Medizinische Fakultät

der

Universität Duisburg-Essen

Aus der Klinik für Gastroenterologie und Hepatologie

The impact of hepatitis B surface antigen on natural killer cells in patients with chronic hepatitis B virus infection

Inauguraldissertation

zur

Erlangung des Doktorgrades der Medizin

durch die Medizinische Fakultät

der Universität Duisburg-Essen

Vorgelegt von

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aus Hubei, China

2020

DOI: 10.17185/duepublico/74388
URN: urn:nbn:de:hbz:464-20210623-112949-4

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Tag der mündlichen Prüfung: 20. April. 2021

List of publication

Du, Y., Anastasiou, E.O., Strunz, B., Scheuten, J., Bremer, B., Kraft A., Kleinsimglinhaus K., Todt, D., Broering, R., Hardtke-Wolenski, M., Wu, J., Yang, D., Dittmer, U., Lu, M., Cornberg, M., Björkström, K.N., Khera, T., and Wedemeyer, H. **The impact of hepatitis B surface antigen on natural killer cells patients with chronic hepatitis B patients**. **Liver Int.** 2021 Apr 1. doi: 10.1111/liv.14885.

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1. Introduction

Hepatitis B virus

The hepatitis B virus (HBV) was first discovered in human sera by Blumberg's group in 1964 (Blumberg et al., 1965), and was referred to as the "Australian antigen" (Alter et al., 1966). In 1970, David Dane isolated the complete virus particle from the serum of patients with Australian antigen-associated hepatitis, referred as "Dane particles", demonstrated by electron microscopy (Dane et al., 1970). HBV is a small-enveloped DNA virus that is blood-borne, has a high transmission rate, and causes both acute and chronic infection. Since its discovery, the study of HBV has progressed at an impressive rate. One of the biggest breakthroughs for HBV was the approval of the hepatitis B vaccine more than three decades ago. Additionally, dramatic progress has been made in the field of antiviral treatments and the accessibility of clinical care for hepatitis infection. However, the global burden of chronic HBV infections (CHB) remains substantial, especially for HBV-associated end-stage liver disease and death. Nevertheless, the World Health Organization (WHO) has endorsed the goal of eliminating HBV by 2030 (Lancet, 2016).

1.1. Global Prevalence and public health burden

HBV is a global health issue that affects approximately 3.5% of the world's population with chronically infecting around 257 million people worldwide (Hutin et al., 2018). Due to the difference in vaccination coverage, the global prevalence of HBV infections varies widely. The WHO reported that the prevalence was the highest in the Western Pacific regions (including China, Japan, South Korea, the Philippines, and Vietnam), at 6.2%, followed by the African region, at 6.1% (WHO, 2017). In highly endemic areas, HBV primarily spreads from mother to child at birth (perinatal transmission), or through exposure to infected blood during early childhood (WHO, 2015). HBV is also spread by unprotected sexual intercourse and intravenous drug use in some low prevalence areas.

From 1990 to 2013, the number of HBV-related deaths due to liver cirrhosis and hepatocellular carcinoma (HCC) increased by 33%, representing approximately 686000 cases in 2013, worldwide (Stanaway et al., 2016). Most of the HBV-associated disease burden is the result of infections acquired through perinatal transmission or early childhood (1–5 years old) horizontal transmission (Seto et al., 2018).

1.2. HBV virology

1.2.1. HBV structure

HBV is a prototypical member of the Hepadnaviridae family, characterized by using a reverse transcriptase to replicate its genome, similar to a retrovirus. The infectious virion of HBV is composed of the HBV genome, nucleocapsid, and the envelope proteins. The HBV genome is a partially double-stranded, relaxed-circular (rc) DNA, approximately 3.2kb in size (Block et al., 2007).The HBV genome has four major open reading frames (ORFs): i) the *preS/S*, which encodes the three viral surface proteins; ii) the *precore/core*, which encodes both the core protein and the precore protein, also known as secreted-e antigen (HBeAg); iii) the *pol* ORF of the viral polymerase, which possesses reverse transcriptase, DNA polymerase and RNase H activities, and the terminal protein; and iv) *X* ORF, which encodes the HBx protein (Block et al., 2007).

Apart from the infectious virus particles (Dane particles), two types of non-infectious particles can be produced by HBV, the spheres and the filaments, which are empty subviral particles (SVPs) without internal capsids (Block et al., 2007). SVPs outnumber infectious virions by 100,000 fold or even greater in host sera (10^{13}/ml), and SVPs are predominately composed of hepatitis B surface antigen (HBsAg) (Cornberg et al., 2017; Tong and Revill, 2016). SVPs display an array of antigenic sequences to the innate immune system, facilitating the subsequent activation of adaptive system, which may be used as a vaccine platform (Ho et al., 2020). In addition, these SVPs have also been

shown to impact HBV host immunity and thus contribute to the persistence of HBV infections (Kondo et al., 2013). Detailed information regarding SVPs is discussed in **Sections 1.5–1.7**.

1.2.2. Viral life cycle

The life cycle of HBV has been well-characterized (**Figure 1**). Upon viral uptake into hepatocytes by sodium taurocholate cotransporting polypeptide (NTCP), the rcDNA is delivered to the nucleus where it is converted into covalently closed, circular DNA (cccDNA). cccDNA then serves as a template for all viral transcripts that are eventually translated into the related viral proteins. Mature nucleocapsid and envelope proteins aggregate in the endoplasmic reticulum where packaging and maturation occur. Then HBV virions or SVPs are secreted into extracellular by budding. Additionally, pre-genomic RNA (pgRNA) is reverse transcribed into new rcDNA within the viral capsid, and nucleocapsids that contains newly transcribed rcDNA are either released as new virions or recycled into the nucleus to maintain cccDNA reservoir (Durantel and Zoulim, 2016; Seeger and Mason, 2015). Understanding of the HBV life cycle plays an important role in the development of anti-HBV treatment which targets different HBV replication processes.

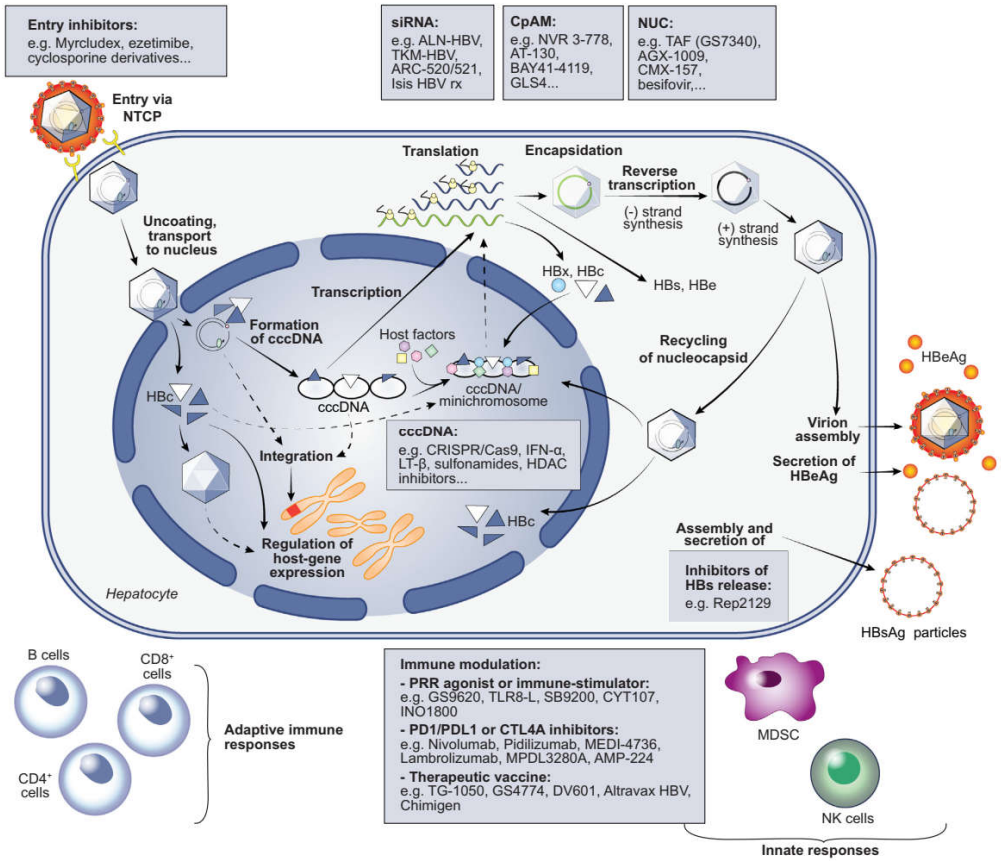

Figure 1. HBV life cycle and novel antiviral strategies in development. The incoming virus binds to the sodium-taurocholate cotransporting polypeptide (NTCP) receptor on the host and enters the cell via receptor-mediated endocytosis. Nucleocapsids pass through the nuclear pore complex and release the HBV genome. The relaxed circular DNA is converted to cccDNA, which serves as a template for HBV mRNA and pre-genomic RNA (pgRNA). The translation of HBV RNA results in the production of HBx protein, HBcAg, HBeAg, HBsAg, and P protein. Finally, viral proteins undergo packaging, maturation, and budding. The pgRNA can then be reverse transcribed into new rcDNA, within the viral capsid, allowing nucleocapsids containing newly transcribed rcDNA are released as new virions or recycled into the nucleus to maintain the cccDNA reservoir. Novel therapeutic drugs that are currently in development target different steps of HBV life cycle or modulate the host immune system as shown. Adapted with permission from Elsevier (Durantel and Zoulim, 2016).

1.3. Natural history and clinical manifestation of HBV infection

1.3.1. Acute HBV infection

HBV can cause both acute and chronic infections. The incubation period for HBV ranges from one to four months, which can make it difficult to determine when and

where transmission occurred. The expression of HBsAg can be detected within 30 to 60 days after exposure. Acute HBV infections in adults are not usually clinically apparent (Likhitsup and Lok, 2019). However, fewer than 30% of patients will develop icteric hepatitis, accompanied by abdominal pain, nausea, jaundice, and other non-specific constitutional symptoms (Stefan Mauss, 2018; Thuener, 2017). During the acute illness alanine and aspartate aminotransferase (ALT and AST, respectively) levels may become elevated above 1,000 U/L whereas bilirubin generally remains within the normal range, especially in patients without icteric hepatitis (Stefan Mauss, 2018; Thuener, 2017). The levels of liver transaminases usually return to normal within one to four months (Stefan Mauss, 2018). The detection of HBsAg will be negative in patients who recover from acute infection.

The rate of progression from acute to chronic HBV is primarily dependent on the age acquired infection (Seto et al., 2018). Most immunocompetent adults could spontaneously recover, displaying HBsAg to anti-HBs seroconversion; however fewer than 5% of adults will develop CHB. On contrast, the risk of developing CHB infection is approximately 90% for perinatally acquired infections and 20% to 50% for infections acquired between the ages of 1 and 5 years old (Ganem and Prince, 2004; McMahon et al., 1985). Patients who recover from acute HBV are unable to completely eradicate the virus, even if they achieve HBsAg seroconversion. In those recovered individuals, HBV DNA may persist in the form of cccDNA, which can be reactivated during immunosuppressive periods, such as after organ transplant (Seto et al., 2017) or during chemotherapy (Hsu et al., 2014).

1.3.2. Chronic HBV infection

Chronic HBV infection is defined by the presence of hepatitis B surface antigen (HBsAg) for duration of 6 months or more. As mentioned above, HBV chronicity occurs in less

than 5% of adult-acquired infections but approximately 90% of perinatally acquired infections. In fact, most CHB patients do not have a history of acute hepatitis.

Most patients with chronic HBV are also clinically asymptomatic. Some patients may present non-specific symptoms such as fatigue. In most cases, significant clinical symptoms only develop if the liver disease progresses to decompensated cirrhosis. Clinical signs of chronic liver disease include splenomegaly, spider angioma, caput medusa, palmar erythema, and gynecomastia (Stefan Mauss, 2018). In patients with decompensated cirrhosis, jaundice, ascites, peripheral edema, and encephalopathy may be observed (Stefan Mauss, 2018).

The natural course of chronic HBV infection involves the dynamic interaction between virus replication and host immune responses, and not all patients with CHB experience a chronic hepatitis phase (EASL, 2017). CHB can be classified into five phases, based on the detection of HBeAg, HBsAg, and HBV DNA levels, ALT values, and the presence or absence of liver inflammation as shown in **Figure 2** (Seto et al., 2018).

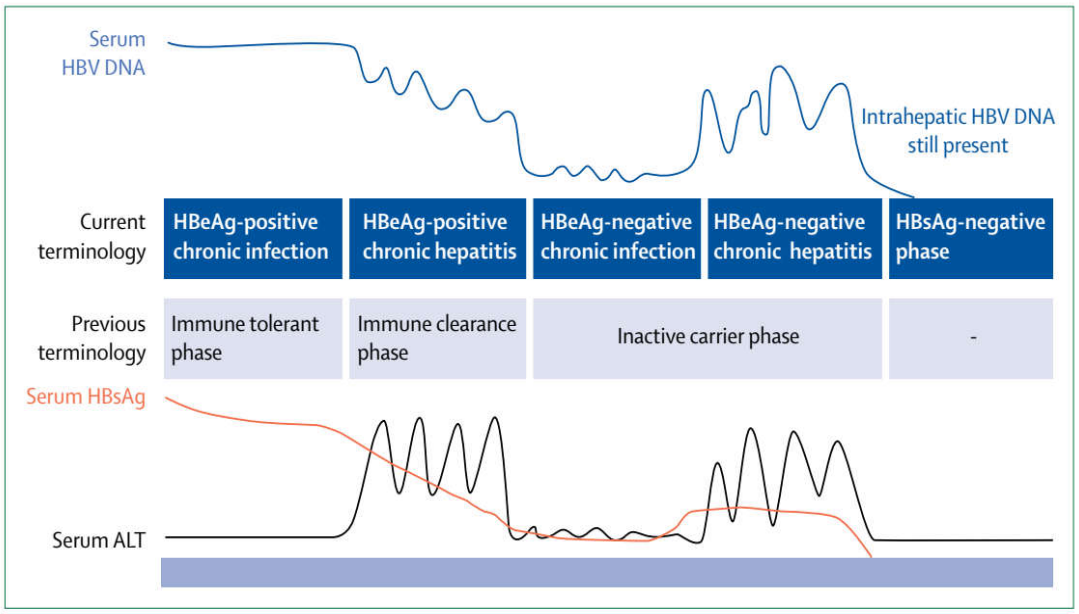

Figure 2. Different phases of chronic HBV infection in relation to the kinetics of serum HBV DNA,

HBsAg, ALT and the presence of HBeAg. Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase. Adapted with permission from Elsevier (Seto et al., 2018).

The first phase represents an HBeAg-positive chronic infection, also known as the immune-tolerant phase, which is characterized by high levels of serum HBV DNA, the presence of serum HBeAg, normal serum ALT levels and near-normal liver histology (EASL, 2017). This phase is more frequent and prolonged among subjects that acquire the infection perinatally and can lasts for10 to 30 years. During this phase, patients are highly contagious because of the high HBV DNA levels, and the rate of spontaneous HBeAg clearance is very low.

The second phase, termed HBeAg-positive chronic hepatitis or immune-clearance phase is characterized by immune-mediated liver necroinflammation, fluctuating serum ALT levels, the presence of serum HBeAg, and high levels of HBV DNA (EASL, 2017). During this stage, most patients can achieve HBV DNA suppression, HBeAg loss, and the appearance of anti-HBe antibodies (HBeAg seroconversion). However, the timing of HBeAg seroconversion is influenced by various factors, including the HBV genotype and the age at which the individual was initially infected with the virus (Liu and Kao, 2013).

The third phase, known as the HBeAg-negtive chronic infection phase and previously referred to as the inactive carrier phase, is characterized by the presence of serum anti-HBe antibodies, low (< 2,000 IU/mL) or sometimes undetectable HBV DNA level and normal ALT levels. Some patients in this phase may have HBV DNA levels >2,000IU/ml (although they are usually < 20,000IU/ml), accompanied by normal ALT levels and minimal hepatic necroinflammation (EASL, 2017). A minority of patients in this stage, who typically present with low levels of serum HBsAg (< 1,000 IU/ml), can spontaneously achieve HBsAg loss (Zeisel et al., 2015).

A proportion of patients can progress to HBeAg-negative chronic hepatitis (the fourth phase). Patients during this stage are characterized by intermittent fluctuations in serum HBV DNA and ALT concentrations and liver necroinflammation (EASL, 2017). Some subjects in this stage have been associated with HBV variants in the precore or basal core promoter regions which impair or abolish HBeAg expression (Lim et al., 2007). Only a minority of patients in this stage can spontaneously achieve HBsAg seroclearance (which is a functional cure for HBV infections) allowing them to enter the last stage (EASL, 2017).

The last phase is the HBsAg-negative phase, which is characterized by negative serum HBsAg and positive anti-HBc antibodies, with or without detectable anti-HBs antibodies. Patients in this stage have normal ALT levels and undetectable HBV DNA levels; however, intrahepatic HBV persists at low replicative and transcriptional levels (Seto et al., 2018). Similar to patients who recover from acute HBV infections, immunosuppression may also lead to HBV reactivation among patients who achieve this final phase of CHB, as cccDNA forms a mini-chromosome in the nuclei of host hepatocytes (EASL, 2017).

1.4. HBV prevention and treatment

1.4.1. Vaccination

Vaccination against HBV at birth remains the primary strategy for the elimination of hepatitis B. The complete hepatitis B vaccine series (a dose at birth plus two additional booster doses) induced protective amounts of anti-HBs antibodies in more than 95% of vaccinated infants (WHO, 2015). As reported in 2015, the global HBsAg prevalence rate decreased from 4.7% to 1.3% in children younger than 5 years old due to effective worldwide vaccination programs (WHO, 2015). However, the prevalence of HBV

remains high among non-vaccinated individuals, and a small number of individuals do not respond to the vaccine. Therefore, the development of effective anti-HBV drugs remains necessary.

1.4.2. Goals of treatment

HBV patients carry a significantly increased risk of life-threatening complications such as liver cirrhosis and HCC. Among untreated CHB patients, the 5-year cumulative incidence of cirrhosis ranges from 8% to 20% (WHO, 2015). Among those with cirrhosis, the 5-year cumulative risk of hepatic decompensation is 20% (EASL, 2017), and the annual risk of HCC has been reported to be approximately 2%–5% (Raffetti et al., 2016). Therefore, the primary therapeutic goal for CHB patients is to improve survival and quality of life by preventing the progression to end-stage liver disease and HCC and preventing mother-to-child transmission (EASL, 2017; Sarin et al., 2016).

Recently, three categories of cures have been defined: complete or sterilizing cures, functional cures and partial cures (Likhitsup and Lok, 2019). A complete or sterilizing cure is defined as the loss of HBsAg and the complete elimination of all forms of replicating HBV, including intrahepatic cccDNA. However, the complete eradication of HBV infection is rarely achievable through current treatment options, even after HBsAg seroconversion. A functional cure refers to the loss of HBsAg or HBsAg seroconversion, which is associated with a very benign prognosis and the negligible risk of progression to liver cirrhosis and HCC (Wedemeyer, 2019). A functional cure is the currently accepted definition of HBV cure and represents the goal for new HBV therapies (Likhitsup and Lok, 2019). Partial cure refers to the suppression of HBV replication to undetectable levels and HBeAg seroconversion but is accompanied by the persistent detection of HBsAg. Although a partial cure is not ideal, it may represent a reasonable intermediate endpoint, particularly if it can be sustained without requiring continued treatment.

1.4.3. Current treatment options and limitations

Currently, two treatment options are available for CHB patients: the immune modulator interferon (IFN)-α and nucleoside or nucleotide analogues (NA), which act as reverse transcriptase inhibitors against the HBV polymerase. The recommended first-line NAs include entecavir and two prodrugs of tenofovir, tenofovir disoproxil fumarate and tenofovir alafenamide (EASL, 2017). These three drugs have a high barrier to resistance, favorable safety and tolerability characteristics (EASL, 2017; Seto et al., 2018), and can achieve a high level of virological suppression in more than 95% patients (Buti et al., 2016; Chang et al., 2010; Marcellin et al., 2013). Long-term treatments have also been associated with the reduction of intrahepatic cccDNA level (Lai et al., 2017), significant histological improvements (Marcellin et al., 2013), and the reduced risk of developing cirrhosis and HCC (Schiff et al., 2011). However, because NA therapy rarely results in HBsAg loss and does not usually eradicate intrahepatic HBV, long-term therapy regimes are necessary for the majority of NA-treated CHB patients, which is associated with the corresponding economic burden, and the increased risk of drug resistance and side effects.

The subcutaneous injection of pegylated IFN-α, for a finite duration of 1 year, is another first-line therapy option. However, the unfavorable side-effect profile and high variability of response make this treatment option cumbersome for many patients (EASL, 2017). Pegylated IFN-α is recommended for certain patient subgroups, including patients with genotype A, coinfection with hepatitis D virus, and young patients who are reluctant to engage in lifelong treatments with NA (Seto et al., 2018). However, off-treatment viral control is low with the use of IFN-α, and only approximately 23% of patients achieve suppressed HBV replication (Marcellin et al., 2009; Buster et al., 2008). In addition, pegylated IFN-α as an add-on therapy to NA did not significantly improve the virological and serological response of NA (Tatsukawa et al., 2018; EASL, 2017; Qiu et al., 2018).

