hepatitis B virus infection and the immune response: the big questions. Best Pract Res Clin Gastroenterol. 2017;31:265–272.
6. Gill US, Pallett LJ, Kennedy PTF, Maini MK. Liver sampling: a vital window into HBV pathogenesis on the path to functional cure. Gut. 2018;67:767–775.
7. Singh AK, Rooge SB, Varshney A, et al. Global micro RNA expression profiling in the liver biopsies of hepatitis B virus infected patients suggests specific miRNA signatures for viral persistence and hepatocellular injury. Hepatology. 2018;67:1695–1709.
8. Piccinino F, Sagnelli E, Pasquale G, Giusti G. Complications following percutaneous liver biopsy. A multicentre retrospective study on 68276 biopsies. J Hepatol. 1986;2:165–173.
9. Goyal R, Mallick SR, Mahanta M, et al. Fibroscan can avoid liver biopsy in Indian patients with chronic hepatitis B. J Gastroenterol Hepatol. 2013;28:1738–1745.
10. Lipp MJ, D'Souza LS, Clain DJ, Jr Bodenheimer HC, Min AD. Trends in the indication and method of liver biopsy for hepatitis B and C. Dig Dis Sci. 2010;55:2971–2976.
11. Castera L. Noninvasive methods to assess liver disease in patients with hepatitis B or C. Gastroenterology. 2012;142:1293–1302.e4.
12. Papatheodoridis GV, Manolakopoulos S, Liaw YF, Lok A. Follow-up and indications for liver biopsy in HBeAg-negative chronic hepatitis B virus infection with persistently normal ALT: a systematic review. J Hepatol. 2012;57:196–202.
13. Demirkiran A, Baan CC, Kok A, Metselaar HJ, Tilanus HW, van der Laan LJ. Intrahepatic detection of FOXP3 gene expression after liver transplantation using minimally invasive aspiration biopsy. Transplantation. 2007;83:819–823.
14. Sprengers D, van der Molen RG, Kusters JG, et al. Flow cytometry of fine-needle-aspiration biopsies: a new method to monitor the intrahepatic immunological environment in chronic viral hepatitis. J Viral Hepat. 2005;12:507–512.
15. Janssen HLA, Brunetto MR, Kim YJ, et al. Safety, efficacy and pharmacodynamics of vesatolimod (GS-9620) in virally suppressed patients with chronic hepatitis B. J Hepatol. 2018;68:431–440.
16. Boeijen LL, Montanari NR, de Groen RA, et al. Mucosal-associated invariant T cells are more activated in chronic hepatitis B, but not depleted in blood: reversal by antiviral therapy. J Infect Dis. 2017;216:969–976.
17. Stegmann KA, Robertson F, Hansi N, et al. CXCR6 marks a novel subset of T-bet(lo)Eomes(hi) natural killer cells residing in human liver. Sci Rep. 2016;6:26157.

18. Cuff AO, Robertson FP, Stegmann KA, et al. Eomeshi NK cells in human liver are long-lived and do not recirculate but can be replenished from the circulation. J Immunol. 2016;197:4283–4291.
19. Yang YM, Seki E. TNFalpha in liver fibrosis. Curr Pathobiol Rep. 2015;3:253–261.
20. Boni C, Vecchi A, Rossi M, et al. TLR7 agonist increases responses of hepatitis B virus-specific T cells and natural killer cells in patients with chronic hepatitis B treated with nucleos(t)ide analogues. Gastroenterology. 2018;154:1764–1777.e7.

SUPPLEMENTARY MATERIAL

- **Supplementary Figure 1ABCD** (Representative flow cytometry plots patient 6)
- **Supplementary Table 1** (Patient characteristics)
- **Supplementary Table 2** (ISG reference list)
- **Supplementary Table 3** (T cell genes list)
- **Supplementary Table 4** (NK cell genes list)
- Extended Methods and supplementary references



Supplementary Figure 1

Supplementary Table 1: summary of chronic HBV patient characteristics

Patient*	Age	Gender	Ethnicity	Fibrosis grade**(Metavir)	HBV genotype	HBeAg
1	53	M	Asian	F0-F1	A	-
2	59	M	Asian	F0-F1	A	-
4	46	F	Asian	F0-F1	B	+
5	63	M	African	F0-F1	E	-
6	29	M	Asian	F0	C	-
8	45	F	Caucasian	F0-F2	unknown	-

*Patient number was based on order of screening.

**Fibrosis grade was measured using transient elastography or liver biopsy.

Abbreviations HBeAg; hepatitis B envelope antigen.

Supplementary Table 2 (ISG): the 139 Interferon Stimulated Genes (ISGs) used as a reference list in this study.

ISG									
1	SEPT2	33	GBP2	65	LGALS3BP	97	PSMB9	129	TRIM56
2	ABCA1	34	GBP3	66	LGALS9	98	RBCK1	130	TRIM6
3	ACTA2	35	GBP4	67	LHFPL2	99	REC8	131	TRIMP1
4	ADAR	36	GBP5	68	LOC129607	100	RHBDF2	132	UBE2L6
5	AIM2	37	GBP6	69	LOC26010	101	RSAD2	133	UNC93B1
6	APOL6	38	HERC5	70	LOC400759	102	RTP4	134	WARS
7	ATF3	39	HERC6	71	LOC401433	103	SAMD9	135	XAF1
8	BATF2	40	HES4	72	LOC554203	104	SAMD9L	136	ZBP1
9	BST2	41	HSH2D	73	LY6E	105	SASP	137	ZC3HAV1
10	BTN3A1	42	IFI16	74	MDK	106	SCO2	138	ZNF684
11	C1QA	43	IFI35	75	MOV10	107	SERPING1	139	ZNFX1
12	CASP1	44	IFI44	76	MT1A	108	SOCS1		
13	CCL8	45	IFI44L	77	MT2A	109	SP100		
14	CEACAM1	46	IFI6	78	MX1	110	SP110		
15	CHMP5	47	IFIH1	79	NBN	111	SP140		
16	COP1	48	IFIT1	80	NCOA7	112	SRBD1		
17	CPT1B	49	IFIT2	81	NMI	113	STAT1		
18	CXCL10	50	IFIT3	82	NT5C3	114	STAT2		
19	DDX58	51	IFIT5	83	NTNG2	115	TAP1		
20	DHRS9	52	IFITM1	84	OAS1	116	TAP2		
21	DHX58	53	IFITM3	85	OAS2	117	TCN2		
22	DRAP1	54	INCA	86	OAS3	118	TDRD7		
23	DYNLT1	55	INDO	87	OASL	119	TIMM10		
24	ECGF1	56	IRF4	88	OTOF	120	TMEM140		
25	EIF2AK2	57	IRF7	89	PARP10	121	TNFAIP6		
26	EPSTI1	58	ISG15	90	PARP12	122	TNFSF10		
27	ETV7	59	ISG20	91	PARP14	123	TRAFD1		
28	FBXO6	60	ISGF3G	92	PARP9	124	TRIM21		
29	FLJ20035	61	KIAA1618	93	PHF11	125	TRIM22		
30	GADD45B	62	LAMP3	94	PLSCR1	126	TRIM25		
31	GALM	63	LAP3	95	PML	127	TRIM38		
32	GBP1	64	LBA1	96	PRIC285	128	TRIM5		

Supplementary Table 3 (T cell genes): a reference list of 177 genes associated with T cell function, used as a reference list in this study.

1	AAK1	41	CST7	81	IL6R	121	PCSK5	161	TARP
2	ACTN1	42	CTLA4	82	IL6ST	122	PCYT2	162	TBX21
3	ACVR2B	43	CTSB	83	IL7R	123	PDCD1	163	TCF7
4	ADA	44	CTSW	84	INPP4A	124	PDE4D	164	TIAM1
5	ADRB2	45	DNAJB1	85	INPP4B	125	PDE9A	165	TNFAIP3
6	ANK3	46	DNASE1L3	86	ITGA6	126	PIK3R1	166	TNFRSF25
7	ANXA1	47	DOCK9	87	ITK	127	PIM1	167	TNFSF8
8	APBA2	48	DPP4	88	ITM2A	128	PLXDC1	168	TNIK
9	AQP3	49	DUSP16	89	ITPKB	129	PRF1	169	TOB1
10	ATP1A1	50	DUSP2	90	KLRB1	130	PRKCA	170	TRA@
11	BAG3	51	FBLN5	91	KLRC1	131	PRKCI	171	TRERF1
12	BCL11B	52	FCGBP	92	KLRG1	132	PRKCQ	172	TXK
13	BIN2	53	FHIT	93	KLRK1	133	PTGER2	173	UPP1
14	BUB1B	54	FLT3LG	94	LAT	134	PXN	174	VIPR1
15	C1orf21	55	FYB	95	LCK	135	RAB43	175	WNT10B
16	C20orf112	56	FYN	96	LCP2	136	RARRES3	176	WWP1
17	CAMK4	57	GABARAPL1	97	LDHA	137	RASGRP1	177	ZAP70
18	CCL5	58	GALT	98	LDHB	138	RBMS1		
19	CCND2	59	GATA3	99	LEF1	139	RGS10		
20	CCR7	60	GBP1	100	LEPROTL1	140	RORA		
21	CD2	61	GBP2	101	LPIN2	141	RUNX2		
22	CD247	62	GFI1	102	LRIG1	142	S100A10		
23	CD27	63	GIMAP2	103	LTBP4	143	S100A8		
24	CD28	64	GIMAP4	104	LYAR	144	S100B		
25	CD3D	65	GNLY	105	MAL	145	SATB1		
26	CD3E	66	GPR56	106	MAN1C1	146	SELPLG		
27	CD3G	67	GPSM3	107	MAPKAPK5	147	SEMA4D		
28	CD4	68	GZMB	108	MAST4	148	SH2D1A		
29	CD40	69	GZMH	109	MATN2	149	SHFM1		
30	CD40LG	70	GZMK	110	MEN1	150	SLC35D2		
31	CD5	71	HAVCR2	111	MLLT3	151	SLC39A8		
32	CD6	72	HOXB2	112	MPP7	152	SLCO3A1		
33	CD69	73	HSPA1L	113	MXI1	153	SNPH		
34	CD80	74	ID2	114	MYBL1	154	SOCS3		
35	CD8A	75	IDO1	115	NELL2	155	SORL1		
36	CD8B	76	IFITM1	116	NGFRAP1	156	SPOCK2		
37	CDC14A	77	IFNG	117	NPDC1	157	STAT4		
38	CDC25B	78	IL18R1	118	NPTXR	158	SYNE2		
39	CDR2	79	IL2RA	119	NR4A2	159	SYT1		
40	CISH	80	IL2RB	120	OPTN	160	TACC3		

Supplementary Table 4 (NK cell genes): a reference list of 59 genes associated with NK cell function, used as a reference list in this study.

1	ADIPOQ	41	NCR3
2	CCL4	42	NCR3LG1
3	CD160	43	PRF1
4	CD1D	44	PTPRC
5	CD244	45	Pmaip1
6	CD38	46	RAB27A
7	CD40LG	47	RAET1G
8	CD48	48	SH2D1A
9	CISH	49	SLAMF6
10	CSF2RA	50	SMAD3
11	CSF2RB	51	STX11
12	CX3CR1	52	SYK
13	DICER1	53	TGFB1
14	ETS1	54	TNF
15	FCGR2A	55	TYK2
16	FCGR3A	56	ULBP2
17	FCGR3B	57	UNC13D
18	FSTL1	58	VAV1
19	HLA-A	59	ZAP70
20	HLA-B		
21	HLA-C		
22	IFNG		
23	IGHG1		
24	IKBKG		
25	IL12A		
26	IL12B		
27	IL15		
28	IL15RA		
29	IL18		
30	IL2		
31	IL21		
32	JAK1		
33	JAK3		
34	KIR3DL1		
35	KLF4		
36	KLRB1		
37	KLRC4		
38	KLRK1		
39	NCR1		
40	NCR2		

EXTENDED METHODS

RNA-sequencing of purified leukocyte populations

Multiplexed libraries were prepared from 2 ng RNA from each sample. All samples included were analyzed using the SMARTer library protocol. RNA-Seq was conducted by EA Genomics (USA). Briefly, using a cDNA input of 1ng, sequencing libraries were created using the Clontech SMART-Seq v4 kit together with the Illumina Nextera XT Library Preparation Kit and sequenced on Illumina HiSeq systems (50bp paired-end sequencing and read depth of 30M reads per sample). Low quality reads, bases, homopolymers, and adapter sequences were removed based on high frequency, clipping of N's at either ends, low average Q score the following rules: 1) Any read that had one particular base at ≥ 95% frequency was removed (homopolymer filtering); 2) Any read with more than 4 Ns within its sequence was removed (filtered). N's at either end of reads are clipped; 3) Any read with an average Q score below 25was removed; 4) Nucleotides with a quality score below 7 at the end of reads were clipped; 5) Sequence corresponding to Illumina sequencing adapters were clipped; 6) If the read resulting from steps 4 or 5 is shorter than 25 nt in length, it was removed.

Alignment of short reads to genome

Clean reads were aligned to human transcriptome using STAR v2.4 with default parameters[6]. Post-alignment, the resulting bam file was fed into the RNA-Seq quantification software RSEM10, version 1.2.14. RSEM uses statistical modeling to determine how best to account for reads that align to multiple genes and/or isoforms. Gene expression was quantified with reads mapped to exonic regions and in a non-strand specific manner.

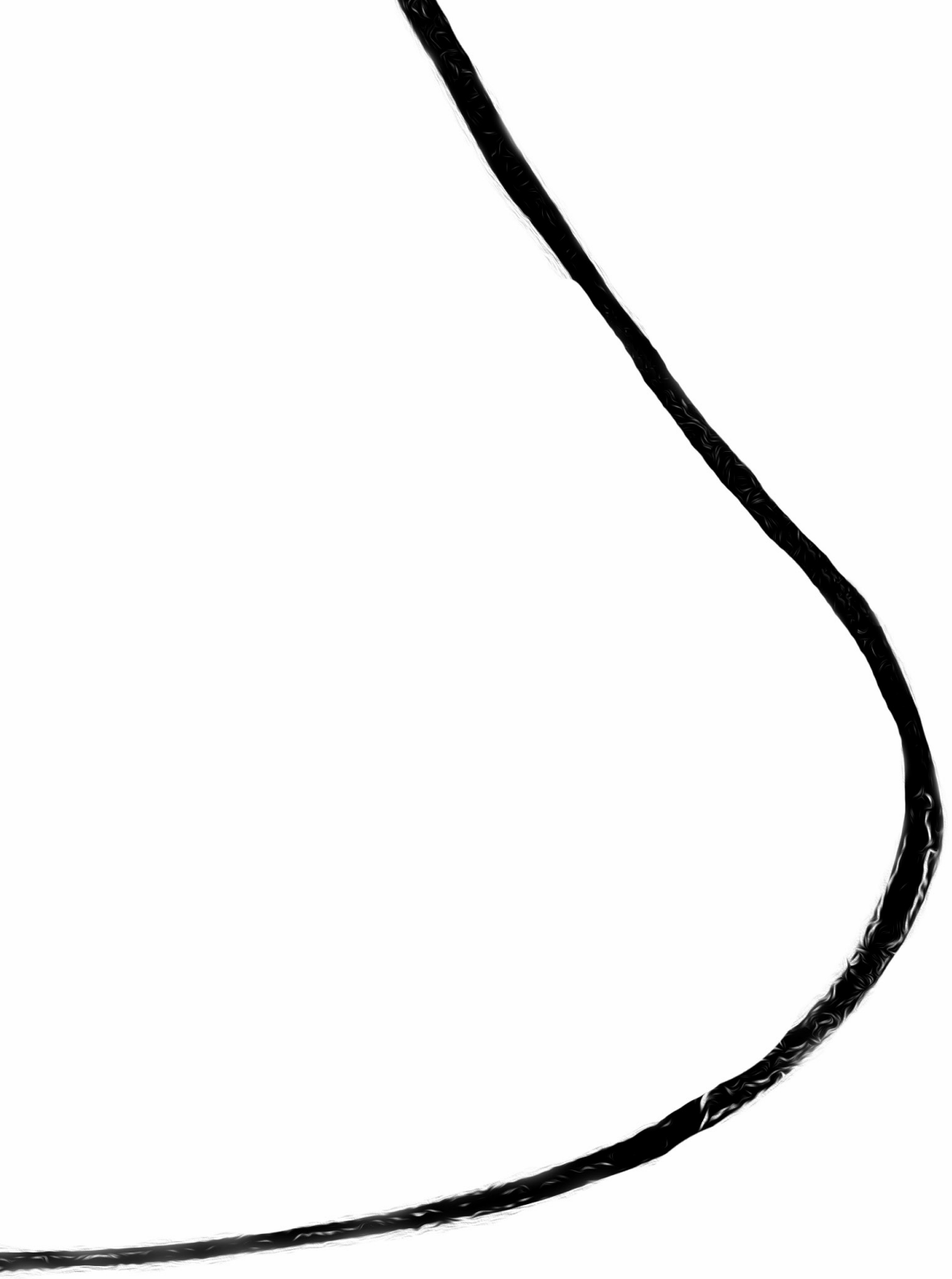
Analysis of expression of genes and pathways

The numbers of detected genes in individual samples were counted as follows: genes with more than 3 reads aligned were counted; and genes with more than one exonic regions/ transcripts aligned by more than 3 reads were counted only once. Lists of genes associated with T cell (**Supplementary Table 3**) and NK cell (**Supplementary Table 4**) activation and function, as well as interferon stimulated genes (ISG, **Supplementary Table 2**) were composed prior to the analysis. Genes were included based on known correlation between gene expression and cell activation/function in at least one published study [1] 2) the correlation was documented in one of molecular interactions databases, including KEGG (http://www.kegg.jp), Ingenuity, Gene Ontology, BioCarta; 3) the gene was differentially expressed in related studies[2-5]. The expression of these gene groups in liver and PBMC was estimated and compared between liver and PBMC. In addition, we analyzed the activation of pathways of interest. Ten signaling pathways were selected based on known relevance for NK/T cell mediated immune responses during viral hepatitis or for general lymphocyte function. The relative activation of each pathway

was determined by comparison of its expression in samples of the same type and origin. For each sample, we verified that the lower number of genes identified in liver samples (**Figure 1E**) did not impact our analysis. The percentage overlap with the original list of genes for each individual pathway was analyzed in each sample (**Table 2**). Pathways with less than 50% of their individual genes expressed in our samples were not considered to be representative for the complete pathway and were excluded.

SUPPLEMENTARY MATERIAL REFERENCES

1. Obermoser, G., Presnell, S., Domico, K., Xu, H., Wang, Y., Anguiano, E., Thompson-Snipes, L., Ranganathan, R., Zeitner, B., Bjork, A., Anderson, D., Speake, C., Ruchaud, E., Skinner, J., Alsina, L., Sharma, M., Dutartre, H., Cepika, A., Israelsson, E., Nguyen, P., Nguyen, Q. A., Harrod, A. C., Zurawski, S. M., Pascual, V., Ueno, H., Nepom, G. T., Quinn, C., Blankenship, D., Palucka, K., Banchereau, J., Chaussabel, D. (2013) Systems scale interactive exploration reveals quantitative and qualitative differences in response to influenza and pneumococcal vaccines. Immunity 38, 831-44.
2. Hou, J., Brouwer, W. P., Kreefft, K., Gama, L., Price, S. L., Janssen, H. L. A., French, P. J., Vanwolleghem, T., Boonstra, A. (2017) Unique intrahepatic transcriptomics profiles discriminate the clinical phases of a chronic HBV infection. PLoS One 12, e0179920.
3. Bezman, N. A., Kim, C. C., Sun, J. C., Min-Oo, G., Hendricks, D. W., Kamimura, Y., Best, J. A., Goldrath, A. W., Lanier, L. L., Immunological Genome Project, C. (2012) Molecular definition of the identity and activation of natural killer cells. Nat Immunol 13, 1000-9.
4. Palmer, C., Diehn, M., Alizadeh, A. A., Brown, P. O. (2006) Cell-type specific gene expression profiles of leukocytes in human peripheral blood. BMC Genomics 7, 115.
5. Dybkaer, K., Iqbal, J., Zhou, G., Geng, H., Xiao, L., Schmitz, A., d'Amore, F., Chan, W. C. (2007) Genome wide transcriptional analysis of resting and IL2 activated human natural killer cells: gene expression signatures indicative of novel molecular signaling pathways. BMC Genomics 8, 230.



5

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.

MATERIALS 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 (IFNγ) is key [2]. In response to stimulation with IFNα, interleukin (IL)-12 or IL-18 released by plasmacytoid dendritic cells, or monocytes, IFNγ 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 IFNγ-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 IFNγ, 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.

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-IFNγ-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 × 107 IU/mL (2.9 × 102–2.2 × 108 IU/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 × 108 and 7.5 × 105 IU/mL, respectively) [13, 14].

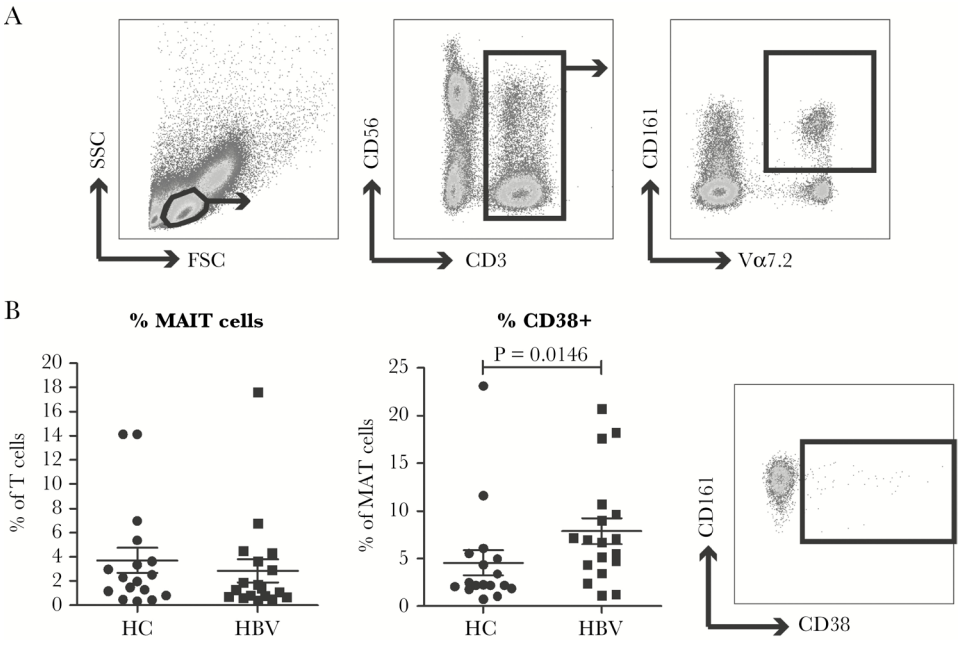

Figure 1. Peripheral CD38⁺ mucosal-associated invariant T (MAIT) cells are more frequent in chronic hepatitis B (cHBV) patients. (A) Representative flow cytometry plots showing the gating strategy for peripheral MAIT cells from a patient chronically infected with cHBV. (B) The percentage of peripheral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

MAIT cells of total T cells in 18 patients with cHBV and matched healthy controls (HC). (Second Panel) The percentage of $CD3^+V\alpha7.2^+CD161^+CD38^+$ 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 IFNγ-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 (IFNγ, 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 IFNγ-positive MAIT cells).

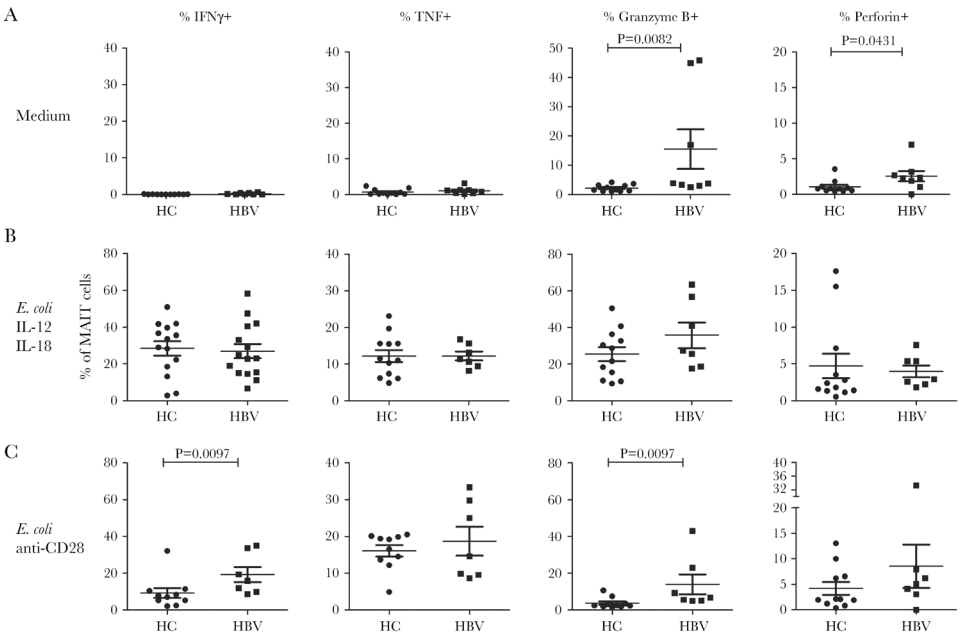

Figure 2. Mucosal-associated invariant T (MAIT) cells of chronic hepatitis B (HBV) patients are not functionally impaired. The percentage of Vα7.2+CD161+ positive for interferon (IFN)ɣ (15 HBV patients), tumor necrosis factor (TNF), granzyme B, and perforin (8 HBV patients) compared with healthy controls (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).

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).

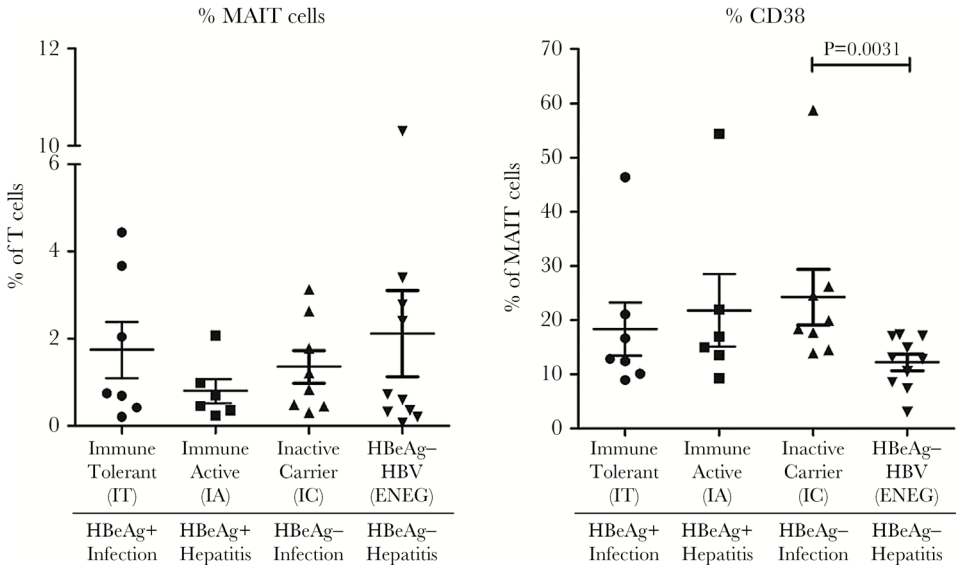

Figure 3. Chronic hepatitis B virus (cHBV) patients in the hepatitis B e-antigen (HBeAg)-negative clinical phase have fewer CD38+ mucosal-associated invariant T (MAIT) cells compared with the inactive carriers. The percentage of CD3+Vα7.2+CD161+ of total T cells (left panel) and CD3+Vα7.2+CD161+CD38+ 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).

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.

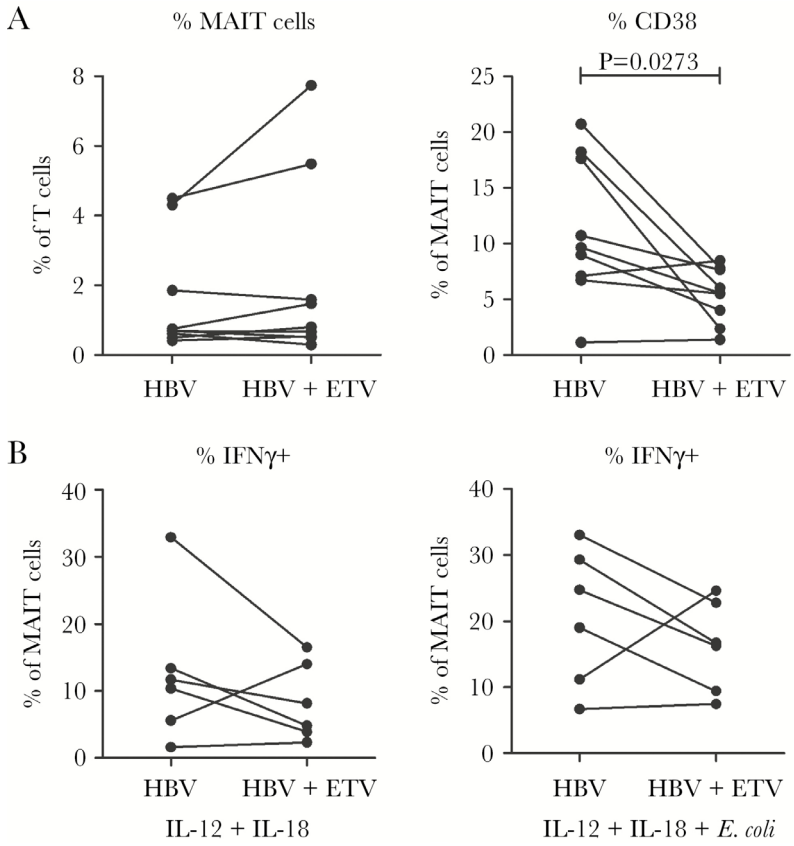

Figure 4. Antiviral therapy for chronic hepatitis B virus (cHBV) reduces mucosal-associated invariant T (MAIT) cell activation to levels of healthy controls, but it does not impact interferon (IFN)γ production by MAIT cells significantly. (A) The percentage of CD3+Vα7.2+CD161+ of total T cells in 9 cHBV 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 IFNγ production stimulation with interleukin (IL)-12/IL-18 (left panel) or IL-12/IL-18/Escherichia coli (right panel) before and on entecavir treatment.

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**).

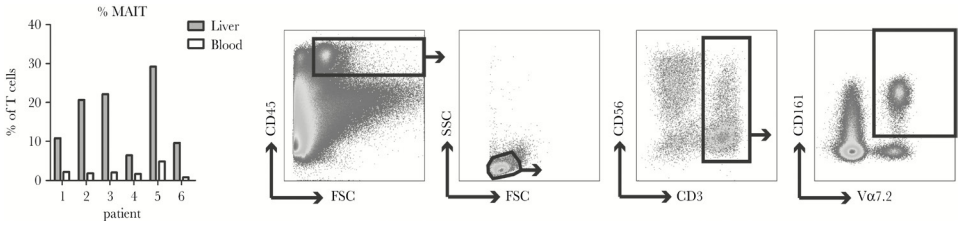

Figure 5. Intrahepatic mucosal-associated invariant T (MAIT) cells are present in high frequencies in the liver compared with blood of chronic hepatitis B virus (cHBV) patients on antiviral therapy. CD3+Vα7.2+CD161+ MAIT cells of total T cells of 6 cHBV patients on continuous nucleoside analog therapy 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 IFNγ 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 IFNγ-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 IFNγ-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 IFNγ-positive MAIT cells between healthy controls and HBV patients, except for E coli/anti-CD28. The latter stimulus resulted in higher frequencies of IFNγ-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, IFNγ production is not impaired compared with healthy controls. The biological relevance of the finding requires more detailed studies to determine whether MAIT cell-derived IFNγ 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 IFNγ-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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REFERENCES

1. European Association for the Study of the Liver. EASL clinical practice guidelines: management of chronic hepatitis B virus infection. J Hepatol 2012; 57:167–85.
2. Phillips S , Chokshi S , Riva A et al. CD8(+) T cell control of hepatitis B virus replication: direct comparison between cytolytic and noncytolytic functions. J Immunol 2010; 184:287–95.
3. Jo J , Tan AT , Ussher JE et al. Toll-like receptor 8 agonist and bacteria trigger potent activation of innate immune cells in human liver. PLoS Pathog 2014; 10:e1004210.
4. van Wilgenburg B , Scherwitzl I , Hutchinson EC et al. MAIT cells are activated during human viral infections. Nat Commun 2016; 7:11653.
5. Dusseaux M , Martin E , Serriari N et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. Blood 2011; 117:1250–9.
6. Tang XZ , Jo J , Tan AT et al. IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. J Immunol 2013; 190:3142–52.
7. Spaan M , Hullegie SJ , Beudeker BJ et al. Frequencies of circulating MAIT cells are diminished in chronic HCV, HIV and HCV/HIV co-infection and do not recover during therapy. PLoS One 2016; 11:e0159243.
8. Hengst J , Strunz B , Deterding K et al. Nonreversible MAIT cell-dysfunction in chronic hepatitis C virus infection despite successful interferon-free therapy. Eur J Immunol 2016; 46:2204–10.
9. Leeansyah E , Svärd J , Dias J et al. Arming of MAIT cell cytolytic antimicrobial activity is induced by IL-7 and defective in HIV-1 infection. PLoS Pathog 2015; 11:e1005072.
10. European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol 2017; 67:370–98.
11. Schoeman JC , Hou J , Harms AC et al. Metabolic characterization of the natural progression of chronic hepatitis B. Genome Med 2016; 8:64.
12. de Groen RA , Hou J , van Oord GW et al. NK cell phenotypic and functional shifts coincide with specific clinical phases in the natural history of chronic HBV infection. Antiviral Res 2017; 140:18–24.
13. Nguyen T , Thompson AJ , Bowden S et al. Hepatitis B surface antigen levels during the natural history of chronic hepatitis B: a perspective on Asia. J Hepatol 2010; 52:508–13.
14. Jaroszewicz J , Calle Serrano B , Wursthorn K et al. Hepatitis B surface antigen (HBsAg) levels in the natural history of hepatitis B virus (HBV)-infection: a European perspective. J Hepatol 2010; 52:514–22.
15. Cho YN , Kee SJ , Kim TJ et al. Mucosal-associated invariant T cell deficiency in systemic lupus erythematosus. J Immunol 2014; 193:3891–901.
16. Eberhard JM , Hartjen P , Kummer S et al. CD161+ MAIT cells are severely reduced in peripheral blood and lymph nodes of HIV-infected individuals independently of disease progression. PLoS One 2014; 9:e111323.
17. Hiejima E , Kawai T , Nakase H et al. Reduced numbers and proapoptotic features of mucosal-associated invariant T cells as a characteristic finding in patients with inflammatory bowel disease. Inflamm Bowel Dis 2015; 21:1529–40.
18. Vanwolleghem T , Hou J , van Oord G et al. Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells. Hepatology 2015; 62:87–100.

19. Grossi G , Viganò M , Loglio A , Lampertico P . Hepatitis B virus long-term impact of antiviral therapy nucleot(s)ide analogues (NUCs). Liver Int 2017; 37(Suppl 1):45–51.

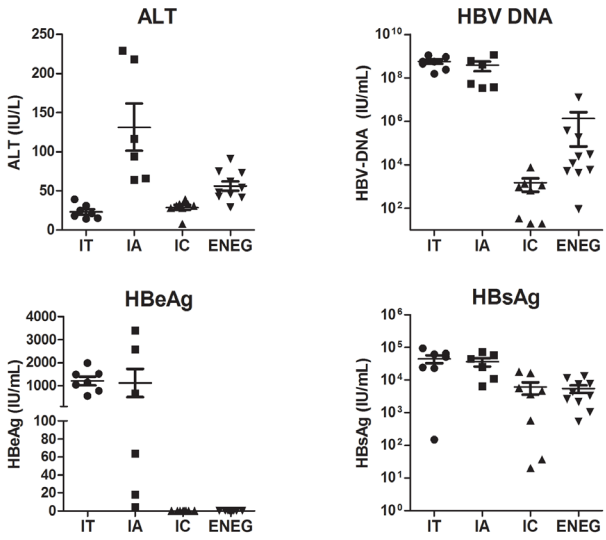
20. Reantragoon R , Corbett AJ , Sakala IG et al. Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells. J Exp Med 2013; 210:2305–20.