Therefore, due to the limitations associated with the two current treatment options, many researchers are devoted to developing novel treatments.

1.4.4. Novel treatments for HBV infection

Recently, numerous novel strategies for HBV treatment have been investigated, which might offer more the potent suppression of HBV replication and, ideally, even HBV eradication (**Figure 1**). Current novel treatment options under pre-clinical and early clinical evaluation can be classified into direct antivirals and immunotherapeutic agents. Direct-acting antivirals target the virus, including virus entry inhibitors, drugs that aim at cccDNA degradation or preventing the formation of cccDNA, siRNA or anti-sense oligonucleotides that target viral transcripts, nucleocapsid assembly modulators, and HBsAg secretion inhibitors (Lok et al., 2017). In contrast, immune modulators aim to restore the HBV-specific immune responses to attain immunological control in conjunction with the profound suppression of HBV replication and HBsAg production. These immunomodulators include pattern recognition receptor (PRR) agonist [e.g., the toll-like receptor (TLR)7 agonist GS96020, and the retinoic acid-inducible gene (RIG)-1 and nucleotide-binding oligomerization domain-containing protein (NOD)2 agonist inarigivir SB9200]，the anti-programmed cell death protein 1 (anti-PD1) antibody nivolumab, cytotoxic T-lymphocyte-associated protein (CTLA)4 inhibitors, and therapeutic vaccines (e.g., GS-4774 and TG-1050) (Gehring and Protzer, 2019; Fanning et al., 2019). Based on these novel treatment strategies, future treatment options for the development of an HBV cure may represent the combination of multiple antiviral drugs that target the various steps of the HBV life cycle or the combination of direct antiviral drugs with host immune modulators. With the optimization treatment strategies based on current antiviral drugs and newly developed antiviral agents, the ultimate cure of HBV infection is likely to be achieved in the foreseeable future.

1.5. Innate immune response

Innate immunity represents the first line of defense against microbial pathogens. Generally, viral infections induce the production of type-I IFNs (IFN-I) and proinflammatory cytokines through TLRs and the RIG-I signaling pathway (Megahed et al., 2020). However, HBV has long been accepted as a "stealth virus", against which the innate immune response-related genes and IFN-α/β were weakly induced within the livers of infected patients (Dunn et al., 2009; Fisicaro et al., 2009). Instead, a recent study demonstrated that HBV can be sensed by TLR2 on hepatocytes, inducing proinflammatory cytokines but not IFNs (Zhang et al., 2020). Evidence suggests that different HBV proteins may suppress the IFN-I response, especially the presence of huge amounts of HBsAg (Lebossé et al., 2017). HBsAg has been shown to inhibit the interaction between interferon regulatory transcription factor 7 (IRF7) and nuclear factor kappa B subunit (NF-κB), preventing translocation of NF-κB to the nucleus and the subsequent production of IFN-I (Megahed et al., 2020).

In addition to the suppression of the IFN-I signaling pathway, HBV may escape the antiviral functions of innate immune cells by modulating their numbers or impairing their functions (Faure-Dupuy et al., 2017). The number of granulocytic subsets of myeloid-derived suppressor cells (MDSCs) has been reported to increase during the immune-tolerant phase of HBV (Pallett et al., 2015) whereas the numbers of $CD14^+$ monocytes, resident Kupffer cells (KCs), dendritic cells (DCs), natural killer (NK) cells, and natural killer T (NKT) cells are not significantly affected throughout the natural course of HBV infections (Faure-Dupuy et al., 2017). HBsAg has been shown to trigger MDSC recruitment or expansion, by acting on the extracellular signal-related kinase (ERK)/interleukin-6/signal transducer and activator of transcription (STAT) 3 pathway (Faure-Dupuy et al., 2017), and these recruited MDSCs inhibited HBV-specific $CD4^+$ and $CD8^+$ T-cell responses by affecting their metabolism (Fang et al., 2015). More

importantly, HBV also impairs the functions of many innate immune cells. Plasmacytoid DCs (pDC) from CHB patients were less capable of producing IFN-α following treatment with a TLR9 agonist and less capable of cross-talking with activation of NK cells (Martinet et al., 2012), whereas they were more likely to induce regulatory T-cells (Hong and Gong, 2008; Martinet et al., 2012). In addition, HBV was able to block absent in melanoma (AIM)2-inflammasome-mediated IL-1β production in freshly isolated KCs, via an HBsAg-mediated mechanism (Zannetti et al., 2016). Furthermore, the impaired functionality of NK cells has also been identified in CHB patients, which is discussed in detail **in Section 1.7**. Therefore, the therapeutic manipulation of innate immunity, including the use of IFN-α, the depletion of MDSCs, and the restoration of monocytes or DCs function may have the potential to control HBV infections (Maini and Gehring, 2016).

1.6. Adaptive immune response

HBV-specific antibody-producing B cells and functional HBV-specific T cell responses are thought to ultimately determine the outcomes of HBV infections. $CD4^+$ and $CD8^+$ T cells that respond specifically to the nucleocapsid (HBcAg and HBeAg), envelope, polymerase, and x proteins can all be induced following HBV infections (Bertoletti and Ferrari, 2016). During acute HBV infections, a multispecific and vigorous HBV-specific T cell response has been associated with viral clearance (Thimme et al., 2003). In contrast, during chronic HBV infections, the HBV-specific T cells have been found to exhibit quantitative and functional defects, also referred to as T cell exhaustion (Thimme et al., 2003), which is a major contributing factor to persistent infections. The exhausted HBV-specific T cells highly express several negative co-inhibitory molecules, such as PD-1, CTLA4, lymphocyte-activation gene-3 (LAG-3), CD160, T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) and 2B4 (Bertoletti and Ferrari, 2016). In addition, exhausted T cells also upregulate the tumor necrosis factor-alpha

(TNF-α)-related apoptosis-inducing ligand (TRAIL)-death receptor, making them more susceptible to TRAIL-dependent NK cell-mediated lysis (Peppa et al., 2013).

Compared with HBV-specific T cell responses, less attention has been paid to the role played by antibody-producing B cells. Anti-HBsAg, which are produced by HBsAg-specific B cells, can block HBV cell entry, neutralize virions, and kill infected cells through Fc-mediated phagocytosis (Neumann-Haefelin and Thimme, 2018). The lack of detectable anti-HBsAg has been observed in both acute and chronic HBV infections prior to HBsAg loss. Studies have suggested two plausible explanations for this phenomenon: the first possibility is that anti-HBs are depleted by the large number of circulating HBsAg-containing SVPs (Maruyama et al., 1993); the second possibility is the occurrence of HBsAg-specific B cell dysfunction, especially in chronic HBV infections (Maruyama et al., 1993). HBsAg-specific B cells that have been isolated from acute and chronic HBV patients are unable to mature into anti-HBs-secreting cells *in vitro*, and these B cells resemble "atypical memory B cells", characterized by the high expression levels of the inhibitory marker PD-1 and the transcription factor T-bet (Salimzadeh et al., 2018). Phenotypic changes and functional impairments also affect the global B cell population in HBV-infected patients, instead of being restricted to HBsAg-specific B cells (Burton et al., 2018). These findings suggest that B cell dysfunction rather than antibody depletion is the main reason for the lack of anti-HBsAg; thus, B cells may represent targets for novel antiviral strategies designed to deliver a functional cure for CHB.

1.7. Natural killer cells in HBV infection

1.7.1. General features of NK cells

NK cells are vital innate effector cells that play important roles in defense against viral infections, tumor immune-surveillance and the regulation of both innate and adaptive

immune responses (Del et al., 2017). Human NK cells are generally defined as a group of lymphocytes that express CD56 but lack the T cell marker CD3. NK cells represent approximately 15% of all peripheral blood lymphocytes and more than 30% of the lymphocytes in the liver (Wu et al., 2015). Two major subsets have been identified based on the density of CD56: $CD56^{bright}$ and $CD56^{dim}$. These two subsets display different phenotypes, differentiation characteristics, tissue distributions and functionalities. $CD56^{dim}$ NK cells express high levels of the low-affinity Fcγ-receptor CD16 and the killer immunoglobulin-like receptor (KIR), whereas $CD56^{bright}$ NK cells do not express CD16 or KIR (Wu et al., 2015). $CD56^{bright}$ NK cells are considered to be predecessors of $CD56^{dim}$ NK cells; within the $CD56^{dim}$ subpopulation, the stages of differentiation have been associated with the increased expression of CD57 and KIR, as well as the loss of NKG2A (Björkström et al., 2010). $CD56^{dim}$ NK cells are largely predominant in the peripheral blood and represent approximately 90% of all NK cells in the blood, whereas $CD56^{bright}$ NK cells constitute less than 10% of all NK cells in the blood and are more frequently found in certain tissues and secondary lymphoid organs (Cooper et al., 2001; Del et al., 2017; Wu et al., 2015). $CD56^{dim}$ NK cells efficiently kill target cells through degranulation but secret low level of cytokines, whereas $CD56^{bright}$ NK cells produce large amounts of cytokines upon stimulation but are less cytotoxic (Björkström et al., 2016). However, $CD56^{bright}$ NK cells exhibit similar or even enhanced cytotoxicity against target cells after prolonged activation compared with $CD56^{dim}$ NK cells (Wu et al., 2015).

Unlike T cells and B cells, NK cells do not express rearranged antigen-specific receptors; instead, they rely on an array of germ line-encoded receptors that allow them to exert rapid cytotoxic and cytokine-producing effects against tumor cells and infected cells (Wu et al., 2015). NK cells can be activated by the integration of inhibitory and activating signals through the activation of cell surface receptors or by cytokine-mediated

stimulation, including IL-12, IL-15, IL-18 and IFN-α/β (Vivier et al., 2008). Upon activation, NK cells exert a degranulation function and secrete cytokines, such as TNF-α, IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF), which in turn exert anti-viral or anti-tumor effects (Vivier et al., 2008).

In addition to direct immunomodulatory effects, NK cells interact with other innate immune cells, through receptor-ligand combinations or regional cytokine secretion. For instance, interactions between NK cells and DCs result in the maturation, activation, and cytokine production of both cells (Thomas and Yang, 2016). On the one hand, DCs induce NK cell activation by secreting soluble cytokines or through direct cell-cell contacts (Ferlazzo and Morandi, 2014). The TLR-mediated recognition of pathogens by DC can stimulate their maturation and the secretion of several cytokines, including IL-12, IL-18, IL-15, IFN-α/β, and prostaglandin E2 (PGE2) (Thomas and Yang, 2016), which can activate NK cells. On the other hand, activated NK cells release profound amount of TNF, IFN-γ which in turn promote DC maturation (Ferlazzo and Morandi, 2014). In addition, human KCs have been reported to promote NK cell activation and IFN-γ production following stimulation by TLR ligands or HBsAg, *in vitro* (Peng and Tian, 2018).

1.7.2. NK cells during HBV infections

NK cells play a controversial role during the acute phase of HBV. Some studies have suggested that NK cells and even innate immunity do not significantly contribute to the initial control of HBV infections (Fletcher et al., 2013; Wu et al., 2015). Other experiments, however, have reported that NK cells display an activated phenotype and enhanced effecter functions during the early stages of HBV infections (Fisicaro et al., 2009; Guy et al., 2008). Although adaptive immune responses are considered to be critical factors for determining the outcomes of HBV infections, NK cell-derived antiviral

cytokines may be responsible for early viral clearance and the regulation of later adaptive immune responses (Peng and Tian, 2018). However, most of these studies have been performed in animal models (Fisicaro et al., 2009; Fletcher et al., 2013; Guy et al., 2008), which may not fully represent the reality in humans. In addition, due to insufficient data from humans, the role played by NK cells during the early phase of HBV infections in patients remains to be elucidated.

During chronic HBV infections, NK cells display abnormal phenotypes and functionality. NK cells have been found to express reduced levels of activating receptors and increased levels of inhibitory receptors, accompanied by a decrease in antiviral cytokines production in patients with chronic HBV infection (Peng and Tian, 2018). Lunemann et.al has also reported that infection with viral hepatitis increase peripheral NK cell number, and NK cells in these patients displayed less activated phenotype and defective functional responses (Lunemann et al., 2014). However, NK cell phenotypic and functional alterations have been equally observed in HBV, HCV and HDV infections, suggesting that the alteration of NK cell phenotype and function depends on disease activity rather than virus-specific factors (Lunemann et al., 2014). The upregulation of the inhibitory receptor NKG2A (Li et al., 2013), and the inhibitory effects of molecules, Tim-3 (Ju et al., 2010) and PD-1 (Wiesmayr et al., 2012), have been observed against NK cells derived from patients with chronic HBV infections. However, the cytolytic activities of NK cells appear to be less affected by HBV infections, and sometimes even enhanced in patients with active CHB (Zhang et al., 2011). In addition, the crosstalk between NK cells and DCs was also found to be impaired during chronic HBV infections. For instance, the ability of myeloid DCs (mDCs) to secret cytokines is severely impaired, which, in turn, disrupts the secretion of adequate amounts of IFN-γ by NK cells (Zhang et al., 2011).

During the natural courses of CHB, NK cell phenotypes and functions may vary depending on the infection stage. Some conflicting results have been reported, likely due to the wide spectrum of different clinical conditions examined (Fisicaro et al., 2019). Compared with healthy controls, patients in the immune-clearance stage and the HBeAg-negative hepatitis stage exhibit the upregulation of peripheral NK cell activation markers whereas patients in the immune-tolerance stage displayed reduced cytokines production (Wang et al., 2019). Another study, however, demonstrated that the compositions, phenotypes and cytolytic activities of peripheral NK cells remained relatively constant except for a few receptor markers (e.g., KIRs, NKp46, and CD57); however, the ability of $CD56^{bright}$ NK cells to produce IFN-γ differs between pre-and post-HBeAg seroconversion clinical phases (de Groen et al., 2017).

During HBV infections, NK cells do not always beneficially clear the virus; the activation of NK cells can also have harmful effects, contributing to the pathogenesis of liver injury. NK cells can promote hepatocellular damage and inflammation by interacting with hepatocytes (Fisicaro et al., 2019). The cytolytic activity of NK cell has been shown to correlate positively with the severity of liver damage during CHB (Zheng et al., 2015). NK cells can also induce the death of hepatocytes through TRAIL or Fas/Fas ligand interactions which have been shown to be upregulated in patients with HBV and inflammation (Wu et al., 2015). Furthermore, HBV-specific $CD8^+$ T cells from CHB patients exhibited the upregulation of TRAIL death receptor, TRAIL-R2, rendering them susceptible to NK cell-mediated death (Peppa et al., 2013). Therefore, the future therapeutic manipulation of NK cells may selectively promote their beneficial effects while simultaneously blocking their pathogenic effects (Maini and Gehring, 2016).

1.7.3. The effects of HBsAg on NK cells

An important feature of HBV is the secretion of large amounts of HBsAg in different

forms by hepatocytes as discussed in **Section 1.1**. However, the significance of these high HBsAg protein levels is not completely understood. Several studies have demonstrated that HBsAg may impact NK cell phenotypes or functions in mice as well as humans. The number of hepatic NK cells decreases with the increased expression of HBsAg, and their cytotoxicity was attenuated in HBV transgenic mice (Peppa et al., 2013). Moreover, a negative correlation was found between the lysis function of peripheral NK cells and HBsAg levels in CHB patients (Yang et al., 2016). HBsAg protein also blocks NK cell activation, cytokine production and cytotoxic granule release in the human NK cell line NK-92 (Yang et al., 2016). In addition, the activation of NK cells has been negatively correlated with patients' HBsAg levels in HBeAg-negtive CHB patients (Tjwa et al., 2014). These lines of evidence suggested that HBsAg exerts a generic immunosuppressive effect against NK cell activation and function. However, most of these studies focused only on a small cohort or were performed in mice or cell lines. Therefore, the specific effects of varying HBsAg quantities on systemic NK cell phenotypes and functions in chronic HBV patients remain to be determined.

2. Objective of the study

This thesis aimed to investigate the phenotypes and functions of NK cells in the peripheral blood of CHB patients associated with varying amounts of HBsAg. The underlying hypothesis of this study is that HBsAg suppresses NK cell activation and function in a concentration-dependent manner. Overall, this study should provide data which may be of relevance for development of novel immunotherapies aiming to achieve functional cure of HBV.

3. Materials and methods

3.1. Materials

3.1.1. Patient material

In this study, a total of 80 individuals were included. These patients with chronic hepatitis B were recruited either in the outpatient clinic of the Department of Gastroenterology and Hepatology at Essen University Hospital in Germany or in the outpatient clinic of Department of Gastroenterology, Hepatology, and Endocrinology at Hannover Medical School in Germany. Patients gave informed consent for the investigation of immunological parameters as part of protocols approved by the ethics committee of University Hospital Essen and Hannover Medical School. All enrolled CHB patients met the following criteria: HBsAg positive for at least 6 month; absence of human immunodeficiency virus (HIV), hepatitis C virus (HCV), or hepatitis D virus (HDV) co-infection; without evidence of decompensated liver cirrhosis and hepatocellular carcinoma (HCC); absence of autoimmune disease, immunosuppressive agents, organ transplant or other comorbid illnesses that may affect immune response; without evidence for alcoholic hepatitis or nonalcoholic fatty liver disease; none of the patients received interferon (IFN)-based antiviral therapy at the time of the present investigation. Patients were classified into four groups according to their HBsAg concentration: HBsAg <100 IU/ml (n = 20), HBsAg 100–1,000 IU/ml (n = 21), HBsAg 1,000–10,000 IU/ml (n = 19) or HBsAg >10,000 IU/ml (n = 20). As control, 30 healthy donors were recruited at Essen University Hospital. The patients' characteristic was shown in **Table 1** and the detailed information of individual patient was shown in attachment.

Table 1. Clinical characteristic of study subjects

Characteristic	Healthy	Chronic hepatitis B patients (HBsAg IU/mL)			
		< 100	100-1000	1000-10000	>10000
Number	30	20	21	19	20
Age (years)	45 (23-71)	52 (22-68)	48 (27-71)	44 (27-69)	38 (24-52)
Gender					
Female	14(46.7%)	8 (40%)	10 (47.6%)	5 (26.3%)	5 (25%)
Male	16(53.3%)	12 (60%)	11 (52.4%)	14(73.7%)	15 (75%)
HBV DNA (IU/mL)	-	<10 (<10-1200)	29 (<10-17090)	140 (<10-331800)	71.5 (<10-414000)
HBV genotype	-	A1/C1/D6/E 1/n.a 11	A4/B1/D8/ n.a 8	A1/C1/D7/F1/ n.a 9	A5/B1/D9/n.a 5
HBsAg (IU/mL)	-	19.65 (0.4-78)	550 (102-998)	2061 (1257-8768)	20956 (10098-124827)
HBeAg pos/neg	-	Neg18/n.a 2	Neg 18/n.a 3	Neg 18/n.a 1	Neg 15/ pos 4/ n.a1
ALT (U/L)	-	27 (14-78)	25 (14-62)	28 (14-156)	39 (21-154)
AST (U/L)	-	28.5 (13-137)	23 (11-51)	24 (13-70)	28 (15-155)
Bilirubin (mg/dL)	-	0.65 (0.4-7)	0.7 (0.2-10)	1.05 (0.5-13)	0.7 (0.4-15)
Fibroscan (kPa)	-	5.8 (3.3-25.1)	5.6 (2.3-11.8)	5.7 (4.3-17)	5.9 (2.2-9.6)

Unless otherwise indicated, values represent median (range). ALT and AST values from 13 individuals were not available (2 in the group with HBsAg <100 IU/ml, 4 in the group with HBsAg 100–1,000 IU/ml, 2 in the group with HBsAg 1,000–10,000 IU/ml, 3 in the group with HBsAg >10,000 IU/ml). Fibroscan data from 19 individuals were not available (3 in the group with HBsAg <100 IU/ml, 7 in the group with HBsAg 100–1,000 IU/ml, 4 in the group with HBsAg 1,000–10,000 IU/ml, 5 in the group with HBsAg >10,000 IU/ml). Abbreviations: ALT, alanine aminotransferase; AST, aspirate aminotransferase; HBeAg, hepatitis e antigen; HBsAg, hepatitis B surface antigen; CHB, chronic hepatitis B.

3.1.2. Cell line

The cell line K562 was kindly provided by Prof. Niklas K. Björkström in Karolinska University Hospital Huddinge, Sweden. The cells were kept in complete RPMI-1640 medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1 mM L-glutamine.

3.1.3. HBV particles

HBV particles were kindly provided by Prof. Mengji Lu from the Institute for Virology, University Hospital Essen, Germany. Briefly, the HBV particles were purified from the supernatant of human HepG2.2.15 hepatoma cell line after culturing for 6 days. A mock control was produced by precipitating supernatants of HepG2 or Huh 7 cells (without HBV particles) under the same condition.

3.1.4. Reagents

Reagent	Cat number	Source
1640 medium	11875-093	Gibco
Pen/Strep	15140-122	Gibco
PBS	14190-094	Gibco
EDTA	A10492-01	Gibco
Fetal calf serum	F7524	Sigma
DMSO	D2650-5X5ML	Sigma
Bicoll separating solution		Biochrom
Erythrocyte lysis buffer	15575-038	Invitrogen
Human recombinant IL-12	200-12	PeproTech
Human recombinant IL-15	200-15	PeproTech
Human recombinant IL-18	B001-5	R&D Systems
Zombie	423101	Biolegend
NK cells isolation Kit	130-092-657	Miltenyi
MACS buffer	130-091-221	Miltenyi
LS column	130-042-401	Miltenyi
Fc blocking buffer	130-059-901	Miltenyi

Fix/permeabilisation diluent	00-5223-56	eBioscience
Fix/permeabilization concentrate	00-5123-43	eBioscience
Permeabilisation buffer 10×	00-8333-56	eBioscience
brefeldin	00-4506-51	eBioscience
Monensin	00-4505-51	eBioscience
PMA		Sigma Aldrich
ionomycin		Sigma Aldrich

3.1.5. Buffers and Solutions

FACS buffer: 500 ml PBS + 2mM EDTA + 2% FCS

Freezing buffer: 90% fetal calf serum + 10% DMSO

1640 complete medium: 1640 RPMI medium + 10% fetal calf serum + 1% Pen /strep + 1% L-Glutamine

3.1.6. Antibodies for flow cytometry

Table 2. Antibody information for flow cytometry

Antibody	Color	Clone	Source
Extracellular staining			
TRAIL	PE	RIK-2	BD Biosciences
CD56	PE-Cy7	NCAM16.2	BD Biosciences
PD1	PE	EH12:1	BD Biosciences
CD4	PE-Cy7	SK3	BD Biosciences
CD161	Brilliant Violet 650	DX12	BD Biosciences
NKG2A	FITC	130-105-646	Miltenyi
CD107a	FITC	130-106-233	Miltenyi
CD158a	PE-Cy5.5	EB6B	Beckman Coulter

CD158b	PE-Cy5.5	GL183	Beckman Coulter
CD56	PE-Dazzle 594	5.1H11	Biolegend
DNAM-1	PE-Cy7	11A8	Biolegend
NKG2D	APC	1D11	Biolegend
CD3	Alexa 700	HIT3a	Biolegend
NKp46	APC-Cy7	9E2	Biolegend
CXCR6	Brilliant Violet 421	SA051D1	Biolegend
CD14	Brilliant Violet 510	63D3	Biolegend
CD19	Brilliant Violet 510	HIB19	Biolegend
CD57	Brilliant Violet 605	QA17A04	Biolegend
CD16	Brilliant Violet 650	3G8	Biolegend
Tim3	FITC	F38-2E2	Biolegend
CCR7	PE	G043G7	Biolegend
CD69	PE-Dazzle 594	FN50	Biolegend
CD38	APC	HB-7	Biolegend
CD8	APC-Cy7	SK1	Biolegend
HLA-DR	Brilliant Violet 421	L243	Biolegend
PD-1	PE	EH12:1	BD Biosciences
TIGIT	APC	A15153G	Biolegend
KLRG1	BV421	14C2A07	Biolegend
Intracellular staining			
Ki-67	Percp	KI-67	Biolegend
IFN-γ	Brilliant Violet 650	4S.B3	Biolegend
TNF	APC	MAb11	Biolegend
MIP-1β	Brilliant Violet 421	D21-1351	BD Biosciences
GM-CSF	PE-Dazzle 594	BVD2-21C	Biolegend
IL-10	Perxp-cy5.5	JES3-9D7	Biolegend

TGF-1β	PE	A15153G	Biolegend
Eomes	FITC	11-4877-41	eBioscience
Granzyme B	PE	561142	BD Biosciences
Helios	PE-Cy7	22F6	Biolegend
PLZF	APC	9E12	eBioscience
T-bet	Brilliant Violet 421	4B10	Biolegend

3.2. Methods

3.2.1. Isolation of human peripheral blood mononuclear cells (PBMC)

(1) For the EDTA/CPDA/heparine tubes containing whole blood samples, centrifuge at 1000g for 10 minutes at 4 ℃ with break.

(2) Transfer the plasma in the upper layer into new cryopreservation tubes and store them in the -80 ℃ refrigerator for further use.

(3) For the blood cells in the bottom, fill each tube with 5 ml room temperature PBS and transfer the diluted samples into new 50 ml Falcon tubes.

(4) Put 15 ml Bicoll into other empty 50 ml Falcon tubes.

(5) Aspire the diluted blood with a sterile serological 25 ml pipette and overlay the Bicoll with the blood. Let the blood slowly down the side of the tube while holding the tube with the Bicoll nearly horizontally.

(6) Centrifuge at 600g for 25 minutes in room temperature without brake.

(7) Afterwards the content is separated into four different phases: the upper one is diluted plasma; the thin white layer beneath is the interphase and contains the PBMCs lying upon; the Bicoll phase and the fourth one is the lower red phase consisting of erythrocytes.

(8) Take off some supernatant in the upper layer and discard it.

(9) Remove the interphase containing the PBMCs carefully with 25 ml serological pipette moving it slowly in circular moves over the interphase and transfer the aspires

interphase into new 50 ml Falcon tube.

(10) Wash the cells with PBS (fill up to 50 ml), centrifuge 300g for 10 minutes (with break).

(11) Discard supernatant, resuspend and pool cells from one individual with 5 ml of erythrocyte lysis buffer, incubate at room temperature for 5 minutes. Wash the cells again with PBS (fill up to 50 ml), centrifuge 300g for 10 minutes (with break).

(12) Discard the supernatant and resuspend cell pellets in PBS. Count the cells by taking 10 µl of the cell suspension and mixing with 1:1 (10 µl) trypan blue buffer (0.2% stock solution), transfer 10 µl into counting slides and count it manually.

(13) Wash cells again with PBS by filling up to 50 ml, centrifuge 300g for 10 minutes (with break). Discard the supernatant and resuspend the cells in freezing buffer (7 million cells/ml).

(14) Transfer the cells into new cryopreservation tubes and store them in -80℃ refrigerator for further use.

3.2.2. Functional NK cell Assays

(1) Cryopreserved PBMCs were thawed and washed once by PBS. Spin down the PBMC and resuspend in complete medium at 5×10^6 cells/ml (or 1×10^6 cells/ml if purified NK cells are used). Seed 200 µl cells (10^6 cells/well) to 96 well U-bottom plate per well. Cells were either rested or prestimulated overnight with 10 ng/ml IL-12 and 100 ng/ml IL-18 at 37 ℃ and 5% CO_2.

(2) On the second day, centrifuge samples at 600g for 5 minutes at room temperature and discard the supernatant. Resuspend the cells in 100 µl complete medium.

(3) Spin down K562 target cells and resuspend at 1×10^6 cells/ml in complete 1640 medium.

(4) Add 100 µl of K562 target cell suspension (10^5 cells/well) into indicated wells in U-bottom plate mentioned in Step (2). So the effector/target ratio is 10:1. Add the 2 µl

FITC anti-CD107a antibody per well into the plate and centrifuge the cells at 30g for 3 minutes.

(5) Incubate at 37 ℃ and 5% CO_2 for 1 hour. 1 hour later, add 20 μl of culture medium supplemented with Golgi Plug (brefeldin) diluted 1:100 and Golgi Stop (Monensin) diluted 1:100 per well. Immediately return the cells to the incubator at 37 ℃ and 5% CO_2 for 5 more hours.

(6) After a total of 6 hours, centrifuge the cells at 600g for 5 minutes and promptly flick off the supernatant. Staining the cell by surface staining and then intracellular staining as described in **3.2.6 section**.

3.2.3. Regulatory function of NK cells

Cryopreserved PBMCs were thawed and washed once by PBS. Cells were stimulated for 6 h with phorbol-12-myristate-13-acetate (PMA; 50 ng/mL) and ionomycin (1 μg/mL, Sigma Aldrich) in the presence of monensin (1 μg/mL) and Brefeldin A (BFA; 1 μg/mL). IL-10 and TGF-1β were detected by flow cytometry after incubation.

3.2.4. NK cells isolation

(1) Cryopreserved PBMCs were thawed and washed once by PBS. Determine cell number and resuspend cell pellet in 40 μl of MACS buffer per 10^7 total cells.

(2) Add 10 μl of NK Cell Biotin-Antibody Cocktail per 10^7 total cells. Mix well and incubate for 5 minutes in 4 ℃ refrigerator.

(3) Add 30 μl of buffer per 10^7 total cells and 20 μl of NK Cell MicroBead Cocktail per 10^7 total cells and mix well. Incubate for 10 minutes in 4 ℃ refrigerator.

(4) Place LS column in the magnetic field of a MACS Separator. Prepare column by rinsing with the 3 ml MACS buffer.

(5) Apply cell suspension onto the column. Collect flow-through containing unlabeled cells, representing the enriched NK cells. Wash column with the 3 ml MACS buffer for three times. Collect unlabeled cells that pass through.

(6) Remove column from the separator and place it on a suitable collection tube. Pipette 5

ml MACS buffer onto the column. Immediately flush out the magnetically labeled non-NK cells by firmly pushing the plunger into the column.

(7) Detect the purity of NK cells by staining with CD56-ECD and CD3-Alexa 700 and analyzed by flow cytometry. Cell debris and dead cells were excluded from the analysis based on scatter signals and Zombie staining. NK cell purity was > 90% all the time (**Figure 3**).

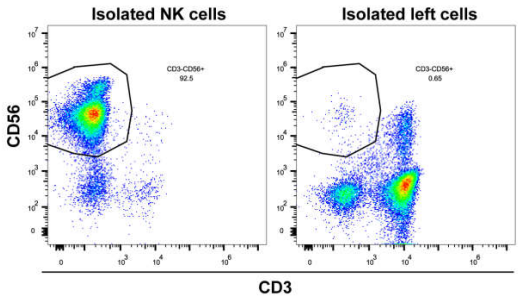

Figure 3. The purity of NK cells after isolation. The left figure shows the percent of $CD3^{-}CD56^{+}$ NK cells in isolated NK cells while the right one shows the NK cells percentage in magnetically labeled non-NK cells population after MACS isolation.

3.2.5. HBV particles stimulation *in vitro*

For purified NK cells, add human recombinant IL-15 to keep the final concentration of 1 ng/ml. Seed 200 ul cells (1×10^5 cells/well) to 96 well U-bottom plate per well. NK cells were stimulated with different dose of HBV particles or same volume of control for 24h. As a control, HepG2 or Huh 7 cell culture media without HBV particles were used. For NK cell functional assay, all the cells were stimulated with 10 ng/ml IL-12 and 100 ng/ml IL-18 for 16h. 24h later, NK cells phenotype and function were detected by flow cytometry.

3.2.6. Flow cytometry

The staining panel was shown in **Table 3**. These panels include phenotype, activation markers, exhaustion markers, transcription factors and functions.

Surface staining

(1) Cryopreserved PBMCs were thawed and washed once by PBS. Resuspend the cells by FACS buffer and adjust the cells concentration to 5×10^6/ml. Pre-incubate the cells with 1ul human Fc receptor binding inhibitor per 100 μl for 10 minutes at 4 ℃. Then aliquot 200 μl of cells (10^6 cells/well) to the 96-well U-bottom plate each well. Centrifuge at 600g for 5 minutes.

Table 3. Staining panels for NK cells

Laser line	conjugates	NK cell phenotype	NK cell activation	NK cell transcription factor	NK cell function	exhaustion marker
Blue 488 nm	FITC	NKG2A	Tim3	Eomes	CD107a	Tim3
	Percp-cy5.5	panKIR		panKIR	Ki-67	
Yellow-green	PE	TRAIL	TRAIL	Granzyme B		PD-1
	PE-Dazzle594	CD56	CD69	CD56	GM-CSF	
	PE-cy7	DNAM1	CD56	Helios	CD56	CD56
Red 633nm	APC	NKG2D	CD38	PLZF	TNF	TIGIT
	Alexa 700	CD3	CD3	CD3	CD3	CD3
	APC-cy7	NKp46	CD16	NKp46	CD16	CD16
Violet 405nm	BV421	CXCR6	HLA-DR	T-bet	MIP-1β	KLRG1
	BV510	Zombie CD14/CD19	Zombie CD14/CD19	Zombie CD14/CD19	Zombie CD14/CD19	Zombie CD14/CD19
	BV605	CD57	CD57	CD57	CD57	CD57
	BV650	CD16	CD161	CD16	IFN-γ	

(2) Prepare the antibodies mix: Combine the recommended quantity of each primary antibody and Zombie in an appropriate volume of FACS buffer. (50 μl of antibodies mix per well).

(3) Discard the supernatant of the plate and add the antibody mix to each well. Pulse vortex gently to mix. Incubate for at least 30 min at 4 ℃ in dark. Wash the cells by adding 150 μl FASC buffer to each well and centrifuge at 600g for 5 minutes. Discard the supernatant.

(4) Resuspend cells in 200 μl of FASC buffer and transfer the cells suspension into FACS

tubes.

Intracellular staining

(1) Stain cell surface markers as mentioned above. Add 100 µl of Fixation/Permeabilization working solution to each well and mix well. Incubate for 45 minutes at room temperature in the dark.

(2) Wash the cells with 100 µl 1× Permeabilization buffer per well and centrifuge samples at 600g for 5 minutes.

(3) Prepare the intracellular staining antibodies mix: combine the recommended quantity of each primary antibody in an appropriate volume of 1× Permeabilization buffer (50 µl of antibodies mix per well).

(4) Discard the supernatant of the plate and add the antibody mix to each well. Pulse vortex gently to mix. Incubate for at least 30 minutes at room temperature in the dark.

(5) Wash the cells by adding 150 µl 1× Permeabilization buffer to each well and centrifuge samples at 600g for 5 minutes at room temperature. Discard the supernatant.

(6) Resuspend the stained cells in an appropriate volume of FACS buffer and transfer the cells into FACS tubes.

FASC data acquisition and analysis

The data were acquired on Beckman Cytoflex and analyzed with Flowjo software version 10.3 (Treestar Inc, Ashland, OR). Cell debris and dead cells were excluded from the analysis based on scatter signals and Zombie staining.

3.2.7. Data analysis

Data were analyzed using GraphPad Prism version 8 (GraphPaD Software, La Jolla, CA). The data sets were first evaluated for normality of the data distribution using the

D'Agostino-Person omnibus normality test. Statistically significant differences among multiple groups were determined as appropriate using the Kruskal-Wallis test followed by Dunn's multiple comparison test, or the ordinary 1-way ANOVA followed by Holm-Sidak's multiple comparisons test for pairwise comparison of unpaired samples. Correlations were analyzed by Spearman's correlations. For creation of correlation matrix, the R-packages, Hmisc v.4.2-0 and corrplot v.0.84 were used in R version 3.6.1. $p<0.05$ was considered significant. Principal component analysis (PCA) was performed using Qlucore Omics Explorer v3.6 (Qlucore AB, Lund, Sweden), when comparing multiple groups in the PCA model an analysis of one-way ANOVA was used, when comparing 2 groups a t-test was used.

4. Results

4.1. Experimental design and patient characteristics

In our cohort, we selected 80 chronic HBV infected patients who were classified into four groups according to their HBsAg concentrations: HBsAg <100 IU/ml (n = 20), HBsAg 100–1,000 IU/ml (n = 21), HBsAg 1,000–10,000 IU/ml (n = 19) and HBsAg >10,000 IU/ml (n = 20). As a control group, 30 healthy donors were included (**Figure 4**). Most of the patients in our cohort showed normal ALT (<40 U/L) and bilirubin (0.2–1.2 mg/dL) levels. Of note, most patients had rather low HBV DNA levels and thus HBV replication was controlled in most patients. In addition, no significant differences were observed for HBV DNA, Fibroscan, ALT, AST, and bilirubin levels among the four patient groups, except for differences in HBsAg levels (**Figure 5A**). Notably, the age distribution differed slightly among the patient groups, with a younger population of patients in the high HBsAg level (>1,000 IU/ml) group compared the population of those groups with low HBsAg levels (<1,000 IU/ml, **Figure 5B**). In line with this result, HBsAg levels were negatively correlated with age (**Figure**

5C). As expected, based on the natural course of HBV infections, our CHB cohort was primarily comprised of patients in the stage of HBeAg-negative chronic infection.

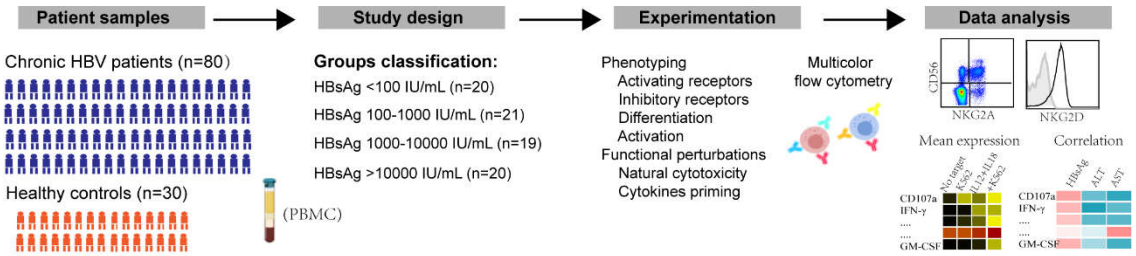

Figure 4. Schematic representation of the study design. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBsAg, hepatitis B surface antigen; CHB, chronic hepatitis B; IFN-γ, interferon gama; IL, interleukin; PBMC, peripheral blood mononuclear cells.

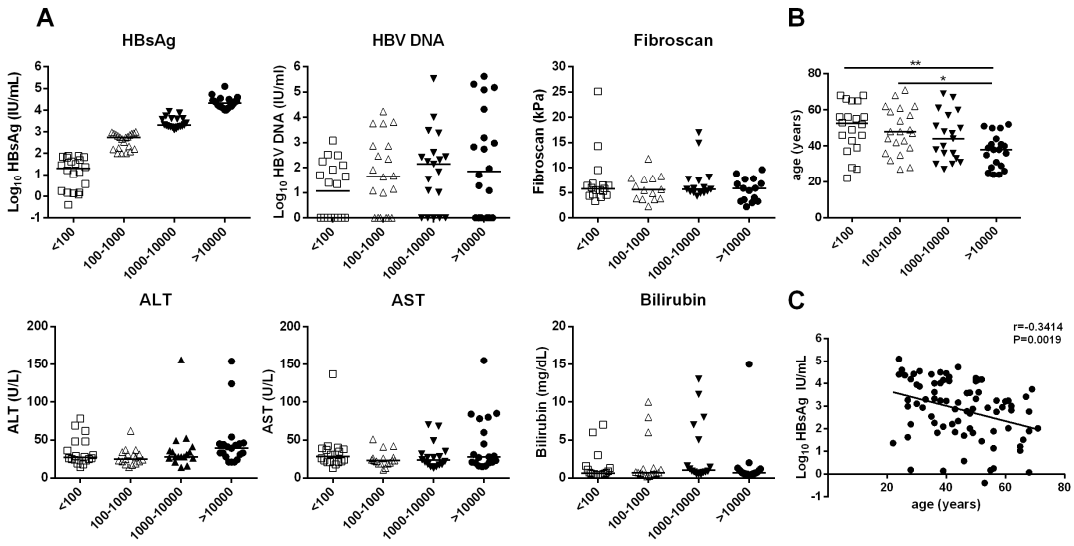

Figure 5. Clinical data for patients with chronic hepatitis B. (A) Summary of hepatitis B surface antigen (HBsAg), HBV DNA, transient elastography (Fibroscan), alanine aminotransferase (ALT); aspirate aminotransferase (AST), and bilirubin levels in each group. (B) Ages of patients in each group. (C) Correlation between patient age and HBsAg level. Spearman's correlation coefficient and the p value are shown in the upper-right corner. $P < 0.05$ was considered significant. The horizontal bar represents the median. Statistical analysis was assessed by the using the ANOVA with Kruskal-Wallis test followed by Dunn's multiple comparison test; * $P < 0.05$, ** $P < 0.01$.

4.2. Increased frequency of CD56^{bright} NK cells in CHB patients with higher HBsAg levels.

Next, we compared the systematic phenotype and functionalities among NK cells derived from CHB patients expressing different concentrations of HBsAg and healthy controls. First, we examined the changes in the compartment of peripheral blood NK cells. Therefore, the frequencies of total NK cells, CD56^{dim}, and CD56^{bright} NK cells, were assessed in CHB patients with varying HBsAg concentrations compared with those in healthy controls. The gating strategy first gated single lymphocytes and then excluded CD14^{+} monocytes, CD19^{+} B cells, and zombie^{+} dead cells. NK cells were identified as the CD3^{-}CD56^{+} population, and the two subsets, CD56^{dim} and CD56^{bright} NK cells, were further identified according to the surface expression levels of CD56 and CD16 (**Figure 6A**). The frequencies of total NK cells and the two subsets, CD56^{dim} and CD56^{bright} NK cells, among total lymphocytes were not significantly different in CHB patients compared with healthy controls (**Figure 6B**). Importantly, the CD56^{bright} subpopulation out of total NK cells was significantly increased in patients with higher HBsAg levels (1,000–10,000 or >10,000 IU/ml) compared with in healthy controls, whereas CD56^{dim} NK cells were significantly reduced in these patients (**Figure 6C**). In addition, we did not observe significant differences of the non-classical NK cell subpopulation, CD56^{neg}CD16^{bright} cells, in patients with different HBsAg titers (**Figure 6D**). In summary, patients with high levels of HBsAg were associated with a shift towards more CD56^{bright} NK cells.