SUPPLEMENTARY DATA

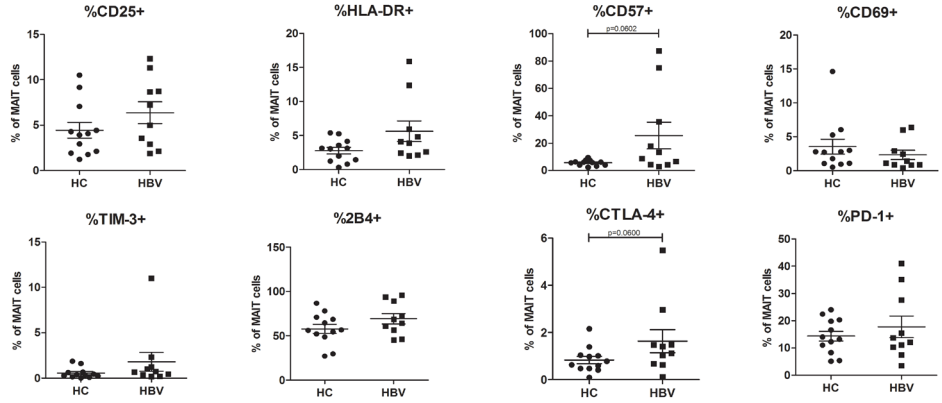
Supplementary Table 1. Percentages of MAIT cells positive for activation and exhaustion markers other than CD38 are not higher in HBV infected patients compared to healthy controls.

		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)

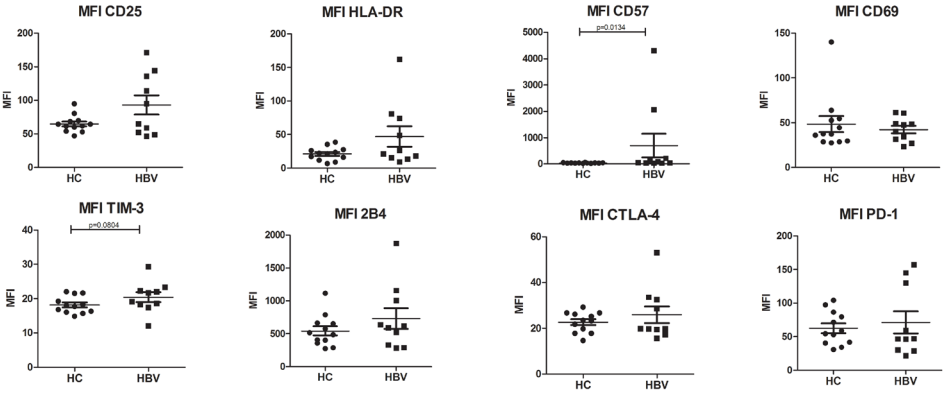
MFI, (Geometric) Mean Fluorescence Intensity. All values are depicted as mean (minimum-maximum). HLA-DR, Human Leukocyte Antigen - antigen D Related; CTLA-4, Cytotoxic T-Lymphocyte-Associated protein 4; PD-1, Programmed cell Death protein 1; TIM-3, T-cell immunoglobulin and mucin-domain containing-3.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



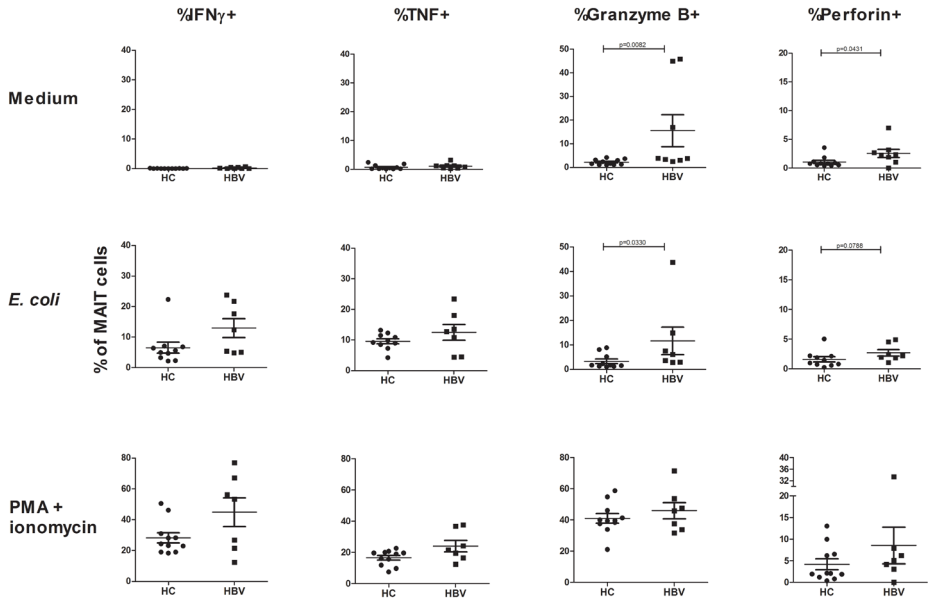
Supplementary Figure 1. cHBV is characterized by 4 phases with different degrees of liver inflammation, HBV replication and HBeAg expression Representation of the patient characteristics of Cohort 2. Based on serum HBV DNA, ALT levels, and HBeAg presence at the time of sampling, patients were categorized 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 HBeAg-negative infection or hepatitis, respectively. HBsAg levels are presented to illustrate lower values among IC and ENEG patients, characteristic for the natural history of cHBV.



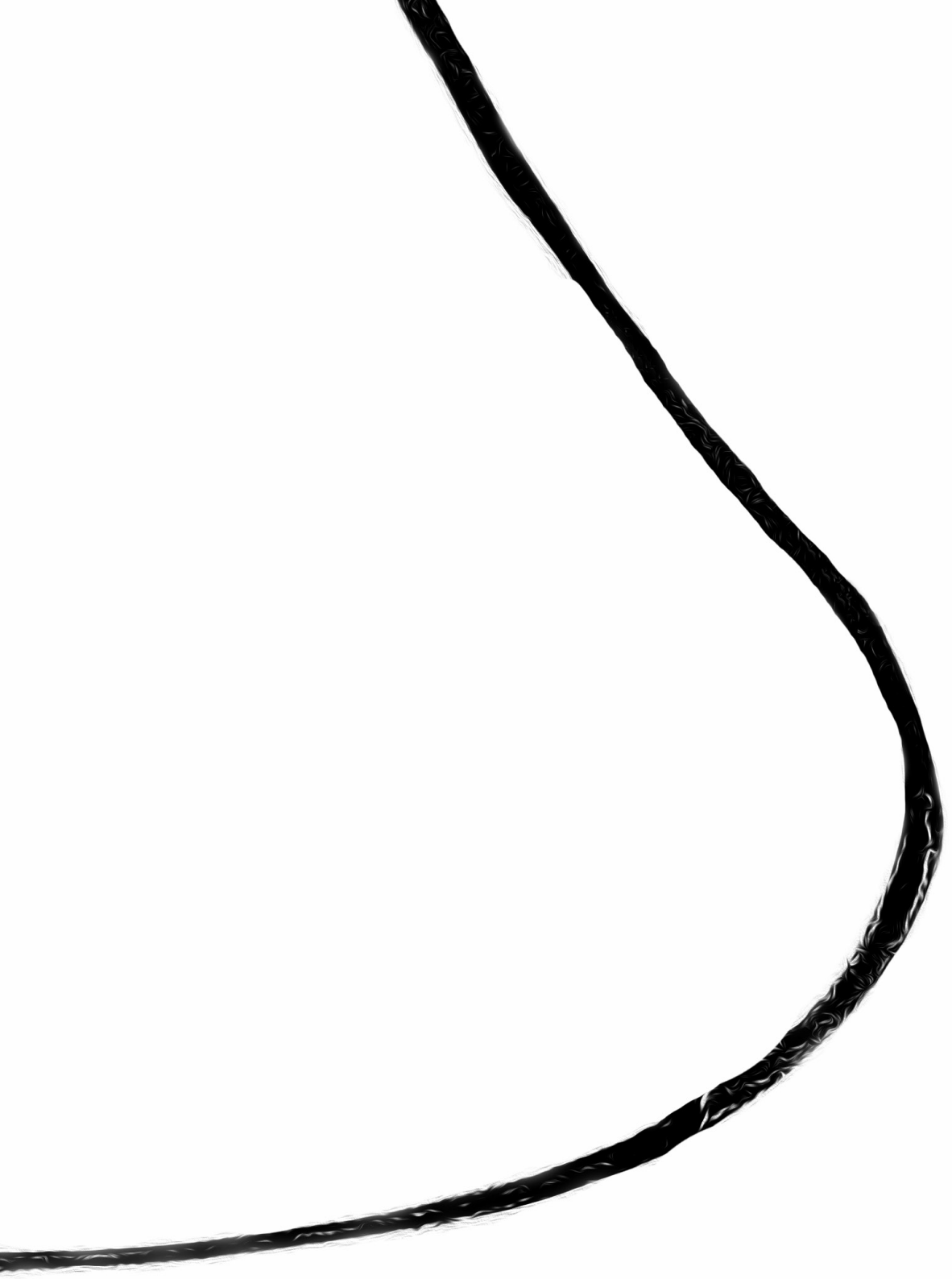
Supplementary Figure 2. Percentages of MAIT cells positive for activation and exhaustion markers other than CD38 are not higher in HBV infected patients compared to healthy controls. Presented are the percentages of CD3+Vα7.2+CD161+ MAIT cells positive for the activation (CD25, HLA-DR, CD57, CD69) and 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 IFNγ, 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.



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The effects of nucleo(s)tide analogs on host immune cells: the baseline for future immune therapy for HBV?

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Antiviral Therapy, provisionally accepted

ABSTRACT

HBV is a non-cytopathic virus, and the progression of liver fibrosis is attributed to the host immune response. Complete suppression of viral replication using nucleotide or nucleoside analogs (NUCs) can prevent most complications related to chronic HBV infection. Unfortunately, antiviral treatment often has to be administered lifelong as HBV persists in the hepatocytes. However, although NUCs are very frequently administered in clinical practice, their effects on vital parts of the host immune response to HBV are not well established. In this review we summarize the currently available data gathered from longitudinal studies that investigated treatment-induced alterations of HBV specific CD4+ and CD8+ T cells, regulatory T cells, and NK cells. These observations are important, as they guide the design and interpretation of studies that investigate the efficacy of new immune therapeutic agents. Novel experimental compounds will likely be added to ongoing NUC treatment, which leads to a cure in only a small minority of patients.

INTRODUCTION

Currently, an estimated 250 million people are chronically infected with the hepatitis B virus (HBV) in spite of the availability of an effective vaccine that prevents transmission. Mortality related to chronic HBV infection remains high and may even be increasing: approximately 800,000 people die from the consequences of the infection annually, which exceeds the yearly death toll from diseases like malaria and HIV [1]. Nucleotide or nucleoside analogs (NUCs) are currently the most frequently applied treatment option for chronic HBV [2]. Antiviral agents like tenofovir and entecavir effectively inhibit replication of HBV in almost all chronic patients [3]. HBV DNA is undetectable in serum within months following the start of treatment and, importantly, NUC treatment greatly reduces the incidence of deadly complications, like end-stage liver disease and hepatocellular carcinoma [4]. Current guidelines [2] advise to start treatment when high serum levels are detected of HBV DNA or alanine aminotransferase (ALT), an enzyme abundant in the cytosol of hepatocytes, released upon lysis. NUC treatment specifically suppresses replication of the viral genome, but does not lead to complete viral eradication as evidenced by the persistence of a viral mini-chromosome, called covalently close circular DNA (cccDNA), and the retention of integrated viral sequence in host DNA in hepatocytes[5]. Both cccDNA and integrated viral DNA can act as templates for the transcription of viral proteins, like HBV surface antigen (HBsAg), which is continuously produced and secreted in serum at all stages of the infection, even during effective treatment with NUCs [6]. Resolution of a chronic HBV infection is defined as the loss of HBsAg from the blood. Unfortunately, NUC treatment only results in HBsAg loss in a minority of patients (0-3%) with a virological response[7-9]. Although pegylated-interferon infections result in a durable loss of HBsAg in 7-17% percent of the individuals[10, 11], NUCs are generally preferred, because they are much better tolerated than weekly interferon injections. With the aim to improve low curative treatment rates for chronic HBV, a multitude of novel compounds are currently under development[12]. The mechanisms of action of these novel experimental antivirals vary greatly, ranging from activation of the host immune system by Toll-like receptor (TLR) ligation to small molecules that block translation of viral proteins, like silencing RNA. The goal of these new compounds is to clear HBsAg from the host serum, as this event is associated with a clear improvement of the prognosis. HbsAg loss is also called a functional cure, because even after HBsAg has become undetectable in serum, HBV reactivation can still occur during periods of immune suppression by renewed transcription of the remaining cccDNA. It is generally accepted that a combined activity of compounds with distinct mechanisms of action are needed to achieve a functional cure. Since NUC treatment is effective in blocking viral replication and is able to limit the spread of HBV, it will likely remain the backbone of these novel therapeutic regimens [13]. To select novel candidates for the combined treatment strategy, it is important to have a detailed understanding of the effects of NUCs on the host immune system, and the immune status of chronic HBV patients on continuous NUC treatment. This goal is particularly

relevant with regard to immune cell populations capable of producing antiviral cytokines or lysing virally infected cells, like virus specific T cells and NK cells, as they are a likely target for future immune therapeutic intervention. In this review, we summarize and discuss the literature regarding effects of NUCs on the cellular immune system, with the aim to guide the development of treatment strategies for chronic HBV that combine antiviral and immunological compounds.

The effect of NUCs on HBV specific T cells

The adaptive immune response plays an important role in viral clearance and disease pathogenesis of HBV infection. This is supported by several key observations. In acute HBV infections, HBV specific T cell responses appear directly after an increase in serum HBV DNA levels [14, 15], and, as in many other viral infections like hepatitis C virus (HCV) and human immunodeficiency virus (HIV), acute HBV infections are characterized by multi-specific and polyfunctional HBV specific T cell responses, while these responses are not observed when viral replication persists [16, 17]. During the chronic phase, the frequency of HBV specific T cells in the blood is usually very low, and in early studies of NUC treatment it was observed that these frequencies improved in the majority of the treated individuals, using limiting dilution analyses. In order to reliably detect and study HBV specific T cells obtained from chronic HBV patients, these cells generally need to be expanded in vitro prior to flow cytometric or functional evaluation by, for example, ELISPOT assays. In addition to their relatively low frequencies in the blood, other host and viral factors have been proposed to explain the weak T cell responses to HBV. These factors include the occurrence of HBV escape mutants [18], the dysfunctional interaction of T cells with natural killer (NK) cells or dendritic cells (DC) together with a weak type I interferon induction [18, 19], exhaustion of T cells as a consequence of continuous high exposure to high viral antigen load [20] and active suppression by regulatory T cells or immunosuppressive cytokines like IL-10 or TGF-β [21]. The observation that virus specific T cells are activated during acute HBV [14, 15] and the abovementioned mechanisms of immune impairment during chronic infection have been followed up by numerous studies that focus on HBV specific CD4+ and CD8+ T cells. The majority of these studies used blood leukocytes from chronic HBV patients who are not on NUC treatment, and therefore display variable levels of serum HBV DNA and ALT, characteristic for the natural history of chronic HBV. However, NUC treated chronic HBV patients are on treatment for many years or even lifelong, and generally have undetectable serum HBV-DNA and normalized ALT levels in the absence of other liver related co-morbidities, yet viral eradication is rarely complete since HBsAg is readily detected in serum of NUC treated patients. Therefore, data on virus specific T cells derived from treated patients, preferably in a longitudinal setting, is of particular relevance.

A number of studies have examined the functionality of CD4+ T cells in chronic HBV patients on NUC therapy [21-28]. As presented in **Table 1**, in the majority of studies involving CD4+ T cells, peripheral blood mononuclear cells (PBMC) were stimulated for 1-6 days using viral

antigen (HBcAg or HBsAg) and IFN-γ production was subsequently quantified using ELISPOT. NUC treatment significantly increased the production of IFN-γ by HBV specific CD4+ T cells in seven longitudinal studies that performed this assay, which suggests that some degree of immune reconstitution does occur. Importantly, three of these studies showed a slight increase in IFN-γ producing HBV specific CD4+ T cells at start of treatment, only to see it decline to pre-treatment levels during [28, 29] or after [24] treatment. One cross-sectional study reported unaltered IFN-γ production by specific CD4+ T cells using a comparable assay [17]. Using a tritium-thymidine based assay, two studies (26, 30) reported that NUC treatment improved the proliferative capacity of HBV specific CD4+ T cells, while this effect was transient in one study [24] (**Table 1**). Nonetheless, there is still a possibility that T cells chronically exposed to high antigen load are long-lasting or permanently damaged and unable to undergo a sustained functional restoration. This possibility is supported by the data in LCMV infected mouse models that transferred impaired CD8+ T cells from a chronically infected mice to a naïve uninfected mice which surprisingly did not restore T cell differentiation [31]. In addition, although NUC treatment can control viral replication, HBV e antigen (HBeAg), but especially high serum levels of HBsAg remain present for a long time. To date, the effect of continuous high antigen load on T cell function is unclear, but it is likely to be a contributing factor in the incomplete recovery of T cells as observed during NUC treatment.

Table 1. Longitudinal studies on the effect of NUCs on HBV-specific CD4+ T cells in the peripheral blood

	Treatment	HBeAg+ / total	Cytokine production	HBV protein used	Proliferation	Reference
Increase of IFN-γ production on treatment by HBV specific CD4+ T cells	48 weeks ADV	15/15	ELISPOT: CD4+IFN-γ ↑ (at 12-16 weeks)	HBcAg HBsAg	NA	Zheng, 2014, J Vir Hep
	48 weeks ADV	13/13	ELISPOT: CD4+ IFN-γ ↑ (at 16 weeks)	HBcAg	Proliferation ↑*	Cooksley, 2008, Antimicrob Agents and Chemother
	78 weeks LAM/ LDT	18/18	ELISPOT: CD4+ IFN-γ ↑ (at 12-16 weeks)	HBcAg HBsAg	NA	Evans, 2008, Hepatology
	48 weeks ADV+/- FTC	30/30	ELISPOT: CD4+ IFN-γ ↑ (at 16 weeks)	HBcAg HBsAg	NA	Lau, 2007, Antivir Ther
Transient increase IFN-γ production on treatment by HBV specific CD4+ T cells	24 weeks ADV	6/12	ELISPOT: CD4+ IFN-γ transient ↑	HBcAg	Proliferation ↑*	Stoop, 2007, Virology
	24 weeks LAM	5/5	ELISPOT: CD4+ IFN-γ transient ↑	HBcAg	Proliferation transient ↑*	Rigopoulou, 2005, Hepatology
	52 weeks LAM	12/12	ICS: CD4+ IFN-γ+ transient ↑	HBcAg	NA	Boni, 2003, J Hepatol

HBeAg, hepatitis B e antigen; ELISPOT, enzyme-linked immunosorbent spot; IFN-γ, interferon-gamma; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; ADV, adefovir; NA, not applicable; LAM, lamivudine; LDT, telbivudine; FTC, emtricitabine; ICS, Intracellular Cytokine Straining. * Tritiated thymidine (^{3}H-TdR) assay; ↓, decrease; -, no change; ↑, increase.