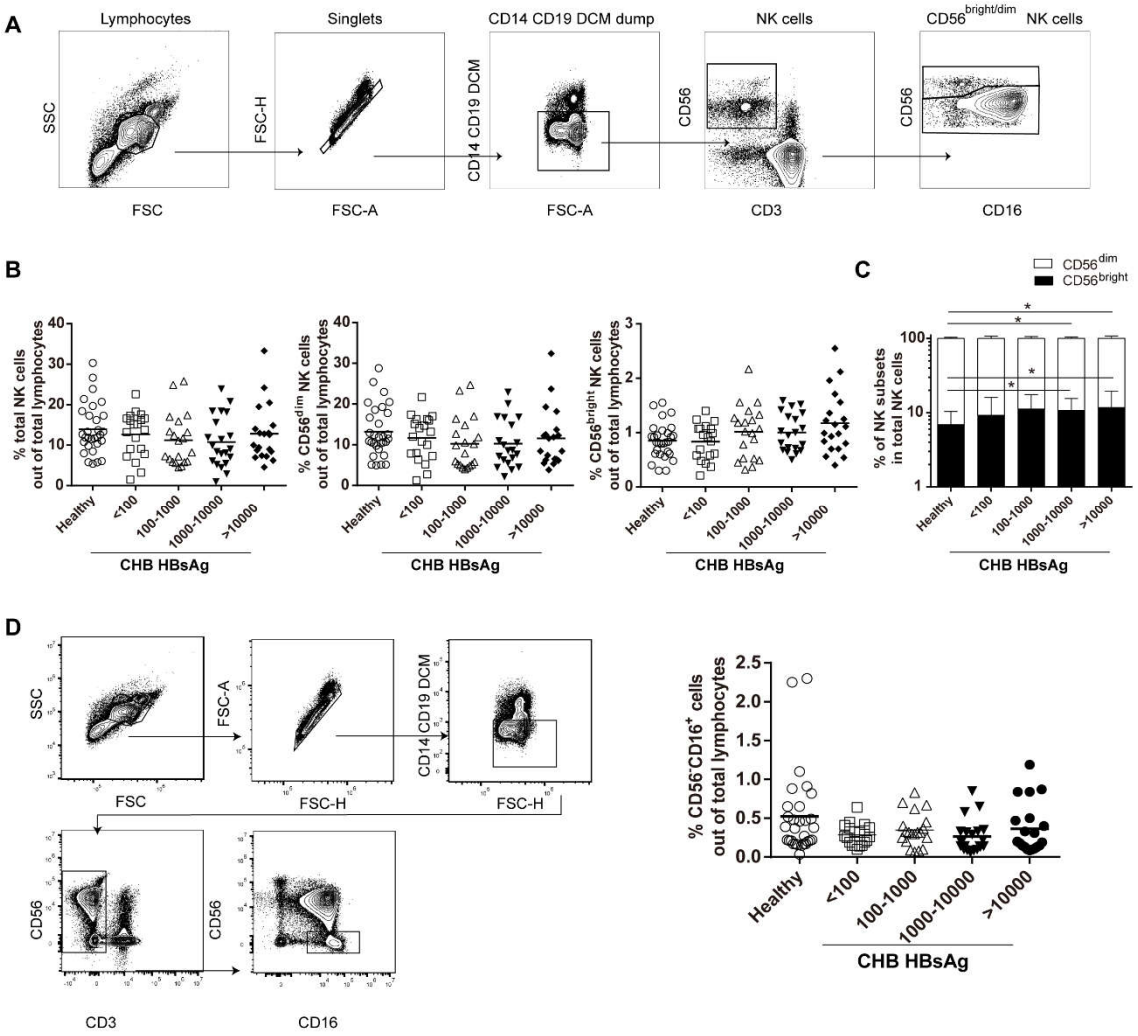

Figure 6. The number of natural killer (NK) cells in chronic hepatitis B patients and healthy donors. (A) Gating strategy used to identify total, $CD56^{dim}$ and $CD56^{bright}$ NK cells. (B)The frequencies of total, $CD56^{dim}$, and $CD56^{bright}$ NK cells among total lymphocytes; and (C) the frequencies of $CD56^{dim}$ and $CD56^{bright}$ subsets out of total NK cells were determined in CHB patients with different HBsAg concentrations compared with those in healthy controls. (D) Gating strategy and the frequencies of non-classical NK cell ($CD56^{-}CD16^{+}$) were summarized. The graph represents the counts for healthy controls (n = 30) and chronic HBV patients with HBsAg <100 IU/ml (n = 20), HBsAg 100–1,000 IU/ml (n = 21), HBsAg 1,000–10,000 IU/ml (n = 19) or HBsAg >10,000 IU/ml (n = 20). Statistical analysis was performed via ANOVA with Kruskal-Wallis test followed by Dunn's multiple comparison test, * $P < 0.05$. The horizontal bars represent mean. Abbreviations: HBsAg, hepatitis B surface antigen; CHB, chronic hepatitis B; DCM, dead-cell marker; FSC, forward scatter; NK, natural killer; SSC, side scatter.

4.3. NK cells exhibited activated phenotype in patients with low HBsAg levels

To analyze the phenotypic characterization of NK cells, we detected the activating receptors (NKG2D, NKp46 and DNAM1), inhibitory receptors (KIR, NKG2A), activation markers (CD38, CD69, HLA-DR, and Granzyme B), exhaustion markers (Tim-3, KLRG1, TIGIT, PD-1) and a proliferation marker (Ki-67) in NK cells from patients with different concentration of HBsAg and healthy controls. Overall, no differences were observed in total NK cells and $CD56^{dim}$ NK cells in terms of the expression of the activating receptors NKG2D, NKp46 and DNAM1 between CHB patients and healthy controls (**Figure 7A**). However, the expression of NKp46 on the $CD56^{bright}$ subpopulation was significantly decreased in patients with low HBsAg (< 100 IU/ml) relative to healthy controls. Interestingly, the expression of CD161 on total NK cells and $CD56^{dim}$ subpopulation significantly increased in patients with relatively low HBsAg levels (100-1000 IU/ml) compared with that in healthy controls (**Figure 7A**). However, this result was not observed for the $CD56^{bright}$ subpopulation. Interestingly, the expression of the exhaustion marker Tim-3 on $CD56^{bright}$ subpopulation was reduced in patients with relatively high HBsAg levels (>10,000 IU/ml) compared to patients with low HBsAg levels (<100 IU/ml) (**Figure 7B**) while the expression of T cell immunoreceptor with Ig and ITIM domains (TIGIT) on total and $CD56^{dim}$ NK cells was significantly increased in patients with highest HBsAg levels relative to the healthy controls (**Figure 7B**). However, the other two exhaustion markers, killer cell lectin-like receptor subfamily G member 1 (KLRG1) and programmed cell death protein 1 (PD-1) were similarly expressed on NK cells and their subsets among the five groups **(Figure 7B)**.

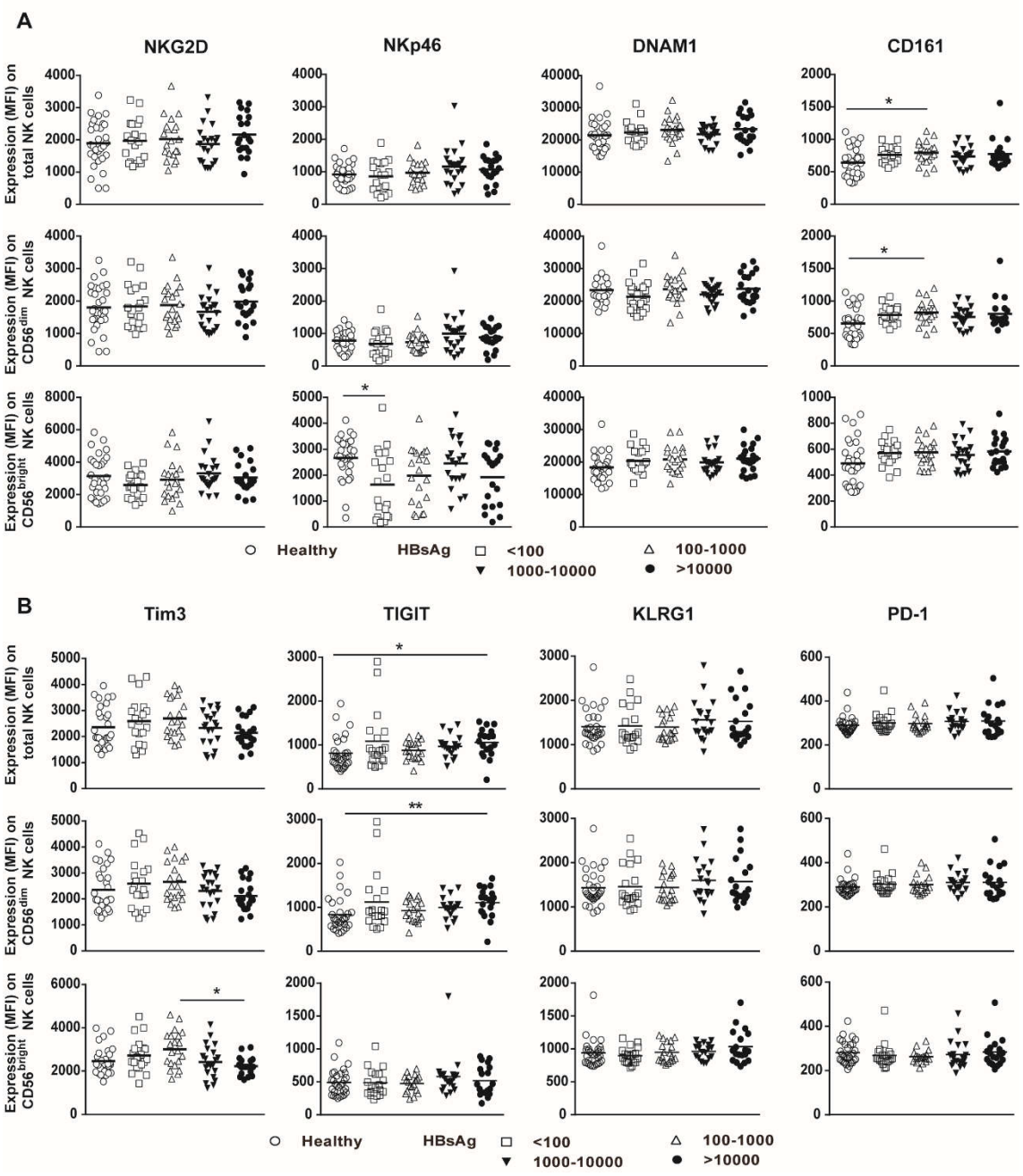

Figure 7. Phenotypic characteristics of NK cells in CHB patients with different HBsAg levels. Scatter plots presenting the mean fluorescence intensity (MFI) of the (A) activating receptors (NKG2D, NKp46, DNAM1), CD161 and (B) exhaustion markers (Tim-3, TIGIT, KLRG1 and PD-1) on total, $CD56^{dim}$ and $CD56^{bright}$ NK cells in healthy controls (n = 30) and CHB patients with HBsAg <100 IU/ml (n = 20), HBsAg 100–1,000 IU/ml (n = 21), HBsAg 1,000–10,000 IU/ml (n = 19) or HBsAg >10,000 IU/ml (n = 20). Statistical analysis was performed via ANOVA with Kruskal-Wallis test followed by Dunn's multiple comparison test, * $P < 0.05$; ** $P < 0.01$. The horizontal bars represent the mean. Abbreviations: HBsAg, hepatitis B surface antigen, CHB, chronic hepatitis B; MFI, mean fluorescence intensity; Tim3, T cell

immunoglobulin and mucin domain-containing protein 3; TIGIT, T cell immunoreceptor with Ig and ITIM domains; KLRG1, killer cell lectin-like receptor subfamily G member 1; PD-1, programmed cell death protein 1.

Importantly, significant increases in activation markers (CD38, Granzyme B) and proliferation marker (Ki-67) were detected in total NK cells and the two subsets of NK cells from CHB patients with lowest HBsAg levels (<100 IU/ml) compared to healthy donors (**Figure 8**). Similarly, CD38 and Ki-67 increased in patients with low HBsAg levels (100–1,000 IU/ml) compared with those in the healthy controls. As for the four patient groups, patients with low HBsAg levels (<100 IU/ml) also exhibited higher expression of Granzyme B and Ki-67 than the other three groups (**Figure 8**). However, TRAIL expressed at similar levels among all patient groups and the healthy controls.

Taken together, although a slight decrease was observed in the expression of activation-associated receptors, such as NKp46 on $CD56^{bright}$ subpopulation, NK cells from patients with low HBsAg concentration exhibited an activated phenotype compared with both in healthy controls and the other three patient groups, with the increased expression of several activation and proliferation markers.

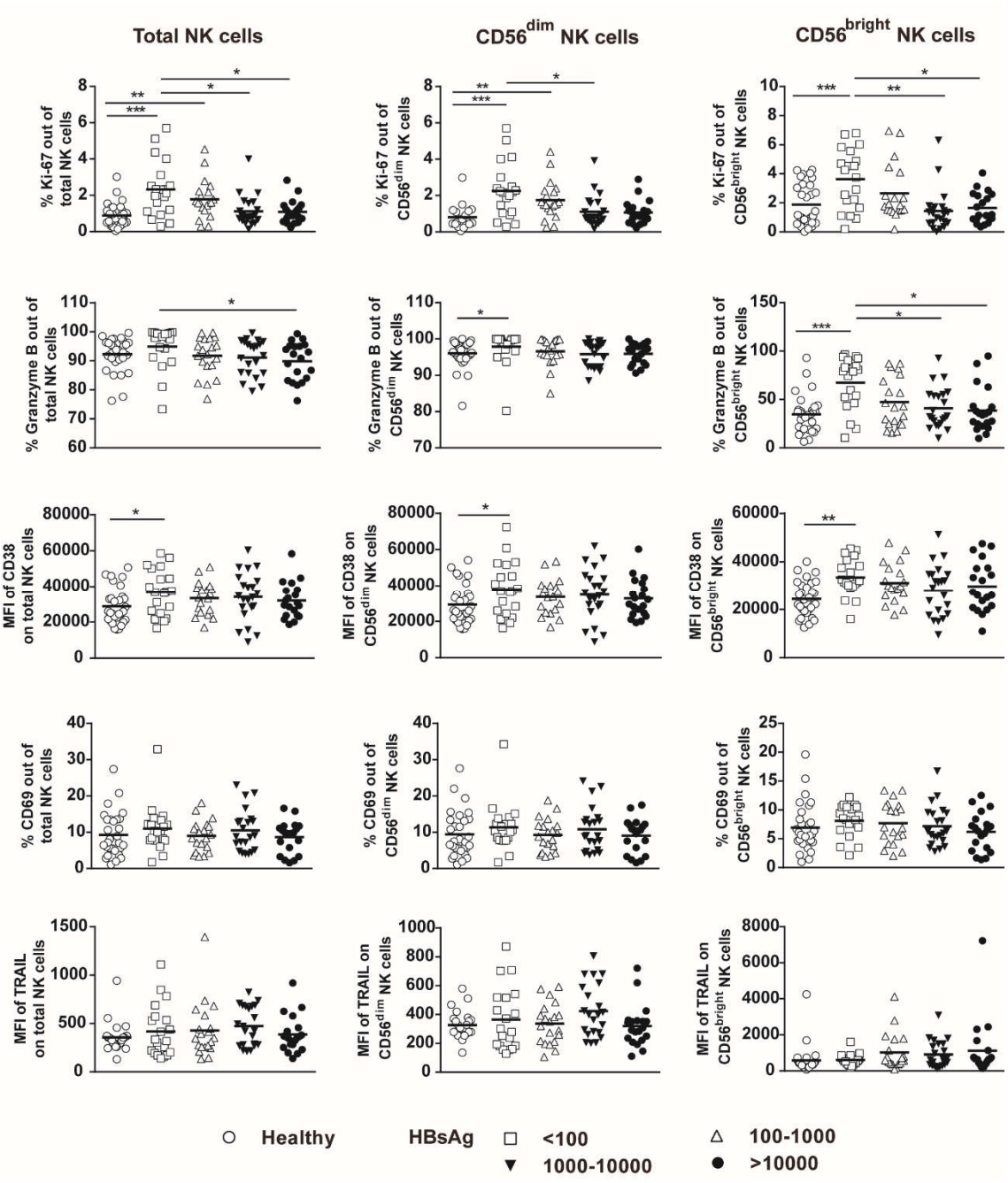

Figure 8. Expression of activation and proliferation markers on NK cells from CHB patients with different HBsAg concentrations. Mean fluorescence intensity (MFI) of activation markers (TRAIL, CD38) and percentages of CD69, granzyme B and proliferation marker Ki-67 on total, CD56^{dim} and CD56^{bright} NK cells in healthy controls (n = 30); CHB patients with HBsAg <100 IU/ml (n = 20), HBsAg 100–1,000 IU/ml (n = 21), HBsAg 1,000–10,000 IU/ml (n = 19) or HBsAg >10,000 IU/ml (n = 20). Statistical analysis was performed via ANOVA with Kruskal-Wallis test followed by Dunn's multiple comparison test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The horizontal bars represent the mean.

4.4. NK cells in CHB patients were less mature than those in healthy controls.

Our observation of increased $CD56^{bright}$ subpopulation among some HBV patients suggests an altered NK-cell differentiation in these patients. A previous study suggested that $CD56^{bright}$ NK cells may represent the predecessors of $CD56^{dim}$ NK cells, and that the loss of NKG2A and sequential acquisition of KIRs and CD57 expression reflect a continued differentiation process that occurs in $CD56^{dim}$ NK cells (Björkström et al., 2010). To determine the role of HBsAg on $CD56^{dim}$ differentiation, we evaluated the expression level of NKG2A, CD57 and pan-KIR on $CD56^{dim}$ NK cells in CHB patients with different HBsAg concentrations, compared with those in healthy controls. Neither NKG2A nor CD57 expression differed between the patient groups and healthy control (**Figure 9A-B**). For patients with relatively high HBsAg (1,000–10,000 or >10000 IU/ml), pan-KIR expression exhibited a trend towards downregulation compared to the healthy controls, but this difference was not significant (**Figure 9A-B**). Similarly, no significant difference was noted in the expression of these three makers on the Boolean analysis (**Figure 9C**). These findings are in line with a previous study, which showed that chronic HBV infections had no general effects on NK cell differentiation, based on the three surface markers (NKG2A, CD57 and pan-KIR) (Lunemann et al., 2014).

To further confirm the impact of HBsAg levels on NK cell differentiation, we detected the transcription factors expressed by $CD56^{dim}$ NK cell subset. We found $CD56^{dim}$ NK cells, both in CHB patients and healthy controls, uniformly lacked the expression of the transcription factor promyelocytic leukemia zinc finger (PLZF) (approximately 0.01%) and Helios (approximately 0.1%) (**Figure 10A-B**), which corroborates a previously published study (Hart et al., 2019).

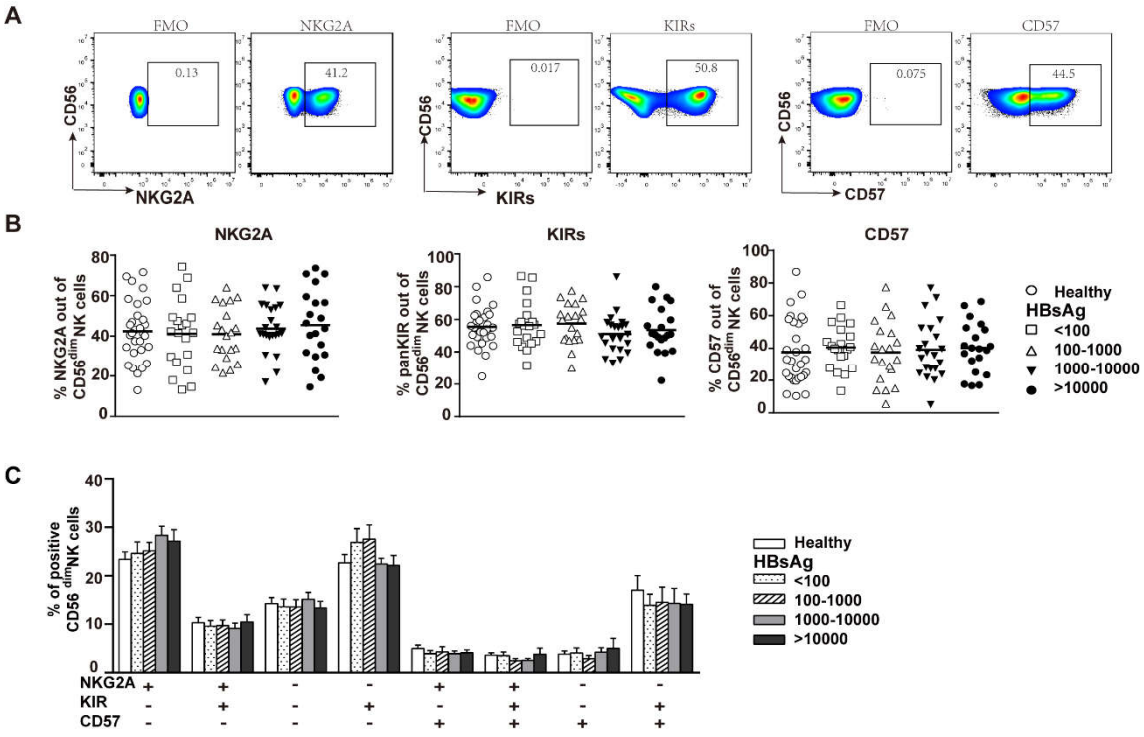

Figure 9. NK cell differentiation was not affected by HBsAg concentration based on surface marker expression. (A) Representative staining for NKG2A, pan-KIR and CD57 from one patient with chronic HBV infection according to fluorescence minus one (FMO) control. (B) The percentage of NKG2A, pan-KIR and CD57 on $CD56^{dim}$ NK cells was summarized for all groups. (C) Boolean analysis was performed for $CD56^{dim}$ NK cells, expressing combinations of NKG2A, pan-KIR and CD57. The graph represents counts of healthy controls (n = 30) and chronic HBV patient with HBsAg <100 IU/ml (n = 20), HBsAg 100–1,000 IU/ml (n = 21), HBsAg 1,000–10,000 IU/ml (n = 19) or HBsAg >10,000 IU/ml (n = 20). Statistical analysis was performed via the ANOVA with Kruskal-Wallis test followed by Dunn's multiple comparison test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The horizontal bars represent the mean. Abbreviations: KIR, killer-cell immunoglobulins-like receptors.

In another study, Collins et al. reported that NK cell maturation is also gradually coupled with a decrease in Eomesodermin (Eomes) and an increase in T-bet expression (Collins et al., 2017). We found that the expression of Eomes was slightly increased in patients with either low (<100 IU/ml) or high (>10,000 IU/ml) HBsAg concentrations compared with that in healthy controls, but the differences were not significant (**Figure 10A-B**). In addition, the expression of T-bet was significantly decreased in patients with moderate

HBsAg concentrations (100–1,000 IU/ml) compared with healthy control (**Figure 10A-B**). Consistently, the percentage of Eomes^{low}T-bet^{high} NK cells showed decreased trend in the four patient groups compared with the healthy controls as shown by the simultaneous analysis of Eomes and T-bet expression patterns on CD56^{dim} NK cells (**Figure 10C**). Taken together, these results showed that NK cells in CHB patients are less mature than those in healthy controls, as demonstrated by the expression patterns of Eomes and T-bet.

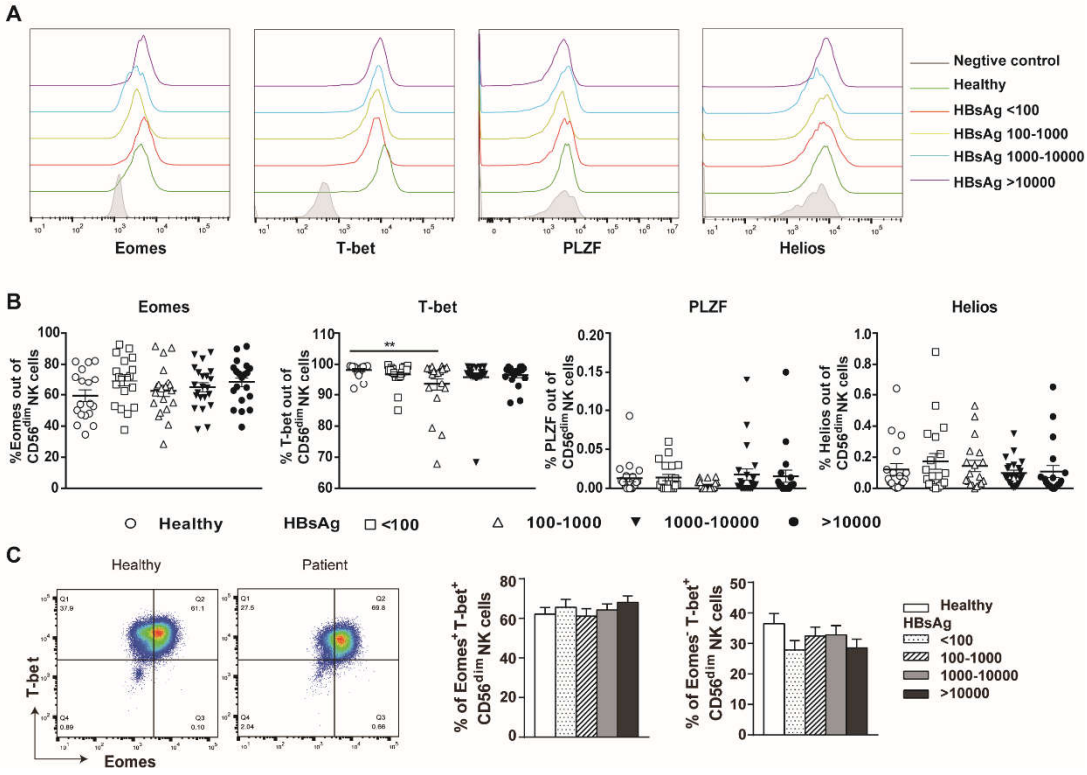

Figure 10. NK cells in CHB patients exhibited less mature phenotype based on the expression of transcription factors. (A) Representative histogram showing the levels of the transcription factors Eomes, T-bet, PLZF and Helios on CD56^{dim} NK cells from one patient in each group. (B) The proportions of CD56^{dim} NK cells expressing the examined transcription factors were summarized for all groups. (C) Representative gating for Eomes and T-bet on CD56^{dim} NK cells and Boolean analysis of CD56^{dim} NK cells expressing combinations of Eomes and T-bet. The graph represents counts of healthy controls (n = 30) and chronic HBV patient with HBsAg <100 IU/ml (n = 20), HBsAg 100 - 1,000 IU/ml (n = 21), HBsAg 1,000–10,000 IU/ml (n = 19) or HBsAg >10,000 IU/ml (n = 20). Statistical analysis was performed via ANOVA with Kruskal-Wallis test followed by Dunn's multiple comparison test, * $P < 0.05$, ** $P < 0.01$. The horizontal bars represent the mean. Abbreviations: PLZF, promyelocytic leukemia zinc finger.

4.5. Defective functional responses of NK cells in patients with low HBsAg levels

To evaluate whether patients with varying amounts of HBsAg displayed different NK cell functionality, we analyzed the capacities of NK cells to functionally respond against different stimulatory inputs in the four patient groups and healthy controls. Five NK cell functions were simultaneously measured within the $CD56^{dim}$ and $CD56^{bright}$ NK cell subpopulations, including the degranulation marker CD107a, cytokines (TNF，IFN-γ and GM-CSF) and chemokine [macrophage inflammatory protein (MIP)-1β]. As shown in **Figure 11**, NK cells responded well against the K562 target cell or in a combinatorial stimulation with IL-12 and IL-18.

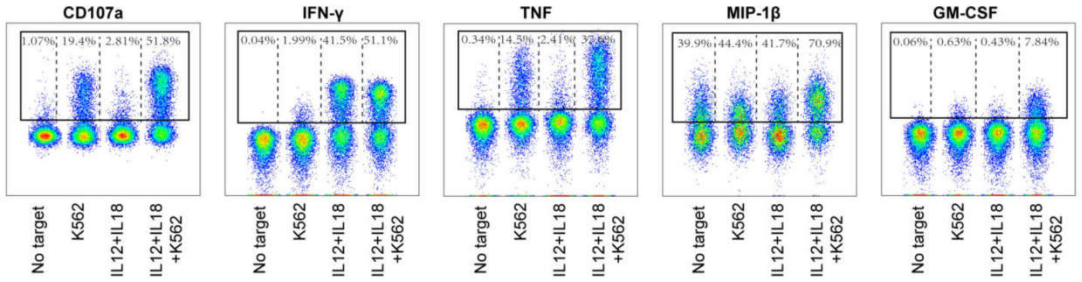

Figure 11. Concatenated flow cytometry plots of NK cells functional responses. Concatenated flow cytometry plots from one representative healthy control showing staining of CD107a, interferon-γ (IFN-γ), tumor necrosis factor (TNF), macrophage inflammatory protein-1β (MIP-1β), and granulocyte-macrophage colony-stimulating factor (GM-CSF) for $CD56^{dim}$ NK cells after the indicated stimulations.

Compared with the healthy control, the major differences in NK cell function were detected in patients with relatively low HBsAg concentrations (<100 or 100–1,000 IU/ml), including reduced responses to MIP-1β and the reduced capability to degranulate within the $CD56^{dim}$ NK cell compartment (**Figure 12A-B**). Consistently, TNF，IFN-γ and GM-CSF production in $CD56^{dim}$ NK cells exhibited a similar trend among patients with low HBsAg levels (< 100 IU/ml) in response to stimulation with K562 cells and IL-12 +

IL-18 (**Figure 12A-B**). Within the four patient groups, patients with relatively high HBsAg levels (1,000–10,000) produced significantly more MIP-1β in response to stimulation with IL-12 + IL-18 compared with patients with low HBsAg levels (<100 IU/ml).

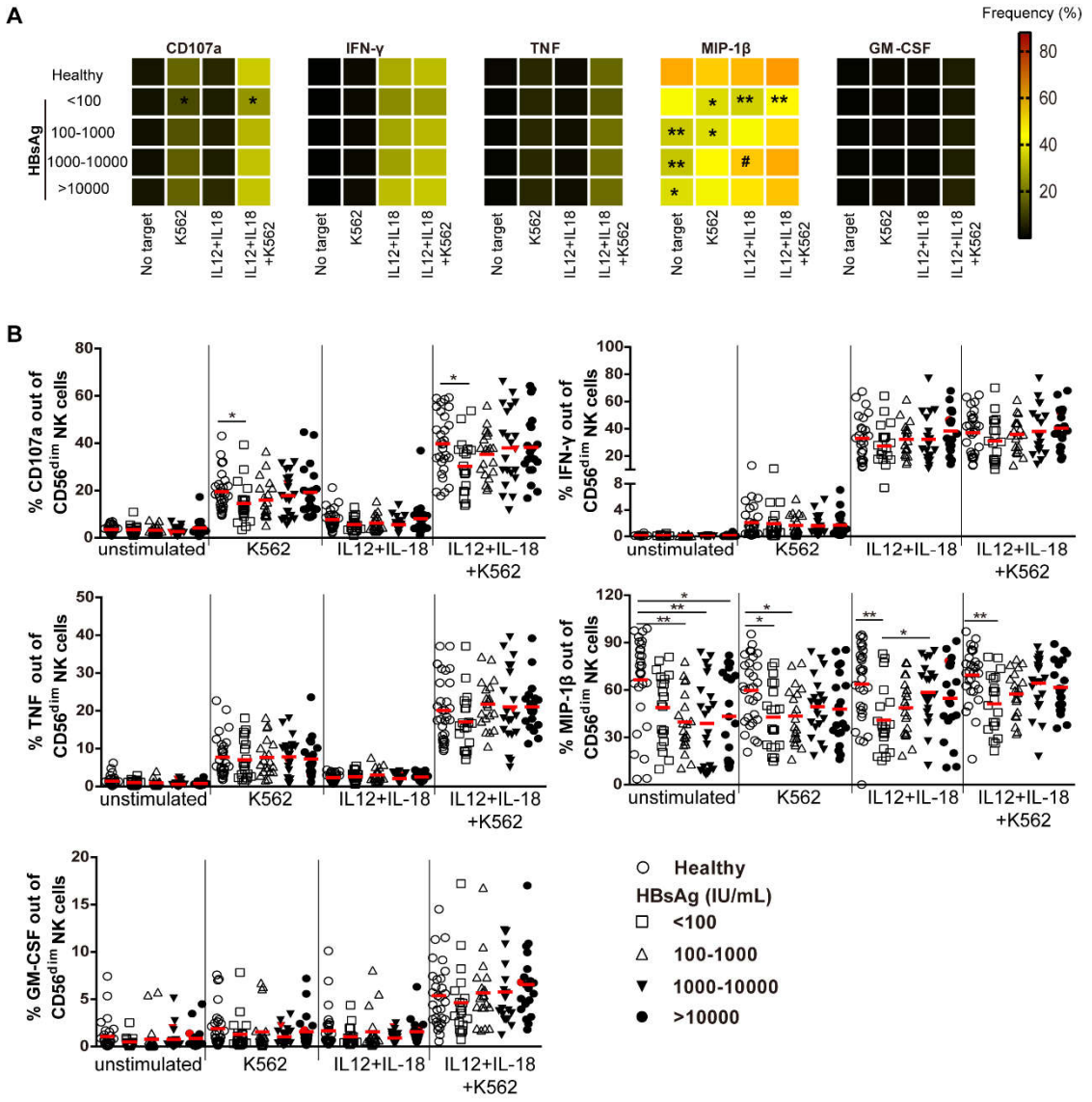

Figure 12. Decreased functional responses of CD56^{dim} NK cells in patients with low HBsAg levels. (A) Summary of the functional data for CD56^{dim} NK cells from healthy controls and different CHB patient groups. Data are presented as the mean. (B) Frequency of CD107a, IFN-γ, TNF, MIP-1β, and GM-CSF out of CD56^{dim} NK cells were summarized. Horizontal bars represent the mean. Counts represent individuals among the healthy controls (n = 30) and chronic HBV patients with HBsAg < 100 (n = 20), 100–1,000 (n =

21), 1,000–10,000 (n=19) or >10,000 IU/ml (n = 20). Statistical analysis was performed via ANOVA with Kruskal-Wallis test followed by Dunn's multiple comparison test; * P< 0.05, ** P<0.01. (A)* indicates significance level compared healthy controls with different patient groups; # P< 0.05, ## P<0.01, indicate significance level when comparing patients of HBsAg <100 IU/ml with other patient groups.

The CD56 ^{bright} subpopulation appears to respond differently from CD56^{dim} NK cells. CD56^{bright} NK cells produced similar levels of IFN-γ, TNF， GM-CSF and CD107a to various stimulatory inputs in both healthy donors and the patient groups, whereas IFN-γ production was significantly decreased in patients with relative high HBsAg levels (1,000–10,000IU/ml) compared to patients with low HBsAg levels (<100 IU/ml) under un-stimulated conditions (**Figure 13A-B**). Of note, MIP-1β production was wide-spread within each group, but the overall production of MIP-1β was markedly lower in patients with relatively low HBsAg levels (<100 or 100–1,000IU/ml) compared with healthy controls in response to stimulation with K562 target cells or K562 cells with IL-12 + IL-18 (**Figure 13A-B**), which was similar to the pattern observed for CD56^{dim} NK cells.

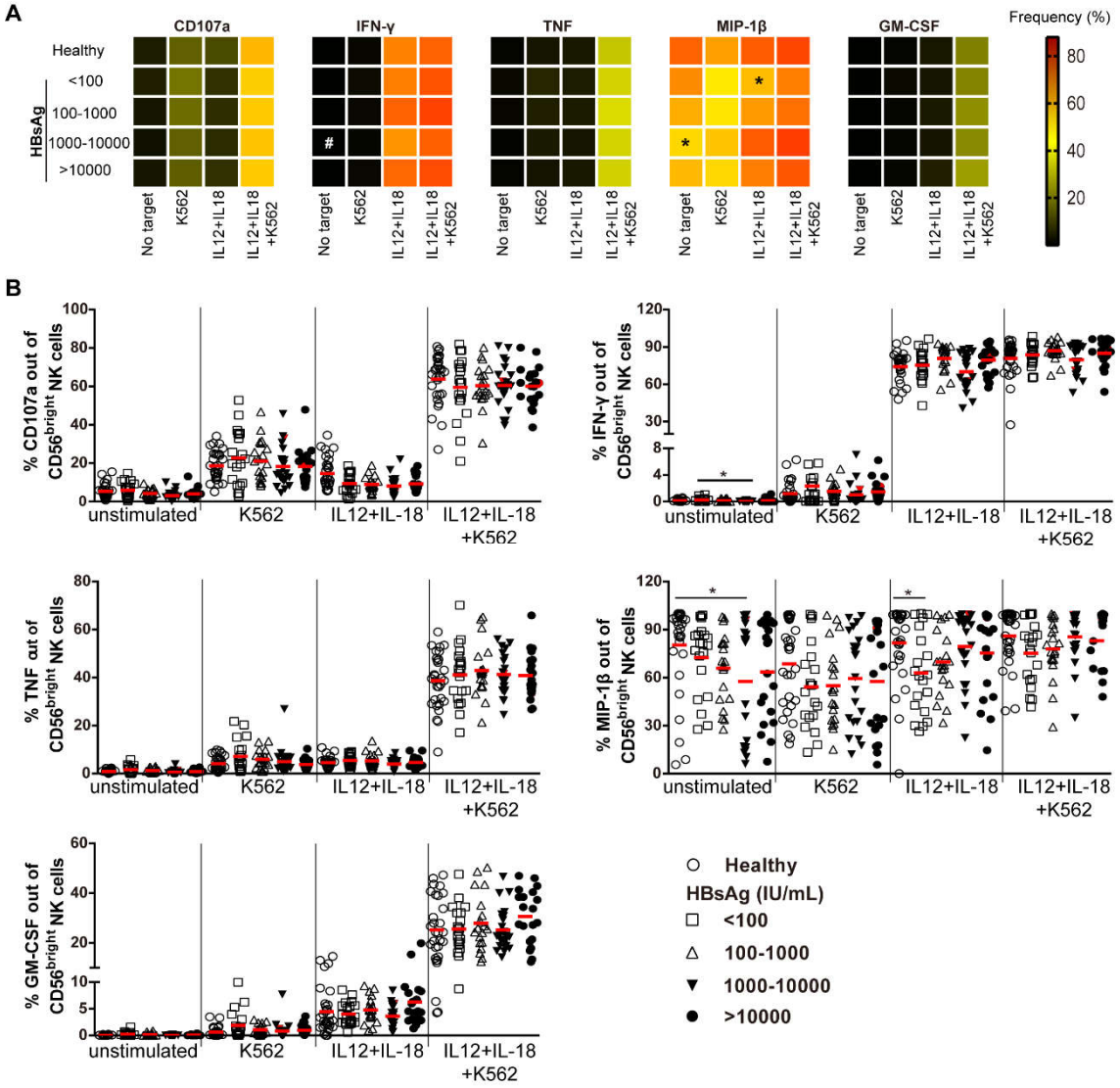

Figure 13. Functional responses of CD56^{bright} NK cells. (A) Summary of functional data for CD56^{bight} NK cells from healthy control and different CHB patient groups were shown by heatmap. Data are presented as the mean. (B) Frequency of CD107a, IFN-γ, TNF, MIP-1β, and GM-CSF out of CD56^{bright} NK cells were summarized. Horizontal bars represent mean. Counts of individuals in the graphs for healthy controls (n = 30) and chronic HBV patient with HBsAg <100 (n = 20), 100–1,000 (n = 21), 1,000–10,000 (n = 19) or >10,000 IU/ml (n = 20). Statistical analysis was performed via ANOVA with Kruskal-Wallis test followed by Dunn's multiple comparison test; * $P < 0.05$. (A)* indicate significance level when comparing healthy control with different patient groups; # $P < 0.05$, indicate significance level when comparing patients of HBsAg < 100 IU/ml with other patient groups.

Consistently, significantly fewer CD56^{dim} NK cells responded with 1, 2, or 3

simultaneous functions following stimulation in patients with low HBsAg levels (<100 or 100–1,000 IU/ml) compared with healthy controls or patients with higher HBsAg concentrations (>1,000 IU/ml) (**Figure 14**). In line with this, the proportions of $CD56^{bright}$ NK cells expressing 1 function were also significantly lower in patients with relatively low HBsAg levels (<100 or 100–1,000 IU/ml) compared with healthy controls (**Figure 14**). In addition, significantly more NK cells responded with 1, 2, or 3 functions simultaneously following stimulation with IL-12 + IL-18 or IL-12 + IL-18 plus K562 target cells in patients with relatively high HBsAg levels (1,000–10,000 or >10,000 IU/ml) compared with those in patients with low HBsAg levels (<100 IU/ml) (**Figure 14**). In addition, CD107a production on NK cells was higher in patients with highest HBsAg levels compared to healthy controls after stimulation of PMA and ionomycin (**Figure 15**). Taken together, NK cells from CHB patients with relatively low HBsAg levels (<100 IU/ml) exhibited blunted functional responses.