Current literature provides strong evidence that virus specific CD8+ T cells are important effectors during spontaneous clearance of HBV from the liver. In this regard, it has been shown that HBV infected chimpanzees display a delayed HBV clearance after CD8+ T cell depletion [32, 33], and that acutely infected patients show an increase of virus specific CD8+ T cells visualized using HLA-A2/HBV tetramers during viral load decline [14]. Comparable to observations for virus specific CD4+ T cells, the frequencies of dextramer positive CD8+ T cells are very low during chronic HBV infection [17, 34], irrespective whether patients are on-treatment or not [17]. Importantly, in patients who experienced loss of serum HBsAg during NUC treatment, the frequency of HBV specific CD8+ T cells was higher, which was an early indication that the frequency of HBV specific CD8+ T cells can improve during treatment [17]. It is also worth noting that the low CD8+ T cell frequency correlates with the activation status directly ex vivo. This could contribute to activation induced apoptosis during the assay which might lower the measured frequency even more [34]. It has been well established by numerous studies in the last decade that during untreated chronic infection, HBV specific CD8+ T cells are functionally impaired, in contrast to CD8+ T cells with other specificities, and that exhaustion markers, such as PD-1 and CTLA-4, are highly expressed [35-37]. Importantly, only few longitudinal studies investigated the effects of NUC treatment on these parameters [22, 24, 25, 28, 38, 39] (**Table 2**). Three studies using ELISPOT assays on CD8+ T cells were conducted, of which only one study reported significant recovery [22]. These studies used different HLA-A2 specific peptides for the stimulatory assays, which is important as the specific targets of HBV specific CD8+ T cells affect their function [40]. One study by Boni et al [17] showed the results of an ELISPOT assay using a broad range of HLA-A2 restricted HBV epitopes in a cross-sectional set up. The authors showed that in those patients who cleared HBsAg following NUC treatment, the production by CD8+ T cells of IFN-γ, IL-2, and TNF was higher than in patients where HBsAg persisted, which suggests restoration of CD8+ T cells in the absence of HBsAg. In addition, the capacity of CD8+ T cells to lyse target cells in an antigen-dependent manner was evaluated in three studies [25, 38, 39] (**Table 2**). Virus specific lysis rates improved during treatment in all three studies, but this positive effect was not durable during the follow up of several months of treatment. With regard to IFN-γ producing HBV specific CD8+ T cells (**Table 2**), an increase at week 14 and 48 was observed in one study [22], but no differences were observed during NUC treatment in two other studies [24, 28]. A study by Rigopoulou et al included 5 individual patients, and detected no increase in IFN-γ production and only a transient improvement of CD8+ T cell proliferation [24]. One study included the analysis of 5 paired liver biopsies [41]. The authors reported a treatment induced increase of intrahepatic CD8+ T cells by examining formalin fixed and paraffin embedded (FFPE) tissue, and of those patients that cleared serum HBeAg on treatment, the increase was more pronounced, which suggests that CD8+ T cells may contribute to this important clinical event, which is associated with an improved long-term prognosis. Unfortunately, no additional functional tests on intrahepatic CD8+ T cells directly ex vivo were performed. Importantly, in several studies discussed in this review, the improvement of CD4+, but

especially CD8+ T cell function was only transient and returned gradually to pre-treatment levels while on NUC induced viral suppression [24, 30]. It is currently unclear what causes this decline, especially since the viral load reduction, the normalization of liver inflammation and cytokine concentrations in the serum are durable. The restoration of weakened functionality of T cells during therapy for chronic viral infection is in line with data in HCV infected patients who received direct-acting antivirals [42] and the combined results of the studies on HBV suggest that T cells could be a target for immune restoration.

Table 2. Publications on the effect of NUCs on CD8+ HBV specific T cells from patients with chronic HBV

	Treatment	HBeAg+ / total	Cytokine production	HBV peptides / stimulus and tetramers	Reference
Increase of IFN-γ production on treatment by HBV specific CD8+ T cells	LAM/LDT 78 weeks	18/18	ELISPOT: CD8+ IFN-γ↑ (32-56 weeks)	HBV c18-27, pentamers c18-27	Evans, 2008, Hepatology
No change IFN-γ production on treatment by HBV specific CD8+ T cells	ADV 48 weeks	16/16	ELISPOT: CD8+ IFN-γ production: no change, tetramer+ CD8+ IFN-γ: no change	ICS: Six HLA-A2 peptides c18–27; s183–191; s335–343; s348–357; p455–463 and p575–583, tetramers c18-27 and s335-343	Lau, 2007, Antivir Ther
	LAM 24 weeks	5/5	ELISPOT: CD8+ IFN-γ production: no change, supernatant IFN-γ no change	peptide c18-27	Rigopoulou, 2005, Hepatology
	Treatment	**HBeAg+**	**Proliferation / Cytotoxicity assay**	**HBV peptides / stimulus and tetramers**	**Reference**
Transient increase specific lysis on treatment by HBV specific CD8+ T cells	LAM 52 weeks	6/6	Transient ↑ % specific lysis ** (12 weeks)	^{51}Cr labeled HLA-A2 matched EBV-B cells pulsed with 26 9-10 mer peptides	Boni, 2003, J Hepatol
	LAM 52 weeks	6/6	Transient ↑ % specific lysis ** (12 weeks)	26 HBV peptides, tetramers c18-27, s183-191, s335-343	Boni, 2001, Hepatology
Transient increase of proliferation on treatment by HBV specific CD8+ T cells	LAM 52 weeks	12/12	Transient proliferation↑*	26 peptides	Boni, 1998, J Clin Invest

HBeAg, hepatitis B e antigen; LAM, lamivudine; LDT, telbivudine; ELISPOT, enzyme-linked immunosorbent spot; ADV, adefovir; s, surface; c, core; p, polymerase; aa, amino acid; *Tritiated thymidine (^{3}H-TdR) assay; **Chromium-51 (^{51}Cr) release assays; ↓, decrease; -, no change; ↑, increase.

The effects of NUCs on regulatory T cells

The importance of regulatory T cells in regulating HBV specific T cell responses is well recognized [43]. Regulatory T cells are CD4+ T cells that express the cell membrane marker CD25 in combination with the transcription factor forkhead box protein P3 (FOXP3). FOXP3 is located in the nucleus of CD4+(CD25+) T cells, and fixation and permeabilization is necessary to definitively

identify these cells. Early studies have convincingly shown that depletion of regulatory T cells in vitro results in improved HBV specific CD8+ T cell function [21, 44, 45]. In vivo, relatively high frequencies of regulatory T cells are inversely correlated with liver inflammation during acute HBV infection [21], suggesting that these can mediate liver inflammation during viral flares. In line with this observation, higher frequencies of intrahepatic regulatory T cells are detected in patients with high viral load [46]. One possible mechanism of this increase is that during liver inflammation and fibrosis progression hepatic stellate cells produce more TGF-beta [47]; this cytokine has been shown to induce differentiation of CD4+ T cells towards a FOXP3+ regulatory phenotype in vitro [48]. Functional tests to determine the capacity of regulatory T cells to suppress the functionality of other T cells are relatively difficult to perform, especially when investigating liver derived cells. Numerous studies have determined the relative frequency of regulatory T cells in the peripheral blood during NUC treatment, albeit not always using the same molecules (CD4+/CD25+/FOXP3+/CTLA+/CD127^{low}) to define regulatory T cells. As presented in **Table 3**, the great majority of studies published today demonstrated that NUC mediated viral load reduction leads to a decrease of regulatory T cell frequency within the CD4+ T cell population [30, 49-58]. This finding is robust and is observed even when using variable definitions of regulatory T cells during cytometric analysis, and is irrespective of percentages of HBeAg positive patients, the degree of viral suppression or treatment duration. It is important to mention that, to the best of our knowledge, no functional assay-based results before and on NUC treatment have been published to date. In addition, as it is proposed that regulatory T cells may exert local immunosuppressive effects on other lymphocyte populations residing in the liver, like HBV specific T cells and NK cells, assessment of liver-derived regulatory T cells before and on NUC treatment is currently needed to create a complete picture of effects related to antiviral treatment. It is possible that regulatory T cells contribute to the improved activity of HBV specific (CD4+) T cells during NUC therapy. Simultaneous assessment of their phenotype/function during NUC treatment would greatly improve the current knowledge on this subject, as well as provide clues to the mechanism of fibrosis regression that has been observed during NUC trials for HBV.

The effect of NUC treatment on NK cells, MAIT cells and NKT cells

Interacting at several stages with antiviral T cells as described above [59], CD56+CD3- NK cells represent an important antiviral effector cell. Unlike HBV specific CD8+ T cells, NK cells have the capacity to lyse infected target cells without prior sensitization and induce non-cytolytic clearance of HBV through IFN-γ release [60]. Their activity depends on the balance of stimulatory and inhibitory receptor expression and ligation, as well as by exposure to inflammatory cytokines (like interferon-α, IL-2, IL-12 and IL-18). Corresponding with observations of acutely infected chimpanzees [61], early research on NK cells in human subjects with acute HBV infection in humans suggested that NK cells have a decline in frequency one to three weeks before HBV DNA starts to decline [14]. However, during chronic HBV, NK cell frequency or activation status does

not seem to correlate strongly with considerable shifts in HBV DNA and ALT, although it should be noted here that longitudinal data is limited. Described as a NK cell 'functional dichotomy' during chronic HBV, NK cells appear to retain their in vitro cytotoxic potential, but have marked dysfunctional TNF and IFN-γ production [62-64]. During NUC treatment, the concentration in blood of several cytokines that affect NK cell phenotype is altered [65, 66] and it seems likely that NK cell phenotype and function is affected likewise. However, when the HBV DNA declines during the natural history of chronic HBV among untreated HBV patients, NK cell frequencies and phenotypes are only minimally affected by fluctuations in viral load and liver inflammation that characterize the natural history of chronic HBV infection [67]. Here, we summarize the existing information of NK cells before and on NUC treatment [56, 64, 68-72], including reports on frequencies, expression of activation markers and functional tests (**Table 4**).

Table 3. Longitudinal studies on the effect of NUCs on regulatory T cells in the peripheral blood

	Definition Treg (in addition to CD4+)	**Treatment**	**HBeAg+ / total**	**Reference**
Decrease of Treg % of total CD4+ T cells	CD25+ FOXP3+	LDT 24 weeks	27/27	Yang, 2017, Medicine
	CD25+	TDF 96 weeks	32/32	Yang, 2016, Cell Mol Imm
	CD25+ $CD127^{low}$	LDT 24 weeks	5/22	Yan, 2015, Mol Med Reports
	CD25+ CTLA4+ CD45RO+	ADV 24 weeks	4/8	Stoop, 2007, Virology
	$CD127^{low}$	LDT 14 weeks	26/44	Yu, 2013, Hepatitis Mon
	CD25+ FOXP3+	TDF 24 weeks	15/30*	Trehanpati, 2011, J Clin Immun
	FOXP3+	ETV/ADV 48 weeks	43/57	Jiang, 2011, Cell Mol Imm
	FOXP3+	LDT 52 weeks	36/36	Li, 2015, Liver Int
Decrease (absolute number of Tregs)	FOXP3+	52 weeks 54	54/54	Ma, 2013, Antimicrobiol Agents Chem
No change	FOXP3+	ETV 48 weeks	28/28	Zhang, 2010, Plos One

HBeAg, hepatitis B e antigen; FOXP3, forkhead box P3; LDT, telbivudine; LAM, lamivudine; TDF, tenofovir; ETV, entecavir; ADV, adefovir. *decrease only observed in HBeAg+ patients. ↓, decrease; -, no change; ↑, increase.

In 4 out of 9 studies on NUC treatment of chronic HBV patients, the NK cell frequencies in blood were not affected. Interestingly, the relative NK cell frequencies did increase in studies that included high numbers of HBeAg positive patients [68-70]. Zheng et al [27] studied five paired FFPE liver biopsies before and on treatment and reported that NK cells increased in the hepatic lobule on treatment. The consequences of the migration of NK cells into the liver or blood following NUC treatment remain unclear, as NK cells have been implicated to play a role in negative regulation of immune responses to HBV, as well as in the (beneficial) mitigation of liver damage possibly by the production of anti-fibrotic IFN-γ [73] or direct lysis of hepatic stellate cells [74]. In general, the selection of NK cell activation markers varied greatly among studies. Three studies studied the inhibitory lectin type receptor NKG2A, and showed that

its expression was downregulated upon treatment (**Table 4**). In one study by Tjwa et al [64], treatment resulted in a downregulation of NKG2A expression, as well as in an increase of the frequency of IFN-γ positive NK cells upon IL-12/IL-18 stimulation. In line with this observation, the expression of the activating lectin type receptor NKG2D is reported to be upregulated upon treatment in two studies. These observations suggest that in those patients where NUCs affect NK cells phenotype, the effect is activating rather than a return to 'resting state' in the complete absence of virus in the blood. Boni et al. [71] reported that among 10 HBeAg negative patients, the percentages of NK cells positive for NKp46, CD38 and Ki67 were downregulated, indicating less proliferation and activation of NK cells during treatment. In contrast, among the studies investigating Asian patient cohorts, Zhao et al and Lv et al. did not detect modulation of Natural Cytotoxicity Receptor expression on blood NK cells (**Table 4**). The capacity of NK cells to produce cytokines upon NUC treatment was measured longitudinally by only three studies. Two studies [71, 72] showed that NUCs did not influence the percentage of IFN-γ positive NK cells (following IL-12/IL-18 or PMA/ionomycin), while one study [64] reported an increase (following IL-12/IL-18). The interaction of NK cells with DC or monocytes in the context of HBV treatment has not been investigated extensively. In one study by Tjwa et al [75], NK cells were collected before and on antiviral treatment, and co-cultured with BDCA-1+ DC for 48 hours. In vivo viral suppression resulted in higher percentages IFN-γ positive NK cells following incubation with DC ex vivo, suggesting NK cell cytokine production is supported, not inhibited, by antiviral treatment. With regard to NK cells, as is the case for many immune parameters measured ex vivo, it is not certain that an increased activation or relative frequency would necessarily improve the clinical course of HBV. As mentioned before, NK cells produce immune stimulatory cytokines which are likely beneficial to patients, but NK cells might also eliminate effector cells, like CD8+ T cells, that are thought to deplete infected host cells thereby hampering HBV clearance [59]. Conversely, increased numbers of IFN-γ producing NK cells in the liver may be beneficial, since IFN-γ has been shown to attenuate liver fibrosis progression [73] as well as clear the virus in a non-cytolytic manner. Among the studies included in this review, the frequency of NK cells decreased only in studies that included a high number of HBeAg positive patients. During the natural history of HBV, HBeAg negative infection has been reported to result in comparable frequencies, and only slightly higher percentages of IFN-γ positive $CD56^{bright}$ NK cells [67]. This was a study with a cross-sectional design, and it seems unlikely that this difference is caused by a direct effect of the viral HBeAg on NK cell differentiation. It is more likely that HBeAg positivity selects for patients that harbor more active HBV infection, which may indirectly affect NK cell function.

Supplementing the work done on NK cells, in recent years, attention has been directed at other innate immune cell populations, like NKT cells, that depend on the major histocompatibility complex class I-like CD1 for development and recognize lipid antigen presented by CD1. Data from a transgenic HBV mouse model indicated that NKT cells, enriched in human liver, support

local T cell-mediated immune responses, which likely contributed to HBV elimination in these animals [60]. Complementing the literature discussed in this review, several studies investigated NKT cells in the context of NUC treatment. Three studies noted an increase in frequency of NKT cells upon treatment [76-78], one study noted a decrease[79], and two studies reported no change [80, 81]. Like the peripheral blood NK cell frequency, NKT cell frequency is decreased predominantly in the studies that included HBeAg positive patients, which may be related to the higher baseline in vivo HBV replication. Another invariant T cell population, the mucosal-associated invariant T cell population, or MAIT cell, is also of interest. Although their role in the context of chronic viral infections like hepatitis B has not been frequently investigated, these cells can produce high amounts of antiviral cytokines and are enriched in the human liver [76, 82-84]. Despite the fact that these cells have an invariant T cell receptor specific for (bacterial) vitamin B metabolites, several indications exist that their frequency and function may alter during chronic viral infection [85]. Unfortunately, MAIT cells have been investigated during NUC treatment in only one study [82], which showed that the MAIT frequency in blood of patients was not different than in healthy controls, but CD38 expression was clearly downregulated during viral suppression by treatment, which may indicate that HBV activates MAIT cells during chronic infection.

Table 4. Longitudinal studies on the effect of NUCs on CD56+ CD3- NK cells in the peripheral blood

	Treatment	HBeAg+ / total	Markers of activation or proliferation	Function	Reference
Increase % CD56+CD3- NK cells / total lymphocytes on treatment	LDT 52 weeks	54/54	2B4↑	NA	Ma 2013 Agents and Chem
	LDT 48 weeks	52/52	NKG2D↑, NKP46↑, NKG2A↓	NA	Chen 2017 Hepatol Int
	LAM 24 weeks	14/14	NA	NA	Duan 2004 J Clin immuno
	TDF 24 weeks	7/12	NKp30-, CD158b-, NKG2A↓, KIR2DL3↓	NA	Lv 2012 Med Inflamm
	LAM/ETV/ADV 176 weeks	0/10	CD38↓, TRAIL↓, Ki67↓	No significant changes in IFN-γ and CD107a expression (IL-12/IL-18)	Boni 2015 Hepatology
No change %CD56+ CD3- NK cells / total lymphocytes on treatment	ETV/TDF 48 weeks	1/11	CD69-, HLA-DR-, NKG2A-, NKG2D-	NA	Tjwa 2016 Antivir Res
	ADV 24 weeks	8/12	CD158b-, NKG2A↓, KIR2DL3↓	NA	Lv 2012 Med Inflamm
	ETV 24 weeks	18/18	NKG2D↓, NKp30↓, NKG2C-, NKp44-, NKp46-, KIR3DL1-, KIR2DL3-, NKG2A-, CD158a-	No change in IFN-γ expression (PMA/ ionomycin), CD107a↓ (K562 stimulation)	Zhao 2013 Clin Exp Pharmacol
	ETV 24 weeks	5/15	NKG2A↓	% IFN-γ positive NK cells ↑ (IL-12/IL-18)	Tjwa 2011 J Hepatol

HBeAg, hepatitis B e antigen; LDT, telbivudine; LAM, lamivudine; TDF, tenofovir; ETV, entecavir; ADV, adefovir; ↓, decrease; -, no change; ↑, increase.

DISCUSSION AND CONCLUSION

From the existing literature on NUC associated immune effects, we summarize that HBV specific CD4+ T cells recover in number and function in blood, but in some patients only transiently, and that treatment improves their proliferative capacity in vitro. HBV specific CD8+ T cells recover their capacity to lyse target cells, albeit transiently, but not their ability to produce antiviral cytokines. In most studies, the most pronounced increase of the specific immune responses to HBV occurs 12-16 weeks after the start of NUCs. Regulatory T cell frequencies consistently and durably decrease on treatment, and NK cell frequencies increase in most studies.

In several studies, the detected enhancement of the virus specific immune response decreased to pre-treatment levels during follow up. Importantly, the specific T cell mediated immune response during long term NUCs does not compare to the polyclonal and strong responses observed in patients who spontaneously clear HBV infection [16]. Regulatory T cell frequencies in blood decreased in almost all NUC treatment studies, along with the sharp decline of HBV DNA in the serum, but functional tests have not been performed longitudinally on these cells. Blood NK cell frequencies recover in most studies included in this review, in particular among HBeAg positive cohorts. Inhibitory markers like NKG2A on NK cells are downregulated but not measured frequently or consistently in a longitudinal setup. NK cell cytokine production upon stimulation in vitro was unaltered by treatment or only slightly improved, which suggests that from an immunological perspective, the innate immune response is minimally altered by NUC treatment, despite clear decreases of viral load and normalization of liver inflammation. However, very few functional assays have been performed on these cells and on other cell types like B cells, MAIT cells and liver derived lymphocytes.

To our best knowledge, this is the first review to focus on the immune effects of NUC treatment for HBV. The studies included in this summary of the existing literature differ greatly, and display considerable variation of important parameters like the type of NUC used, the duration of treatment, and HBeAg status of the included subjects. Therefore, for this review, given the striking heterogeneity of the studies, we consider a systematic review or meta-analysis more prone to inaccurate conclusions than the current more narrative approach. Also, the studies included in this review report mainly on relative lymphocyte frequencies, and information on the absolute number of T cells during NUC treatment are currently not available. Furthermore, several immune cells that have been previously proposed to play a role during viral control of HBV were not included in this review as little or no published studies evaluated their phenotype before and on NUC treatment. Peripheral blood B cells for example, shown to associate with fluctuation of viral load [86] and relevant for sustained control of HBV replication, have not been investigated in the context of NUC treatment for HBV. Similarly, few reports have been published

on treatment effects on other lymphocyte populations like MAIT cells [82] and DC [87]. Therefore, to complement the abovementioned conclusions, future work should include evaluation of these populations. In addition, despite several publications on liver derived immune cells [27, 28, 88, 89], both from fresh and FFPE biopsies, the possibility to improve our knowledge on immune responses in the liver is a promising one. This is particularly interesting in the context of NUC treatment for HBV, as liver inflammation is reduced in almost all treated patients upon treatment, and subtle changes to the host immune response not detectable in the blood might become detectable during suppression of the virus.

This review has included findings on studies with patients on NUC treatment for HBV, while also several studies have been published on the effect of NUC discontinuation. In these studies, patients were sampled on treatment and after stopping NUCs. In some countries, stopping NUC therapy is the standard of HBV care [90], as the reimbursement ends after a set period of antiviral suppression. It has been demonstrated that cessation of NUC treatment enhances the HBV specific T cell response, and that stronger T cell responses associate with the absence of clinical flares [41]. This suggests that, although the effects on HBV viral load are oppositional, both might boost virus specific immune responses, at least in the short term.

Overall, the available data suggests that the NUC-induced functional improvement of CD8+ T cells in the blood might be temporary with a peak between 12 and 16 weeks after the start of NUCs. These findings are important, but -since a large number of patients take NUCs for a many years without interruption- are not easy to exploit when further boosting the responses with immunotherapeutic agents. In addition to this information, knowledge of the memory or effector T cell phenotype of the target cell could further guide the design of future trials. For HBV treatment, however, it is not clear which subsets of CD4+ or CD8+ T cells display the most pronounced reconstitution during the suppression of viral replication. This is in contrast to the curative treatment with direct acting antivirals for HCV during which a phenotypical shift towards functional memory CD8+ T cell phenotype was observed in successfully treated patients, which corresponds to the changes after spontaneous resolution of acute HCV infections [91]. This information aids current HBV research, as it guides the choice and treatment regimens for specific immunotherapeutic agents. Given the short window of opportunity for optimal combination of antiviral and immune therapy, it may prove beneficial to apply experimental treatment within months following the start of NUCs, as the immunological benefits are not always lifelong, while for many patients, treatment is.

REFERENCES

1. Graber-Stiehl I. The silent epidemic killing more people than HIV, malaria or TB. Nature. 2018; 564(7734):24-6.
2. European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol. 2017; 67(2):370-98.
3. Han Y, Zeng A, Liao H, Liu Y, Chen Y, Ding H. The efficacy and safety comparison between tenofovir and entecavir in treatment of chronic hepatitis B and HBV related cirrhosis: A systematic review and Meta-analysis. Int Immunopharmacol. 2017; 42:168-75.
4. Papatheodoridis GV, Chan HL, Hansen BE, Janssen HL, Lampertico P. Risk of hepatocellular carcinoma in chronic hepatitis B: assessment and modification with current antiviral therapy. J Hepatol. 2015; 62(4):956-67.
5. Tu T, Budzinska MA, Shackel NA, Urban S. HBV DNA Integration: Molecular Mechanisms and Clinical Implications. Viruses. 2017; 9(4).
6. Idilman R, Gunsar F, Koruk M, et al. Long-term entecavir or tenofovir disoproxil fumarate therapy in treatment-naive chronic hepatitis B patients in the real-world setting. J Viral Hepat. 2015; 22(5):504-10.
7. Zoutendijk R, Hansen BE, van Vuuren AJ, Boucher CA, Janssen HL. Serum HBsAg decline during long-term potent nucleos(t)ide analogue therapy for chronic hepatitis B and prediction of HBsAg loss. J Infect Dis. 2011; 204(3):415-8.
8. Dienstag JL, Schiff ER, Wright TL, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med. 1999; 341(17):1256-63.
9. Lai CL, Gane E, Liaw YF, et al. Telbivudine versus lamivudine in patients with chronic hepatitis B. N Engl J Med. 2007; 357(25):2576-88.
10. van Zonneveld M, Honkoop P, Hansen BE, et al. Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. Hepatology. 2004; 39(3):804-10.
11. Buster EH, Flink HJ, Simsek H, et al. Early HBeAg loss during peginterferon alpha-2b therapy predicts HBsAg loss: results of a long-term follow-up study in chronic hepatitis B patients. Am J Gastroenterol. 2009; 104(10):2449-57.
12. Bertoletti A, Le Bert N. Immunotherapy for Chronic Hepatitis B Virus Infection. Gut Liver. 2018; 12(5):497-507.
13. Revill PA, Chisari FV, Block JM, et al. A global scientific strategy to cure hepatitis B. Lancet Gastroenterol Hepatol. 2019.
14. Webster GJ, Reignat S, Maini MK, et al. Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. Hepatology. 2000; 32(5):1117-24.
15. Fisicaro P, Valdatta C, Boni C, et al. Early kinetics of innate and adaptive immune responses during hepatitis B virus infection. Gut. 2009; 58(7):974-82.
16. Ferrari C, Penna A, Bertoletti A, et al. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. J Immunol. 1990; 145(10):3442-9.
17. Boni C, Laccabue D, Lampertico P, et al. Restored function of HBV-specific T cells after long-term effective therapy with nucleos(t)ide analogues. Gastroenterology. 2012; 143(4):963-73.e9.
18. Di Lello FA, Ridruejo E, Martinez AP, Perez PS, Campos RH, Flichman DM. Molecular epidemiology of hepatitis B virus mutants associated with vaccine escape, drug resistance and diagnosis failure. J Viral Hepat. 2019; 26(5):552-60.
19. Dunn C, Peppa D, Khanna P, et al. Temporal analysis of early immune responses in patients with acute hepatitis B virus infection. Gastroenterology. 2009; 137(4):1289-300.

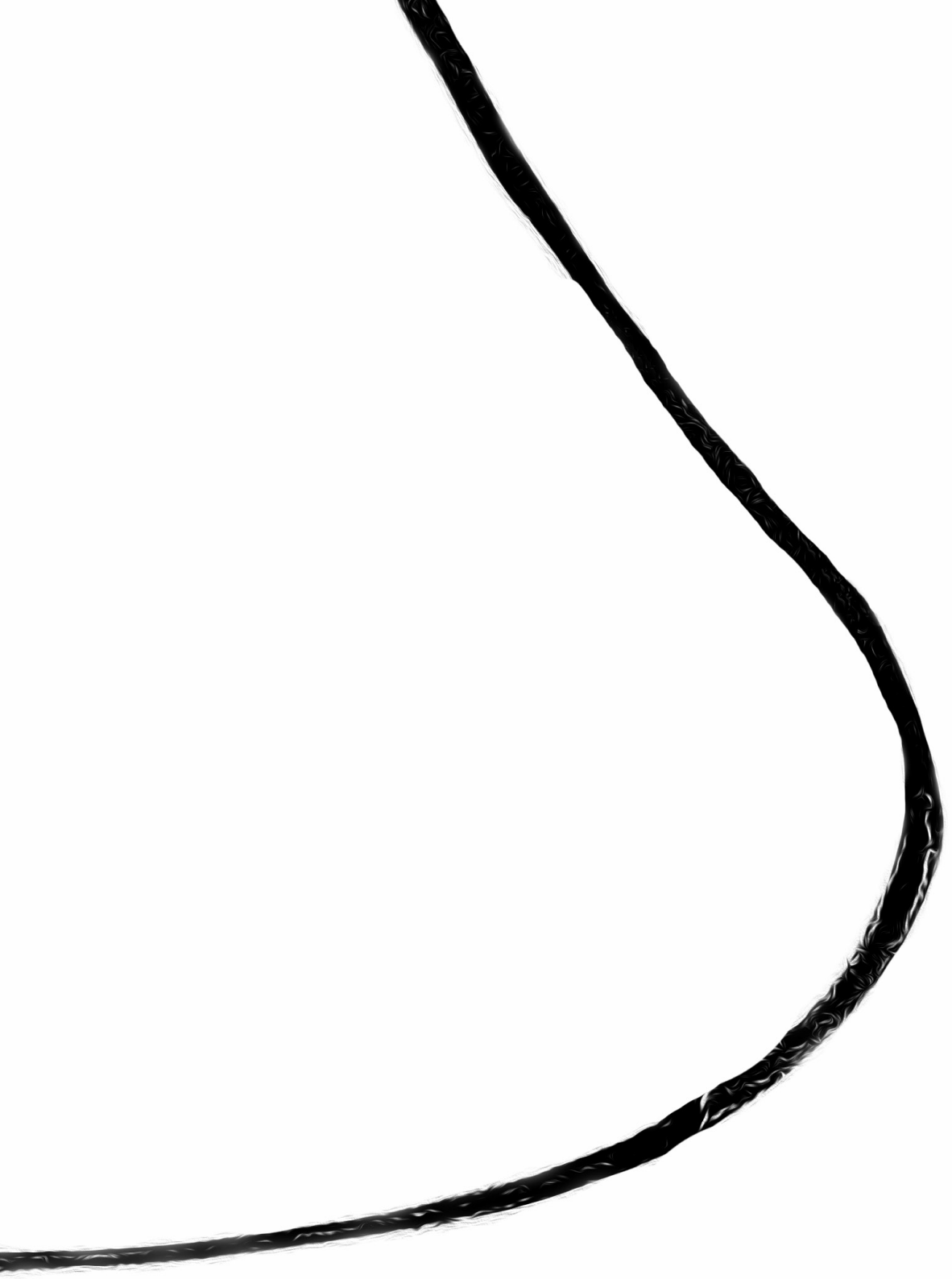
20. Wang Q, Pan W, Liu Y, et al. Hepatitis B Virus-Specific CD8+ T Cells Maintain Functional Exhaustion after Antigen Reexposure in an Acute Activation Immune Environment. Front Immunol. 2018; 9:219.
21. Xu D, Fu J, Jin L, et al. Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. J Immunol. 2006; 177(1):739-47.
22. Evans A, Riva A, Cooksley H, et al. Programmed death 1 expression during antiviral treatment of chronic hepatitis B: Impact of hepatitis B e-antigen seroconversion. Hepatology. 2008; 48(3):759-69.
23. Sprengers D, Stoop JN, Binda RS, et al. Induction of regulatory T-cells and interleukin-10-producing cells in non-responders to pegylated interferon-alpha therapy for chronic hepatitis B. Antiviral Therapy. 2007; 12(7):1087-96.
24. Rigopoulou EI, Suri D, Chokshi S, et al. Lamivudine plus interleukin-12 combination therapy in chronic hepatitis B: Antiviral and immunological activity. Hepatology. 2005; 42(5):1028-36.
25. Boni C, Penna A, Bertoletti A, et al. Transient restoration of anti-viral T cell responses induced by lamivudine therapy in chronic hepatitis B. Journal of Hepatology. 2003; 39(4):595-605.
26. Cooksley H, Chokshi S, Maayan Y, et al. Hepatitis B virus e antigen loss during adefovir dipivoxil therapy is associated with enhanced virus-specific CD4+ T-cell reactivity. Antimicrob Agents Chemother. 2008; 52(1):312-20.
27. Zheng Q, Zhu YY, Chen J, et al. Decline in intrahepatic cccDNA and increase in immune cell reactivity after 12 weeks of antiviral treatment were associated with HBeAg loss. J Viral Hepat. 2014; 21(12):909-16.
28. Lau GK, Cooksley H, Ribeiro RM, et al. Impact of early viral kinetics on T-cell reactivity during antiviral therapy in chronic hepatitis B. Antivir Ther. 2007; 12(5):705-18.
29. Zheng BJ, Zhou J, Qu D, et al. Selective functional deficit in dendritic cell--T cell interaction is a crucial mechanism in chronic hepatitis B virus infection. J Viral Hepat. 2004; 11(3):217-24.
30. Stoop JN, van der Molen RG, Kuipers EJ, Kusters JG, Janssen HLA. Inhibition of viral replication reduces regulatory T cells and enhances the antiviral immune response in chronic hepatitis B. Virology. 2007; 361(1):141-8.
31. Wherry EJ, Barber DL, Kaech SM, Blattman JN, Ahmed R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. Proc Natl Acad Sci U S A. 2004; 101(45):16004-9.
32. Thimme R, Wieland S, Steiger C, et al. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J Virol. 2003; 77(1):68-76.
33. Rehermann B, Fowler P, Sidney J, et al. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med. 1995; 181(3):1047-58.
34. Maini MK, Boni C, Ogg GS, et al. Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. Gastroenterology. 1999; 117(6):1386-96.
35. Schuch A, Salimi Alizei E, Heim K, et al. Phenotypic and functional differences of HBV core-specific versus HBV polymerase-specific CD8+ T cells in chronically HBV-infected patients with low viral load. Gut. 2019; 68(5):905-15.
36. Sung PS, Park DJ, Kim JH, et al. Ex vivo Detection and Characterization of Hepatitis B Virus-Specific CD8(+) T Cells in Patients Considered Immune Tolerant. Front Immunol. 2019; 10:1319.
37. Schuch A, Hoh A, Thimme R. The role of natural killer cells and CD8(+) T cells in hepatitis B virus infection. Front Immunol. 2014; 5:258.
38. Boni C, Penna A, Ogg GS, et al. Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: New perspectives for immune therapy. Hepatology. 2001; 33(4):963-71.

39. Boni C, Bertoletti A, Penna A, et al. Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. Journal of Clinical Investigation. 1998; 102(5):968-75.
40. Hoogeveen RC, Robidoux MP, Schwarz T, et al. Phenotype and function of HBV-specific T cells is determined by the targeted epitope in addition to the stage of infection. Gut. 2019; 68(5):893-904.
41. Rivino L, Le Bert N, Gill US, et al. Hepatitis B virus-specific T cells associate with viral control upon nucleos(t)ide-analogue therapy discontinuation. J Clin Invest. 2018; 128(2):668-81.
42. Spaan M, van Oord G, Kreefft K, et al. Immunological Analysis During Interferon-Free Therapy for Chronic Hepatitis C Virus Infection Reveals Modulation of the Natural Killer Cell Compartment. J Infect Dis. 2016; 213(2):216-23.
43. Manigold T, Racanelli V. T-cell regulation by CD4 regulatory T cells during hepatitis B and C virus infections: facts and controversies. Lancet Infect Dis. 2007; 7(12):804-13.
44. Franzese O, Kennedy PT, Gehring AJ, et al. Modulation of the CD8+-T-cell response by CD4+ CD25+ regulatory T cells in patients with hepatitis B virus infection. J Virol. 2005; 79(6):3322-8.
45. Stoop JN, van der Molen RG, Baan CC, et al. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. Hepatology. 2005; 41(4):771-8.
46. Stoop JN, Claassen MA, Woltman AM, et al. Intrahepatic regulatory T cells are phenotypically distinct from their peripheral counterparts in chronic HBV patients. Clin Immunol. 2008; 129(3):419-27.
47. Ichikawa S, Mucida D, Tyznik AJ, Kronenberg M, Cheroutre H. Hepatic stellate cells function as regulatory bystanders. J Immunol. 2011; 186(10):5549-55.
48. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. J Immunol. 2004; 172(9):5149-53.
49. Yang X, Li J, Liu J, Gao M, Zhou L, Lu W. Relationship of Treg/Th17 balance with HBeAg change in HBeAg-positive chronic hepatitis B patients receiving telbivudine antiviral treatment: A longitudinal observational study. Medicine (Baltimore). 2017; 96(23):e7064.
50. Yan Z, Zhou J, Zhang M, Fu X, Wu Y, Wang Y. Telbivudine decreases proportion of peripheral blood CD4+CD25+CD127low T cells in parallel with inhibiting hepatitis B virus DNA. Mol Med Rep. 2014; 9(5):2024-30.
51. Yang J, Sheng G, Xiao D, et al. The frequency and skewed T-cell receptor beta-chain variable patterns of peripheral CD4(+)CD25(+) regulatory T-cells are associated with hepatitis B e antigen seroconversion of chronic hepatitis B patients during antiviral treatment. Cell Mol Immunol. 2016; 13(5):678-87.
52. Zhang JYS, C. H.; Shi, F.; Zhang, Z.; Fu, J. L.; Wang, F. S. Decreased ratio of Treg cells to Th17 cells correlates with HBV DNA suppression in chronic hepatitis B patients undergoing entecavir treatment. PLoS One. 2010; 5(11):e13869.
53. Yu XP, Guo RY, Su ML, et al. Dynamic Changes of Treg and Th17 Cells and Related Cytokines Closely Correlate With the Virological and Biochemical Response in Chronic Hepatitis B Patients Undergoing Nucleos(t)ide Analogues Treatment. Hepat Mon. 2013; 13(12):e15332.
54. Trehanpati N, Kotillil S, Hissar SS, et al. Circulating tregs correlate with viral load reduction in chronic HBV-treated patients with tenofovir disoproxil fumarate. J Clin Immunol. 2011; 31(3):509-20.
55. Li CZ, Hu JJ, Xue JY, et al. Viral infection parameters not nucleoside analogue itself correlates with host immunity in nucleoside analogue therapy for chronic hepatitis B. World J Gastroenterol. 2014; 20(28):9486-96.

56. Ma L, Cai YJ, Yu L, et al. Treatment with telbivudine positively regulates antiviral immune profiles in chinese patients with chronic hepatitis B. Antimicrob Agents Chemother. 2013; 57(3):1304-11.
57. Jiang Y, Li W, Yu L, et al. Enhancing the antihepatitis B virus immune response by adefovir dipivoxil and entecavir therapies. Cell Mol Immunol. 2011; 8(1):75-82.
58. Okulicz JF, Le TD, Agan BK, et al. Influence of the Timing of Antiretroviral Therapy on the Potential for Normalization of Immune Status in Human Immunodeficiency Virus 1-Infected Individuals. Jama Internal Medicine. 2015; 175(1):88-99.
59. Peppa D, Gill US, Reynolds G, et al. Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion. J Exp Med. 2013; 210(1):99-114.
60. Kakimi K, Guidotti LG, Koezuka Y, Chisari FV. Natural killer T cell activation inhibits hepatitis B virus replication in vivo. J Exp Med. 2000; 192(7):921-30.
61. Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. Science. 1999; 284(5415):825-9.
62. Oliviero B, Varchetta S, Paudice E, et al. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. Gastroenterology. 2009; 137(3):1151-60, 60 e1-7.
63. Peppa D, Micco L, Javaid A, et al. Blockade of immunosuppressive cytokines restores NK cell antiviral function in chronic hepatitis B virus infection. PLoS Pathog. 2010; 6(12):e1001227.
64. Tjwa ETTL, Van Oord GW, Hegmans JP, Janssen HLA, Woltman AM. Viral load reduction improves activation and function of natural killer cells in patients with chronic hepatitis B. J Hepatol. 2011; 54(2):209-18.
65. Kurihara M, Tsuge M, Murakami E, et al. The association between serum cytokine and chemokine levels and antiviral response by entecavir treatment in chronic hepatitis B patients. Antivir Ther. 2018; 23(3):239-48.
66. Zhao J, Li Y, Jin L, et al. Natural killer cells are characterized by the concomitantly increased interferon-gamma and cytotoxicity in acute resolved hepatitis B patients. PLoS One. 2012; 7(11):e49135.
67. de Groen RA, Hou J, van Oord GW, et al. NK cell phenotypic and functional shifts coincide with specific clinical phases in the natural history of chronic HBV infection. Antiviral Res. 2017; 140:18-24.
68. Chen T, Zhu L, Shi A, et al. Functional restoration of CD56^{bright} NK cells facilitates immune control via IL-15 and NKG2D in patients under antiviral treatment for chronic hepatitis B. Hepatol Int. 2017; 11(5):419-28.
69. Lv J, Jin Q, Sun H, et al. Antiviral treatment alters the frequency of activating and inhibitory receptor-expressing natural killer cells in chronic hepatitis B virus infected patients. Mediators Inflamm. 2012.
70. Duan XZ, Wang M, Li HW, Zhuang H, Xu D, Wang FS. Decreased frequency and function of circulating plasmocytoid dendritic cells (pDC) in hepatitis B virus infected humans. J Clin Immunol. 2004; 24(6):637-46.
71. Boni C, Lampertico P, Talamona L, et al. Natural killer cell phenotype modulation and natural killer/T-cell interplay in nucleos(t)ide analogue-treated hepatitis e antigen-negative patients with chronic hepatitis B. Hepatology. 2015; 62(6):1697-709.
72. Zhao P-W, Jia F-Y, Shan Y-X, et al. Downregulation and altered function of natural killer cells in hepatitis B virus patients treated with entecavir. Clin Exp Pharmacol Physiol. 2013; 40(3):190-6.
73. Weng HL, Wang BE, Jia JD, et al. Effect of interferon-gamma on hepatic fibrosis in chronic hepatitis B virus infection: a randomized controlled study. Clin Gastroenterol Hepatol. 2005; 3(8):819-28.
74. Gur C, Doron S, Kfir-Erenfeld S, et al. NKp46-mediated killing of human and mouse hepatic stellate cells attenuates

liver fibrosis. Gut. 2012; 61(6):885-93.

75. Tjwa ET, van Oord GW, Biesta PJ, Boonstra A, Janssen HL, Woltman AM. Restoration of TLR3-activated myeloid dendritic cell activity leads to improved natural killer cell function in chronic hepatitis B virus infection. J Virol. 2012; 86(8):4102-9.
76. Li M, Zhou ZH, Sun XH, et al. The dynamic changes of circulating invariant natural killer T cells during chronic hepatitis B virus infection. Hepatol Int. 2016; 10(4):594-601.
77. Shi TD, Zhang JM, Wang XF, et al. Effects of antiviral therapy with Telbivudine on peripheral iNKT cells in HBeAg(+) chronic hepatitis B patients. Clin Exp Med. 2012; 12(2):105-13.
78. Jiang X, Zhang M, Lai Q, et al. Restored circulating invariant NKT cells are associated with viral control in patients with chronic hepatitis B. PLoS One. 2011; 6(12):e28871.
79. Diao H, He J, Zheng Q, et al. A possible role for NKT-like cells in patients with chronic hepatitis B during telbivudine treatment. Immunol Lett. 2014; 160(1):65-71.
80. Cannizzo ES, Tincati C, Binda F, et al. Unconventional T cells in chronic hepatitis B patients on long-term suppressive therapy with tenofovir followed by a Peg-IFN add-on strategy: A randomized study. J Viral Hepat. 2018; 25(4):381-90.
81. Arasli M, Ustundag Y, Delikanli B, Harmandar F, Buyukuysal C. Peripheral blood lymphocyte dynamics and viral kinetics in patients with chronic active hepatitis B virus infection treated by tenofovir. Hepatogastroenterology. 2012; 59(115):851-7.
82. Boeijen LL, Montanari NR, De Groen RA, et al. Mucosal-Associated Invariant T Cells Are More Activated in Chronic Hepatitis B, but Not Depleted in Blood: Reversal by Antiviral Therapy. J Infect Dis. 2017; 216(8):969-76.
83. Beudeker BJB, van Oord GW, Arends JE, et al. Mucosal-associated invariant T-cell frequency and function in blood and liver of HCV mono- and HCV/HIV co-infected patients with advanced fibrosis. Liver Int. 2018; 38(3):458-68.
84. Spaan M, Hullegie SJ, Beudeker BJ, et al. Frequencies of Circulating MAIT Cells Are Diminished in Chronic HCV, HIV and HCV/HIV Co-Infection and Do Not Recover during Therapy. PLoS One. 2016; 11(7):e0159243.
85. van Wilgenburg B, Scherwitzl I, Hutchinson EC, et al. MAIT cells are activated during human viral infections. Nat Commun. 2016; 7:11653.
86. Vanwolleghem T, Hou J, van Oord G, et al. Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells. Hepatology. 2015; 62(1):87-100.
87. Op den Brouw ML, Binda RS, van Roosmalen MH, et al. Hepatitis B virus surface antigen impairs myeloid dendritic cell function: a possible immune escape mechanism of hepatitis B virus. Immunology. 2009; 126(2):280-9.
88. Boeijen LL, van Oord GW, Hou J, et al. Gene expression profiling of human tissue-resident immune cells: Comparing blood and liver. J Leukoc Biol. 2019; 105(3):603-8.
89. Tjwa ET, Zoutendijk R, van Oord GW, et al. Similar frequencies, phenotype and activation status of intrahepatic NK cells in chronic HBV patients after long-term treatment with tenofovir disoproxil fumarate (TDF). Antiviral Res. 2016; 132:70-5.
90. Van Hees S, Bourgeois S, Van Vlierberghe H, et al. Stopping nucleos(t)ide analogue treatment in Caucasian hepatitis B patients after HBeAg seroconversion is associated with high relapse rates and fatal outcomes. Aliment Pharmacol Ther. 2018; 47(8):1170-80.
91. Martin B, Hennecke N, Lohmann V, et al. Restoration of HCV-specific CD8+ T cell function by interferon-free therapy. J Hepatol. 2014; 61(3):538-43.



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Discussion and future perspectives

The work in this thesis characterizes lymphocytes during the various phases of chronic HBV, and advances our understanding of the differences between liver and blood immune profiles. In addition, it provides a practical evaluation of different methods that can be used to investigate immune mediated liver diseases like chronic HBV.

CONCLUSIONS

Due to the lack of a suitable model for HBV and unavailability of unbiased methods to investigate material from human patients, it was only possible to detect very large shifts in immune cell phenotype using immunohistochemistry and 8-color flow cytometry. With the advent of high-resolution biomedical techniques like RNA sequencing, as well as developments in the analysis of liver material, it is possible to gain more detailed information on immune profiles of patients infected with chronic HBV. In this thesis we explore the application of several techniques on blood and liver tissue. Using next generation RNA sequencing, we mapped the transcriptome of NK cells during all phases of chronic HBV, foregoing the necessity of researcher-based selection of markers. We detected few immune related genes differentially expressed during these phases when compared to healthy controls, while HIV and HCV significantly affected NK cell gene expression. Another immune cell with innate properties, the MAIT cell, was not affected by the various phases of HBV, while its activated phenotype was normalized by antiviral treatment. In addition, we optimized the information output of core liver biopsies available for research, and show that both flow cytometry and RNA sequencing can be performed on fresh liver biopsies, detecting several liver specific genes, and liver specific effects of experimental immune therapy. Based on the consistently identified patient and organ specific immune profiles, we propose that the most sensitive biomedical techniques available, like single cell transcriptomics, best applied in a longitudinal setting, may detect more subtle changes in the host immune response during HBV. Identification and association of these changes with the natural history of HBV or antiviral treatment, could prove to be an essential step to better understand the complex pathogenesis of HBV.

THE HBV VIRAL LOAD FLUCTUATES, BUT HOST RESPONSES MIGHT NOT

In chapter 3 and 5 of this thesis, we detected changes in the innate immune response associated with virological changes. In previous work, de Groen et al[1] characterized peripheral blood NK cells during the various phases of HBV, associated with fluctuations of HBV DNA and ALT levels. Surprisingly, there were no clear correlations of NK cell activation with replication of HBV, despite the great difference in degree of liver inflammation and the viral load between the HBV cohorts.

However, NK cells showed a slight increase during progression of HBV phases in IFN-γ production upon in vitro stimulation with IL-12/IL-18. In another study[2], we investigated the NK cells from liver aspirates paired with blood cells after rapid HBV viral load decline induced by nucleotide analog treatment. Compared to baseline, liver and blood NK cell frequency and activation (assessed by changes of CD25 or CD69 expression) were not affected by complete suppression of viral replication. It remains possible, that more subtle immunological changes which might be detected using other biomedical techniques, occurred during HBV viral load shifts either induced by antiviral treatment or clinical phase progression.

To address the possibility of NK cells being affected during the clinical phases of HBV infection, we performed bulk RNA sequencing on peripheral blood NK cells of patients during several phases. While using a highly sensitive and unbiased technique (next generation RNA sequencing), we detected only few differences in the NK cell transcriptome. In contrast to flow cytometry, this approach does not require a researcher-based preselection of markers of interest: all sequenced mRNA is compared to that of healthy controls. Chronic HCV or HIV viremia did clearly induce activating signaling pathways and expression of ISGs, in contrast to NK cells from chronic HBV patients. The results of these studies combined raise the possibility that NK cells, evaluated by flow cytometry or RNA sequencing, are not at all affected by viral load fluctuations during the chronic phases of HBV. Alternatively, it is possible that tissue resident NK cells may display variations that are not occurring in the blood[3]. In contrast to chronic HBV, it is clear from the sparse studies on acute HBV[4] that NK cells are activated several weeks following transmission of the virus. The NK cell compartment might be altered during the transition from acute HBV to a chronic viremic infection. But during the chronic phase, the phenotypical NK cell shifts could be too subtle to be detected in cross-sectional comparison with a cohort of healthy controls. CMV infection in humans results in a clear and lasting enrichment for NKG2C+CD57+ memory-like NK cells[5]. Irrespective of renewed CMV exposure, this skewing of NK cell subsets is fixed. A recent study published by Cuff et al[6] showed that NK cells remain present in the successfully transplanted liver, even 13 years after transplantation (proven by haplotyping). This suggests that certain subpopulations of NK cells (in this study expressing high levels of Eomes, a transcription factor that governs NK cell differentiation and function) may be long-lived and unresponsive to episodes of liver inflammation or future viral infection. Currently it remains unknown how dynamic the intrahepatic NK cell compartment is during HBV, as longitudinal liver samples are rare. Ideally, to investigate immunological changes attributed to HBV itself, one would need to sample individual human subjects before HBV was transmitted and additionally sampled once chronic infection has been established. Even though the disease is usually cleared by the host, allowing chronic HBV to establish without intervention is unethical. However, it might be possible to use material previously collected during prospective follow up of individuals at risk for HBV transmission in order to investigate these immunological changes.

SMALL CHANGES OF IMMUNE CELL ACTIVITY ARE DETECTED DURING CHRONIC HBV

In contrast to NK cells during treatment with nucleotide analogs[2], we did detect phenotypic alterations of Valpha7.2+CD161+MAIT cells after viral load suppression by antiviral treatment. MAIT cells are T cells that express an invariant TCR[7] specific for bacterial antigens. Their role in viral infection is not well understood, but during HCV[8] and HIV[9, 10] infections, as well as some other inflammatory diseases, the frequency of MAIT cells in the blood is reduced. Reduced MAIT cell frequency could be related to migration, activation induced apoptosis, or impaired differentiation of these cells, but the exact mechanisms are currently unclear. MAIT cells themselves are unlikely to be activated by viral particles, because of the invariant T cell receptor specific for bacterial products. Interestingly, the percentage of granzyme B and perforin positive MAIT cells was higher in response to stimulation with fixed E. coli among patients with chronic HBV. This may reflect increased sensitivity to inflammatory signals in the blood or differential interaction with other immune cells that may be activated during the HBV infection, like Kupffer cells and monocytes[11]. When the HBV viral load in the blood of patients was lowered by treatment with nucleotide and nucleoside analog treatment, MAIT cell CD38 expression dropped to healthy control levels. This result corresponds to HIV treatment effects on CD38 expression by MAIT cells[12]. CD38 has a role in cell adhesion of lymphocytes, their signal transduction and calcium signaling, and is considered a marker of activation for CD8+ T cells, NK cells and MAIT cells. Although the origin of the correlation of this marker with HBV viral load is unclear, associations of CD38 with viral load is not unique to HBV. T cells that express CD8 and CD38 are even used as a marker of residual HIV replication during antiretroviral treatment[13]. MAIT cells are exposed to high concentrations of bacterial antigens via the portal vein in the liver, possibly affecting their activation status. Whether the viral load mediated suppression of MAIT cells also occurs in the liver remains to be determined. In general, this observation does emphasize that a longitudinal approach to sample HBV can detect interactions between the virus and the host immune response that might be missed or related to patient specific factors (other than viral factors like the degree of viral replication). The downregulation of CD38 during antiviral therapy is one of the first observations described on MAIT cells in this clinical context. However, the effects of nucleotide analogs for HBV have been investigated for other immune cell types, like virus specific T cells, and also innate immune cells[14]. We evaluated these longitudinal studies that investigated nucleotide analog effects, or the artificial lowering of the viral load, on the host immune cells. We concluded that HBV specific CD4+ T cell IFN-γ production improved slightly after 3 months of treatment in most studies, but that CD8+ T cell improvements were transient, which is an observation that could be exploited in the design of trials that include experimental treatment for HBV[15]. Considering recent improvement of biomedical techniques like multicolor flow cytometry, mass spectrometry and RNA sequencing, future studies can now

expand on these observations, to better understand the effects of treatment on the host immune response.

STUDIES ON HBV INFECTED LIVER DERIVED CELLS ARE RARE

Considerable progress has been made on what we know of the intrahepatic immune response using liver from patients with liver diseases other than HBV. It has been consistently shown in explanted livers with HCC, that both tumor infiltrating T cells and intrahepatic T cells (from liver tissue adjacent to the tumor) produce less cytokines and have high expression of exhaustion markers like PD-1 and CTLA-4[16]. Based on tumor tissue from HCC patients, it was shown that NK cell frequency and activity correlated with survival of these patients[17, 18]. Interestingly, another comparable study showed that liver derived NK cells had lower cytotoxic potential, but that their function could be restored by IL-2 stimulation in vitro[19]. In a study of core biopsies derived from HCV infected patients before and on direct acting antivirals, it was shown that in patients who successfully cleared HCV, IFN signaling was more pronounced at the start of treatment compared to those where the virus reactivated. This may indicate that the increased IFN signaling supports the direct acting antiviral therapy, or that active replication of HCV suppresses the host immune response[20]. In HBV research, unfortunately, the current knowledge on host – pathogen interactions in the liver is limited, and based on a relatively small number of studies that used flow cytometry to evaluate human liver resident immune cells[2, 21-23].

MINIMALLY INVASIVE BIOPSIES CAN USED TO SAMPLE HBV INFECTED LIVER DERIVED CELLS

Our knowledge of immune responses in the liver is still mainly based on the study of formalin fixed and paraffin embedded (FFPE) liver tissue, not on biopsies taken directly ex vivo during non-cirrhotic HBV infection. The need for fresh liver material from HBV patients is more pressing compared to that from HCC patients for example, due to the rapid development of less invasive diagnostic applications like transient elastography to evaluate the stage of HBV related liver disease[24]. While reducing the number of complications related to core biopsies, these innovations also reduce the amount of residual liver biopsy material available for research. Fortunately, considerable progress has been made using immunohistochemistry of liver biopsies from HBV patients, by assessment of several membrane markers using flow cytometry, and by RNA sequencing of FFPE liver biopsies. Moreover, several studies have combined information on the liver compartment with measurements of paired blood samples. In previous studies by

Vanwolleghem et al[25], and Hou et al[26], the blood gene expression profile of cross-sectional cohorts of chronic HBV patients at different clinical phases was compared, and importantly, this was combined with micro-array analysis of FFPE liver biopsies. Distinctive gene signatures were observed with variable activity of interferon stimulated genes (ISG), NK cell-related genes and B cell-related genes in specific HBV clinical phases. This was a novel observation linking B cells and NK cells to the progression of HBV phases. Another study by Lebossé et al[27] also analyzed FFPE biopsies and showed that innate immune regulatory pathways are downregulated in comparison to a control group of patients undergoing elective abdominal surgery. In one study, Suslov et al[28] compared liver biopsies from chronic HBV patients by immunohistochemistry and PCR, directly after the biopsy procedure. No increase of ISG transcription was observed in HBV patients, compared predominantly to liver biopsies from patients with fatty liver disease, which may not reflect a healthy state. By these cross-sectional studies comparing substantially different patient cohorts, subtle changes in gene expression profiles might be missed since the population of chronic HBV patients is highly heterogeneous and good control liver biopsies are rarely available. Furthermore, it is not possible to correct for important confounding factors, including gender, ethnicity or age, which again favors serial biopsy from the same patient. Therefore, given the complication rate of diagnostic core liver biopsies for patients and healthy controls, a longitudinal (repeated) evaluation of fresh tissue solely for research purposes would further increase this risk.

Few studies have combined the collection of liver material with a longitudinal study setup. Using dual FFPE liver biopsies, Zheng et al[29] showed that antiviral treatment increased the intrahepatic CD8+ T lymphocytes frequency in the liver, but only in those patients with HBeAg loss during follow up. The possibility to isolate RNA of reasonable quality from FFPE liver biopsies[25, 26] represents a promising approach to study the effect of viral load and ALT shifts in an unbiased manner using previously stored material. Fixed liver biopsies are more readily available than fresh liver material. Alternatively, reducing the risk of the biopsy procedure, by -for example- using a smaller sized needle, is also an attractive option that could provide fresh and viable liver cells.

The use of a minimally invasive biopsy technique, the fine needle aspiration biopsy used for cytology as opposed to histology, has proven useful in mapping intrahepatic immune responses during chronic HBV. This technique is more suitable for research, as it is associated with minimal risk for the patient, in contrast to core biopsy procedures[30]. Much smaller than the needles used for the diagnostic core biopsies (14-18 Gauge), an aspiration needle (22-25 Gauge) can be used to sample liver immune cells and possibly parenchymal cells for flow cytometric evaluation. Using this technique, Sprengers[21] et al showed in 2005 that virus specific CD8+ T cells infiltrate the liver during HBV infection. Interestingly, three months after a functional cure for HBV, intrahepatic HBV-specific CD8+ T cells remained frequent in the liver. The technique

has also been applied to HCV infected patients, effectively sampling regulatory T cells[31] and NK cells[32] derived from the liver. In a more recent study, we investigated the effect of long-term suppression of HBV DNA and ALT normalization by nucleotide analogs treatment on HBV infected liver derived NK cells taken directly ex vivo[2]. Surprisingly, NK cell frequency and phenotype, which differed considerably between liver and blood, was not affected by treatment in this cohort, even after years of viral suppression. Although this study was unique, as it applied flow cytometry to multiple fresh liver aspiration biopsies and paired blood samples, additional changes might be detected by assessing additional parameters in a larger cohort. Another possibility is that the decline of ALT (and viral load) is not associated with activation of liver resident NK cells, or that modulation of NK cell function and/or phenotype are not detected by the techniques used in this project.

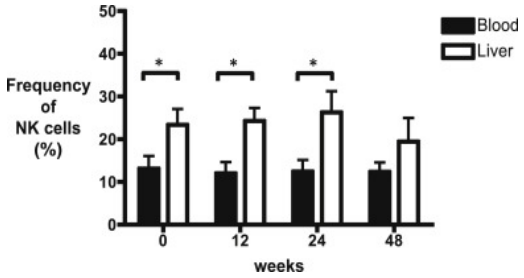


Figure 2. The frequency of liver resident and blood derived NK cells does not change upon treatment. Black bars: blood. White bars: liver obtained as fine needle aspirates. Adapted from Tjwa et al 2016 [2].

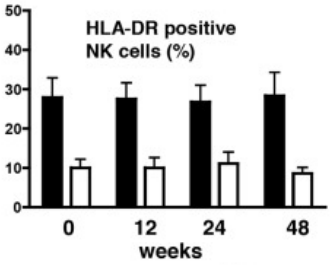


Figure 3. The expression of the activation marker HLA-DR differs between liver and blood, but is not significantly changed upon treatment with nucleotide analogs (0 weeks = untreated). Black bars: blood. White bars: liver. Adapted from Tjwa et al 2016 [2].

In summary, despite considerable advances in the field of liver immunology and recent progress in the characterization of immune responses to HBV, additional information on intrahepatic immune responses to HBV, especially using direct ex vivo biopsies, remain essential to better understand the disease. In addition, the variation and dynamic clinical presentation of HBV patients require us to sample patients in a longitudinal fashion and use biomedical techniques that can detect subtle changes, like RNA sequencing.

IMMUNOLOGICAL CHARACTERIZATION OF LIVER AND BLOOD: FUTURE PERSPECTIVES

The combination of fluorescence assisted cell sorting (FACS) and RNA sequencing on fresh liver material is a method to perform a detailed characterization of organ specific immune cells, but requires fresh (liver) biopsy material, which is rarely available for research purposes. In chapter 4 of this thesis, we used bulk sorted liver derived immune cells (CD3+ or CD56+) and subsequently performed next generation RNA sequencing. We had the opportunity to sort these liver biopsies at two different time points, using material from the same patients. This allowed us to detect the induction of ISG, as well as several other immune related genes, by treatment with the TLR7 agonist GS-9620. Some of these genes, like CD8a and PRF1, were differentially expressed in the liver derived cells, but not in the blood. This study increased the immunological information gained from the analysis of fresh biopsy material by isolating RNA from fresh liver immune cells. We could not trace back the origin of the RNA fragments to individual immune cells, as we performed RNA-sequencing of an entire population of cells. This results in an average of the abundance of transcripts of a single gene in a particular population of immune cells. Recently, it has become possible to isolate RNA from single immune cells[33, 34]. By analyzing the transcriptomes of single cells instead of the entire population, the heterogeneity among liver resident cells (for example hepatocytes, Kupffer cells, or lymphocytes) can be uncovered. Mapping the single cell transcriptome can distinguish different cell populations with related gene expression profiles, possibly identifying novel immune cell subpopulations specific to the infected liver. Subtle changes in blood and liver that occur during the progression of HBV phases or during the reactivation of HBV after cessation of therapy, as well as additional detail on blood liver differences might be uncovered. In addition, the potential immune suppressive effects of HBsAg on different immune cells might be unraveled when patients with different levels of HBsAg are tested this way [35, 36]. It may even be possible to sequence viral and host transcriptome simultaneously, derived from the same (infected) cell. This would provide the most direct information on the interaction between pathogen and host. Ongoing efforts on single cell RNA sequencing in combination with fine needle aspirations biopsy or core biopsy derived cells of HBV patients have not been published to date. Given the risk of complications for patients associated with core biopsies, the

use of the minimally invasive fine needle biopsy technique is preferred. Still, it is important to select the single cell RNA sequencing platform most suitable for liver cytology acquired using FNAB. Only a few comparisons on the currently available techniques have yet been published, but these studies did not include liver FNAB or HBV patient samples. However, considerable progress has been made on other types of material from patients with other diseases. Single cell RNA sequencing data has already been gathered on various human tissues like blood, lung and liver, with the 10x Genomics Chromium platform[37], a droplet based single cell isolation technique. A mixture of patient derived single cells, reverse transcription reagents, gel beads containing barcoded oligonucleotides, and oil are combined on a microfluidic chip and form reaction vesicles called Gel Beads in Emulsion or GEMs. Each GEM contains a single cell, subsequently used for reverse transcription and (barcoded) library generation. Other droplet based single cell systems, like Drop-Seq[38], have also been shown to result in high information yield characterizations of the host immune system. Another promising single cell RNA sequencing platform is Seq-Well[39], which uses uniquely barcoded mRNA capture beads pre-loaded into microscopic pico wells, enabling entry, cell lysis and mRNA capture of a single cell. The VyCAP single cell isolation technique is also of interest as it allows for visual selection of single cells[40]. Finally, laser capture microdissection uses a precise laser to cut out a cell of interest from tissue, and subsequently isolate cell specific RNA[41]. To date, these techniques have not been applied to liver or blood of mono infected HBV patients, but data has been published on liver material of patients with other liver diseases. For example, combining two single cell sequencing platforms (10X Genomics and SmartSeq2) on lymph node and hepatocellular carcinoma derived CD45+ cells, Zhang et al[42] showed that LAMP3, a lysosomal membrane protein specific to mature dendritic cells (DCs), marked a population of DC present in lymph node and liver. This DC subset may have the potential to migrate from the tumor to lymph nodes, and facilitate the presentation of tumor antigens. Progress has also been made using biopsies from patients with different stages of liver fibrosis: Rachmandran et al[43] used the 10X Genomics Chromium platform, and detected a subpopulation of macrophages (TREM2+CD9+) to be associated with the degree of fibrosis, expressing several additional genes potentially involved in the fibrogenesis, providing new information on this incompletely understood pathological process. Using laser capture microdissection, a recent paper has been published on liver derived cells form HIV/HBV coinfected human liver tissue[44]. Although the results remain to be confirmed using HBV mono-infected liver, the authors detected that during untreated HBV, more than 95 percent of the hepatocytes in the biopsy were infected with HBV. In addition, viral replication was estimated by quantification of the pregenomic viral RNA present in each cell. Interestingly, as has been proposed before by others[45], pregenomic RNA did not correlate with cccDNA levels, suggesting that HBV replication is dependent on other intracellular factors. These very detailed characterizations of liver cells elegantly show the wealth of information that can be gained using single cell transcriptomics of liver tissue and emphasize the potential for translational HBV research.

REFERENCES

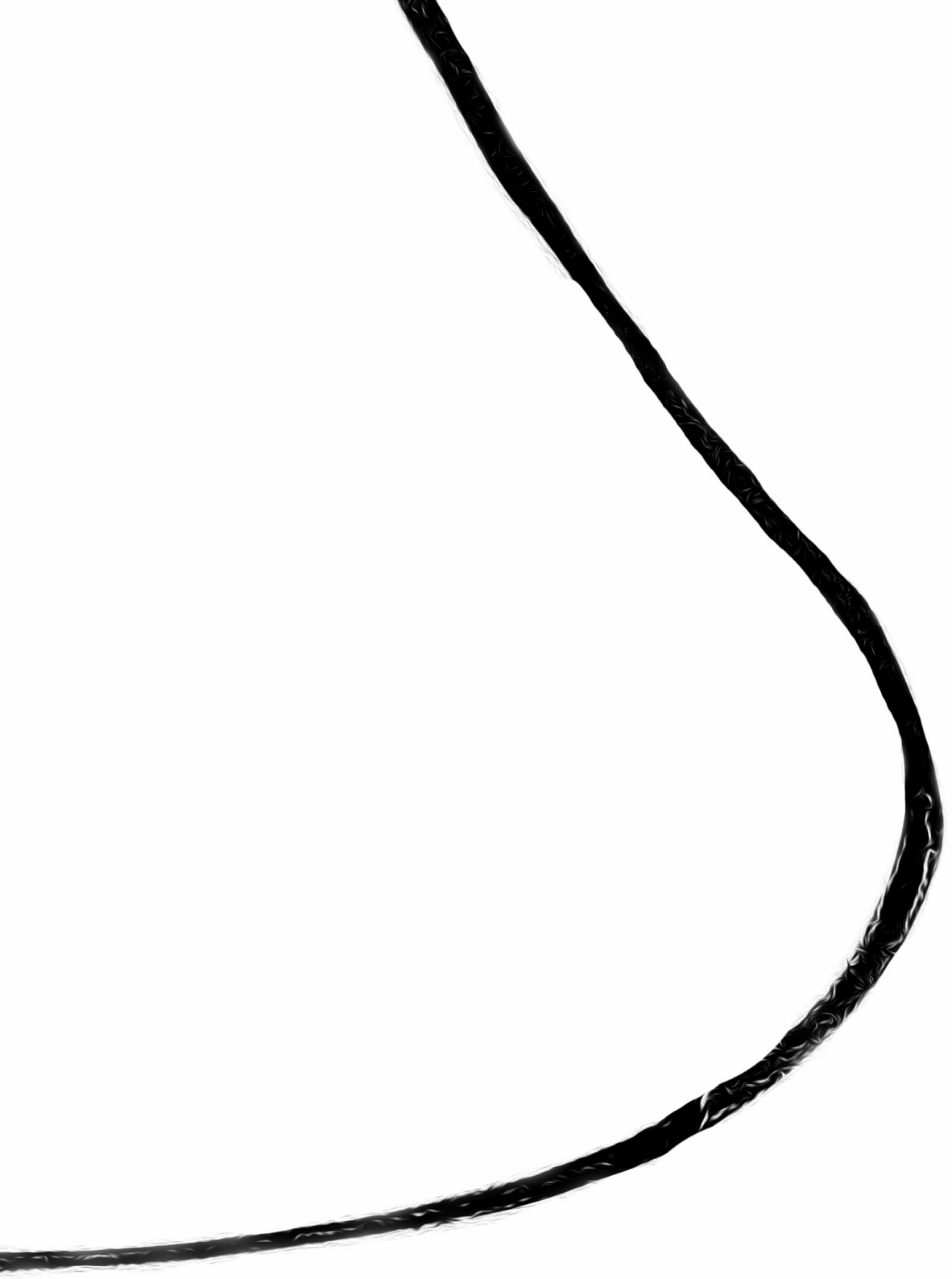
1. de Groen, R.A., et al., NK cell phenotypic and functional shifts coincide with specific clinical phases in the natural history of chronic HBV infection. Antiviral Res, 2017. 140: p. 18-24.
2. Tjwa, E.T., et al., Similar frequencies, phenotype and activation status of intrahepatic NK cells in chronic HBV patients after long-term treatment with tenofovir disoproxil fumarate (TDF). Antiviral Res, 2016. 132: p. 70-5.
3. Freud, A.G., et al., The Broad Spectrum of Human Natural Killer Cell Diversity. Immunity, 2017. 47(5): p. 820-833.
4. Webster, G.J., et al., Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. Hepatology, 2000. 32(5): p. 1117-24.
5. Pahl, J.H.W., A. Cerwenka, and J. Ni, Memory-Like NK Cells: Remembering a Previous Activation by Cytokines and NK Cell Receptors. Front Immunol, 2018. 9: p. 2796.
6. Cuff, A.O., et al., Eomeshi NK Cells in Human Liver Are Long-Lived and Do Not Recirculate but Can Be Replenished from the Circulation. J Immunol, 2016. 197(11): p. 4283-4291.
7. Franciszkiewicz, K., et al., MHC class I-related molecule, MR1, and mucosal-associated invariant T cells. Immunol Rev, 2016. 272(1): p. 120-38.
8. Spaan, M., et al., Frequencies of Circulating MAIT Cells Are Diminished in Chronic HCV, HIV and HCV/HIV Co-Infection and Do Not Recover during Therapy. PLoS One, 2016. 11(7): p. e0159243.
9. Khaitan, A., et al., HIV-Infected Children Have Lower Frequencies of CD8+ Mucosal-Associated Invariant T (MAIT) Cells that Correlate with Innate, Th17 and Th22 Cell Subsets. PLoS One, 2016. 11(8): p. e0161786.
10. Beudeker, B.J.B., et al., Mucosal-associated invariant T-cell frequency and function in blood and liver of HCV mono- and HCV/HIV co-infected patients with advanced fibrosis. Liver Int, 2018. 38(3): p. 458-468.
11. Boltjes, A., et al., Kupffer cells interact with hepatitis B surface antigen in vivo and in vitro, leading to proinflammatory cytokine production and natural killer cell function. J Infect Dis, 2015. 211(8): p. 1268-78.
12. Eberhard, J.M., et al., CD161+ MAIT cells are severely reduced in peripheral blood and lymph nodes of HIV-infected individuals independently of disease progression. PLoS One, 2014. 9(11): p. e111323.
13. Benito, J.M., et al., CD38 expression on CD8 T lymphocytes as a marker of residual virus replication in chronically HIV-infected patients receiving antiretroviral therapy. AIDS Res Hum Retroviruses, 2004. 20(2): p. 227-33.
14. van Bommel, F. and T. Berg, Antiviral therapy of chronic hepatitis B. Intervirology, 2014. 57(3-4): p. 171-80.
15. Boni, C., et al., HBV Immune-Therapy: From Molecular Mechanisms to Clinical Applications. Int J Mol Sci, 2019. 20(11).
16. Prieto, J., I. Melero, and B. Sangro, Immunological landscape and immunotherapy of hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol, 2015. 12(12): p. 681-700.
17. Cariani, E., et al., HLA and killer immunoglobulin-like receptor genes as outcome predictors of hepatitis C virus-related hepatocellular carcinoma. Clin Cancer Res, 2013. 19(19): p. 5465-73.
18. Wu, Y., et al., Monocyte/macrophage-elicited natural killer cell dysfunction in hepatocellular carcinoma is mediated by CD48/2B4 interactions. Hepatology, 2013. 57(3): p. 1107-16.
19. Ishiyama, K., et al., Difference in cytotoxicity against hepatocellular carcinoma between liver and periphery natural killer cells in humans. Hepatology, 2006. 43(2): p. 362-72.
20. Alao, H., et al., Baseline Intrahepatic and Peripheral Innate Immunity are Associated with Hepatitis C Virus Clearance

During Direct-Acting Antiviral Therapy. Hepatology, 2018. 68(6): p. 2078-2088.

21. Sprengers, D., et al., Flow cytometry of fine-needle-aspiration biopsies: a new method to monitor the intrahepatic immunological environment in chronic viral hepatitis. J Viral Hepat, 2005. 12(5): p. 507-12.
22. Boeijen, L.L., et al., Mucosal-Associated Invariant T Cells Are More Activated in Chronic Hepatitis B, but Not Depleted in Blood: Reversal by Antiviral Therapy. J Infect Dis, 2017. 216(8): p. 969-976.
23. Gill, U.S., et al., Fine needle aspirates comprehensively sample intrahepatic immunity. Gut, 2019. 68(8): p. 1493-1503.
24. Brener, S., Transient Elastography for Assessment of Liver Fibrosis and Steatosis: An Evidence-Based Analysis. Ont Health Technol Assess Ser, 2015. 15(18): p. 1-45.
25. Vanwolleghem, T., et al., Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells. Hepatology, 2015. 62(1): p. 87-100.
26. Hou, J., et al., Unique intrahepatic transcriptomics profiles discriminate the clinical phases of a chronic HBV infection. PLoS One, 2017. 12(6): p. e0179920.
27. Lebosse, F., et al., Intrahepatic innate immune response pathways are downregulated in untreated chronic hepatitis B. J Hepatol, 2017. 66(5): p. 897-909.
28. Suslov, A., et al., Hepatitis B Virus Does Not Interfere With Innate Immune Responses in the Human Liver. Gastroenterology, 2018. 154(6): p. 1778-1790.
29. Zheng, Q., et al., Decline in intrahepatic cccDNA and increase in immune cell reactivity after 12 weeks of antiviral treatment were associated with HBeAg loss. J Viral Hepat, 2014. 21(12): p. 909-16.
30. West, J. and T.R. Card, Reduced mortality rates following elective percutaneous liver biopsies. Gastroenterology, 2010. 139(4): p. 1230-7.
31. Claassen, M.A., et al., Retention of CD4+ CD25+ FoxP3+ regulatory T cells in the liver after therapy-induced hepatitis C virus eradication in humans. J Virol, 2011. 85(11): p. 5323-30.
32. Spaan, M., et al., Immunological Analysis During Interferon-Free Therapy for Chronic Hepatitis C Virus Infection Reveals Modulation of the Natural Killer Cell Compartment. J Infect Dis, 2016. 213(2): p. 216-23.
33. Tang, F., et al., mRNA-Seq whole-transcriptome analysis of a single cell. Nat Methods, 2009. 6(5): p. 377-82.
34. Vieth, B., et al., A systematic evaluation of single cell RNA-seq analysis pipelines. Nat Commun, 2019. 10(1): p. 4667.
35. Yu, D.M., et al., N-glycosylation mutations within hepatitis B virus surface major hydrophilic region contribute mostly to immune escape. J Hepatol, 2014. 60(3): p. 515-22.
36. Salpini, R., et al., Hepatitis B surface antigen genetic elements critical for immune escape correlate with hepatitis B virus reactivation upon immunosuppression. Hepatology, 2015. 61(3): p. 823-33.
37. Zheng, G.X., et al., Massively parallel digital transcriptional profiling of single cells. Nat Commun, 2017. 8: p. 14049.
38. Macosko, E.Z., et al., Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. Cell, 2015. 161(5): p. 1202-1214.
39. Gierahn, T.M., et al., Seq-Well: portable, low-cost RNA sequencing of single cells at high throughput. Nat Methods, 2017. 14(4): p. 395-398.
40. Stevens, M., et al., VyCAP's puncher technology for single cell identification, isolation, and analysis. Cytometry A, 2018. 93(12): p. 1255-1259.
41. Nichterwitz, S., et al., Laser capture microscopy coupled with Smart-seq2 for precise spatial transcriptomic profiling.

Nat Commun, 2016. 7: p. 12139.

42. Zhang, Q., et al., Landscape and Dynamics of Single Immune Cells in Hepatocellular Carcinoma. Cell, 2019. 179(4): p. 829-845.e20.
43. Ramachandran, P., et al., Resolving the fibrotic niche of human liver cirrhosis at single-cell level. Nature, 2019. 575(7783): p. 512-518.
44. Balagopal, A., et al., Single Hepatocyte Hepatitis B Virus Transcriptional Landscape in HIV Co-infection. J Infect Dis, 2019.
45. Zhang, X., et al., In situ analysis of intrahepatic virological events in chronic hepatitis B virus infection. J Clin Invest, 2016. 126(3): p. 1079-92.



8

Samenvatting (NL)

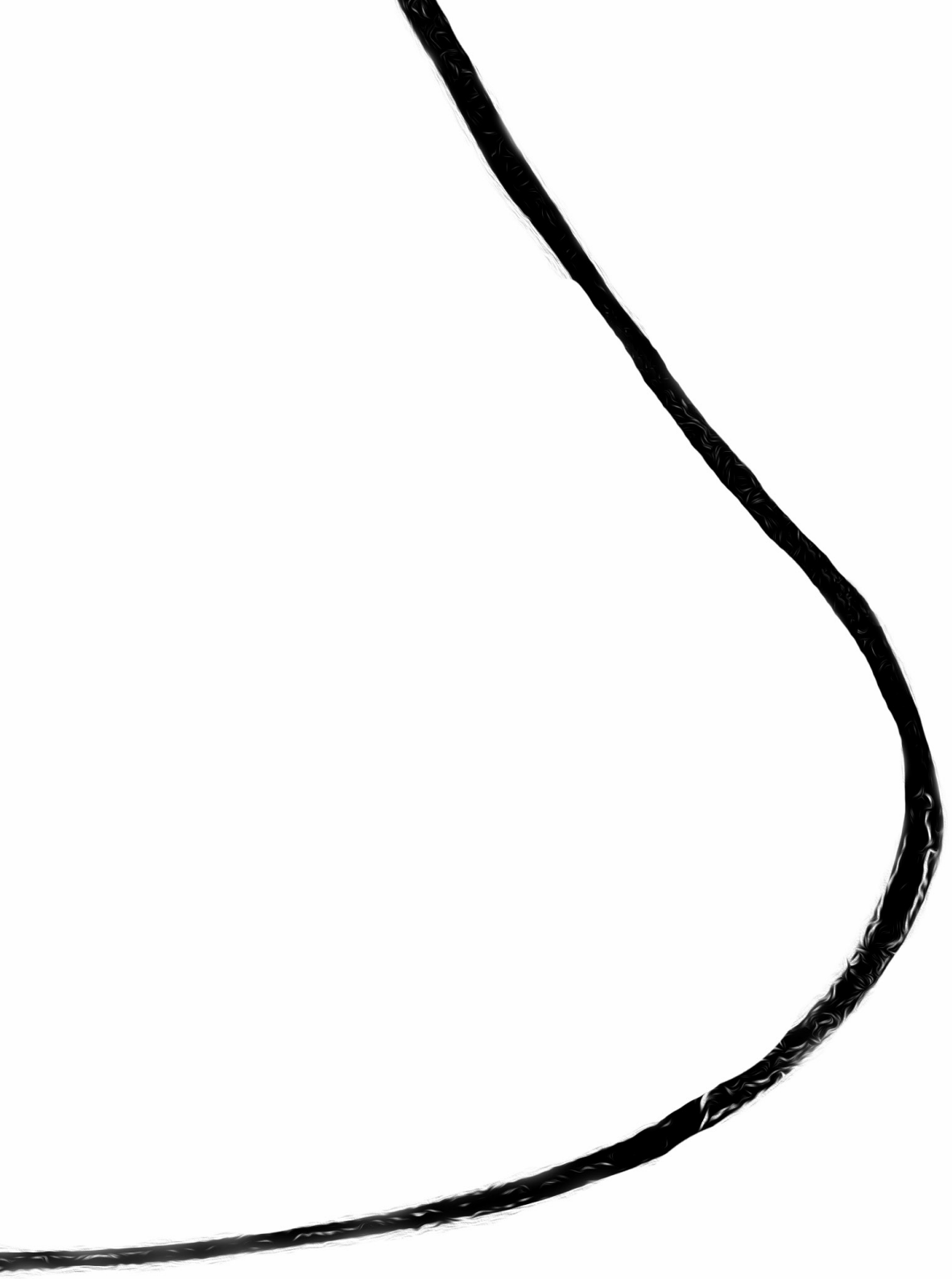
Het hepatitis B virus (HBV) leidt tot een virusinfectie van de lever. Ondanks de beschikbaarheid van een effectief vaccin dat een aanhoudende infectie kan voorkomen, zijn wereldwijd 250 miljoen individuen chronisch geïnfecteerd met HBV. Chronische HBV infectie kan na vele jaren leiden tot verbindweefseling van de lever (levercirrose) en leverkanker. Jaarlijks overlijden 800.000 mensen aan deze gevolgen van HBV, wat het één van de meest dodelijke infectieziekten ter wereld maakt. Een belangrijke eigenschap van HBV is dat er in de loop van de jaren vaak wisselingen optreden van de virustiter (HBV DNA) in het bloed, alsook van de mate van leverontsteking (gemeten via alanine aminotransferase of ALT in het bloed). De oorzaken van deze forse veranderingen gedurende chronische HBV, die bij elke patiënt in wisselende mate optreden, zijn helaas onbekend. Wel is duidelijk dat een infectie met HBV niet direct schadelijk is voor de levercel of hepatocyt, maar dat de verbindweefseling en celsterfte optreedt als gevolg van de afweerreactie tegen het virus. Anderzijds wordt juist ook de genezing van het virus tijdens een acute of chronische infectie toegeschreven aan deze immuunrespons. HBV blijft dus wereldwijd een groot probleem waarvoor een nieuwe behandeling nodig is om meer chronische patiënten volledig te genezen, omdat de huidige behandeling het virus alleen onderdrukt. Experimentele therapieën kunnen mogelijk, eventueel met de gebruikelijke en gelijktijdig gegeven onderdrukkende therapie, de immuunreactie van de gastheer versterken om tot blijvende genezing te komen. Toch resten er nog belangrijke vragen over immuun responsen van de mens tegen HBV. Zo zijn de verschillen tussen chronische HBV infecties, gepaard gaande met de genoemde wisselingen van HBV DNA in het bloed, nog onbegrepen.

In dit proefschrift richten we ons daarom op de volgende vragen, om de interactie tussen HBV en de mens beter te begrijpen:

1. Hoe beïnvloedt de progressie van de variërende fasen van leverontsteking en virustiters, kenmerkend voor chronische HBV, de immuuncellen van de patiënt, zoals de NK cellen en MAIT cellen? Verschilt dit effect tussen lever en bloed?
2. Hoe beïnvloedt onderdrukking van het virus door antivirale behandeling de immuuncellen van chronische HBV patiënten?

Daarnaast stellen we de toenemende schaarste aan leverbiopten die gebruikt kunnen worden voor wetenschappelijk onderzoek aan de orde door te verkennen hoe de informatie gewonnen uit dit materiaal vergroot kan worden. Door gebruik te maken van gevoelige technieken (o.a. RNA-sequencing) hebben we NK-cellen onderzocht gedurende de verschillende klinische fasen van HBV, met hoge en lage virustiters en zeer verschillende mate van leverontsteking. NK cellen zijn immuuncellen die belangrijk zijn voor onze aangeboren afweer tegen virusinfecties, in en buiten de lever. We ontdekten hier dat NK cellen tussen de verschillende fasen van HBV zeer weinig verschilden, zelfs wanneer de klinische presentatie dus sterk varieerde. Los van de verschillende virustiters en ontstekingswaarden, bleek een chronische infectie met HBV op zichzelf slechts

minimale veranderingen te veroorzaken in NK cellen in het bloed van patiënten. Dit was een opvallend resultaat, omdat andere chronische virusinfecties wel duidelijke effecten hadden op NK cellen. Zo vonden we een versterkte expressie van ISG (Interferon Stimulated Genes) van NK cellen tijdens HCV en HIV infecties, passend bij immuunreacties tegen virussen, terwijl dat niet gold voor NK cellen van HBV patiënten. Ook toonden we in dit proefschrift aan dat MAIT cellen, een subtype van T cellen die nog weinig onderzocht is in de context van virusinfecties, óók niet reageert op de fase wisselingen tijdens chronische HBV. Onderdrukking van de virustiter met de gangbare behandeling voor HBV zorgde wél voor een verandering van de activiteit van MAIT cellen, in tegenstelling tot schommelingen van de virustiters tijdens het natuurlijke beloop van de ziekte. Tot slot laten we in dit proefschrift zien dat de informatie-opbrengst van leverbiopten, bij HBV patiënten en mogelijk andere ziekten, vergroot kan worden. We toonden met een combinatie van moderne analyse technieken aan dat sommige effecten van immuuntherapie alléén in de lever detecteerbaar zijn, en niet in het bloed van patiënten.



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Appendices:

- PhD portfolio
- Bibliography
- Curriculum Vitae
- Dankwoord (NL)

PHD PORTFOLIO

Name PhD student : Lauke Leonard Boeijen
Promotor : Prof.dr. H.J. Metselaar
Co-promotor : Dr. A. Boonstra
Affiliation : Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center Rotterdam
PhD period : 2015-2019

COURSES

2017 Research Integrity
2017 EDC Research Coordinator Training
2016 BROK ('Basiscursus Regelgeving Klinisch Onderzoek')
2016 Molecular Medicine course 'Biomedical Research Techniques'
2016 EASL Course 'T-cell responses in viral hepatitis' (Freiburg, Germany)
2016 Preceptorship HBV cure (Lyon, France)
2016 Molecular Medicine Introduction in GraphPad Prism
2016 Molecular Medicine Workshop on Photoshop and Illustrator CC
2015 Systematic literature search

OTHER CONFERENCES

2018 Liver Day
2018 Molecular Medicine (MolMed) Day
2018 National Hepatitis Day
2018 Nederlandse Vereniging voor immunologie
2018 European Conference for Immunology (Amsterdam)
2018 Molecular Medicine (MolMed) Day (*Oral Presentation*)
2017 Nederlandse Vereniging voor immunologie
2017 European Association For Study of the Liver (Amsterdam)
2017 Molecular biology of HBV conference (Washington DC, USA)
2016 Dutch Liver Retreat (*Oral Presentation*)
2016 European Association For Study of the Liver (Barcelona, Spain)
2016 Nederlandse Vereniging voor Immunologie
2016 Liver day
2016 NVVI/BSI Congress 2016 (Liverpool, UK)
2016 Nederlandse Vereniging voor Gastro-Enterologie
2016 Liver day
2015 Molecular biology of HBV conference (Dolce Bad Neuheim, Germany)

MONITORING CLINICAL RESEARCH

2015-2016 DAHHS-2 study monitor

TEACHING AND SUPERVISING

Master Thesis Supervision Noe Axel Rico Montanari (2017)

Master Thesis Supervision Pritha Biswas (2019)

Supervision Junior Med students (2016)

Lecturing: 'MDL en Chirurgie: the best of both worlds': HBV research today

BIBLIOGRAPHY

1. Boeijen L.L., Spaan M., Boonstra A., The effects of nucleo(s)tide analogs on host immune cells: the baseline for future immune therapy for HBV? Antiviral Therapy, provisionally accepted, 2020.
2. Boeijen, L.L., et al., Gene expression profiling of human tissue-resident immune cells: Comparing blood and liver. J Leukoc Biol, 2019. 105(3): p. 603-608.
3. Boeijen, L.L., et al., Persistent Replication of HIV, Hepatitis C Virus (HCV), and HBV Results in Distinct Gene Expression Profiles by Human NK Cells. J Virol, 2019. 93(9).
4. Boeijen, L.L., et al., Mucosal-Associated Invariant T Cells Are More Activated in Chronic Hepatitis B, but Not Depleted in Blood: Reversal by Antiviral Therapy. J Infect Dis, 2017. 216(8): p. 969-976.
5. Boeijen, L.L., et al., Hepatitis B virus infection and the immune response: The big questions. Best Pract Res Clin Gastroenterol, 2017. 31(3): p. 265-272.
6. Tjwa, E.T., et al., Similar frequencies, phenotype and activation status of intrahepatic NK cells in chronic HBV patients after long-term treatment with tenofovir disoproxil fumarate (TDF). Antiviral Res, 2016. 132: p. 70-5.
7. Arends, J.E., et al., Natural history and treatment of HCV/HIV coinfection: Is it time to change paradigms? J Hepatol, 2015. 63(5): p. 1254-62.

CURRICULUM VITAE

Lauke Boeijen was born on July 12th 1989 in Nijmegen, The Netherlands. He attended the Stedelijk Gymnasium Nijmegen, and graduated cum laude in 2007. In order to combine his interest in biology and health care, he started to study Medicine at the University of Utrecht. As a medical student, he performed several research projects related to infectious diseases, such as Gram-negative bacteremia and HIV / HCV co-infection. During his studies, he spent 6 months in Montpellier to study French language and culture. Lauke Boeijen received his medical degree from the University of Utrecht in 2014 in The Netherlands. To acquire experience as an MD, he spent 18 months as a medical resident (Internal Medicine) in the Tergooi hospital. In 2015, he started his work as research physician and PhD-candidate at the Erasmus MC Rotterdam, under supervision of Andre Boonstra and Herold Metselaar, focusing on translational work regarding immune responses to chronic viral infections (HBV, HCV, HIV). Under supervision of Rob de Knegt, his projects include the characterization of intrahepatic immune cells, derived from Fine Needle Aspiration Biopsy (FNAB) of the liver, as well as the larger core biopsies. In January 2020, he started his residency microbiology (AIOS Medische Microbiologie) at the UMC Utrecht.

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heb heel erg genoten van het informele en vrolijke contact de afgelopen jaren! Ik heb me meteen thuis gevoeld en herinner me het allereerste begin, het congres in Dolce Bad Neuheim nog als de dag van gisteren. BOTMers, de Cadzand vakanties waren fantastisch, hopelijk kunnen we dat nog vaker doen, liefst zonder diefstal door zeewasberen, paars or otherwise. **Vincenzo**, erg jammer dat we niet meer dagelijks het lunch gesprek kunnen domineren met nieuwe vis strategieën, ik kijk wel uit naar ons volgend fishing adventure !

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