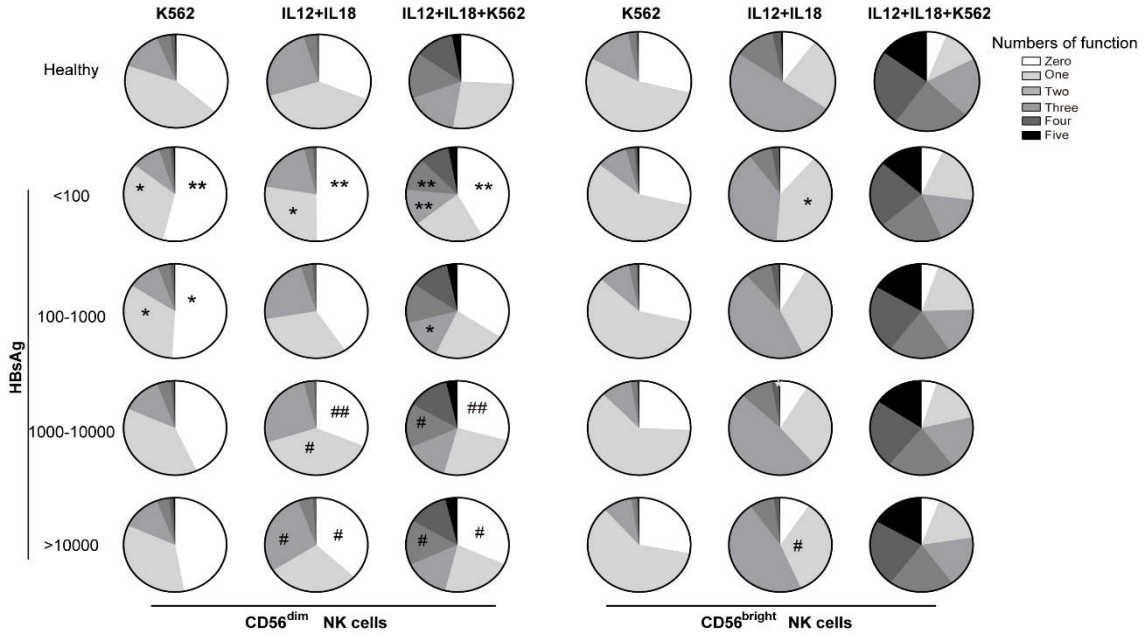

Figure 14. Multifunctional NK responses in chronic hepatitis B patients. The number of functions, as defined by Boolean gating, simultaneously exhibited by $CD56^{dim}$ or $CD56^{bright}$ NK cells from healthy control or different patient group. The pie charts indicate the proportions of cells exhibiting each number of simultaneous functions. Mean values are shown. Statistical analysis was performed via ANOVA with

Kruskal-Wallis test followed by Dunn's multiple comparison test. * P < 0.05, ** P < 0.01, indicate significance level when comparing healthy control with different patient groups; # P < 0.05, ## P < 0.01, indicate significance level when comparing patients of HBsAg <100 IU/ml with other patient groups.

To evaluate the regulatory function of NK cells, the expression of IL-10 and TGF-1β was measured after stimulating with PMA and ionomycin. We found significant increase of IL-10 secretion on NK cells in several patient groups compared to that of healthy controls (**Figure 15**). However, no major differences were found when comparing the four patient groups. In addition, TGF-1β production on NK cells also did not exhibit any significant differences among the five groups (**Figure 15**).

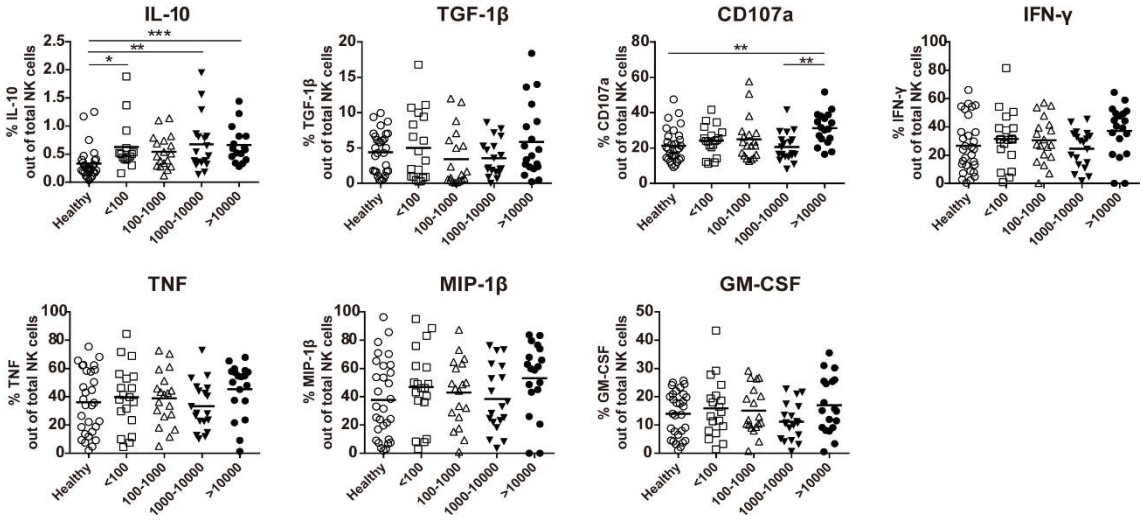

Figure 15. Functional responses of NK cells after stimulating by PMA and ionomycin. PBMCs were thawed, washed, and later stimulated with 1μg/ml ionomycin and 50 ng/ml phorbol myristate acetate (PMA) for 6 h. The expression of immunoregulatory cytokines (IL-10 and TGF-1β) and five functional markers were detected by intracellular staining. Horizontal bars represent mean. Counts of individuals in the graphs for healthy controls (n = 30) and chronic HBV patient with HBsAg <100 (n = 20), 100-1,000 (n = 21), 1,000-10,000 (n = 19) or >10,000 IU/ml (n = 20). Statistical analysis was performed via Kruskal-Wallis test followed by Dunn's analysis with correction for multiple comparisons; * p<0.05, ** p<0.01, ** p<0.001.

4.6. Correlation between NK cell parameters with virological and biochemical parameters

As a next step, we set out to explore the potential correlation between alterations in NK cell parameters with virological and biochemical parameters. As activation and functional markers exhibited significant differences, we correlated these markers with HBsAg levels and ALT levels. We found that the exhaustion marker Tim3 and the activation markers Granzyme B and CD69 in the $CD56^{bright}$ subpopulation negatively correlated with HBsAg levels (**Figure 16A**). On the contrast, MIP-1β production by total and $CD56^{dim}$ NK cells after IL-12/IL-18 or IL-12/IL-18 plus K562 target cell stimulation was positively correlated with HBsAg levels (**Figure 16B**). Of note, several functional markers CD107a, IFN-γ and GM-CSF on total and $CD56^{dim}$ NK cells were negatively correlated with patient age, either stimulated by IL-12/IL-18 or IL-12/IL-18 plus K562 target cell (**Figure 16B**). Other function markers also exhibited positive correlation trend with HBsAg levels but without statistical significance.

For correlation in each patient group, negative correlation between CD107a and HBsAg levels was only observed in patients with low HBsAg levels when stimulated with IL-12/IL-18 plus K562 target cell (**Figure 16C**). Consistently, most other function markers also exhibited negative correlation trend with HBsAg levels in low HBsAg group (<100 IU/ml) while displaying positive correlation trend in other three patient groups. In contrast, the correlation between NK cell parameters and liver enzyme (ALT) showed opposite trend. Most function markers on total or $CD56^{dim}$ NK cells exhibited positive correlation trend with ALT levels in low HBsAg group (<100 IU/ml) while displaying negative correlation trend in other three patient groups (**Figure 16C**). In addition, a difference between low HBsAg (<100 IU/ml) and high HBsAg (>10,000 IU/ml) could be observed when we assessed correlation between different markers on NK cells (**Figure 17**). As a result, both HBsAg and ALT levels may influence NK cell

activation and function; and the correlation pattern differs between patients with low HBsAg (<100 IU/ml) and high HBsAg levels (>10,000 IU/ml).

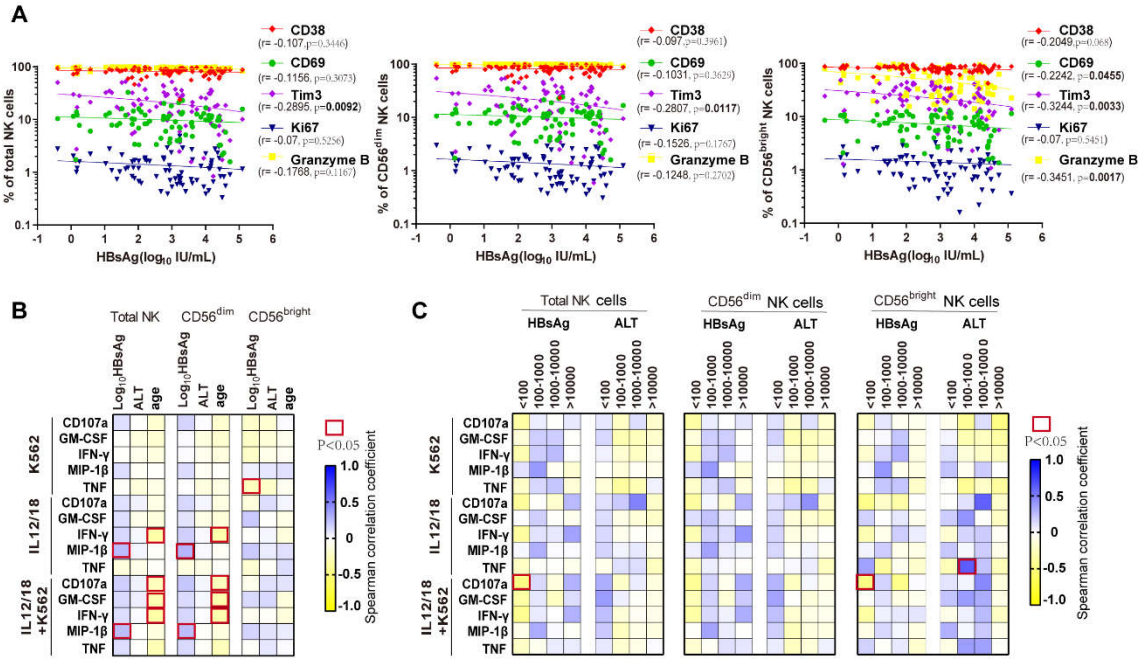

Figure 16. Correlations between NK cell parameters with virological and biochemical indicators. (A) Correlation analyses between HBsAg levels and the proportions of NK cells expressing various markers (n = 80). (B) Pairwise Spearman correlations between the indicated NK cell parameters in CHB patients and HBsAg levels (n = 80), ALT levels (n = 71) and patient age (n = 80). (C) Pairwise Spearman's correlations between the indicated NK cell parameters in CHB patients and HBsAg (n = 20, 21, 19 and 20 for HBsAg concentrations <100, 100–1,000, 1000–10,000 and >1000 IU/ml, respectively), and ALT levels (n = 18, 17, 17, and 19 for HBsAg concentrations <100, 100–1,000, 1,000–10,000 and >10,000 IU/ml, respectively). (B-C) Color intensities indicate Spearman's rank correlation coefficients as described in the legend. Significant (P < 0.05) correlations are highlighted with red squares.

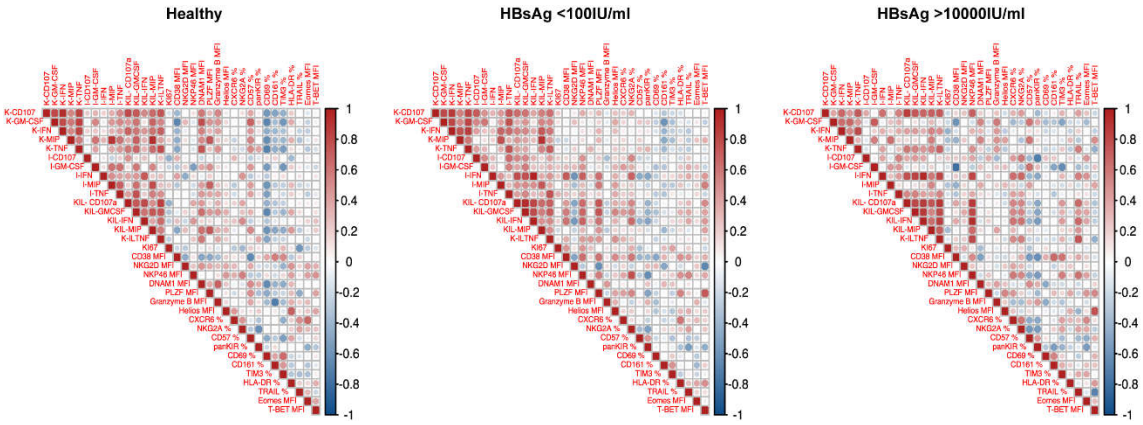

Figure 17. **Different correlation pattern between patients with low HBsAg levels and patients with high HBsAg levels.** Correlation matrices of different NK cell parameters in the respective CHB patient groups. Red circles indicate positive correlation and blue circles indicate negative correlation, and circle size indicates strength of correlation. K represents K562 cell stimulation; I represents IL-12+IL-18 stimulation; and KIL represents the stimulation of IL-12+IL-18+K562.

4.7. Correlations between NK cell parameters and other clinical data

To investigate the influences of treatment and gender on NK cell response, we performed principal component analysis (PCA), based on all parameters in total NK cells. As shown in **Figure 18A**, no distinct clustering was observed for patients with NA-treated and treatment-naïve patients (**Figure 18A**). Similarly, no distinct clustering was observed male and female patients (**Figure 18B**). These results suggested that neither treatment nor gender significantly impacted NK cell responses.

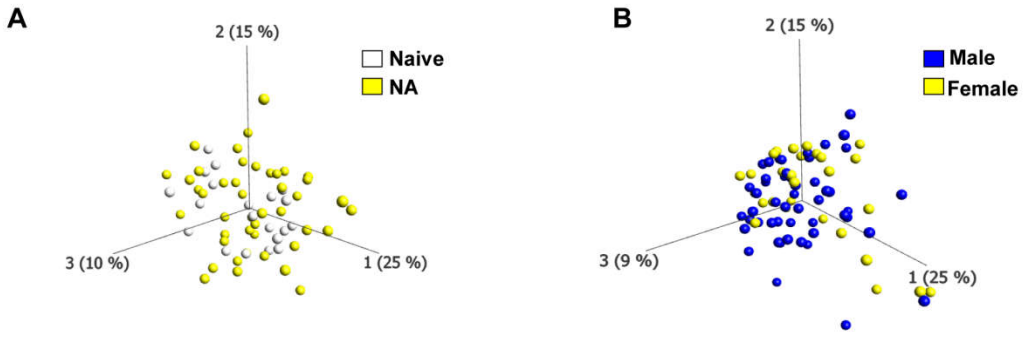

Figure 18. The influence of treatment and gender on NK cells. Principal component analysis (PCA) performed for total NK cells from chronic HBV patients according to treatment (A) and gender (B). The

PCA plot was calculated using all functional and the phenotypic markers for total NK cells and showed how the patients clustered together on basis of their treatment and gender. For treatment, nucleos(t)ide analogue (NA) treated patients n = 44, naïve patients n = 20; for gender, male n = 52, female n = 28; Multiple Student's t-test were performed.

4.8. HBV particles suppressed NK cell function *in vitro*

To identify whether HBsAg has direct effects on NK cells activation and function, we stimulated purified NK cells, derived from healthy controls and HBV patients, with various doses of HBV particles, collected from the supernatants of HepG2.215 cell lines. HBsAg concentration increased in a time-dependent manner in HepG2.215 cells (Zhao et al., 2011). These HBV particles were primarily composed of HBsAg, with a few HBV virions and HBeAg. The study design is shown in **Figure 19**. Purified NK cells were incubated with HBV particles for 24 hours in the presence of a low concentration of IL-15 (1 ng/ml). To perform the NK cell functional assays, NK cells were also stimulated with 10 ng/ml IL-12 and 100 ng/ml IL-18 for 16 hours. We found that the activating receptors NKG2D, NKp46, DNAM1, the inhibitory receptors NKG2A and pan-KIR, the differentiation markers CD16 and CD57, and CXCR6 and TRAIL were expressed at similar levels in mock control cells and cells treated with different doses of particles (**Figure 20**), indicating that HBV particles had no impact on NK cell phenotypes.

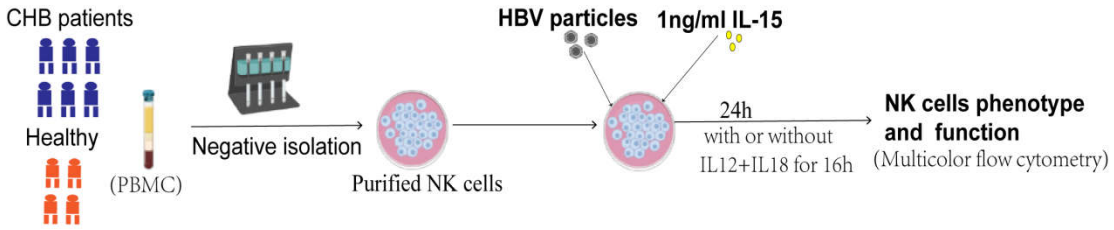

Figure 19. *In vitro* experimental design. Purified NK cells were isolated from the PBMCs of healthy donors or chronic hepatitis B patients, using magnetic separation. Then NK cells were stimulated with different doses of HBV particles in the presence of 1ng/ml IL-15 for 24 h. HBV particles were purified from the supernatant of the HepG2.2.15 cell line after culturing for 6 days. As a control, HepG2 or Huh 7

cell culture media without HBV particles were used. For functional assays, 10 ng/ml IL-12 and 100 ng/ml IL-18 were added for 16 h. Abbreviations: HBV, hepatitis B surface virus; CHB, chronic hepatitis B; NK, natural killer; PBMC, peripheral blood mononuclear cells.

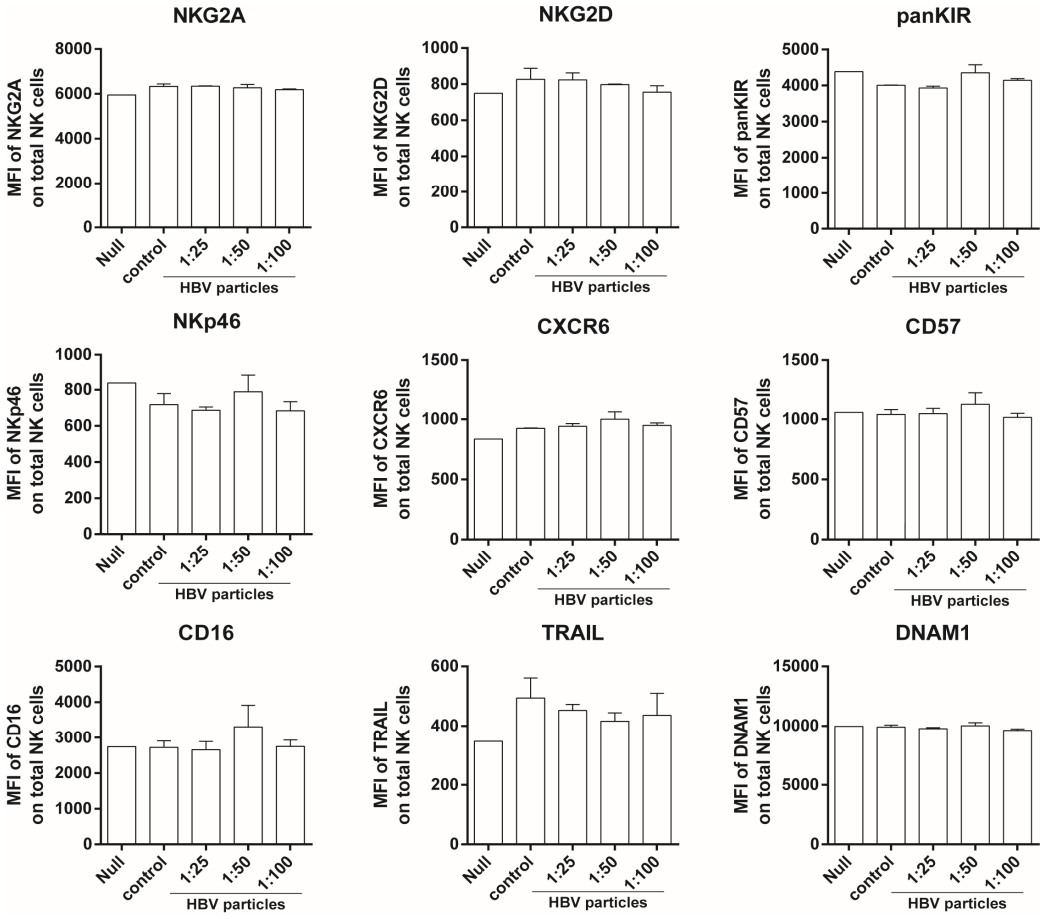

Figure 20. HBV particles had no impacts on NK cell phenotype *in vitro*. Purified NK cells were stimulated with different doses of HBV particles or control medium in the presence of 1 ng/ml IL-15 for 24h. The expression (MFI) of surface markers on NK cells was determined. Data are displayed as the mean ± SEM of triplicates experiments. Statistical analysis was performed via the Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviations: HBV, hepatitis B surface virus; KIR, killer-cell immunoglobulins-like receptor; MFI, mean fluorescence intensity; TRAIL, tumor necrosis factor related apoptosis-inducing ligand.

When examining NK cell function, we found that high doses of HBV particles (1:25 ratio) suppressed the cytokine production of NK cells derived from healthy controls, with

significant decreases in IFN-γ, TNF, and GM-CSF expression (**Figure 21A-B**). Consistently, MIP-1β exhibited a trend towards downregulation after stimulation with high doses of HBV particles (1:25 ratio). Moreover, low doses of HBV particles (1:100 ratio) also suppressed the expression of CD107a on NK cells compared with the control treatment, and a similar trend was observed for treatment with medium and high concentrations of HBV particles. However, we did not a reduced trend for the cytokines production when stimulating with low (1:100 ratio) or medium concentrations of HBV particles (1:50 ratio) (**Figure 21A-B**).

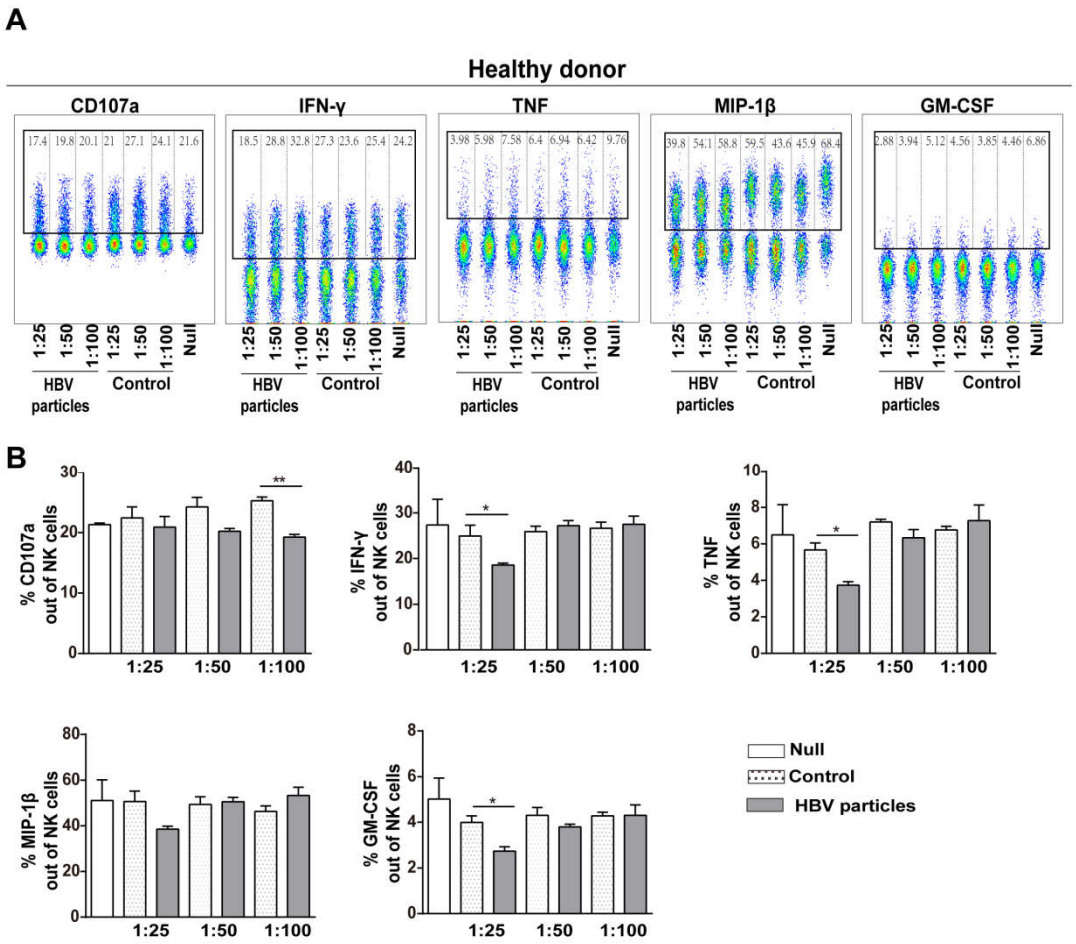

Figure 21. HBV particles suppressed the function of NK cells from healthy donors *in vitro*. The study design is shown in Figure 15. (A) Concatenated flow cytometry plots, showing the representative staining of CD107a, IFN-γ, TNF, MIP-1β, and GM-CSF in total NK cells from one healthy control following the indicated stimulations. The numbers indicate the percentage. (B) The percentage of cells expressing these

five cytokines in total NK cells from one healthy control was determined after the indicated stimulations. One representative experiment, out of three independent experiments, is presented. Data are displayed as mean ± SEM of triplicates experiments. Statistical analysis was performed via paired Student's t tests, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

To investigate whether HBV particles have the same suppressive effects on NK cell functions in patients who have experienced HBsAg, NK cells isolated from CHB were also assayed. Only minor differences were found in NK cells from HBV patients, including the reduced production of IFN-γ after stimulation with a low dose of HBV particles (1:100 ratios) (**Figure 22A-B**). However, other functional markers, such as CD107a, TNF, MIP-1β and GM-CSF, were expressed at similar levels in the mock control and HBV particle-treated groups (**Figure 22A-B**). In summary, HBV particles significantly suppressed the functions of NK cells derived from healthy donors, but this inhibitory effect was much weaker against NK cells derived from CHB patients with previous HBsAg exposure.

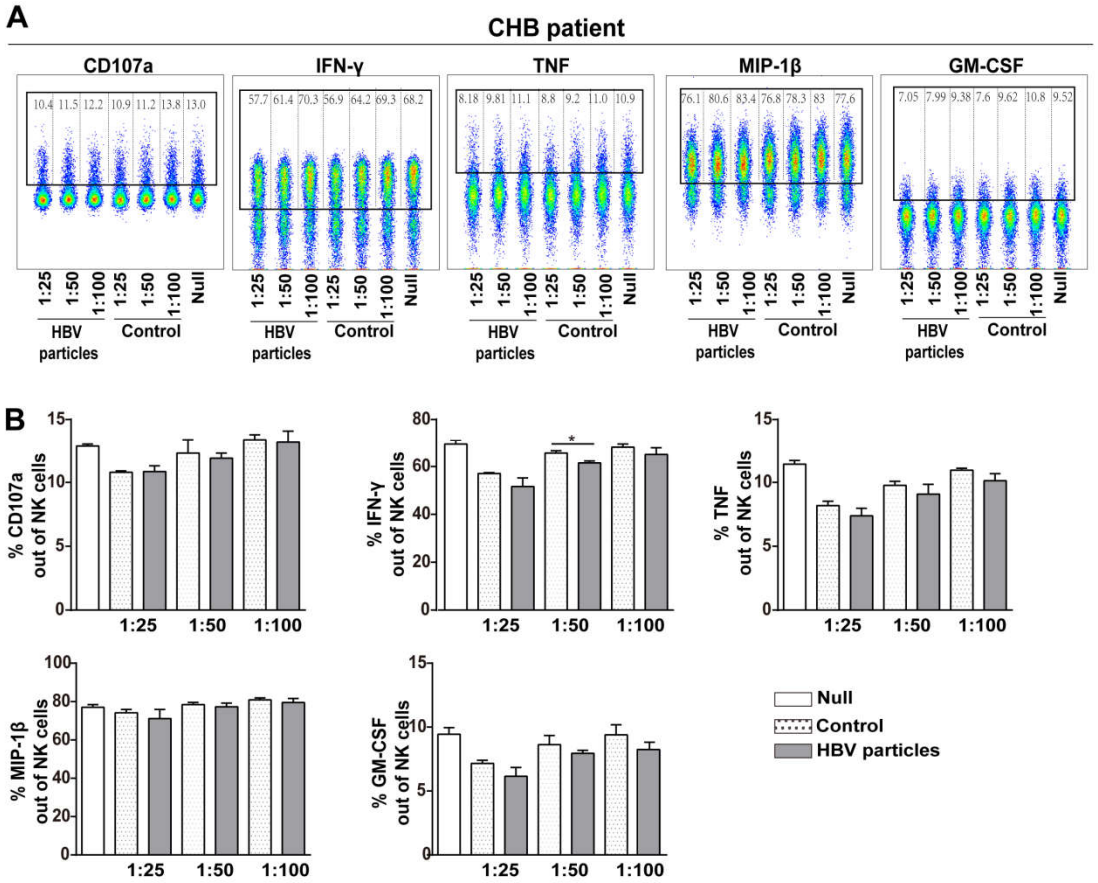

Figure 22. Functional responses of NK cells from chronic hepatitis B patients *in vitro*. (A) Concatenated flow cytometry plots showing the representative staining of CD107a, IFN-γ, TNF, MIP-1β and GM-CSF in total NK cells. Numbers indicate the percentage. (B) The percentage of cells expressing these five markers in total NK cells from one CHB patient was determined after the indicated stimulations. One representative experiment out of three independent experiments is presented. Data are displayed as the mean ± SEM. Triplicates were shown. Statistical analysis was performed via paired Student's t test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

5. Discussion

This comprehensive analysis of NK cell phenotypes and functions in CHB patients expressing varying amounts of HBsAg revealed distinct features of NK cells. Overall, our data suggested that the reshaping of NK cell pool occurs during CHB infections towards increased population of $CD56^{bright}$ NK cells in patients expressing high HBsAg levels. NK cells displayed less mature phenotypes based on the expression pattern of

Eomes^{-}T-bet^{+} identified in CHB patients. Importantly, patients with low HBsAg levels (<100 IU/ml) displayed NK cells with activated phenotypes, presenting the increased expression of the proliferation marker Ki-67 and the activation markers CD38 and Granzyme B whilst simultaneously exhibiting defective NK cell function compared to healthy controls and patients with high HBsAg levels. The levels of the activation markers CD69 and Granzyme B in CD56^{bright} NK cells were negatively correlated with HBsAg levels. No major differences were observed in either NK cell phenotypes or functions when comparing patients with high HBsAg levels to healthy controls, except for Tim-3 expression, which was slightly lower in patients with high HBsAg levels (>1,000 IU/ml) than in healthy controls. In addition, HBV particles, which are primarily composed of HBsAg, were able to suppress the function of NK cells derived from healthy donors but did not affect the phenotype *in vitro*. However, the suppression effect was much weaker in NK cells derived from patients who had experienced HBsAg exposure.

In our study, we observed the restructuring of the NK cell compartment with a relative increase of CD56^{bright} NK cell subset in patients with relatively high HBsAg levels. The increased CD56^{bright} NK cell subset may hint towards changes in the maturation status of NK cells among these patients because CD56^{bright} NK cells are considered as the predecessors of CD56^{dim} NK cells (Björkström et al., 2010). NK cell differentiation within CD56^{dim} NK cells is associated with the increased expression of KIRs and CD57, and the loss of NKG2A (Del et al., 2017), coupled with a decrease in Eomes expression and an increase in T-bet expression (Collins et al., 2017). Previous study indicated that the differentiation of the CD56^{dim} NK cell subset was not affected in HBV, HCV, and HDV infections, as assessed by the surface expression of KIR, CD57 and NKG2A (Lunemann et al., 2014). In line with this, our current results did not reveal any differences among these three markers in patients with CHB. However, the percentage of

Eomes^{low}T-bet^{high} CD56^{dim} NK cells significantly decreased in CHB patients, which is consistent with an increased CD56^{bright} compartment. Previously, the CD56^{bright} subset has been shown to positively correlate with patients' ALT levels, suggesting a possible role for this cell population in maintaining liver inflammation (Boni et al., 2015). This assumption was not studied here as most of patients in our cohort had normal ALT levels or were received anti-viral therapy.

An earlier study has reported that NK cells display inflammatory phenotypes with increased expression of CD38, Ki-67, and TRAIL in HBeAg-negative CHB patients and that NA treatment was associated with the progressive normalization in the expression of these three markers (Boni et al., 2015). Consistently, we also found that NK cells in patients with low HBsAg levels displayed a similar activated phenotype, with increased CD38, CD69, Granzyme B, and Ki-67 but not the increased expression level of the death receptor TRAIL. However, the levels of expression of these markers were similar when comparing treatment-naïve patients with NA-treated patients among the low HBsAg group. These differences between the past study and our study may due to different patient characteristics. Most of the CHB naïve patients in the cohort from the previous study presented with elevated ALT levels and high HBV DNA levels, whereas the treatment-naive patients in our study predominantly presented normal ALT levels and very low levels of HBV DNA. Another study has reported that HBsAg seroclearance after stopping the therapy was associated with an upregulation of CD38 expression on CD56^{dim} NK cells (Zimmer et al., 2018) and that very low serum HBsAg levels (<100 IU/ml) can be used to identify patients with a high probability of spontaneous HBsAg clearance (Cornberg et al., 2017). Importantly, in line with a previous study (Tjwa et al., 2014), we found that CD69 and Granzyme B of CD56^{bright} NK cells were negatively correlated with HBsAg levels. Overall seems likely that the activation of NK cells might be associated with spontaneous HBsAg clearance in patients with low HBsAg levels.

Furthermore, we noted that both $CD56^{dim}$ and $CD56^{bright}$ NK cells presented a functional impairment in patients with low HBsAg levels, which appears to contradict the observed activated phenotype. In fact, the phenotypic and functional characteristics of NK cells from CHB patients appear to vary from study to study. Previous studies have shown that chronic HBV infection may cause a broad functional impairment and overall inhibited phenotype of NK cells (Lunemann et al., 2014; Zimmer et al., 2018). However, Oliviero et al. demonstrated that NK cells in CHB patients displayed a functional dichotomy, with enhanced cytolytic activity and dysfunctional cytokine production (Lunemann et al., 2014; Zimmer et al., 2018). In our current study, the functional impairment primarily affected cytotoxicity and the production of the chemokines MIP-1β, while few differences were noted in the capacity of NK cells to produce cytokines. It has been reported that NK cell cytotoxicity is negatively correlated with HBsAg level (Lunemann et al., 2014; Zimmer et al., 2018) but positively correlated with ALT levels (Zheng et al., 2015), possibly indicating that NK cell cytotoxicity may accelerate liver damage. In line with these findings, in our study, among patients with low HBsAg levels, CD107a responses to K562 cell + IL-12 + IL-18 stimulation were negatively correlated with HBsAg levels. Consistently, both NK cell functional responses and the ALT levels in patients with extremely lower HBsAg levels (< 10 IU/ml, n = 13) were relatively higher than those of patients with HBsAg (10-100 IU/ml, n=7) (**Figure 23**). These findings may indicate that the differential NK cell functional responses may indeed reduce some liver inflammation which would be required to clear HBsAg.

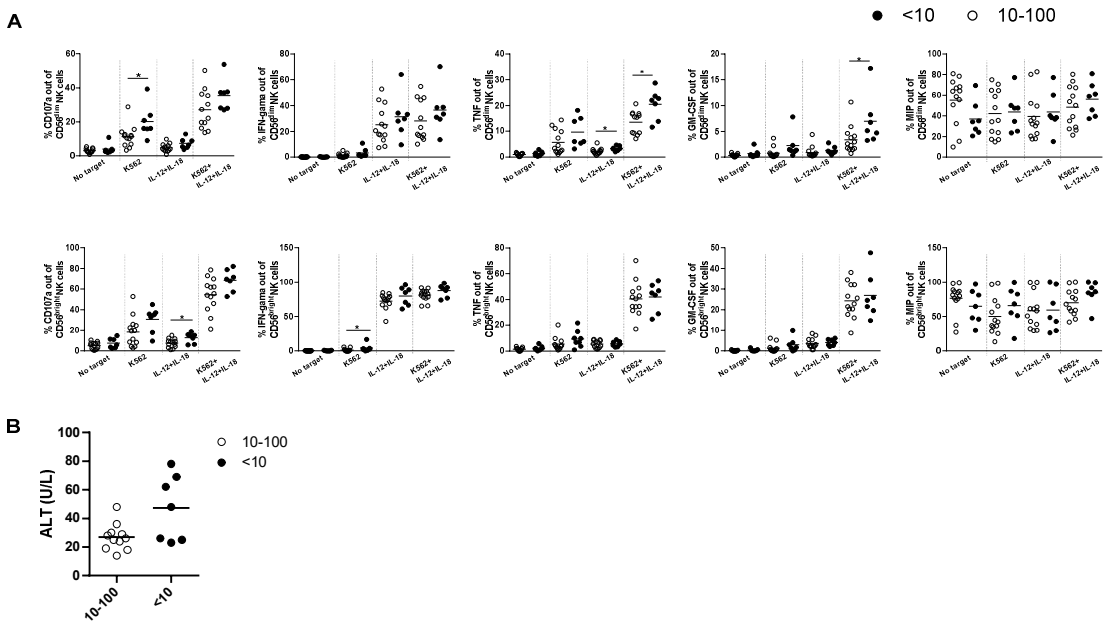

Figure 23. Relatively higher NK cell functional responses in patients with extremely lower HBsAg levels. (A) Frequency of CD107a, IFN-γ, TNF, MIP-1β, and GM-CSF out of $CD56^{dim}$ and $CD56^{bright}$ NK cells in patients with HBsAg <10 IU/ml and 10–100 IU/ml were summarized. (B) The ALT level was summarized. Horizontal bars represent mean. Counts of individuals in the graphs for chronic HBV patient with HBsAg <10 (n = 13) or 10–100 (n = 7). Statistical analysis was performed via Mann-Whitney test when comparing two subgroups; * $p<0.05$, ** $p<0.01$.

A recent study also reported that the duration of HBsAg exposure rather than quantity of HBsAg associates with the level of HBV-specific T cells response (Le Bert et al., 2020). Similarly, in our study, patient age which corresponds to the duration of infection, is negatively correlated with HBsAg levels, and CD107a and cytokines production of $CD56^{dim}$ NK cells against K562 and IL-12/18 stimulation was negatively correlated with patient age. Consistently, several functional markers of NK cells were significantly lower in patients older than 44 compared to the younger patients in the group of lowest HBsAg levels (**Figure 24E**). Therefore, the defective functional responses of NK cells might associate with the duration of HBsAg. However, age had minute effect on NK cell phenotype (**Figure 24D**), indicating that the duration of HBsAg mainly links with NK cell function.

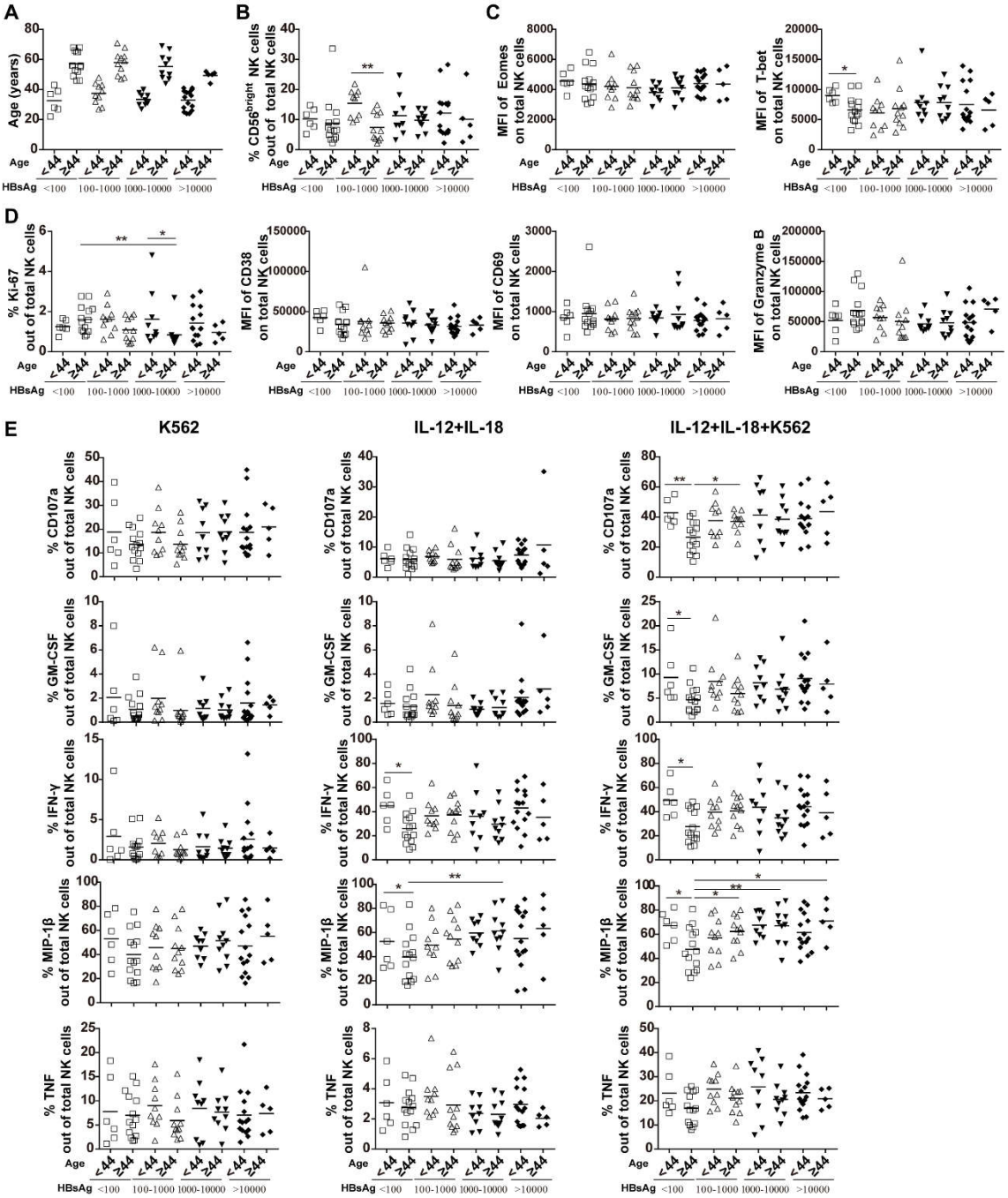

Figure 24. Few NK cell parameters were significantly different between younger patients and patients older than 44 in patient with similar HBsAg levels. (A) Summary of patient age in each patient group. (B) The frequency of CD56bright NK cells was compared between CHB patients younger than 44 and that of older than 44 in each patient group. (C) Mean fluorescence intensity (MFI) of transcription factors Eomes and T-bet on total NK cells was summarized in each patient group. (D) The expression of Ki-67, CD38, CD69, and Granzyme B on total NK cells was summarized in patients with similar HBsAg levels but different age group. (E) The percentage of functional markers on total NK cells

after different stimulation was summarized. The horizontal bars represent mean. Statistical analysis was performed via Mann-Whitney test when comparing two subgroups with similar HBsAg levels or Kruskal-Wallis test followed by Dunn's analysis with correction for multiple comparisons when comparing multiple groups with similar age but different HBsAg levels. * $p<0.05$, ** $p<0.01$.

NK cell cytotoxicity is dependent on the interaction between target cell ligands and a series of stimulatory receptors expressed by NK cells (Chester et al., 2015). In our study, the natural cytotoxicity receptor NKp46 receptor were significantly decreased on $CD56^{bright}$ NK cells in patients with low HBsAg levels. This might be associated with the defective functional responses observed in the $CD56^{bright}$ NK cells. Unexpectedly, the exhaustion marker Tim-3 decreased in patients with high HBsAg concentration, which contradicts the previously reported findings (Chester et al., 2015). Indeed, a recent study demonstrated that Tim-3 upregulation is a common end-result of NK cell activation by various stimuli and is not an independent marker of NK exhaustion (So et al., 2019). Therefore, the reduced expression of Tim3 on NK cells from patients with high HBsAg levels may be associated with the less activated NK cell phenotype observed in those patients. Another interesting finding in our study was the universal upregulation of CXCR6 on NK cells in CHB patients. Several studies have reported that CXCR6 expression serves as specific markers for liver-resident NK cells (Hydes et al., 2018). The upregulation of CXCR6 on circulating NK cells may be associated with NK cells homing to the liver where HBV is reproduced.

The most recent study examining the role of NK cells in CHB patients suggested no significant differences in the expression levels of 33 NK cell markers between patients with low (<1,000 IU/ml, n = 14) and high levels of HBsAg (>10,000 IU/ml, n = 13) including CD57, NKG2D, CD38, CD161, Granzyme B, HLA-DR, Tim3, and several functional markers (Le Bert et al., 2020). Similarly, in our study, we did not observe any differences in the expression of CD57, NKG2D and CD161 between NK cells from

patients with low and high levels of HBsAg. However, the expression of Granzyme B, Tim-3, and several functional markers differed in our study between patients with low HBsAg levels (<100 IU/ml) and those with high HBsAg levels (>10,000 IU/ml). The different results may be due to differences in group classification and the number of patients included in the analysis. In our study, we classified patients with HBsAg concentration below 1,000 IU/ml into two groups: <100 IU/ml and 100–1,000 IU/ml. Most of the difference was observed for patients with lowest HBsAg levels (<100 IU/ml) instead of for patients with HBsAg levels between 100–1000 IU/ml. In addition, our cohort included a larger sample, approximately 20 in each group, which allows the real state to be better determined.

Previously, both HBsAg and HBeAg were shown to suppress cytokine production in NK-92 cell lines (Yang et al., 2016). In line with this, we observed that HBV particles significantly impaired the functional responses of NK cells from healthy donors. However, this phenomenon was less obvious in NK cells from HBV patients, which may be due to the HBV-specific NK cell memory, as previously reported (Wijaya et al., 2020). NK cells that have previously experienced HBV infections develop a special memory; therefore, these memory NK cells exhibited higher cytotoxic and proliferation responses when encountering HBV particles again. This memory response may explain why NK cells from HBV patients did not exhibit decreased functional responses following HBV exposure compared with the controls. In addition, defective NK cells responses were only observed in patients with low HBsAg levels *in vivo*, whereas only high doses of HBV particles were able to suppress cytokine production in NK cells from healthy donors *in vitro*. These findings may be explained by one of the following: i) the concentration of HBsAg in HepG2.215 cell line-derived HBV particles may be too low after dilution in 1:100 ratio; ii) 24h of HBsAg exposure was too short to impact the NK cell responses. In addition, the HBV particles we used for this experiment were not purified HBsAg;

therefore, other components, such as HBV DNA, HBeAg, may also have affected the NK cells functional responses. Therefore, these inhibitory effects might need to be further confirmed by using of purified human HBsAg.

This study has several strengths and limitations as discussed below. Firstly, a large cohort of patients with varying amounts of surface antigen was studied. Secondly, patients were selected from two clinical centers, which are more representative than the single center. Thirdly, patients in our cohort were well distributed, with almost equal number of patients in each group. Lastly, most of our patients were HBeAg negative allowing for analysis of a homogeneous cohort. However, certain limitations of our study should also be considered. First, in our cohort, the number of previously treated vs naïve patients was not balanced for each group. Future work with larger studies should accommodate for this. Second, it is uncertain that the NK cells responses observed towards the golden standard NK cell target cell line K562 or upon IL-12/18 stimulation are translatable to the physiological setting. Third, our study mainly focused on the circulating NK cells and did not include liver samples. Certainly, a paired analysis of NK cells phenotype and function in blood and liver would be ideal to confirm our findings. However, it is challenging because patients might be unwilling to undergo liver biopsy for research purposes solely and the quantity of intrahepatic lymphocytes for our analysis also impose severe practical limitations. Lastly, future studies should also address NK-T cell crosstalk in relation to HBsAg levels.

In conclusion, this comprehensive study investigating potential relationship between different surface antigen levels and NK cell functions and phenotype suggests that NK cells may indeed play a role in controlling the HBV infection. Our findings may provide important insights for future immunotherapy aiming at HBV functional cure.

6. Summary

Suppressed natural killer (NK) cell functionality potentially contributes to hepatitis B virus (HBV) persistence during chronic hepatitis B, although the underlying mechanism is not entirely clear. A peculiar feature of HBV is the secretion of large amounts of hepatitis B surface antigen (HBsAg) by hepatocytes. However, the effects of varying HBsAg quantities on the phenotype and function of systemic NK cells remains to be determined.

In the present study, we selected 80 patients expressing varying amounts of HBsAg and categorized them into four groups according to HBsAg quantity. We measured and compared the NK cell phenotypes and functions among these groups and in healthy controls. Overall, we found the following: (i) the NK cell pool was reshaped during CHB infections with increasing $CD56^{bright}$ NK cells observed in patients with high HBsAg levels; (ii) NK cells were less mature in CHB patients as assessed by the presence of $Eomes^{-}T\text{-}bet^{+}$; (iii) NK cells in patients with low HBsAg levels (<100 IU/ml) displayed the activated phenotype with the increased proliferation of Ki-67 and the activation markers CD38 and Granzyme B whilst exhibiting defective functional responses; (iv) $CD56^{bright}$ NK cell activation markers were negatively correlated with surface antigen levels; and (v) HBsAg suppressed the NK cell functional responses but not the NK cell phenotype *in vitro*.

In conclusion, this comprehensive study investigating potential relationship between different surface antigen levels and NK cell functions and phenotype suggests that NK cells may indeed play a role in controlling the systemic HBV infection.

Zusammenfassung

Die unterdrückte Funktionalität natürlicher Killerzellen (NK) trägt möglicherweise zur Persistenz des Hepatitis B-Virus (HBV) während einer chronischen Hepatitis B bei, obwohl der zugrunde liegende Mechanismus nicht ganz klar ist. Ein besonderes Merkmal von HBV ist die Sekretion großer Mengen verschiedener Formen des Hepatitis B-Oberflächenantigens (HBsAg) durch Hepatozyten. Jedoch müssen die Auswirkungen variierender HBsAg-Mengen auf den Phänotypen und die Funktion systemischer NK-Zellen noch untersucht werden.

In der vorliegenden Studie wurden 80 Patienten, die unterschiedliche Mengen an HBsAg exprimieren, ausgewählt und entsprechend der HBsAg-Menge in vier Gruppen eingeteilt. Es wurden die Phänotypen und Funktionen der NK-Zellen zwischen diesen Gruppen sowiebei gesunden Kontrollen gemessen und miteinander verglichen. Folgendes wurde beobachtet: (i) Der NK-Zellpool wurde während einer CHB-Infektionen umgeformt, wobei bei Patienten mit hohen HBsAg-Spiegeln vermehrt $CD56^{bright}$-NK-Zellenbeobachtet wurden, (ii) NK-Zellen waren bei CHB-Patienten weniger reif, was durch das Vorhandensein von Eomes-T-bet + beurteilt wurde; (iii) NK-Zellen bei Patienten mit niedrigen HBsAg-Spiegeln (<100 IU/ml) zeigten den aktivierten Phänotypen mit der erhöhten Proliferation von Ki-67 und den Aktivierungsmarkern CD38 und Granzyme B, während sie fehlerhafte funktionelle Reaktionen aufweisen; (iv) $CD56^{bright}$ NK-Zellaktivierungsmarker korrelierten negativ mit den Oberflächenantigenspiegeln; und (v) HBsAg unterdrückte die funktionellen Reaktionen der NK-Zellen, jedoch nicht den Phänotyp der NK-Zellen *in vitro*.

Zusammenfassend lässt sich sagen, dass diese umfassende Studie, in der die potentielleBeziehung zwischen verschiedenen Oberflächenantigenspiegeln und den Funktionen und dem Phänotyp der NK-Zellen untersucht, darauf schließen, dass NK-Zellen tatsächlich eine Rolle bei der Kontrolle chronischer HBV-Infektionen spielen könnten.

7. Reference

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8. Attachment

8.1. Individual characteristics of CHB patients

ID	Gender	HBV genotype	ALT (IU/mL)	AST (IU/mL)	HBsAg (IU/mL)	HBeAg	HBV-DNA (IU/mL)	Treatment (NA)
1	F	C	48	40	1.37	neg	25	ETV
2	M	n.a	69	31	1. 51	neg	<10	IFN(past)
3	M	D	78	39	1. 45	neg	<10	TDF
4	M	n.a	25	21	10.8	neg	<10	ETV
5	M	D	24	18	70.9	neg	126	IFN(past)
6	M	n.a.	19	21	23	neg	<20	naive
7	F	n.a.	48	22	42	neg	<10	TDF
8	M	E	n.a	n.a	65	n.a	1200	naive
9	F	n.a.	26	31	66.26	n.a	50	naive
10	F	n.a.	n.a	n.a	78	neg	310	naive
11	F	D	30	38	13.8	neg	44	naive
12	F	D	14	13	16.3	neg	335	naive
13	M	A	18	26	49.5	neg	175	naive
14	M	n.a	62	137	0.4	neg	22	naive

15	F	D	25	23	1.17	neg	<10	ETV
16	M	n.a	28	35	32.5	neg	<10	ETV
17	M	n.a	29	24	28.1	neg	<10	TDF
18	M	D	23	37	1.77	neg	83	naive
19	F	n.a	26	21	3.73	neg	<10	TDF
20	M	n.a	36	42	76.6	neg	<10	TDF
21	M	D	24	27	884	neg	10	ETV
22	F	D	25	20	844	neg	17090	naive
23	F	n.a	17	19	998	neg	<10	TDF
24	M	A	34	13	122	neg	44	naive
25	M	D	37	23	581	neg	5740	naive
26	M	D	37	26	216	neg	<10	naive
27	F	n.a	62	51	102	neg	<20	Yes (NA)
28	M	B	n.a	n.a	159	n.a	83	n.a
29	F	n.a.	22	19	343	neg	740	naive
30	M	n.a	n.a	n.a	471	neg	47	n.a
31	F	D	18	23	567	n.a	6500	naive
32	F	D/F	n.a	n.a	652	n.a	<20	ETV
33	M	n.a.	n.a	n.a	742	neg	<20	LAM
34	M	A	31	42	178	neg	14	naive
35	F	D	14	11	952	neg	5656	naive
36	M	n.a	19	22	156	neg	291	naive
37	F	n.a	31	41	550	neg	<10	ETV
38	M	n.a	27	19	103	neg	<10	TDF
39	F	A	22	24	104	neg	223	IFN(past)
40	F	A	23	23	836	neg	<10	TDF
41	M	D	30	26	724	neg	732	naive

42	M	A	26	35	2570	neg	<10	ETV
43	M	D	36	21	1695	neg	<10	TDF
44	M	n.a	156	68	2061	neg	331800	naive
45	M	n.a	31	49	5479	n.a	34	ETV
46	M	D	28	15	1597	neg	<10	ETV
47	M	n.a	41	22	8768	neg	12	TDF
48	M	D	52	32	1663	neg	179	TDF
49	M	D	34	24	3736	neg	<10	TDF
50	F	n.a	16	13	2078	neg	204	naive
51	M	C	28	18	1706	neg	2411	n.a
52	M	n.a.	21	17	1257	neg	260	n.a
53	M	D	n.a	n.a	1417	neg	10000	naive
54	M	n.a.	26	27	1739	neg	400	naive
55	F	n.a.	28	18	1834	neg	3000	naive
56	F	n.a	34	27	1939	neg	270	naive
57	F	D	49	70	3888	neg	67	TDF
58	F	n.a	n.a	n.a	4056	neg	140	naive
59	M	F	28	28	7299	neg	neg	ETV
60	M	D	14	15	4082	neg	<10	TDF
61	M	D	33	21	53311	neg	<10	TDF
62	M	A	33	23	12444	neg	<10	ETV
63	M	D	43	78	14810	neg	51	ETV
64	F	A	28	16	27774	neg	<10	TDF
65	M	A	45	29	34937	neg	20750	n.a
66	W	n.a	21	28	24806	n.a	1562	n.a
67	M	A	54	31	31001	neg	<10	TDF
68	M	D	24	15	25441	neg	954	n.a

69	M	D	154	60	25954	neg	19	ETV
70	M	D	33	20	10098	neg	<10	TDF
71	F	n.a	n.a	n.a	10134	neg	92	naive
72	M	n.a.	31	27	16863	pos	<10	yes
73	M	n.a	46	84	15262	neg	202800	naive
74	M	n.a	39	80	22721	neg	150100	IFN (past)
75	F	B	21	21	19191	pos	12	ETV+TDF
76	M	D	45	45	39288	neg	550	TDF
77	F	D	44	85	124827	pos	121800	TDF
78	M	D	41	16	13837	neg	414000	naive
79	M	D	124	155	14432	pos	670	TDF
80	M	A	21	20	12583	neg	<10	TDF

n.a: not available. Abbreviations: LAM, lamivudine; ETV, entecavir; TDF, tenofovir.

8.2. List of abbreviations

AIM Absent in melanoma

ALT Alanine aminotransferase

APC Antigen-presenting cell

AST Aspartate aminotransferase

CD Cluster of differentiation

CHB Chronic hepatitis B

cccDNA Covalently closed circular DNA

cDC Conventional dendritic cell

CTLA-4 Cytotoxic T-lymphocyte antigen 4-immunoglobulin

DC Dendritic cell

EDTA Ethylene diamine tetraacetic acid

ERK Extracellular signal-related kinase

FACS	Fluorescence activated cell sorting
Fas L	Fas Ligand
FCS	Fetal calf serum
FMO	Fluorescence minus one
GM-CSF	Granulocyte-,acrophage colony-stimulating factor
HBcAg	Hepatitis core antigen
HBeAg	Hepatitis e antigen
HBsAg	Hepatitis surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDV	Hepatitis delta virus
HIV	Human immunodeficiency virus
HLA	Human leukocytes antigen
IFN	Interferon
IL	Interleukin
IRF7	interferon regulatory transcription factor 7
ISG	Interferon stimulating gene
KC	Kupffer cell
KIR	Killer-cell immunoglobulin-type receptor
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LAG-3	Lymphocytes activation gene-3
mDC	Myeloid dendritic cell
MDCS	Myeloid-derived suppressor cell
MFI	Mean fluorescence intensity
MIP	Macrophage inflammatory protein
NA	Nucles(t)ide analogues

NF-κB	Nuclear factor kappa B subunit
NK	Natural killer
NOD	Nucleotide-binding oligomerization domain
NTCP	Sodium taurocholate cotransporting polypeptide
ORF	Open reading frame
PBS	Phosphate buffer solution
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid pre-dendritic cell
PGE2	Prostaglandin E2
PLZF	Promyelocytic leukemia zinc finger
pgDNA	Pregenomic RNA
rcDNA	Relaxed circular DNA
RIG	Retinoic acid-inducible gene
siRNA	Small interfere RNA
SVP	Subviral particle
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
Tim-3	T cell immunoglobumin and mucin domain containing molecule-3
TIGIT	T cell immunoreceptor with Ig and ITIM domins
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand

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8.5. Statement of permission

Some patient samples in our cohort were collected from the Department of Gastroenterology, Hepatology and Endocrinology in Hannover Medical School. All the experiments were performed in our lab at University Hospital Essen. Clinical data from AG Wedemeyer at Department of Gastroenterology, Hepatology and Endocrinology in Hannover Medical School are presented here with their permission.

8.6. Publications during MD period

- 1. **Du, Y**, Anastasiou, E.O, Strunz, B., Scheuten, J., Bremer, B., Kraft A., Kleinsimglinhaus K., Todt, D., Broering, R., Hardtke-Wolenski, M., Wu, J., Yang, D., Dittmer, U., Lu, M., Cornberg, M., Björkström, K.N., Khera, T., and Wedemeyer, H. **The impact of hepatitis B surface antigen on natural killer cells patients with chronic hepatitis B patients**. **Liver Int.** 2021 Apr 1. doi:

10.1111/liv.14885.

- 2. **Du Y**, Yan H, Zou S, et al. Natural Killer Cells Regulate the Maturation of Liver Sinusoidal Endothelial Cells Thereby Promoting Intrahepatic T-Cell Responses in a Mouse Model. **Hepatology Communications** 2021 Feb 5. doi: 10.1002/hep4.1676.
- 3. **Du Y**, Yang X, Li J, et al. Delivery of toll-like receptor 3 ligand poly(I:C) to the liver by calcium phosphate nanoparticles conjugated with an F4/80 antibody exerts an anti-hepatitis B virus effect in a mouse model. **Acta Biomater.** 2021 Feb 1;S1742-7061(21)00073-8.
- 4. Khera T, **Du Y**, Todt D, et al. Long-lasting Imprint in the Soluble Inflammatory Milieu despite Early Treatment of Acute Symptomatic Hepatitis C. **J Infect Dis** 2021 Jan 31; jiab048.
- 5. Zou, S.,*, **Du, Y.,***, Huang, S., *et al. Activation of intrahepatic type I interferon signaling at an early stage leads to hepatitis B virus persistence in a mouse model*. (*Equally contributing authors, submit to *Journal of Virology*)

In preparation

- Khera, T., Madsen, LW., **Du, Y.,** Wedemeyer, H. and Christensen, PB. *Systemic inflammatory mediators identify ultra-short term HCV treatment.*

8.7. Participation at Scientific Meetings

- ✧ NK cells regulate liver sinusoidal endothelial cells to promote HBV-specific T cell responses- *29th Annual Meeting of the Society for Virology, 2019- Düsseldorf, Germany,* (**Oral Presentation**).
- ✧ NK cells regulate liver sinusoidal endothelial cells to promote HBV-specific T cell responses- *German Association for the Study of the Liver (GALS) Annual Meeting 2020- Mainz, Germany,* (**Oral Presentation**).

9. Acknowledgement

Firstly, I would like to express my sincere gratitude to **Prof. Heiner Wedemeyer,** my supervisor, for his constant encouragement and excellent academic guidance. He has given me great instructions throughout the process of selecting the research topic, improving the outline and argumentation, and writing the thesis. His profound insight and accurateness about my paper taught me so much that they are engraved on my heart.

I also would like to express my deep appreciation **to Dr. Tanvi Khera** for being the closest mentor and friend in the lab. I thank her for her insightful comments on every draft of the thesis which have inspired me to a great extent. As a friend, she also gives me a lot help in life, which makes me feel less homesick.

I would like to thank **Dr Anke Kraft**, **Karolina Kleinsimlinghaus, Dr Olympia evdoxia Anastasiou and Birgit Bremer** who have helped us collecting and transferring the patient samples, and collecting the clinical data.

I am also grateful to **Prof. Niklas Björkström and Dr Benedikt Strunz** at the Department of Medicine Huddinge in Karolinska University Hospital for their insightful suggestions for the paper.

I would also like to thank **Dr Ruth Bröring, Dr Matthias Hradtke-Wolenski and Dr Martin Trippler** for their academic suggestions. I would like to thank **Sabrina and Xufeng** for their excellent technical assistance. Sincere thanks to **Natalie and Alex** for their help to translate the summary into German.

My sincere thanks go to **Prof. Ulf Dittmer and Prof. Mengji Lu** at the Institute for Virology in University Hospital Essen for their long-term support and insightful

academic suggestions. I also thank for the China office which provides me the scholarship to cover my stay here. Sincere thanks to **Ursula Schrammel** for her thoughtful help in my life here.

Thanks to all the U2 residents **Xueyu, Xiao, Xue, Baoxiao, Xin, Chen** for their friendship and support at all times and making life fun while working.

Finally, I am very grateful to my families and my boyfriend **Hu** for their understanding and selfless support.

10. Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten