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Expression of CD38, CD226, CD39, PD1 on T cells in chronic hepatitis B and their correlation with serum vitamin D levels.

Dissertation

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1. Introduction

1.1. Epidemiology of HBV infection

The earliest studies on viral hepatitis started in 1966, Baruch Blumberg[1], who received the Nobel Prize for his work on HBV, discovered the Australia antigen in the serum of an Australian, which was later identified as hepatitis B surface antigen (HBsAg). From the invention of HBV vaccine in the early 1980s[2] until now more than 100 coutries were benefited from the vaccination programs. With the development of antiviral treatment, the HBV carriers, as well as HBV caused hepatocellular carcinoma (HCC) dropped dramaticly worldwide, especially in Taiwan and Japan[3]. However, HBV infection is still a global public health concern. About one third of the world population has serologic evidence of past or present HBV infection, more than 360 million individuals worldwide are chronic infection[4,5]. Prevalence of HBV ranges from over 10% in Asia and Africa, where HBV exposure tends to occur during the perinatal period, to under 0.5% in the United States and western Europe, where HBV exposure mainly occurs in adults via sexual transmission[6]. Progression to cirrhosis and/or HCC, which is the sixth most frequently diagnosed cancer globally and the third leading cause of cancer death[7], is the greatest threat to human health caused by chronic hepatitis B. A report from World Health Organization (WHO) shows that, HBV related death is approximately 600,000 people each year worldwide[8]. HBV is currently divided into 10 genotypes (A-J) based on the variation of total nucleotide sequence of the genome[9].

1.2. Virological characteristics of HBV

HBV is a non-cytopathic member of Hepadnaviridae family, and partially double-stranded deoxyribonucleic acid (DNA) virus[10]. Its virion is about 42 nm in diameter, consists of an out lipid envelope, which contains embedded proteins HBsAg, and an icosahedral nucleocapsid core, which encloses HBV DNA and DNA polymerase[11]. The viral genome consists of a full coding strand, which is 3020-3320 nucleotides long, and a incomplete non-coding strand, which is 1700-2800 nucleotides long[10]. The genome encodes four overlapping open reading frames (ORFs), known as S, C, P and X. Gene S, which is divided into three different size sections, pre-S1, pre-S2 and S, codes the viral

envelope proteins HBsAg; gene C codes hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg); P gene, which is the largest and overlaps the other three, codes the viral DNA polymerase from the 3,5 kb ribonucleic acid (RNA); as the shortest ORF, the function of X gene and its coding product hepatitis B X protein (HBx) from 0.7 kb RNA is not fully known, however, X gene has now been demonstrated to be a transcriptional transactivator[12]. **Figure 1.**

The unique life cycle of HBV ensures a massive viral replication, while not directly kill the infected hepatocytes. As its high degree of species and tissue specificity, cellular entry of the HBV virion, which is poorly defined owing to lack of a proper in vitro culture system, is now presumably mediated by pre-S1 domain of HBV envelope binding to sodium taurocholate cotransporting polypeptide (NTCP), which is a multiple transmembrane transporter mainly expressed in the liver[13]. After being endocytosed, uncoated capsid was transported to the nucleus, where cellular repair enzymes are involved in completing the second strand of the open circular genome into covalently closed circular DNA (cccDNA), which is the transcriptional template of the virus[14,15], and cccDNA was then reverse transcribed to different funtional messenger ribonucleic acids (mRNAs)[16] in the cytoplasm. The precore mRNA is translated and further processed in endoplasmic reticulum (ER) as secreted HBeAg. The pregenomic mRNA is encapsulated by the core and polymerase proteins to form HBV RNA-containing capsids, and reversely transcribed by the viral polymerase to produce the first single-strand DNA (ssDNA, negative strand), which serves as the template for second-strand DNA (positive strand) synthesis[17]. Finally, the DNA-containing capsid migrates either to the ER menbrane encouting with the envelope proteins to produce virions that are transported out of the cell, or recycled back to the nucleus to establish a pool of cccDNA[12] to produce even more copies.

1.3. HBV Genotypes

The genetic diversity of HBV is influenced by the presence of selective pressure, such as host immune system and antiviral treatment, together with use of reverse transcription to copy its genome, mutant viral genomes emerge frequently[18,19]. The different genotypes have not only distinct geographical

distribution, but also affect the clinical course of liver disease, complications, response to antiviral therapy and possibly vaccination[20,21].

HBV is traditionally divided into four major serotypes, adr, adw, ayr and ayw, according to the variation in the antigenic epitopes presented on the surface proteins of the virions and subviral particles[22,23]. With the development of more molecular approaches, a genetic classification of HBV has first identified as genotypes A to D, in 1988 by Okamoto et al., based on an inter-group divergence in nucleotide sequence of 8% or more[24]. It was reported that genotypes B and C are predominant in Asia and Oceania, whereas genotypes A and D are frequent in Western Europe and India[25]. Studies from Taiwan[26], Japan[27,28] and India[29] have confirmed that HBV genotype C and D are associatied with more severe course of disease, more prevalent of cirrhosis and occurrence of HCC, compared to genotype A and B. However, genotype A and B responed better to an interferon therapy[30,31].

By sequencing the variations of the S-gene of HBV within the major four subtypes, four new genotypes of HBV designated with E, F[32], G[33], and H[34] were identified. Genotype E is mainly restricted in Africa, and genotypes F and H in south America, whereas G in the USA[35]. The inter-group divergence in nucleotide sequence in the F genotype was identified as 14% compare to other HBV genomes sequence. Thus the F genotype is considered as the most divergent HBV genome so far characterized[23].

As a result of mutations and recombinations, HBV has evolved two putative genotypes (I and J). Genotype I, which was recently found in southeastern Asia, is a genetic recombinant of genotypes A, G, and C[36,37,38]. Genotype J, which was isolated from a Japanese patient with hepatocellular carcinoma, is a genetic variant of HBV divergent from known human and ape genotypes, however, it shows no recombinant genomic sequence with any of the nine human and/or four ape genotypes[39].

1.4. Pathogenesis and transmission of HBV

As a noncytopathic hepatotropic virus, HBV is spread by contact with infected blood and body fluids. However, HBV infection is a leading cause of acute and chronic diseases of the liver, which are predominantly immune-mediated, in

order to clear virus[40]. Long-term chronic inflammation of hepatocytes leads to the collaps of liver structure, formation of pseudolobules, further development of liver fibrosis and cirrhosis, and therefore loss of liver function. The dysplastic liver cell masses eventually develop hepatocellular carcinoma.

Acute hepatitis B is usually mild or asymptomatic in younger ages, although later severe liver injury and jaundice can occur in adults or even fulminant hepatitis may develop. However, Chronic hepatitis B is a progressive disease with fatal complications. 2.1% of patients with chronic hepatitis B will progress to cirrhosis each year. The annual incidence of HBV-related HCC in patients with cirrhosis is ranging from 2% to 5%[41]. Asymptomatic carriers or patients with chronic hepatitis B may also develop HCC, even without cirrhosis, with an annual incidence of 0.1% and 1% respectively.

As other infections, an infection with HBV activates first the innate immune responses to defend the host. Immature antigen-capturing dendritic cells (DCs) from the innate immune system mature and migrate to lymphoid organs, where they present viral peptides on human leukocyte antigen (HLA) class I and class II molecules to CD8 and CD4 T cells, polarising the CD4 T cell response in Type 1 helper T cells (Th1) or Type 2 helper T cells (Th2) direction. Th2 cells secrete, for example, interleukin (IL) 4, 5, and 6 supporting the B cell response. Th1 cells secrete, for example, interferon gamma (IFNγ) and IL-2, which support macrophages and cytotoxic T lymphocytes (CTLs) to kill intracellular pathogens. CTLs either induce apoptosis of the infected cell directly through perforin- and/or Fas L- mediated pathway or “cure” the hepatocytes from virus by antiviral cytokines[40]. It is clear that the responses of CTLs play a central role in viral clearance.

A strong adaptive immune response to HBV antigens may eliminate the HBV-infected hepatocytes, therefor, results in acute hepatitis. The symptoms may be mild or asymptomatic, if the immune response starts before a large number of hepatocytes are infected[42]. In chronic HBV infection, the multispecifc T cell response, especially the virus-specific CTL response against epitopes within HBV core, polymerase, and envelope proteins are greatly attenuated, may be the pivotal determinant influence of the course and the onset of liver disease in HBV infection[43,44].

Infectious blood or body fluids containing virions are the most important medium of HBV transmission. For children, the possible forms of transmission usually include vertical transmission from infected mother to child during the time of birth, or acquiring from contact with infected family members[45]. Without intervention, there is 20% risk of vertical transmission from a HBsAg positive mother to her offspring If the mother is also HBeAg positive the risk is as high as 90%. Among adults and adolescents the possible forms of transmission include sexual contact[46,47] and injecting drug with re-using of contaminated needles and syringes[48], blood or other human blood products transfusions[49]. However, there still remains unidentified risk factors and transmission froms of HBV.

1.5. Persistence of HBV

HBV-associated liver diseases vary greatly from person to person[50]. The clinical outcomes of HBV infection range from virus clearance without evident liver desease, to acute inflammation of the liver but resolved without lang-term clinic sequelae and to chronic hepatitis. Both viral factors and the host immune responses are responsible for the pathogenesis and clinical outcomes of HBV infection[43]. Neonates and infants who acquire HBV infection perinatally carry the highest risk of HBV persistence due to the inability of their immature immune system[51]. Approximately 90% or more of neonates exposed to HBV at birth will develop chronic hepatitis, whereas the virus persists in 5% to 10% immunocompetent adults infected with HBV[52]. Numerous studies show that the outcome of HBV infection depends on the interactions between the virus and the host, which mainly include the innate and adaptive immune response attempting to eradicate infection, but also inducing liver damage.

1.5.1. Virus factors in HBV persistence

Generally in the early phase of virus infection, the infected cells produce antiviral cytokines such as type I IFN[53], which inhibits the replication of many DNA and RNA viruses, and trigger the innate immune system. In contrast with most viruses, HBV does not induce any genes in the liver during entry and expansion[54]. This lack of early innate defense reveals it as a stealth virus that can successfully escape the innate immune response.

Although in most cases the adaptive immune response, especially T cell mediated cellular immune response can inhibit viral replication and kill infected cells. The unique HBV genome organisation plays an important role in undermining the adaptive immune response. As the prototype serological marker of HBV infection, HBsAg present in 10^3-10^6-fold excess over whole virions[16]. This large amounts of HBsAg may cause a T cell hyporesponsiveness, tolerance and anergy[55]. The soluble, secreted HBeAg, which is produced in large excess, but not involved in viral replication, may play an important role in viral persistence in neonates infection. Meanwhile HBeAg may establish core-specific T helper cell tolerance to both HBeAg and HBcAg in $HBeAg^+$ adults[56], leading to the failure of resolution and seroconversion of HBV antigens in CHB[57]. HBcAg could also induce immune tolerance toward HBV by stimulating IL-10 production, which is a potent immunosuppressive cytokine[58]. It was proposed that HBV escapes the initial defence by sharing the common replicase system in nucleus with target cells. HBx protein, which is essential to HBV replication, integrates viral DNA into the host's genome and inhibits antigen processing and presentation, leading to viral persistence[59,60].

1.5.2. Host factors in HBV persistence

Defect of HBV target cells and immune tolerance to HBV are the major host factors leading to HBV persistence. Several animal models have shown that virus-specific CTLs in the peripheral blood are detectable as early as 2-3 weeks after HBV infection. Meanwhile these adaptive immune cells from the liver can be detected 2-3 months later after exposure with HBV or hepatitis C virus (HCV)[61,62]. This delayed immune response in infected liver may be attributed to the few expression of major histocompatibility complex (MHC) in hepatocytes[63].

T cell tolerance to HBeAg may be a crucial mechanism responsible for the hyporesponsiveness of an antiviral immune system[64,65]. Viral clearence mainly depends on CD8 T lymphocyte response with the help of CD4 T lymphocytes[66,67].The weak and nonspecific response of CD8/CD4 T lymphocytes in chronic HBV infection results in viral persistence and disease progression[40,68,69]. This T cell tolerance is to a certain extent regulated by the

virus factors. However, extrinsic and intrinsic regulatory mechanisms of the immune system control the virus specifc immune responses in order to regulate the degree of activation as well as to prevent massive tissue damage or autoimmune disease. Regulatory T cells (Tregs)[70,71], the imbalance of costimulatory and coinhibitory receptors on T cells[72] play an important role in anergy and exhaustion of an initially vigorous T cell response.

1.5.2.1. Tregs in chronic HBV inffection

Tregs are described as a subpopulation of T cells that suppress the activation, proliferation, differentiation, and effector functions of many cell types, and express the IL-2 receptor-α-chain (CD25) constitutively[73–75]. Their role in immune regulation is to maintain self-tolerance and immune homeostasis, as well as to regulate immune response to pathogens, establishing viral persistence, but also limiting immune mediated liver damage[76]. Tregs are divided into natural Treg cell population that develop in the thymus and diverse populations of induced or adaptive Tregs that develop from conventional T cells in the periphery[77]. Tregs suppress activation and proliferation of T cells either through contact-dependent mechanisms, which may involve expression of glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR) family related protein, cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and programmed cell death protein 1 (PD1)[78,79] or through the secretion of anti-inflammatory cytokines, such as transforming growth factor beta 1 (TGFβ1) by Th3 cells or IL 10/TGFβ1 by T regulatory type 1 (Tr1) cells[74,80,81]. However, these mechanisms are incompletely known. In chronic viral infection, $CD4^+$ $CD25^-$ $FOXP3^-$ naïve T cells continuous exposure to low dose antigen but high concentration of TGFβ, leading its conversion into suppressive regulatory T cells[82,83], induced $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells can expand as well[84]. Studies have shown higher frequencies of circulating $CD4^+$ $CD25^+$ /$CTLA4^+$ $FOXP3^+$ T cells in CHB and positive correlation with HBV DNA loads[85,86]. The function and frequence of HBV-specifc effector T cells are supressed by increased $CD4^+$ $CD25^+$ Tregs in chronic HBV infection and reversed by depletion of $CD4^+$ $CD25^+$ Tregs[85,87]. $CD39^+$ Tregs may suppress the HBV-specific CD8 CTL effect or function through the CD39/adenosine pathway, thereby maintaining immune tolerance to the invasive pathogens[88]. However,

Franzese et al.[89] found no differences in quantity or suppressive function of $CD4^+$ $CD25^+$ Tregs in peripheral blood of CHB patients and that of persons spontaneously recovered from HBV infection.

1.5.2.2. HBV and co-signalling receptors

Besides Tregs, a bunch of molecules present at the T-cell surface also regulate the degree of activation of T cell response by setting thresholds for T-cell receptor (TCR) signalling[90,91]. These molecules are known as costimulators and coinhibitors. Costimulatory molecules deliver positive signals to T cells after binding to their ligands and counter-receptors on antigen-presenting cells (APCs), inducing T cells proliferation, differentiation and cytokine production, enhancing cytotoxic function. Contrary, co‑inhibitory molecules deliver negative signals to T cells, inducing T cells tolerance, exhaustion and apoptosis[72].

CD38 is a suface receptor, which is expressed on more than 80% of medullary thymocytes and on most resting T cells in the tissues, but rarely on circulating T cells[92]. The main function of CD38 on T lymphocytes is enhancing differentiation and proliferation, activation and cytokine production[93,94]. Given its role in the immune system, CD38 is associated with many pathogenesis of diseases, such as leukemia[95,96], cancer[97], diabetes[98] and AIDS[99,100]. Studies on human immunodeficiency virus (HIV) infection have shown that expression of CD38 and HLA-DR on CD8 T cells could be a marker for ongoing viral replication[101,102].

CD226, also known as DNAX accessory molecule-1 (DNAM-1), was identified as an adhesion molecule expressed on the majority of T cells, Natural Killer (NK) cells, monocytes and a subset of B cells[103]. CD226 is involved in NK and CTL-mediated cytotoxicity through its ligands CD155 and CD112[104]. Expression of CD226 on CD4 T lymphocytes may strongly promote Th1 and Th17 differentiation, but not Th2, enhancing IFNγ production by naïve T cells[105]. Therefore, CD226 is a possible therapeutic target in many diseases[106]. A deficiency of CD226 on CD8 T cells delayed viral clearance in vivo[107], and leaded to CD8 T cell exhausion in chronic HIV infection[108]. Similarly, expression of CD226 on activating NK cells correlated to the outcome of

treatment in HCV infection[109]. However, the effect of CD226 molecules on T cells is not well demonstrated in chronic hepatitis, especially in HBV infection.

CD39 molecule was identified on activated B cells incubated with Epstein–Barr virus (EBV), and has been demonstrated on activated T lymphocytes, NK cells, but not on resting immune cells[110]. CD8 T cells, which express CD39 molecule, showed stronger specific killer activity than CD39 negative CD8 T cells[111]. Contrarily, CD39 was recently identified as a biomarker of the suppressive functional regulatory T cells by studies on cancer, infecious diseases and autoimmune diseases[112–114].

Nowadays growing evidences indicate that CTLA4 and PD1, as immune inhibitory receptors within the CD28 superfamily, negative regulate T cell responses through suppressing IL-2 production and limiting cell cycle progression[115–118], as well as promoting suppresive function of Tregs[119,120], thereby contributing to immunological tolerance. Increased CTLA4 and PD1 expression is reported in chronic HBV infection, and the function of HBV-specific CD8 T cells can be reversed by PD1 single or PD1/CTLA4 double blocked[121,122]. An over expression of PD1 antigen on Tregs both from liver and peripheral blood of patients with chronic HBV infection has been reported[123,124].

1.6. HBV Treatment

Most HBV infections do not require treatment, while more than 90–95% of adults clear the infection spontaneously and develop neutralizing antibodies[125]. Less than 1% patients with fulminant or severe hepatitis need early antiviral treatment or even liver transplantation[126]. Currently, the eradication of HBV is impossible due to the persistence of cccDNA in the nucleus of infected hepatocytes[127]. However, treatment of chronic infection can improve quality of life and survival by reducing the risk of cirrhosis and liver cancer.

Conventional or pegylated interferon alpha (IFN or PEG-IFN) and six nucleoside/ nucleotide analogues (NAs) are two different types of drugs that are licensed for treatment of CHB. The response of treatment depends on medications and HBV genotypes. HBV patients with genotype C and D

showed lower HBeAg seroconversion rate than with genotype A and B by interferon treatment[31,128]. Whereas, sustained virological response in HBV genotypes E (36%), F and H (50%) was higher than in genotype G (20%), therefor genotypes E, F and H appeared to be sensitive to IFN-alpha[129]. HBV genotype does not influence the virological response to any NA[130]. However, only entecavir and tenofovir are confirmed with a high barrier to resistance[131,132].

1.7. Vitamin D as a novel immunomodulator

Vitamin D has received particular attention in recent years as it has an unexpected and crucial interaction with both innate and adaptive immune responses in highly specific ways.

1.7.1. Metabolism of Vitamin D3

Ultraviolet (UV) exposure, diet and vitamin supplement are the main source of our vitamin D3. After synthesized from 7-dehydrocholesterol in the skin, vitamin D3 then converted to 25-dihydroxyvitamin D3 [25(OH)D3] by the enzyme 25-hydroxylase in the liver. In the kidney, 25(OH)D3 is hydroxylated by 1a-hydroxylase to become 1,25-dihydroxyvitamin D3 [1,25$(OH)_2$D3], the most physiologically active vitamin D3 metabolite, then reaches the blood where it has multiple systemic effects[133]. Finally, 1,25$(OH)_2$D3 is catabolized to inactive calcitroic acid by the enzyme 24-hydroxylase, and then excreted in the bile[134].

1.7.2. 1,25$(OH)_2$D3 and immune system

Immune cells, including macrophages, DCs, T and B cells express the sets of the key enzymes, cytochrome P (CYP)27A1 and/or CYP27B1, which encode 25-hydroxylase and 1a-hydroxylase respectively, enabling synthesis of active 1,25$(OH)_2$D3[133,135]. 1,25$(OH)_2$D3 acts on immune cells in an autocrine or paracrine manner by binding to the vitamin D receptor (VDR). The systemic effects of 1,25$(OH)_2$D3 mediated by VDR include calcium and phosphate regulation, and the local effects include reducing cell proliferation and increasing cell differentiation. The immune target cells are DCs, T cells, monocytes, macrophages and B cells[136]. The overall immunomodulatory function of local 1,25$(OH)_2$D3 is strengthening innate immune responses, by

contrast, restraining adaptive immune responses[137]. 1,25(OH)$_2$D3 could enhance the function of macrophages and DCs, and stimulates the synthesis of antimicrobial peptides (AMPs), thereby promoting innate immune response, contrarily[137]. In vitro, 1,25(OH)$_2$D3 negatively affects adaptive immune reponse, such as inhibits T cell proliferation and CD8 T cell-mediated cytotoxicity, suppresses the expression of IL-2[138,139], IFNγ and costimulatory molecules in T cells[140–142]. Overall, the direct effects of 1,25(OH)$_2$D3 on adaptive immune system are promoting the development of Th2 cells and Tregs, while inhibiting Th1 and Th17 cells responses[143,144], decreasing B cells proliferation, plasma cells differentiation and immunoglobulin G (IgG) secretion[145].

1.7.3. Vitamin D and diseases

Considering its potential immunomodulatory properties, vitamin D and its metabolites or its analogues have been already studied in diseases including cancer, insulin resistance, inflammatory and autoimmune disease, confirming that low serum vitamin D and/or its metabolites level is a risk factor in these diseases and supplement of vitamin D or its metabolites or its analogues could improve these diseases evidently[146–151]. Vitamin D induces apoptosis of tumor cells and inhibits proliferation mainly through VDR signals causing an inhibition of mitogen activated protein kinase (MAPK) activity, but not immunological pathways. In diseases such as psoriasis and multiple sclerosis，which are characterized by increased Th1 and Th17 cells[146], may benefit from the immunosuppressive effects of vitamin D that induces Tregs[147,152,153] and reduces the induction of Th1 and Th17 effector cells[154,155]. Allergic asthma, which is Th2 cell driven disease, can still benefit from vitamin D supplementation[156,157], even though, theoretically, vitamin D may stimulate Th2 type immune response. This probably because the sesitization and severe inflammatory reaction are suppressed by vitamin D[153]. Moreover, in contrast to its suppressive effect on adaptive immune response, vitamin D supplementation also benefits infectious diseases[158,159,160], due to the induction of AMPs and promotion of the chemotactic and phagocytic capacity of macrophages, enhancing innate immune response and meanwhile prevent immune-mediated tissue injury[161]. Interestingly, supplement of 1,25(OH)$_2$D3 analogues that exert immunomodulation didn’t cause significant

hypercalcemia[143]. 1,25$(OH)_2$D3 helps to prevent transplant rejection without, however, significant interference with protective immune responses[162].

1.7.4 Vitamin D and Chronic hepatitis C

Vitamin D deficiency is universal among patients with chronic hepatitis C (CHC)[163,164]. In HCV infection, a certain correlation between vitamin D and prognosis has been shown. Deficiency of vitamin D3 are associated with considerably lower rates of sustained virologic response (SVR) in HCV infected patients, while the probability of achieving an SVR following antiviral treatment can be improved by vitamin D supplementation, especially in difficult-to-treat patients[165,166] and no cytotoxicity was found in vivo[167]. This possibly because, vitamin D has an antiviral activity which is mediated by its active metabolite, 1,25$(OH)_2$D3[168,169]. Matsumura T, et al.[167] demonstrated that 25(OH)D3 affects HCV life cycle at the assembly step. 25(OH)D3 indeed augmented IFN-induced HCV core antigen reduction, but did not induce the expression of IFN-stimulated genes(ISGs). A study found that the expression of Myxovirus resistance protein A (MxA), which has the strongest antiviral activity of the IFN-induced antiviral proteins, in HCV-infected cells increased by treatment of both vitamin D3 and 1,25$(OH)_2$D3 (calcitriol) in a dose-dependent manner[168]. Vitamin D not only affects the response of antiviral treatment in CHC, low vitamin D levels may be also associated with severe liver injury and fibrosis in HIV/HCV co-infected patients[170,171]. However, a study in CHC genotype 1 infection found no correlation between vitamin D status and fibrosis stage or SVR[172]. Moreover, VDR gene polymorphisms may be related to the response to PEG-IFN plus ribavirin (RBV) therapy in CHC[173,174].

1.7.5 Vitamin D and Chronic hepatitis B

The effects of vitamin D in pathogenesis, treatment and outcome in patients with chronic hepatitis B get more attention. Similarly, vitamin D deficiency is also noted in patients with chronic hepatitis B[175]. One of the reasons could be the function of liver, where 25(OH)D3 is synthesized, was impaired in hepatitis virus infection. It was observed that low 25(OH)D3 serum levels are associated with high levels of HBV replication in patients with CHB[176]. In addition, it has shown that spontaneous HBsAg seroclearance was associated with a normal

vitamin D level (>20 ng/mL), as well as age, HBeAg negativity and low viral load[177]. It has been confirmed that adaptive immune response involve HBV-specific CD8 cytotoxic T lymphocytes and CD4 helper T lymphocytes palys main role in inhibition of HBV replication. Nonetheless, studies demonstrated that HBV replication could also be inhibited by activated innate immune cells such as NK cells, NK Tcells[178], APCs[152] and toll-like receptors (TLRs)[153] in vivo. Therefore, vitamin D has the possibility of inhibiting HBV replication by activating innate immune response. The relationship of vitamin D level and severity of inflammation provid the possibility that vitamin D modulates the inflammatory process in viral hepatitis by inhibiting the activity of cyclooxygenase (COX)-2[181,182] and the synthesis of prostaglandins (PGs)[183,184]. Genetic studies showed that VDR gene polymorphisms are associated with distinct clinical phenotypes[185] and outcomes[186,187] in HBV infection in Asia and Africa[188]. However, the relationship among vitamin D, immune response and CHB is less well characterized and the mechanisms of this effect have not yet been elucidated.

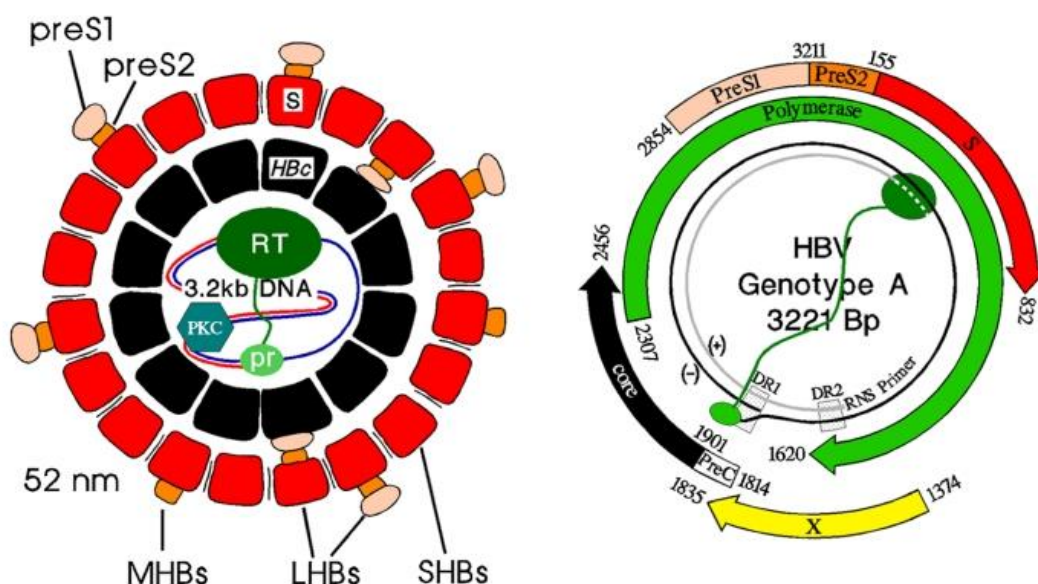


Figure 1. Structural components of HBV (left) and open readingframes (ORF) for encoding proteins in the covalently closed form of HBV DNA. Adapted from ***Gerlich WH et al. Virology Journal 2013, 10:239***

1.8. Aims

The aims of the present study were to analyze the profile of peripheral blood T lymphocyte subpopulations in chronic hepatitis B patients, and to assess their function by measuring their expression of activation/exhaustion markers (CD38, HLA-DR, CD226, CD39/CTLA4, PD1). Further investigated serum vitamin D levels and its immunomodulatory function of T cells in chronic HBV infection. This is also the first study that investigates the correlation between serum vitamin D levels and the expression of activation/exhaustion markers on T cells in patients with chronic hepatitis B.

Thus, we focus on following parameters:

1. Serum 25(OH)D level in patients with chronic HBV infection.

2. T cell subsets analysis:
Analyze the frequence of $CD8^{+}$, $CD4^{+}$, $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs and their subpopulations from peripheral blood of patients with CHB.

3. Reasonable classification of Treg subtypes separated

a. by the expression of FOXP3 and CD45RA: Resting Tregs (rTregs): $CD45RA^{+}FOXP3^{lo}$; actived Tregs (aTregs): $CD45RA^{-}FOXP3^{hi}$; cytokine-secreting $CD45RA^{-}FOXP3^{lo}$ non-suppressive T cells (non-Tregs).

b. by the expression of CD62L and CD45RA: Naïve Tregs (TregN): $CD62L^{+}CD45RA^{+}$; central memory Tregs (TregCM): $CD62L^{+}CD45RA^{-}$; preterminally differentiated effector memory Tregs (TregPreEM): $CD62L^{-}CD45RA^{-}$; terminally differentiated effector memory Tregs (TregTerEM): $CD62L^{-}CD45RA^{+}$.

c. Comparison of the expression of activation/exhaustion markers on these subpopulations in healthy controls and hepatitis B patients.

4. Comprehensive analysis of CD8+ T cells and its subtypes.

a. CD8+ T cell subtypes by the expression of CD62L and CD45RA: Naïve CD8 T cells (CD8N): $CD62L^{+}CD45RA^{+}$; central memory CD8 T cells (CD8CM): $CD62L^{+}CD45RA^{-}$; preterminally differentiated effector memory CD8 T cells (CD8PreEM): $CD62L^{-}CD45RA^{-}$; terminally

differentiated effector memory CD8 T cells (CD8TerEM): $CD62L^{-}CD45RA^{+}$.

b. Comparison of the expression of activation/exhaustion markers on CD8+ T cells and its subtypes.

5. Correlation among the expression of activation/exhaustion markers on T cell subsets and vitamin D levels and HBV DNA levels.

2. Patients, materials and methods

2.1. Patients:

Table 2.1. Characteristics of CHB patients and healthy donors

Characteristics	All CHB patients (n=45)		Healthy controls (HC), (n=18)	*P* value
	Treatment naïve (CHBn) (n=28)	Under treatment (CHBt), (n=17)		
Mean age	44.40±13.90 (20-71)	45.24±10.89 (28-67)	36.06±9.27 (22-51)	*>0.05*
Gender (m/f)	13/15	10/7	10/8	*>0.05*
ALT (IU/L)	28.68±25.33	31.00±14.19	NA*	*>0.05*
Missing, n	9	3		
AST(IU/L)	22.84±20.62	24.36±7.63	NA*	*>0.05*
Missing, n	9	3		
Cholesterol (mg/dl)	194.73±33.78	182.56±45.83	NA*	*>0.05*
Missing, n	13	8		
TSH (mU/L)	1.44±0.87	1.60±0.91	NA*	*>0.05*
Missing, n	12	4		
25(OH)D (ng/mL)	21.76±8.56[a]	19.77±8.92[a]	31.37±14.54	*<0.05*
Missing, n	0	0	8	
>30	4(14%)	3(18%)	5(50%)	*<0.05*
30≥->10	23(82%)	12(70%)	5(50%)	*<0.05*
≤10	1(4%)	2(12%)	0	*<0.05*
HBV DNA Load (Log, IU/mL)	3.59±1.92[b]	1.33±0.55[b]	NA*	*<0.001*
Missing, n	9	4		
HBeAg positive, n(%)	2 (7.15%)	2 (11.76%)	NA*	*>0.05*
Missing, n	11	3		
HBsAg (×10^3 IU/mL)	9.81±12.40[b]	4.33±3.99[b]	NA*	*<0.05*
Missing, n	10	4		

NA* not applicable; a comparasion with HC; b comparation of CHBn and CHBt.

2.2. Materials:

Table 2.2.1. Laboratory Equipment

Equipment	Supplier
Pipette (100-1000 µl, 20-200 µl, 2-20 µl, 0.5-10 µl, 0.1-2,5 µl)	Eppendorf AG, Hamburg, Germany
Pipetman 8-5010	NeoLab Migge Laborbedarf-Vertriebs GmbH, Heidelberg, Germany
Measuring cylinder 2 L	Eppendorf AG, Hamburg, Germany
Schott bottle 1L	Eppendorf AG, Hamburg, Germany
Haemocytometer Neubauer Counting Chamber, Improved	Karl Hecht GmbH, Sondheim/Röhn, Germany
Stratacooler	Stratagene Corp. La Jolla, CA, USA
Vortexer, Model MS 3 basic	IKA® Werke GmbH & Co. KG, Staufen, Germany
Microscope Olympus CK2	Olympus Europa SE, Hamburg, Germany
Waterbath, Typ 1008	Gesellschaft für Labortechnik GmbH, Burgwedel, Germany
Eppendorf-Centrifuge 5810 R	Eppendorf AG, Hamburg, Germany
Heraeus Labofuge 400	Heraeus, Hanau, Germany
KS15 Laminar Flow	Thermo Fisher Scientific GmbH, Schwerte, Germany
BD FACS Fortessa	BD Biosciences, Heidelberg, Germany

Table 2.2.2. Laboratory Consumable

Equipment	Supplier

Plastic Filter Tips (1000 µl, 200 µl, 10 µl)	Sarstedt, Nümbrecht, Germany
Serological Pipette Tips (25 ml, 10 ml, 5 ml)	BD FALCONTM, Heidelberg, Germany
Falcon Tubes (50 ml, 15 ml)	Sarstedt, Nümbrecht, Germany
Safe-lock tubes 2 ml, 1,5 ml	Eppendorf AG, Hamburg, Germany
Micro-tube 2 ml	Sarstedt, Nümbrecht, Germany
FACS tubes	Sarstedt, Nümbrecht, Germany
Nunc Cryotubes 1,8 ml	Thermo Fisher Scientific GmbH, Schwerte, Germany
BD Vacutainer® CPT^{TM} tube with Sodium Citrate	BD Biosciences, Heidelberg, Germany

Table 2.2.3. Solutions, Media and Reagents

Materials	**Supplier**
ddH_2O	Eppendorf AG, Hamburg, Germany
Hydrochloric acid (HCl) 25%	Th. Geyer, Renningen, Germany
Fetal Calf Serum (FCS)	Biochrom AG, Merck, Darmstadt, Germany
PBS (Phosphate Buffered Saline 1×)	Gibco® Life Technologies, Darmstadt, Germany
Trypan blue 0,4%	Gibco® Life Technologies, Darmstadt, Germany
RPMI 1640 medium, L-GlutaMAX	Gibco® Life Technologies, Darmstadt, Germany
DMSO	Gibco® Life Technologies, Darmstadt, Germany
NaN_3	Sigma Aldrich, St. Louis, USA

Kalium chloride (KCl) p.a.	Th. Geyer, Renningen, Germany
Kaliumhydrogenphosphat (KH_2PO_4) p.a.	Th. Geyer, Renningen, Germany
Natriumchlorid (NaCl) p.a.	Carl Roth, Karlsruhe, Germany
Dinatriumhydrogenphosphat dihydrate ($Na_2HPO_4 \cdot 2H_2O$) p.a.	Th. Geyer, Renningen, Germany
FOXP3 Fix/Perm buffer 4×	Biolegend®, Fell, Germany
FOXP3 Perm buffer 10×	Biolegend®, Fell, Germany
Fc Blocking Reagent	Biolegend®, Fell, Germany
Aqua LIVE/DEAD marker (from Aqua LIVE/DEAD Fixable Dead Cell Stain Kit)	Gibco® Life Technologies, Darmstadt, Germany

Table 2.2.4. Mixed Solutions

Solutions	Ingredients
NaN_3 9%	ddH_2O NaN_3 9%
PBS pH 7,4	ddH_2O NaCl 8% KCl 0,2% $Na_2HPO_4 \cdot 2H_2O$ 1,442% KH_2PO_4 0.2%
FCFKSN3	PBS pH 7,4 FCS 1% NaN_3 9% 1%
Freezing Media	FCS 50%

	RPMI 30%
	DMSO 20%

Table 2.2.5. Kits

Kit	Supplier
25-OH vitamin D ELISA test kit	EUROIMMUN AG, Lübeck, Germany

Table 2.2.6. Antibodies

Specificity	Species	Isotype	Fluorophore	Clone	Supplier
CD3	Mouse-anti human	IgG1, κ	PerCP/Cy5.5	SK7	BD Biosciences, Heidelberg, Germany
CD4	Mouse-anti human	IgG1, κ	BV570	RPA-T4	Biolegend®, Fell, Germany
CD8	Mouse-anti human	IgG1, κ	BV785	RPA-T8	Biolegend®, Fell, Germany
CD25	Mouse-anti human	IgG1, κ	BV605	2A3	BD Biosciences, Heidelberg, Germany
CD38	Mouse-anti human	IgG1, κ	APC	HIT2	Biolegend®, Fell, Germany
CD39	Mouse-anti human	IgG1, κ	BV421	A1	Biolegend®, Fell, Germany
CD45RA	Mouse-anti human	IgG1, κ	BV711	HI100	Biolegend®, Fell, Germany
CD62L	Mouse-anti human	IgG1, κ	BV650	DREG-56	Biolegend®, Fell, Germany
CD226	Mouse-anti human	IgG1, κ	PE	11A8	Biolegend®, Fell, Germany
PD1 (CD279)	Mouse-anti human	IgG1, κ	BV421	EH12.2H7	Biolegend®, Fell, Germany
PD1 (CD279)	Mouse-anti human	IgG1, κ	BV605	EH12.2H7	Biolegend®, Fell, Germany
CTLA4	Mouse-anti human	IgG2a, κ	APC	BNI3	BD Biosciences, Heidelberg, Germany

FOXP3	Mouse-anti human	IgG1, κ	FITC	206D	Biolegend®, Fell, Germany
Granzyme B	Mouse-anti human	IgG1, κ	FITC	GB11	BD Biosciences, Heidelberg, Germany
HLA-DR	Mouse-anti human	IgG1, κ	PE	G46-6	BD Biosciences, Heidelberg, Germany
Perforin	Mouse-anti human	IgG2b, κ	PE	DG9	Biolegend®, Fell, Germany

Table 2.2.7. Isotypecontrols

Isotypecontrols (IsCo)	Species	Isotype	Clone	Supplier
IsCo, PerCP/Cy5.5	Mouse-anti human	IgG1, κ	MOPC-21	Biolegend®, Fell, Germany
IsCo, BV570	Mouse-anti human	IgG1, κ	MOPC-21	Biolegend®, Fell, Germany
IsCo, BV785	Mouse-anti human	IgG1, κ	MOPC-21	Biolegend®, Fell, Germany
IsCo, BV605	Mouse-anti human	IgG1, κ	MOPC-21	Biolegend®, Fell, Germany
IsCo, APC	Mouse-anti human	IgG1, κ	MOPC-21	Biolegend®, Fell, Germany
IsCo, BV421	Mouse-anti human	IgG1, κ	MOPC-21	Biolegend®, Fell, Germany
IsCo, BV711	Mouse-anti human	IgG1, κ	MOPC-21	Biolegend®, Fell, Germany
IsCo, BV650	Mouse-anti human	IgG1, κ	MOPC-21	Biolegend®, Fell, Germany
IsCo, FITC	Mouse-anti human	IgG1, κ	MOPC-21	Biolegend®, Fell, Germany
IsCo, PE	Mouse-anti human	IgG1, κ	MOPC-21	Biolegend®, Fell, Germany

2.3. Methodes:

Table 2.3.1. Surface staining

Sample	Isotypecontrols [µl]									
#	IsCo, PerCP/Cy5.5	IsCo, BV570	IsCo, BV785	IsCo, BV605	IsCo, APC	IsCo, BV421	IsCo, BV711	IsCo, BV650	IsCo, FITC	IsCo, PE
1	5	3	1	3	4	2	1	2	2,5	7,5

Sample	Specificantibodies [µl]									
#	CD3, PerCP/Cy5.5	CD4, BV570	CD8, BV785	CD25, BV605	CD38, APC	CD39, BV421	CD45RA, BV711	CD62L, BV650	FOXP3, FITC	HLA-DR, PE
2	5	3	1	3	2	4	2	2	-	15

Sample	Specificantibodies [µl]									
#	CD3, PerCP/Cy5.5	CD4, BV570	CD8, BV785	CD25, BV605	CD45RA, BV711	CD62L, BV650	CD226, PE	PD1, BV421	CTLA4, APC	FOXP3, FITC
3	5	3	1	3	2	2	15	3	5	-

Sample	Specificantibodies [µl]								
#	CD3, PerCP/Cy5.5	CD4, BV570	CD8, BV785	CD45RA, BV711	CD62L, BV650	PD1, BV605	CTLA4, APC	Granzyme B, FITC	Perforin, PE
4	5	3	1	2	2	5	5	-	-

5	Noantibodies

Table 2.3.2. Intracellular staining

Sample	Isotypecontrols [µl]									
#	IsCo, PerCP/Cy5.5	IsCo, BV570	IsCo, BV785	IsCo, BV605	IsCo, APC	IsCo, BV421	IsCo, BV711	IsCo, BV650	IsCo, FITC	IsCo, PE
1	-	-	-	-	-	-	-	-	-	-

Sample	Specificantibodies [µl]									
#	CD3, PerCP/Cy5.5	CD4, BV570	CD8, BV785	CD25, BV605	CD38, APC	CD39, BV421	CD45RA, BV711	CD62L, BV650	FOXP3, FITC	HLA-DR, PE
2	-	-	-	-	-	-	-	-	5	-

Sample	Specificantibodies [µl]									
#	CD3, PerCP/Cy5.5	CD4, BV570	CD8, BV785	CD25, BV605	CD45RA, BV711	CD62L, BV650	CD226, PE	PD1, BV421	CTLA4, APC	FOXP3, FITC
3	-	-	-	-	-	-	-	-	-	5

Sample	Specificantibodies [µl]								
#	CD3, PerCP/Cy5.5	CD4, BV570	CD8, BV785	CD45RA, BV711	CD62L, BV650	PD1, BV605	CTLA4, APC	Granzyme B, FITC	Perforin, PE
4	-	-	-	-	-	-	-	20	4

5	Noantibodies

2. Patients, materials and methods

2.1. Patients:

The patient collective consistet of 45 out-patients with chronic hepatitis B (CHB) (treatment naïve, n=28; under treatment, n=17), attending the Department of Medicine, University Medical Center Hamburg-Eppendorf (Germany), from January to October 2013, were enrolled into the study. The clinical diagnosis of CHB was based on the persistence of HBsAg lasting for more than 6 moths, alanine aminotransferase (ALT) elevated, and HBV DNA was steady positive in the serum[189]. Patients in CHBt group were treated with NAs, entecavir or tenofovir. All patients were negative for hepatitis C, D and HIV antibodies and for other markers of viral hepatitis. Patients with non-viral hepatitis, such as alcoholic hepatitis, toxic and drug-induced hepatitis, autoimmune hepatitis and metabolic liver disease, were excluded. None of the patients were treated with immunosuppressive therapy.

For comparison of T-cell subpopulations and serum vitamin D level with CHB patients, 18 healthy individuals who were free of HBV infection and alcohol consumption less than 60g/d, served as the control group (HC). 10 were male, 8 were female, mean age 36.06±9.27 (rang 22-51). Patients’ and uninfected individuals’ biochemical and virological features are summaried in **table 2.1**.

2.2. Materials:

See **table 2.2.1 — table 2.2.6**.

2.3 Methods:

2.3.1. Liver enzymes, Hepatitis Serology and vitamin D evaluation:

Liver enzymes (ALT, AST), HBV markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb IgM), anti-HCV, anti-delta and anti-HIV antibodies were measured by standard biochemical tests, acquired from patient care inspection. 25(OH)D is the major metabolite of vitamin D in the liver and is widely used as an indicator of serum vitamin D status[190]. The level of 25(OH)D in patients with CHB were determined by specific 25-hydroxyvitamin D ELISA test kit according to the manufacturer’s instructions (EUROIMMUN). The calibrators/controls and

heparin plasma of CHB patients and healthy controls were diluted 1:26 in 1:100 biotin, incubated for 10 minutes. 200 µl of each sample were removed into each of the antibody-coated microplate wells, incubated for 2 hours. The wells were then emptied and washed 3 times using 1:10 wash buffer. 100 µl of enzyme conjugate was added into each of the well and incubated for 30 minutes. After wash as described above, 100 µl of chromogen/substrate solution was added into each of the well and incubated for 15 minutes protected from direct sunlight. The above procedure was performenced at room temperature (18°C to 25°C) The reaction was stopped using stop solution. Measurement of samples were carried out fully automatically using the EUROIMMUN ELISA analysis device.

2.3.2. Isolation of PBMCs from fresh blood:

PBMCs were isolated from heparinized blood,which was collected by standard technique for BD Vacutainer® Brand Blood collection tubes, by standard density gradient centrifugation. Blood samples were centrifuged at 2000×g for 20 minutes at room temprature (15-25 ℃). The lower layer, comprised of mononuclear cells and platelets, was then collected into a 50 ml conical tube. After adjusting the volume to 50ml by PBS, cells were centrifuged at 4 ℃ for 10minutes at 450×g, the supernatant was discarded. 5 ml 4 ℃ Roswell Park Memoria Institute (RPMI) medium was used to resuspend cells. After adding 5ml 4℃ freezing medium, PBMCs were cryopreserved in 2ml cryotubes, which were then placed in a precooled (4 ℃) Stratacooler and frozen in a -80 ℃ freezer. Ultimately all cryotubes were preserved in prelabled boxes in liquid nitrogen tanks.

2.3.3. Thawing of PBMCs:

30 ml RPMI 1640 was transferred into a 50 ml Falcon tube and warmed up to 37 ℃ in a water-bath. Cryotube with PBMCs was thawed in warm water at 37 ℃ for 1 minute and cells were immediately transferred into the 50 ml tube with warmed RPMI 1640. And then centrifuged at 4℃ for 8 minutes at 450×g, discarded supernatant, resuspended in 1 ml PBS (pH 7.4) for further staining.

2.3.4. Flow cytometry:

2.3.4.1. Live/Dead staining of PBMCs:

To determine the population and percentage of alive mononuclear cells, the cells were incubated with Aqua Live/Dead for fluorescence activated cell sorting (FACS). 1 μl Aqua was added into 50 ml tube with thawed PBMCs (at least 5×10^6) for 30 minutes at 4℃ shielded from light. After centrifugation at 450×g for 8 minutes, the supernatant was discarded, and the pellet resuspended in 0.5 ml FCFKSN3 for further staining.

2.3.4.2. Fc receptors Blocking:

In order to reduce nonspecific immunofluorescent staining, the cells were incubated with Fc blocking reagent before immunophenotypic staining. The cell suspension in 0.5 ml FCFKSN3 was pipetted into 5 FACS tubes (100μl/tube). 5 μl Fc blocking reagent was added into each tube for 10 minutes at 4℃ shielded from light.

2.3.4.3. Surface staining of peripheral blood T lymphocytes:

After Fc bloking, to characterize T lymphocyte subsets, 5 PBMC samples in FACS tubes were stained with appropriate fluorochrome-conjugated antibodies against surface markers respectively according to **table 2.3.1** for 30 minutes at 4℃ shielded in the dark. After surface staining, cells were washed once with 1 ml FCFKSN3, then centrifuged for 8 minutes at 4℃ and 450×g, after discarding supernatant intracellular staining was started.

2.3.4.4. Intracellular cytokines staining:

For intracellular cytokine staining, surface-stained cells were permeabilized by using FOXP3 Staining Buffer Kit following the manufacturer’s instructions. 1 ml FOXP3 FixPerm Buffer (1:4, diluted with PBS pH7.4) was added to the washed cell pellet in each FACS tube and the tube vortexed immediately. Samples were incubated for 20 minutes at room temprature (15-25 ℃) in the dark. After permeabilization, cells were washed with 1 ml FCFKSN3 and 1 ml FOXP3 Perm buffer (1:10, diluted with PBS pH7.4) successively, several cells were re-suspend in 1ml FOXP3 Perm buffer, and incubated at room

temperature in the dark for 15 minutes, centrifuged for 8 minutes at room temperature and 450×g, the supernatant was discarded and the pellet resuspended in 100 ul of FOXP3 Perm buffer. Flurochrome-conjugated antibodies for intracelluar cytokines were added into each FACS tube according to **table 2.3.2**, and incubated at room temperature in the dark for 30 minutes. Samples were washed once with 1 ml FCFKSN3, then centrifuged for 8 minutes at room temperature and 450×g. After discarding the supernatant the cell pellet was resuspended in 0.5 ml FCFKSN3 and then analyzed with flow cytometer with BD LSR Fortessa machine using FACS Diva version 5 (BD Biosciences, Heidelberg Germany).

2.3.4.5. T lymphocyte subsets data acquisition:

The prepared samples were analyzed with flow cytometer performed on BD LSR Fortessa machine using FACS Diva version 5 in strict accordance with the manufacturer's instructions. The obtained data was analyzed using FCS express 4 software (De Novo Software, Los Angeles, CA).

Lymphocytes were analyzed using a gate set on forward scatter versus side scatter, and further gated according to their physical parameters. T cell subsets were examined as a percentage of the their superset population, and the mean fluorescence intensity (MFI) on the activation (CD38, CD39, CD226, HLA-DR) /exhaustion (PD1 and CTLA4) markers on antigen positive cells.

CD4 T lymphocytes were furhter selected either by CD25 and FOXP3 as $CD4^{+}CD25^{+}FOXP3^{+}$ regulatory T cells (Tregs) or by CD45RA and FOXP3 as $CD45RA^{+}FOXP3^{lo}$ resting Tregs (rTregs), $CD45RA^{-}FOXP3^{hi}$ activated Tregs (aTregs), and cytokine-secreting $CD45RA^{-}FOXP3^{lo}$ nonsuppressive Tcells (non-Tregs)[191].

$CD4^{+}CD25^{+}FOXP3^{+}$ Tregs and the CD8 T cells were then sorted by CD45RA and CD62L antibodies into four subsets: naïve cells (N), which were $CD45RA^{+}CD62L^{+}$; central memory cells (CM), which were $CD45RA^{-}CD62L^{+}$; and two effector memory populations: preterminally differentiated effector memory cells (PreEM), which were $CD45RA^{-}/CD62L^{-}$ and terminally differentiated effector memory cells (TerEM), which were $CD45RA^{+}$ $CD62L^{-}$[192].

CD8 T cells and the four subsets of memory cells were further determined by intracellular staining with fluorochrome conjugated Granzyme B and Perforin.

2.3.5. Statistical analysis:

All flow-cytometric data were collected using FCS express 4 software. Statistical analysis was carried out using Sigma Plot® 12 software (Systat Software, Erkrath, Gemany). Descriptive statistics were used to determine the characteristics of each group (CHBn, CHBt and HC), such as age, gender, ALT and AST, cholesterol, TSH, 25(OH)D, serum HBV DNA load and HBsAg quantity, as well as to summarize T cell subpopulations for further analysis. Bivariate analysis was performed by an independent *t* test, and for comparison of more than two variables one-way ANOVA. The correlation among serum 25(OH)D level, HBV DNA replication and activation/exhaustion markers on T lymphocyte subpopulations were investigated only in CHBn cohort. Pearson's correlation was performed for the above bivariate correlation analyses. All data was expressed as means ± standard deviations (sd) or median and 25%-70% range. A final P-value less than 0.05 was considered statistically signifcant.

3. Results

3.1. 25(OH)D insufficiency in patients with CHB.

Although 1,25$(OH)_2$D is the most physiologically active vitamin D metabolite, clinical measurement to quantify 1,25$(OH)_2$D is normally unstable and unreliable. 25(OH)D is the major metabolite of vitamin D in the liver and is therefore widely used as an indicator of serum vitamin D status. 25(OH)D concentrations of <10 ng/mL is defined as deficiency, 10-30 ng/mL as insufficiency, and >30 ng/mL is considered as adequate[193,194]. 82% (23/28) patients in CHBn group were insufficent of vitamin D, 70% (12/17) in CHBt and 50% (5/10) in HC cohorts (CHBn vs HC P=0.013999; CHBt vs HC P= 0.026325). Deficiency of vitamin D was found in 1(4%) and 2(12%) individuals in CHBn and CHBt cohorts respectively, but not in healthy cohort. A lower serum level of 25(OH)D was shown in CHBn (21.76±8.56 ng/mL) and CHBt cohorts (19.77±8.92 ng/mL) compared to healthy controls (31.37±14.54 ng/mL). See **Table 2.1** and **Figure 3.1**.

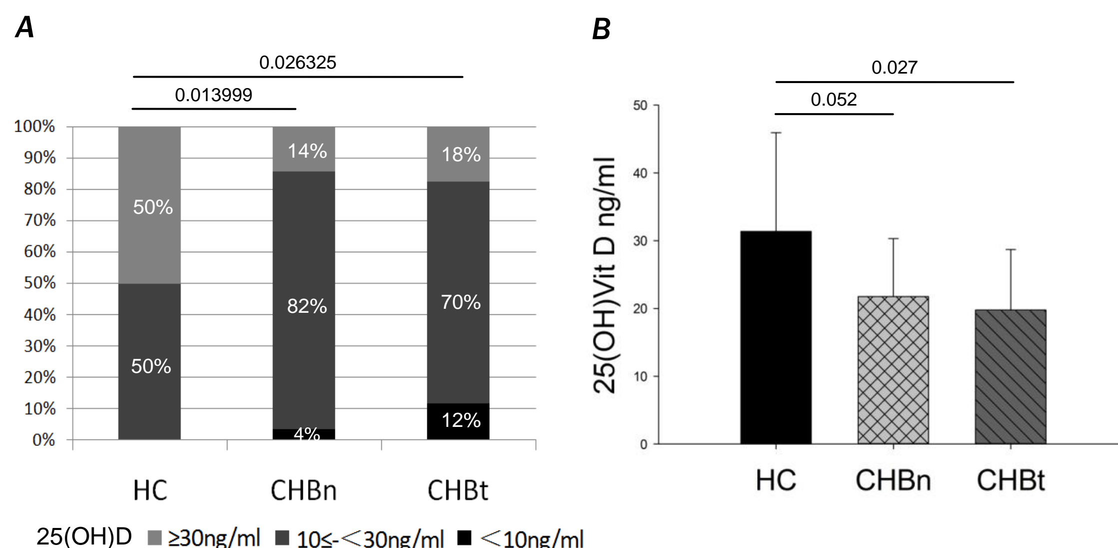


Figure 3.1. 25(OH)D serum level is lower in patients with chronic HBV infection compared with healthy controls. (A) Proportion of vitamin D insufficient and deficient individuals in HC (n=10), CHBn (n=28) and CHBt (n=17) cohorts. (B) Comparison of 25(OH)D serum concentrations in CHB groups and HC cohort. Data are presented as mean±sd.

3.2. Proportions of CD8, CD4 T cells and $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs in peripheral blood showed no difference between patients with chronic HBV infection and healthy controls.

Clearence of hepatitis B virus is mainly dependent on the activation of CD8 CTLs, which with the help of CD4 T cell polarised Th1, induce apoptosis of the infected cells or clearing virus from the hepatocytes by antiviral cytokines[40]. Due to their cytotoxic effect, the activation of CD8 T cells results in liver injury while clearing the virus. In oder to limit tissue damage, Tregs are needed to inhibite the T cell proliferation, cytokine secretion and CTL activity[195].

Arising regulatory T cells are closely associated with immune tolerance[196]. Increased Tregs, decreased CD4 non Tregs and defective functions of CD8 T cells were widely reported in chronic HBV infection[197–200]. However these anomalies were found more in the liver, the location of infection, than in peripheral blood.

This study analyzed CD8, CD4 T cells and $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs in peripheral blood of all cohorts. Frequencies of CD8 T cells were 15.38±6.40%, 13.09±4.73% and 14.19±4.13% in CHBn, CHBt and HC cohorts respectively, and those of CD4 T cells were 28.76±8.23%, 27.24±10.62% and 33.26±5.42%, and those of $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs were 1.15±0.51%, 1.37±1.29% and 1.54±0.95%. No significant differences were detected in all groups ($p>0.05$). See **Figure 3.2**.

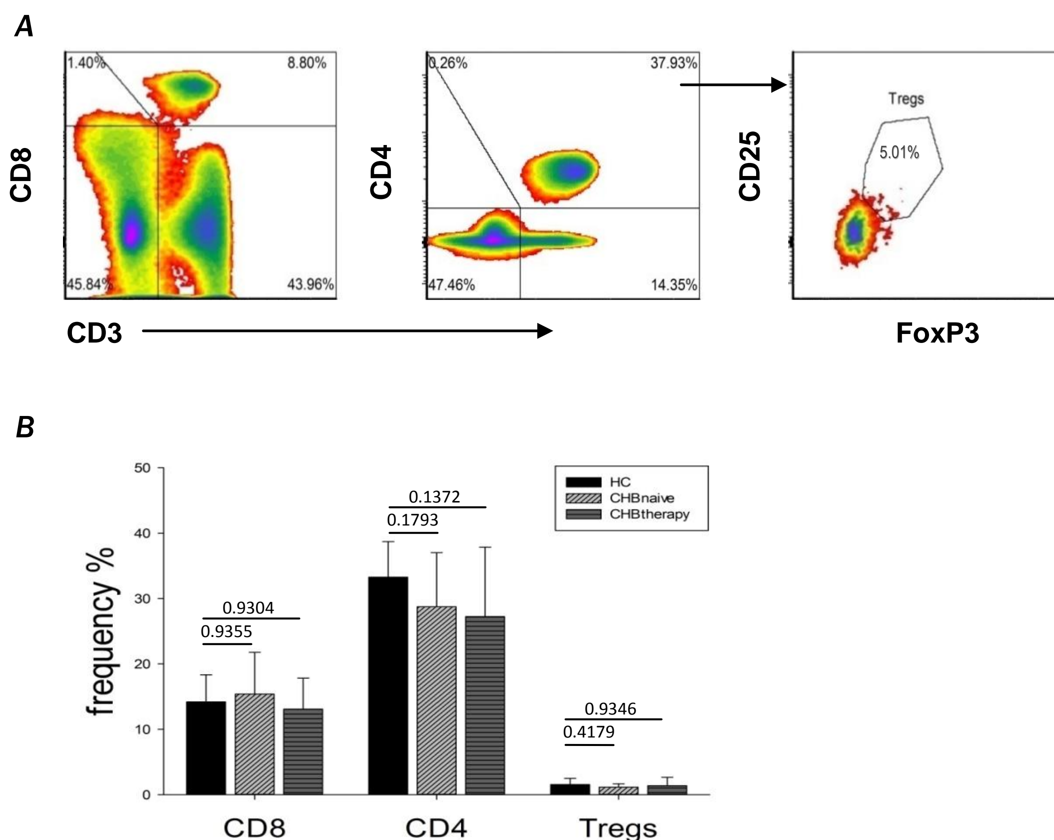


Figure 3.2. Frequencies of CD8, CD4 T cells and $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs in peripheral blood of all cohorts. (A) Representative FACS plots of CD8, CD4 T cells, $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs gating strategy in PBMCs. (B) Histogram of CD8, CD4 T cells, $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs frequencies in PBMCs in HC (n=18), CHBn (n=28) and CHBt (n=17). Data are presented as mean±sd, all groups P>0.05.

3.3. Subpopulations of CD4 Tregs separated by the expression of FOXP3, CD45RA and CD62L.

Traditionally regulatory T cells are defined as $CD4^{+}CD25^{+}FOXP3^{+}$ T cells. But some studies identified both CD25 and FOXP3 can be induced during the activation of non-T regulatory cells, without convertion to a regulatory phenotype[201–203]. Moreover, one naïve ($CD45RA^{+}$) and two memory phenotypes ($CD62L^{-}$) of Tregs were defined in several studies[204–206].

In this study, $CD4^{+}FOXP3^{+}$ Tregs were furhter selected by CD45RA and FOXP3 as $CD45RA^{+}FOXP3^{lo}$ resting Tregs (rTregs), $CD45RA^{-}FOXP3^{hi}$ activated Tregs (aTregs), and cytokine-secreting $CD45RA^{-}FOXP3^{lo}$ nonsuppressive T cells (non-Tregs)[191]. See **Figure 3.3. A**. The expression of

CD39 and HLA-DR were lower in rTregs (CD39 17.21±11.58%; HLADR 12.45±9.98%) and non-Tregs (CD39 38.96±20.61%; HLADR 17.64±7.88%) of all the cohorts, but higher in aTregs(CD39 70.43±24.08%; HLADR 49.45±12.91) (p<0.001). See **Figure 3.3. B, C**.

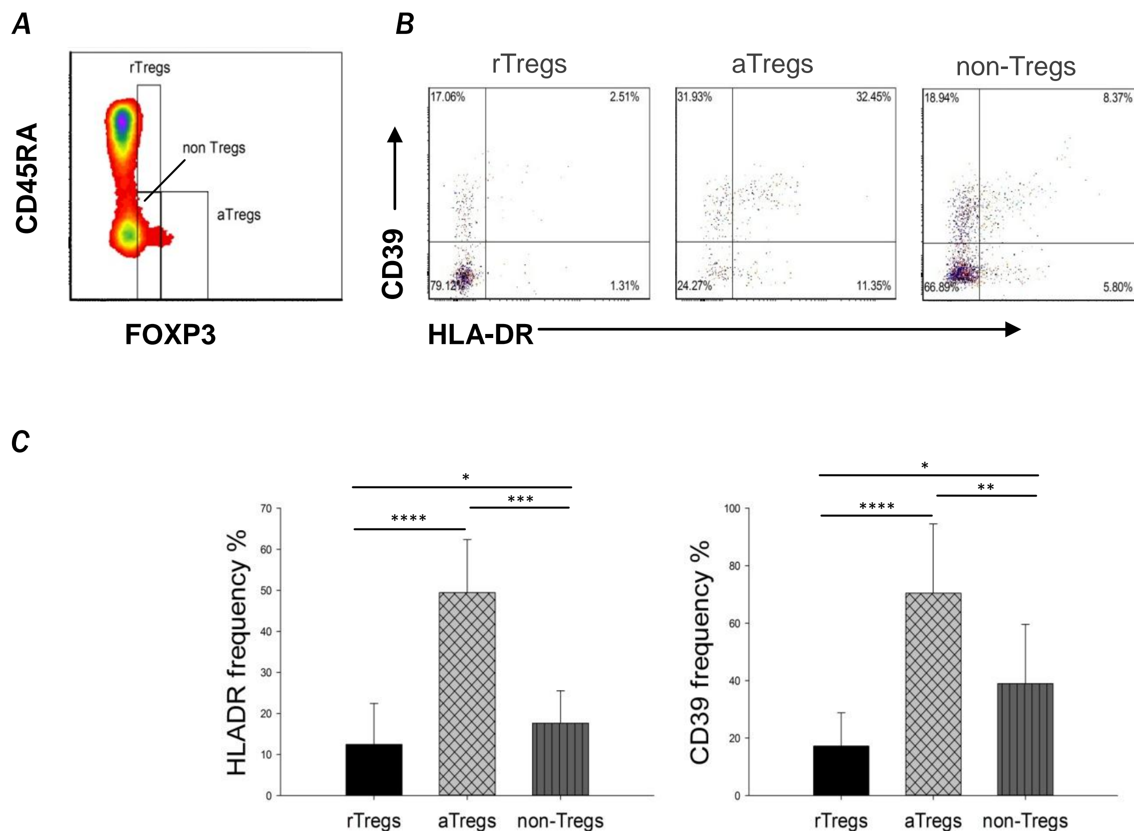


Figure 3. 3. Three subpopulations of circulating CD4$^+$FOXP3$^+$ Tregs in PBMCs of all individuals. (A) Representative FACS plots displaying the gating strategy for rTregs, aTregs and non-Tregs within the CD4 T cell population. (B) Representative FACS plots of HLA-DR and CD39 expression on rTregs, aTregs and non-Tregs. (C) Comparison of HLA-DR and CD39 frequencies of rTregs, aTregs and non-Tregs in all cohorts. Data are presented as mean±sd. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

CD4$^+$CD25$^+$FOXP3$^+$ Tregs were then sorted by CD45RA and CD62L antibodies into four subsets: naïve cells (N), which were CD45RA$^+$CD62L$^+$; central memory cells (CM), which were CD45RA$^-$CD62L$^+$; and two effector memory populations: preterminally differentiated effector memory cells (PreEM), which were CD45RA$^-$CD62L$^-$ and terminally differentiated effector memory cells (TerEM), which were CD45RA$^+$CD62L$^-$[192]. See **Figure 3.4. A**. Similarly, CD45RA$^+$ regulatory T cells (TregN and TregTerEM) expressed small amounts of CD39 (TregN 51.14±28.38%; TregTerEM 32.52±28.28%) and

HLA-DR (TregN 25.79±21.67%; TregTerEM 15.45±23.95%), whereas $CD45RA^-$ regulatory T cells (CM and PreEM regulatory T cells) expressed the largest amounts of CD39 (TregCM 71.75±25.08%; TregPreEM 66.27±25.62%) as well as HLA-DR (TregCM 47.93±15.09%; TregPreEM 34.59±13.61%), ($p<0.001$).See **Figure 3.4. B, C**.

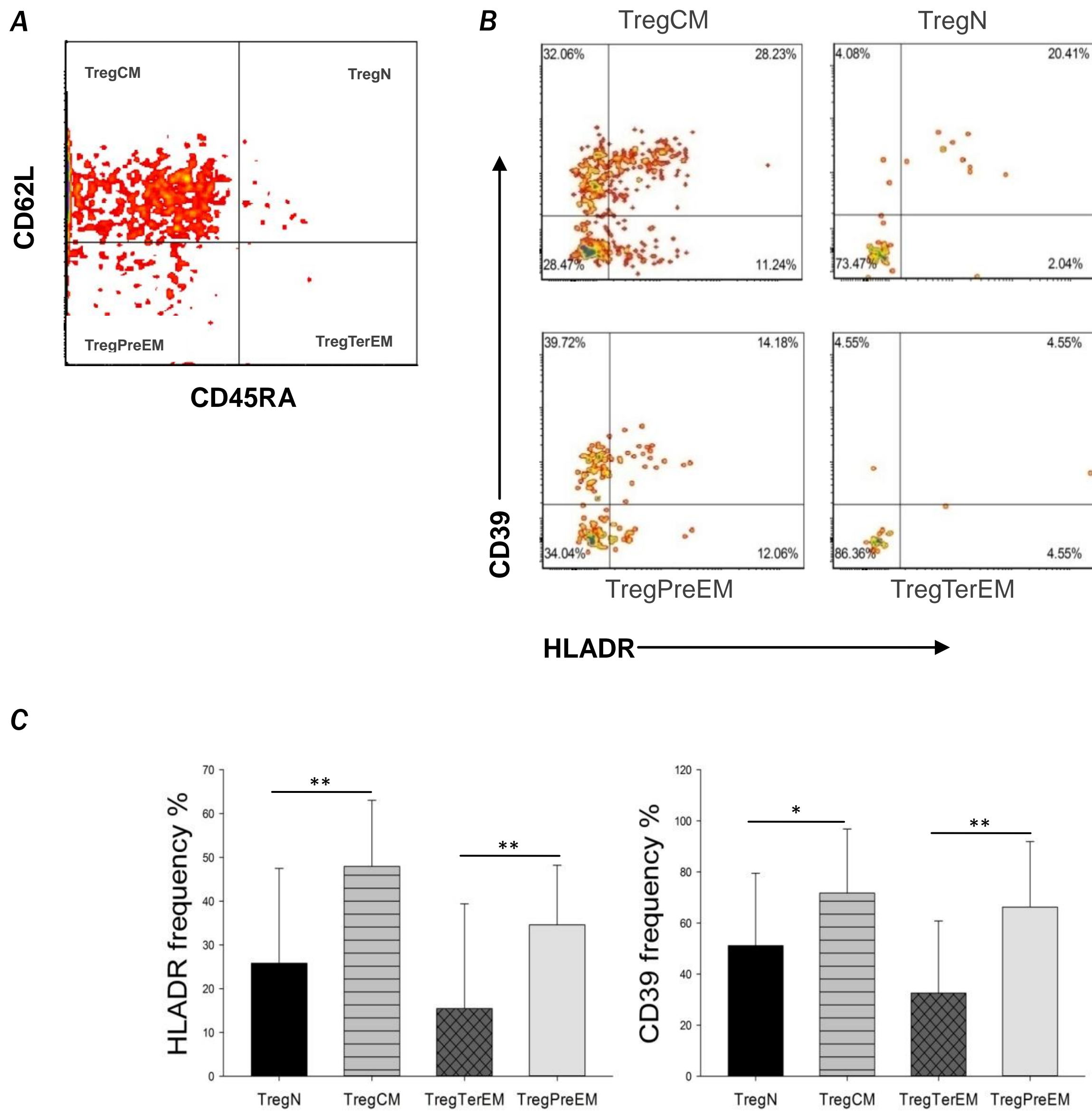


Figure 3.4. Subpopulations of circulating $CD4^+CD25^+FOXP3^+$ Tregs in PBMCs of all individuals. (A) Representative FACS plots displaying the gating strategy for TregN, CM, PreEM and TerEM within the $CD4^+CD25^+FOXP3^+$ Tregs population. (B) Representative FACS plots of HLA-DR and CD39 expression on TregN, CM, PreEM and TerEM. (C) Comparison of HLA-DR and CD39 frequencies of $CD45RA^+$ Tregs (N, TerEM) and $CD45RA^+$ Tregs (CM, PreEM) in all cohorts. Data are presented as mean±sd. *$P<0.05$, **$P<0.01$.

3.4. Comprehensive analysis of expression of CD38, HLA-DR, CD226, CD39, PD1 on circulating T cells in CHB.

Costimulatory and coinhibitory molecules presented on the T cell surface play an important role on modulating the activation of T cells, as well as the development of T cell tolerance. Better understanding of these molecules could improve the treatment for certain diseases, such as to enhance tolerance in autoimmune diesease and grafting or break tolerance in infectious disease and cancer, from the perspective of pharmacology[207–209]. Therefore, the expression of the following activation/exhaustion markers (CD38, HLA-DR, CD226, CD39, PD1, CTLA4) on different T cell subsets in periferal blood were analyzed in a cohort of CHB patients. Among them, expression of CTLA4 in T cell subsets was too low to be analyzed. CTLA4 frequency of CD8 T cells, CD4 nonTreg and Tregs were only 0.035±0.039%, 0.052±0.042% and 0.151±0.271% respectively. More than 50% of all samples displayed a CTLA4 frequency of (nearly) zero within the other T cell subsets. Therefore, CTLA4 was not analyzed further.

3.4.1. Higher expression of PD1 on Tregs in CHB patients.

Among Tregs and its subpopulations the expression of activation markers (CD38, HLA-DR, CD226, CD39) and exhaustion markers (PD1), only the PD1 MFI, but not the frequency, was higher on $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs in the CHBn group (median 1415.73, 25%-70% range 1104.35-2365.96) and the CHBt group (median 1485.44, 25%-70% range 1090.59- 1946.96) compared to healthy controls (median 957.59, 25%-70% range 842.84-1174.11). On Treg subpopulations, a higher expression of PD1 was observed in aTregs in the CHBt group (median 1026.76, 25%-70% range 853.98-1210.03) compared to healthy controls (median 827.90, 25%-70% range 724.86-975.07, p=0.058593), howerver difference was not statistically significant. No difference of Tregs on the expression of PD1 was found between the CHBn and CHBt groups. See **Figure 3.5**.

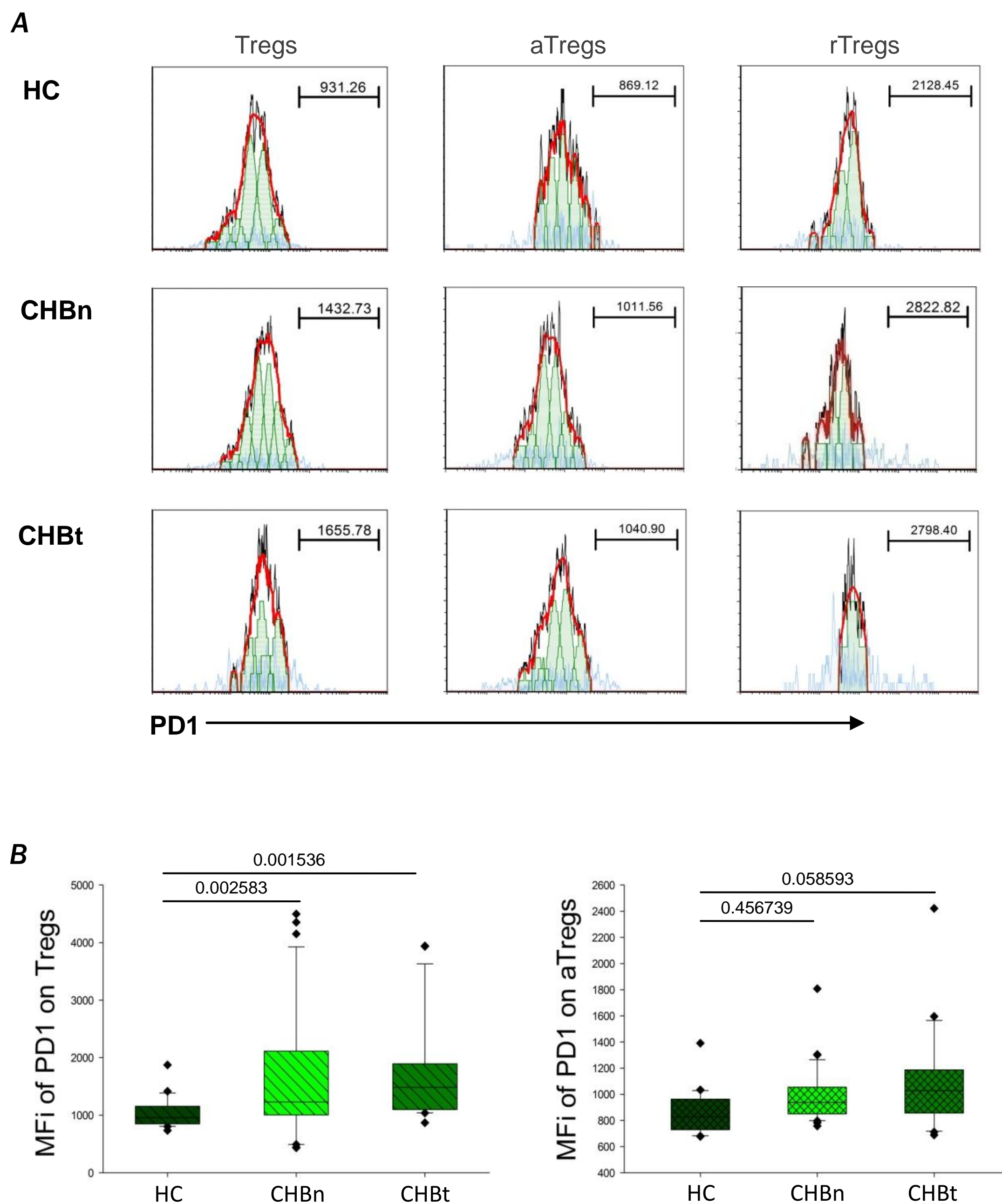


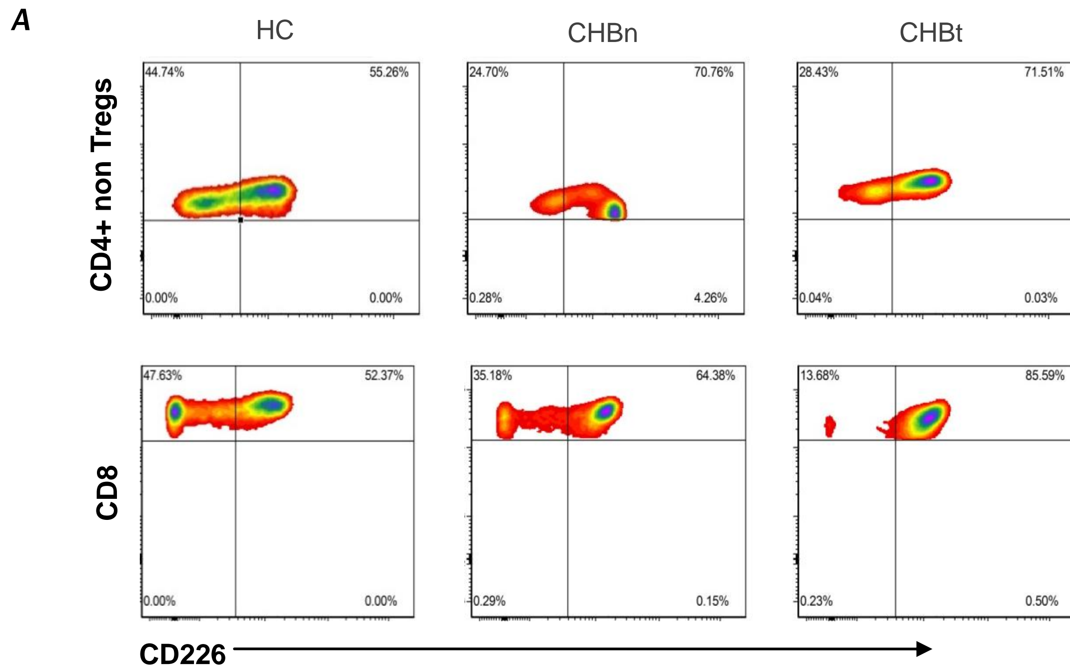
Figure 3.5. Comprehensive analysis of expression of PD1 on CD4⁺ CD25⁺ FOXP3⁺ Tregs, aTregs and rTregs in PBMCs of CHB patients. (A) Representative FACS histogram of the MFI of PD1⁺ cells within CD4⁺ CD25⁺ FOXP3⁺ Tregs, aTregs and rTregs in HC, CHBn and CHBt groups. (B) Comparison of PD1 MFI within CD4⁺ CD25⁺ FOXP3⁺ Tregs and aTregs in HC, CHBn and CHBt groups. Data is presented as median and 25%-75% rang. Tregs, CD4⁺ CD25⁺ FOXP3⁺ Tregs. HC, healthy controls. CHBn, chronic hepatitis B patients treatment naïve. CHBt, chronic hepatitis B patients under therapy.

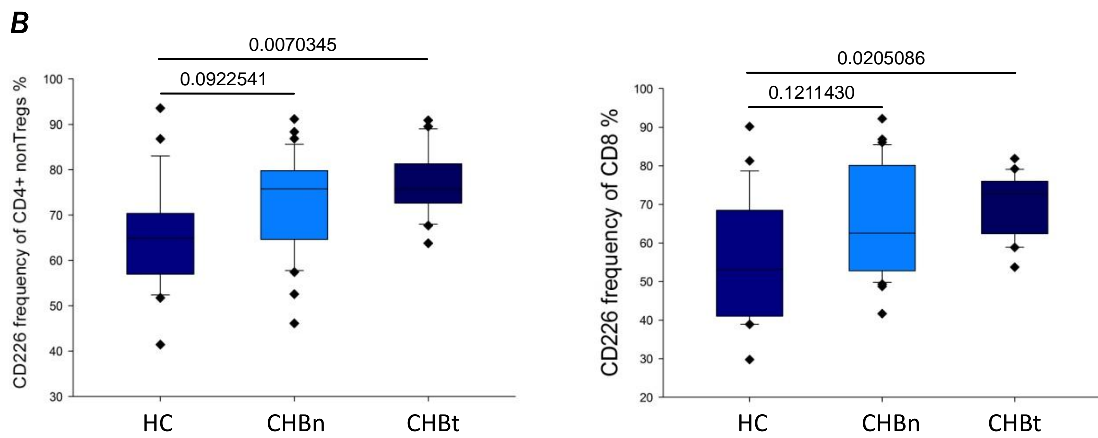
Previous studies showed a different expression of CD39$^+$ Treg cells in periferal blood of patients with different stages of chronic HBV infection (asymptomatic HBV carrier, chronic active hepatitis B, acute-on-chronic liver failure and liver cirrhosis) compared to healthy controls[210,211]. In this study, CD39 frequency and MFI of Tregs and its subphonotypes showed no difference in peripheral blood among CHBn, CHBt and HC groups. Frequency of CD39$^+$ Tregs were 69.88±23.97%, 72.53±21.51% and 62.87± 29.24% (F=0.799, p=0.499), MFI were 1577.46±581.87, 1846.30±906.49 and 1648.99±701.58 (F=0.634, p=0.596) in CHBn, CHBt and HC cohorts respectively. No significant difference of CD38$^+$, CD226$^+$ and HLA-DR$^+$ Tregs was observed among all groups. (data not shown)

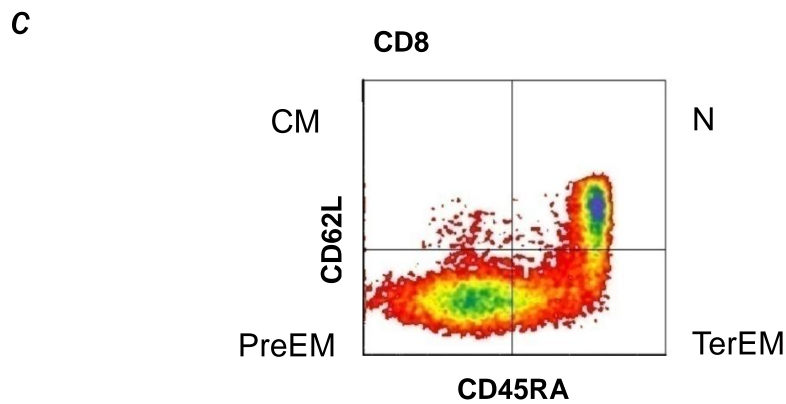
3.4.2. Higher expression of CD226 on CD4 nonTreg cells and CD8 T lymphocytes in peripheral blood of CHB patients.

The surface expression of CD226 molecules on CD4 and CD8 T lymphocytes and their subsets was determined by flow cytometry with various cell-specific surface markers. CD226 frequencies of CD4 nonTreg cells (median 75.67%, 25%-70% range 72.24%-82.71%) and CD8 T lymphocytes (median 72.82%, 25%-70% range 61.79%-74.47%) in the CHBt cohort were higher than in healty controls (median CD4 nonTreg 64.89%, 25%-70% range 56.63%-71.30%, p=0.0070345; median CD8 T 53.12%, 25%-70% range 40.80%-69.41%, p=0.0205086). No such difference was detected in the CHBn cohort (median CD4 nonTreg 75.74%, 25%-70% range 64.33%-79.90%, p=0.0922541; median CD8+ T 62.55%, 25%-70% range 52.58%-80.17%, p=0.1211430) compared with healthy controls. See **Figure 3.6. A, B**.

We furher analyzed the expression of CD226 on CD8 T cell subsets (CD8N, CD8CM, CD8PreEM and CD8TerEM). Higher CD226 frequencies of CD45RA$^+$ CD8 T cells, CD8N (median 62.27%, 25%-70% range 47.38%-75.31%) and CD8TerEM (median 79.60%, 25%-70% range 66.58%-84.23%), were observed in the CHBt group compared with healthy controls (median CD8N 36.05%, 25%-70% range 29.93%-57.41%, p=0.0199018; median CD8TerEM 55.69%, 25%-70% range 40.80%-69.20%, p=0.0168195). See **Figure 3.6. C, D,E**.


A
HC
CHBn
CHBt
CD4+ non Tregs
44.74%
55.26%
0.00%
0.00%
24.70%
70.76%
0.28%
4.26%
28.43%
71.51%
0.04%
0.03%
CD8
47.63%
52.37%
0.00%
0.00%
35.18%
64.38%
0.29%
0.15%
13.68%
85.59%
0.23%
0.50%
CD226


B
0.0070345
0.0922541
CD226 frequency of CD4+ nonTregs %
0.0205086
0.1211430
CD226 frequency of CD8 %
HC
CHBn
CHBt


C
CD8
CM
N
CD62L
PreEM
TerEM
CD45RA

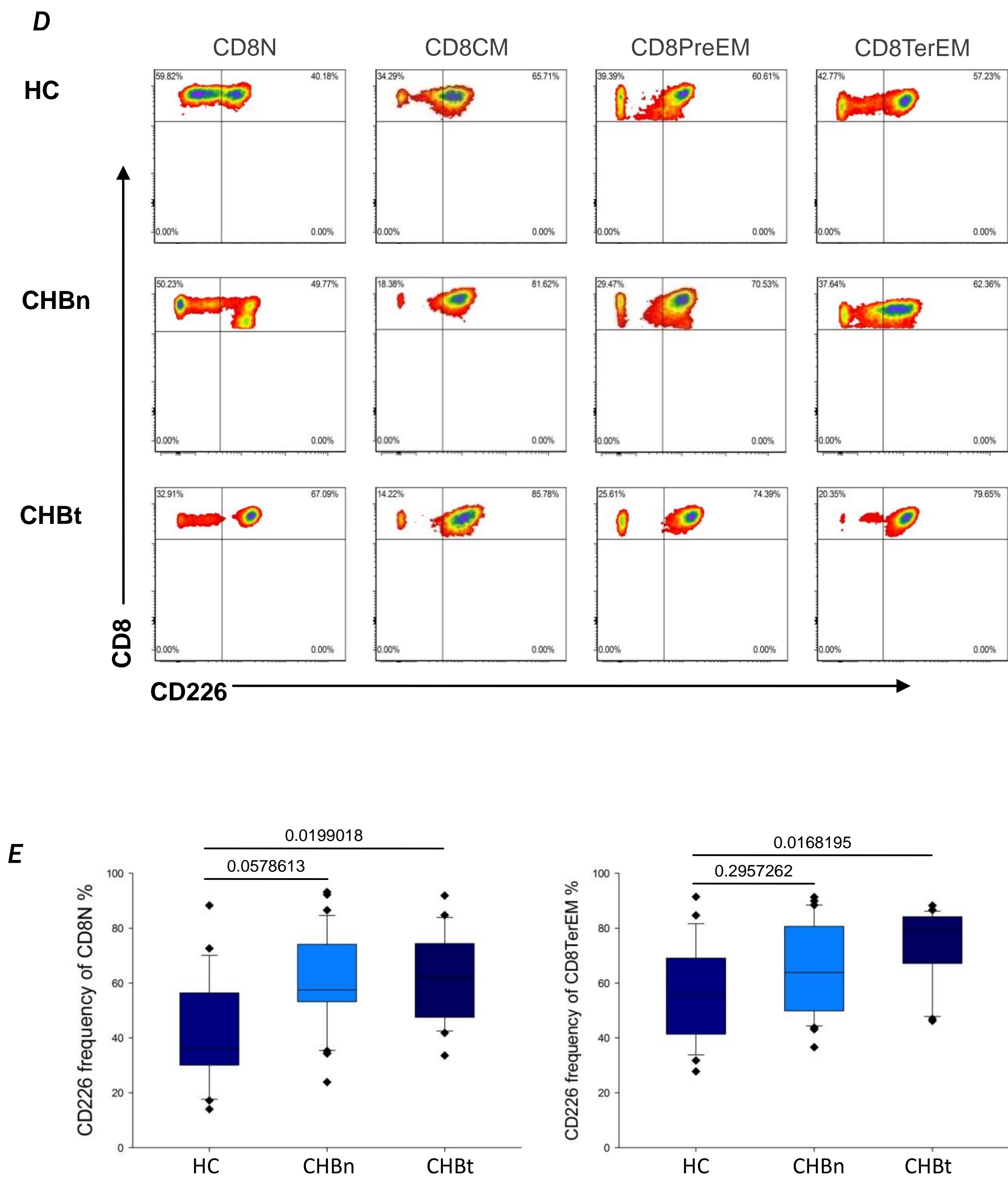
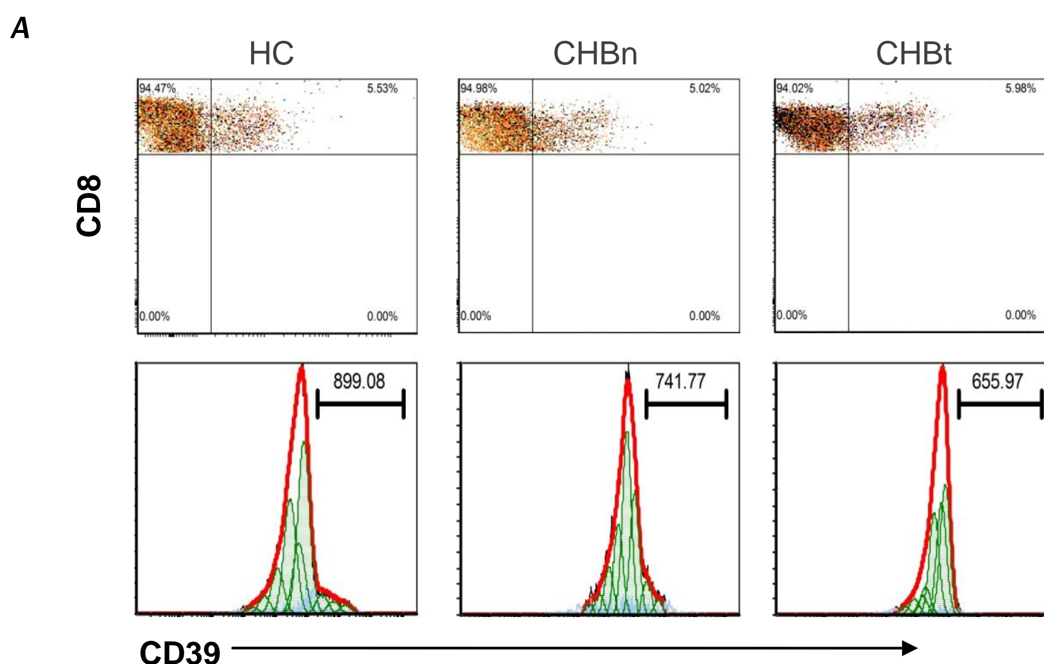


Figure 3.6. Comprehensive analysis of expression of CD226 on CD4 nonTregs, CD8 T cells and its subpopulations in PBMCs of CHB patients. (A) Representative FACS density plots of CD226 frequencies of CD4 nonTregs and CD8 T cells in all cohorts. (B) Comparison of CD226 frequencies of CD4 nonTregs and CD8 T cells in the HC, CHBn and CHBt groups. (C) Representative FACS plots displaying the gating strategy for CD8N, CM, PreEM and TerEM within the CD8 population. (D) Representative FACS plots of CD226 expression on CD8N, CM, PreEM and TerEM. (E) Comparison of CD226 frequencies of CD8N, TerEM in all cohorts. Data are presented as median and 25%-75% rang. N naïve cells. CM central memory cells. PreEM preterminally differentiated effector memory cells. TerEM terminally differentiated effector memory cells.

3.4.3. Lower expression of CD39 on CD8Pre/TerEM T cells in peripheral blood of CHB patients.

In peripheral blood, the frequencies of CD39 positive CD8 T cells and its subsets were no significantly different among CHBn, CHBt and HC groups (all p>0.05). However, compared to the HC cohort (MFI CD39 median CD8 T 943.66, 25%-70% range 742.56-1517.27; median TerEM 560.35, 25%-70% range 459.45-911.03; median PerEM 625.91, 25%-70% range 537.60-859.21), the expression of CD39 on both total CD8 T cells (MFI median CHBn 713.74, 25%-70% range 570.72-820.00, p=0.01472; median CHBt 670.62, 25%-70% range 457.61-830.48, p=0.01239) and CD8TerEM (MFI median CHBn 439.59, 25%-70% range 393.97-487.00, p=0.001216; median CHBt 651.91, 25%-70% range 440.46-1518.05, p=0.01305) were significantly lower in the two CHB groups. Meanwhile a lower MFI of CD39 on CD8PreEM was observed in CHBn cohort (median 503.73, 25%-70% range 441.44-589.96, p=0.04393) compared with healthy controls. They were not significantly different between CHBn and CHBt groups. See **Figure 3.7**.

CD38, HLADR and PD1 as both frequency and MFI showed no significant difference on CD4 nonTregs, CD8 and its subsets among all groups. (data not shown)



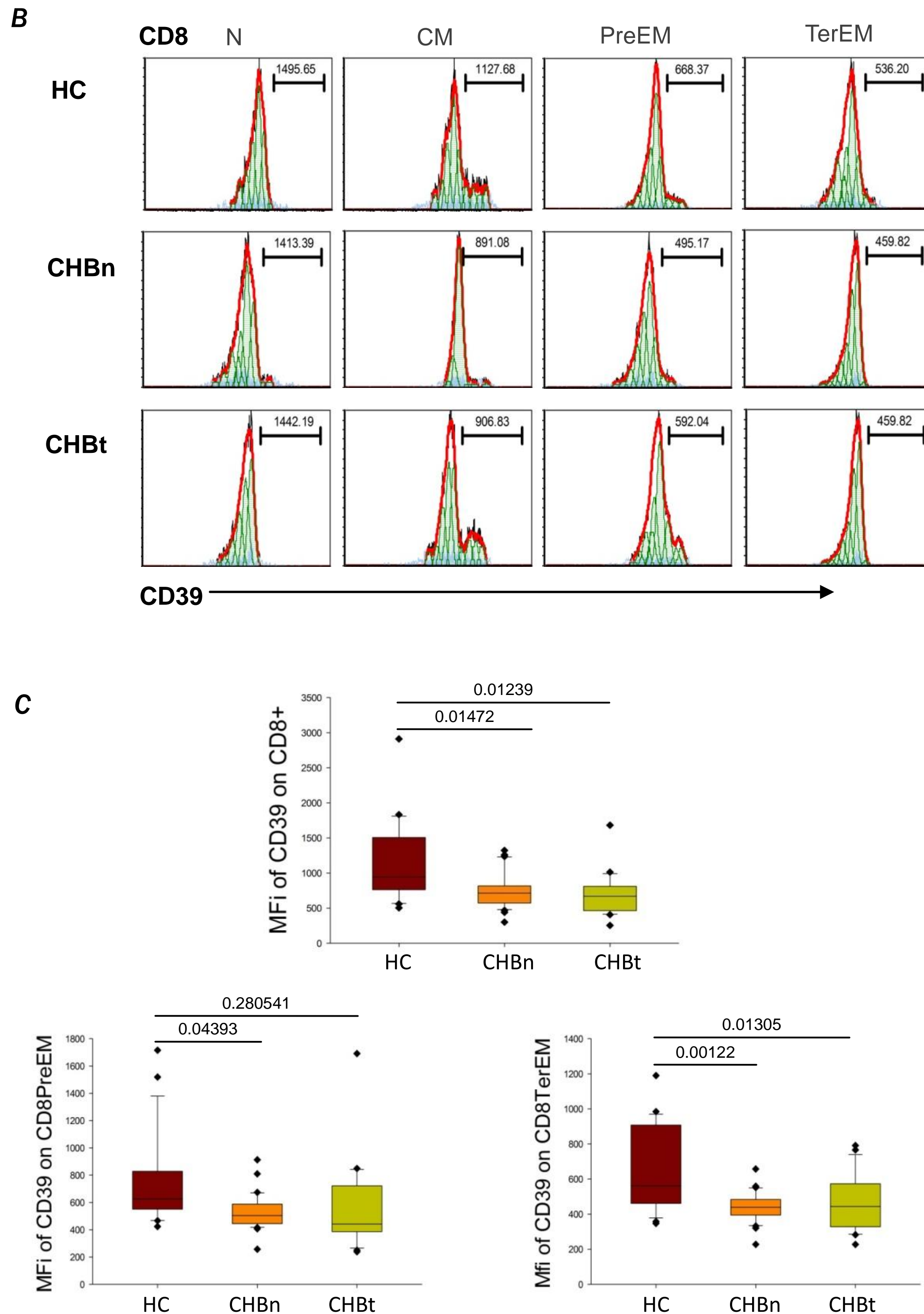


Figure 3.7. Comprehensive analysis of CD39 expression on CD8 T cells and its subpopulations (N, CM, PerEM and TerEM) in PBMCs of CHB patients. (A) Representative FACS dotplots and histogram of CD39 as frequency and as MFI within CD8 T cells in all cohorts. (B) Representative FACS histogram of CD39 as MFI within subpopulations of CD8 T cells in all cohorts.(C) CD39 as MFI within CD8 T cells and its subpopulations in HC, CHBn and CHBt groups. Data are presented as median and 25%-75% rang. HC, healthy controls. CHBn, chronic hepatitis B treatment naïve. CHBt, chronic hepatitis B under therapy.

3.5. Correlations between HBV DNA replacation and T cell subsets in treatment naïve CHB patients.

Among T cells and their subpopulations, including CD8 and its subsets CD8N, CD8CM, CD8PreEM, CD8TerEM T cells and CD4 non-Tregs, $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs and its subsets TregN, TregCM, TregPreEM, TregTerEM cells and rTregs, aTregs, non-Tregs, the frequencies of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs (r=0.47, p=0.042), and rTregs (r=0.47, p=0.0429) were correlated with HBV DNA serum levels in CHBn group. See **Figure 3.8**. No significant correlation was observed in CD8 and CD4 non-Tregs with HBV viral loads in the peripheral blood of the CHBn group (all p>0.05).

Correlation analysis between ALT serum levels and the above T cell subsets was performed in CHBn group as well. The results showed no significant correlation between the frequencies, MFI of above T cell subsets with ALT serum levels (all p>0.05).

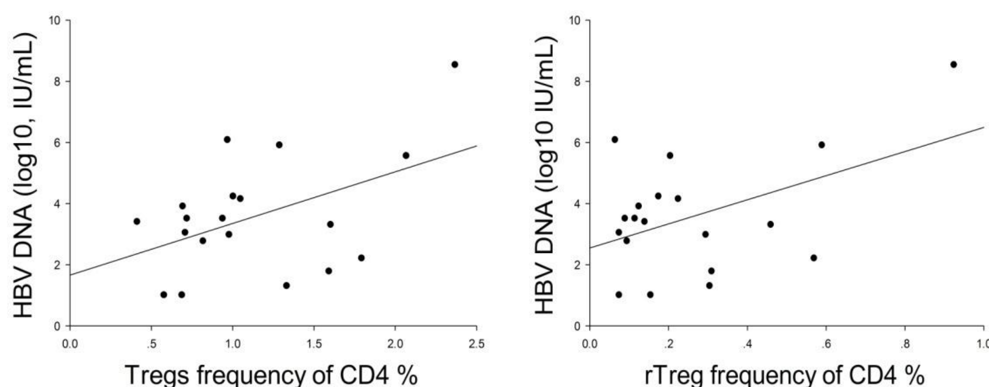


Figure 3.8. Correlation analysis of HBV DNA serum concentration with T lymphocytes and its subsets in PBMCs of CHBn patients. Serum HBV viral loads are positively correlated with Treg (r=0.47, p=0.042) and rTreg frequencies of CD4 T cells (r=0.47, p=0.0429) in CHBn cohort. Treg $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells. rTreg resting regulatory T cells.

3.6. Correlations between serum HBV DNA levels and the expression of activation/exhaustion markers on T cells in CHBn patients.

Several previous studies demostrated that in chronic HBV infection not only the quantities, but also the qualities of CD8, CD4 T cells and Tregs may decrease because of the high frequency of expression of costimulatory/coinhibitory molecules.

We analyzed the correlation between HBV viral loads and the expression of activation markers (CD38, HLA-DR, CD226, CD39) and the exhaustion marker (PD1) on T cells and its subpopulations in the CHBn group. As shown in **Figure 3.9-10**, the expression levels of CD38 and PD1 were significantly increased with the increasing replication of HBV. Previous studies showed that CD39 might be a sensitive marker of the suppressive function of Tregs and $CD39^+$ Tregs associated with the extent of liver injury [211,210]. Our study has demostrated that the expression of CD39 on CD8, CD8PreEM and CD8TerEM T cells were decreased in CHBn group compared to healthy controls (see above **3.4.3**.). We observed, however, no significant correlation between the expression of CD39 on CD8, CD8PreEM, CD8TerEM T cells and Tregs with HBV DNA loads in peripheral blood (all $p>0.05$).

Meanwhile, we also carried out a correlation analysis between ALT serum levels and the expression of the above markers on T cells in CHBn group. No significant correlation was demostrated between both the frequencies and MFI of the above markers with ALT serum levels (all $p>0.05$).

3.6.1. Positive correlation between HBV DNA levels and CD38 frequencies.

CD38 is a marker for continuous T cell activation and cytokine production. Therefore, the expression of CD38 on CD8 T cells was concerned as a marker for ongoing viral replication. Although HBV DNA levels have not shown strong correlation to the frequency of CD38+ CD8 T cells ($r=0.42$, $p=0.0768$), a significant positive correlation between HBV DNA levels and $CD38^+$ CD8PreEM T cells ($r=0.61$, $p=0.0055$) was identified in the CHBn group. See **Figure 3.9. A**.

As shown in **Figure 3.9. B**, CD38 frequencies on Tregs, especially on aTregs (r=0.57, p=0.010) and effector memory Tregs (PreEM r=0.46, p=0.048; TerEM r=0.46, p=0.046), were positively correlated with HBV serum loads.

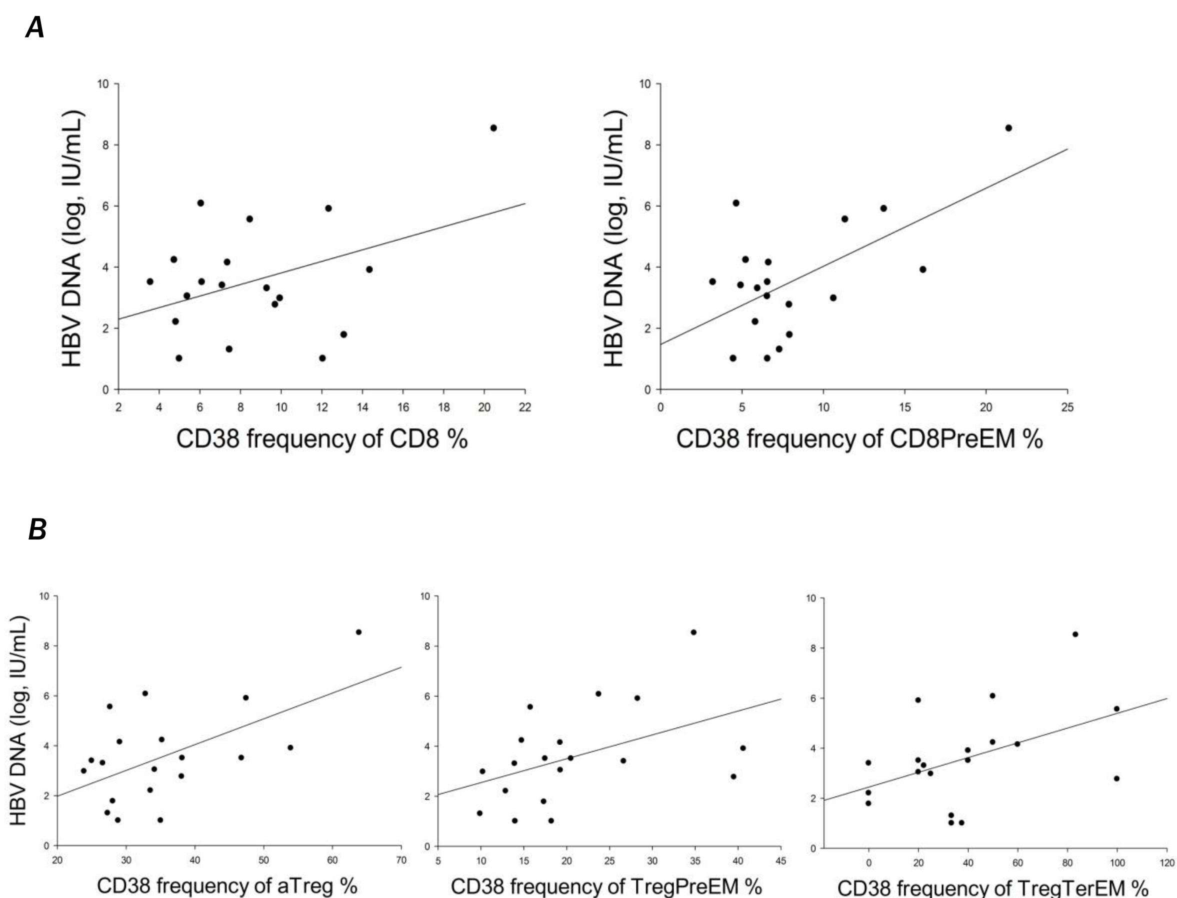


Figure 3.9. Correlation analysis of HBV DNA serum concentration with costimulatory molecules on T lymphocytes and its subsets in PBMCs of CHBn patients. Serum HBV viral loads are positively correlated with CD38 frequencies of (A) CD8 (r=0.42, p=0.0768), CD8PreEM (r=0.61, p=0.0055) and of (B) regulatory T cell subsets (aTregs r=0.57, p=0.010; TregPreEM r=0.46, p=0.048; TregTerEM r=0.46, p=0.046) in the CHBn cohort.

3.6.2. Positive correlation between HBV DNA levels and frequencies of PD1+ aTregs.

Our study demostrated an up-regulation of PD1 expression on Tregs in the CHB group in comparision with healthy controls (see above **3.4.1.).** Frequencies of PD1+ aTregs and HBV DNA serum levels showed a positive correlation (r=0.46, p=0.049), but no correlation with ALT levels in peripheral blood (r= -0.119, p=0.628). See **Figure 3.10**.

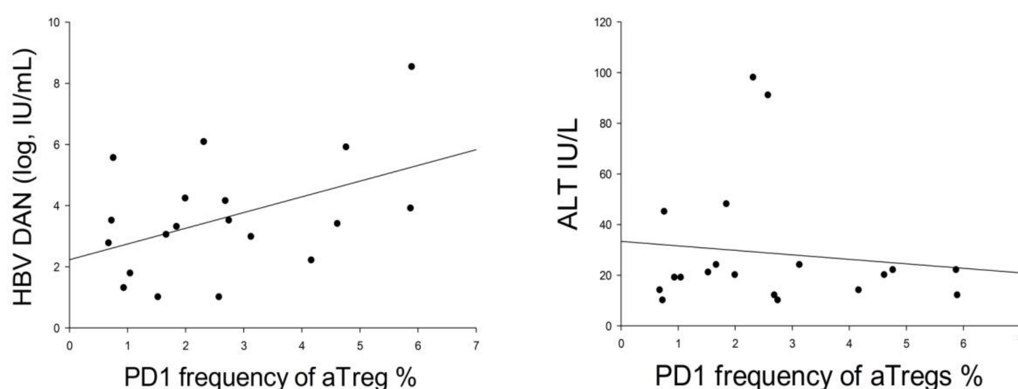


Figure 3. 10. Correlation analysis of HBV DNA serum concentrations and serum ALT levels with coinhibitory molecules on T lymphocytes and its subsets in PBMCs of CHBn patients. Serum HBV viral loads are positively correlated with PD1 frequencies of aTregs (r=0.46, p=0.049). Serum ALT levels are not correlated with PD1 expression (r= -0.119, p=0.628).

3.7. Correlation between T cell subsets and their expression of activation/exhaustion markers with serum 25(OH)D levels in CHBn patients.

Because of the immune modulatory effects of PEG-IFN and the direct antiviral effects of NAs[212,213], and their possible effects on vitamin D metabolism[214,215], the following correlations analyses were performed in treatment naïve CHB patients, and were compared with that in the HC group.

3.7.1. Correlation between T cell subsets and serum 25(OH)D levels.

We performed comprehensive correlation analysis of T cells (CD8 and its

subsets CD8N, CD8CM, CD8PreEM, CD8TerEM T cells and CD4 non-Tregs, $CD4^+CD25^+FOXP3^+$ Tregs and its subsets TregN, TregCM, TregPreEM, TregTerEM cells and rTregs, aTregs, non-Tregs) and serum 25(OH)D levels. The frequency of CD8 T cells and 25(OH)D serum concentrations showed a tendency of inverse correlation, but no statistically significant difference (r= -0.37, p=0.055) was observed. However, we observed a positive relationship between 25(OH)D serum levels and propotions of CD8N T cells (r=0.38, p=0.044) in the CHBn group. While no such correlation between 25(OH)D serum levels and propotions of CD8N T cells was shown in HC (r= -0.02, p=0.952). See **Figure 3.11**. No significant correlation was observed in the frequency of $CD4^+CD25^+FOXP3^+$ Tregs (r=0.1, p=0.6084) and rTregs (r= -0.09, p=0.6551) with serum 25(OH)D levels in peripheral blood, even though our study suggested that they were positively correlated with HBV DNA levels in CHBn group (see above **3.5.**).

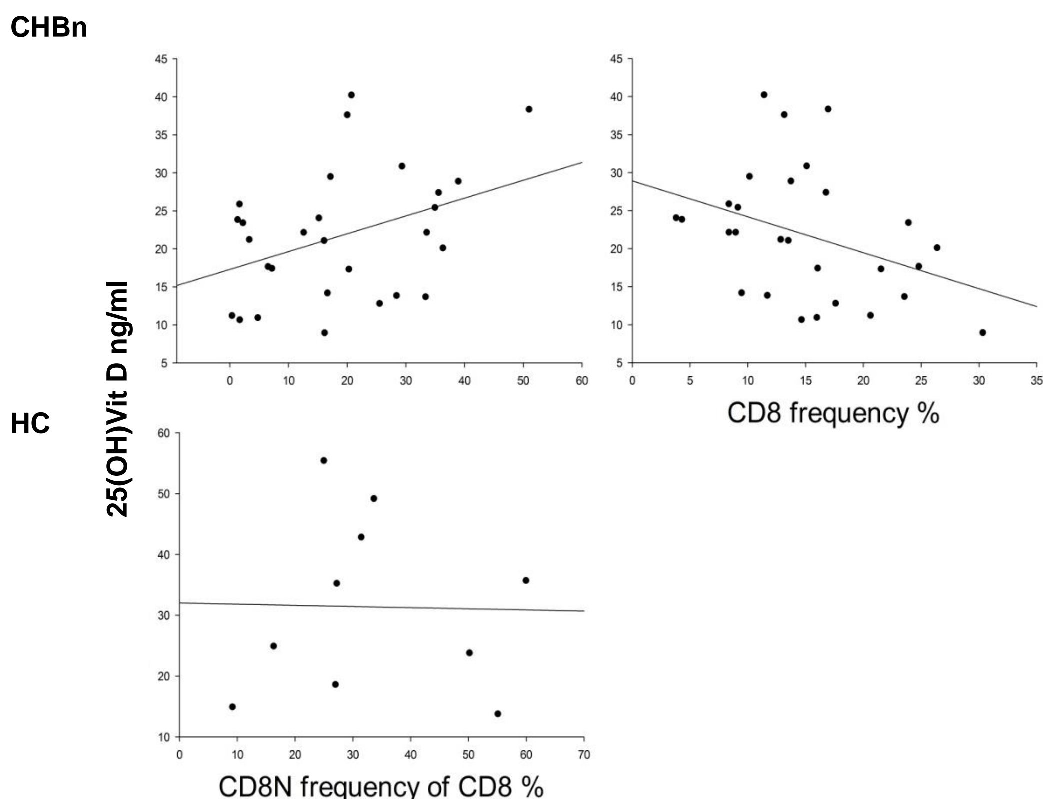


Figure 3.11. Correlation analysis of 25(OH)D serum concentration with T lymphocytes and its subsets in PBMCs of CHBn patients. Serum 25(OH)D levels are negatively correlated with CD8N frequencies of CD8 T cells in CHBn cohort (r=0.38, p=0.044), but not in healthy controls (r= -0.02, p=0.952). CD8N naïve CD8 T cells. HC healthy control. CHBn chronic hepatitis B treatment naïve.

3.7.2. Correlation between serum 25(OH)D levels and the expression of activation/exhaustion markers on T cells.

As an immunomodulatory agent, vitamin D was comprehensively studied in vast diseases. But the mechanism by which it benifits these diseases is still not well understood. Although there is a paper which demostrated that vitamin D level may be inversely correlated with HBV viral load. The relationship between vitaminD metabolism and CHB is still less well characterized. So far, the relation of serum vitamin D3 levels with the variation of activation/exhaustion markers on T cells have not been studied in patients with chronic HBV infection.

3.7.2.1. Expression of CD226 on CD8CM is negatively correlated to 25(OH)D serum concentrations.

Although a significant increase of CD226 expression on CD8 T cell subsets was observed in CHBt group, but not in the CHBn group, the expression of CD226 on CD8CM was still strongly associated with low 25(OH)D serum levels in the CHBn group (r= -0.48, p=0.009). While no such correlation was shown in HC (r= -0.46, p=0.178). See **Figure 3.12**.

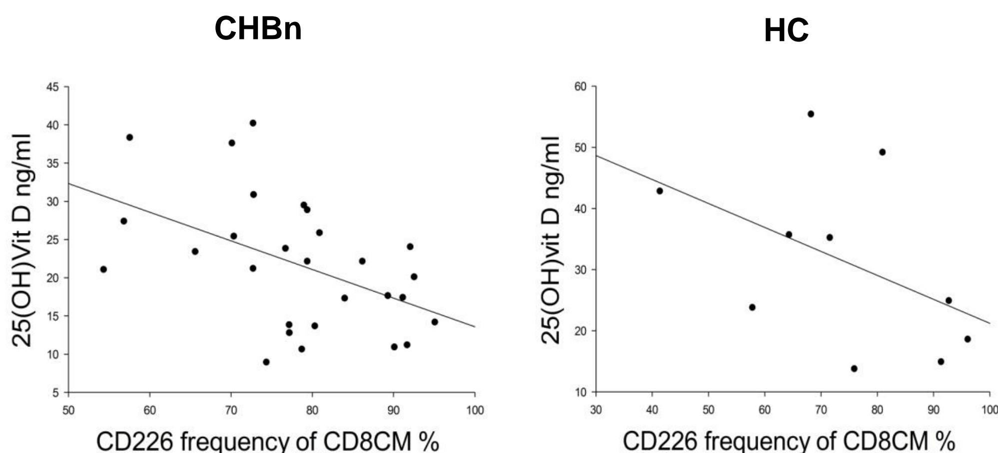


Figure 3.12. Correlation analysis of 25(OH)D serum concentration with costimulatory molecules on T lymphocytes and their subsets in PBMCs of CHBn patients. Serum 25(OH)D levels are negatively correlated with CD226 frequencies of CD8CM in the CHBn cohort (r= -0.48, p=0.009), but not in HC (r= -0.46, p=0.178). CD8CM central memory CD8 T cells.

Our study demostrated that neither CD38+ regulatory T cells (including aTregs, TregPreEM and TregTerEM) nor CD38+ non-Tregs (include CD8+ T cells and CD8PreEM cells), which were positively correlated with HBV DNA levels in the CHBn group (see above **3. 5.**), was correlated with serum 25(OH)D levels in peripheral blood (all p>0.05).

3.7.2.2. Positive correlation between 25(OH)D serum levels and the expression of PD1 on CD8PreEM T cells.

PD1 can broadly and efficiently inhibit the activation of T cells, thereby negatively regulating immune responses. In concord with the putative immunomodulatory function of vitamin D, PD1 frequencies of functional CD8PreEM was positively correlated with serum 25(OH)D levels in peripheral blood in CHBn patients (r=0.47, p=0.011). No significant correlation was found between 25(OH)D serum levels and PD1 both as frequency and as MFI in HC (r=0.31, p=0.383). See **Figure 3.13**.

Expression of PD1 on $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs and aTregs, which were identified as being positively correlated with HBV DNA serum levels and increased in CHB patients, did not correlate 25(OH)D serum levels (Tregs r= -0.25, p=0.2054; aTregs r= -0.16, p=0.411).

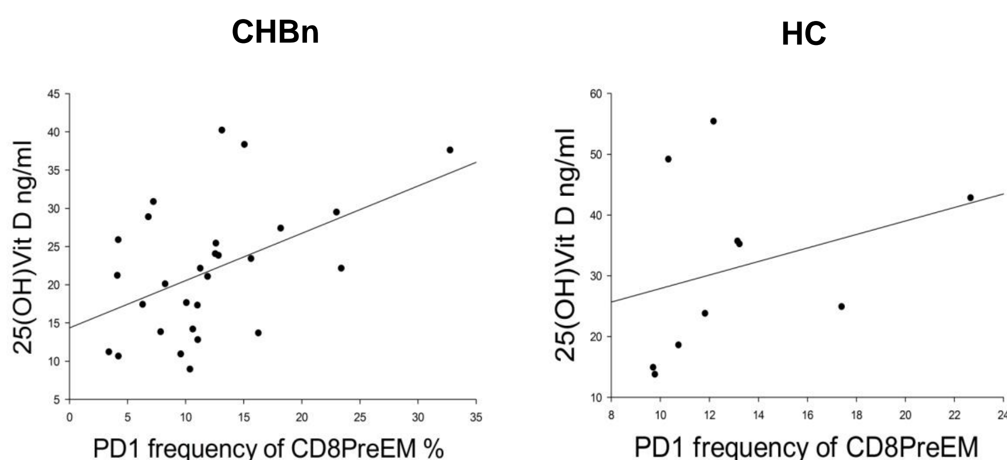


Figure 3.13. Correlation analysis of 25(OH)D serum concentration with coinhibitory molecules on T lymphocytes and its subsets in PBMCs of CHBn patients. Serum 25(OH)D levels are positively correlated with PD1 frequencies of CD8PreEM in CHBn cohort (r=0.47, p=0.011), but not in HC (r=0.31, p=0.383). CD8PreEM preterminally differentiated effector memory CD8 T cells.

3.7.2.3. 25(OH)D serum levels negatively correlated with HLA-DR expression on rTregs.

The direct effects of 1,25$(OH)_2D3$ on T cells is promoting the development of Th2 cells and Tregs. In contrast, in our study on 25(OH)D in patients with chronic HBV infection, we haven't found a widespread, significant relationship between 25(OH)D serum levels and Tregs as well as the activation /exhaustion markers on them. Only a inverse correlation between expression of an activation marker, HLA-DR, on rTregs and 25(OH)D serum levels was shown in peripheral blood (r= -0.47, p=0.012). No correlation was found between 25(OH)D serum levels and HLA-DR on Tregs both as frequency and as MFI in HC (r=0.24, p=0.507). See **Figure 3.14**.

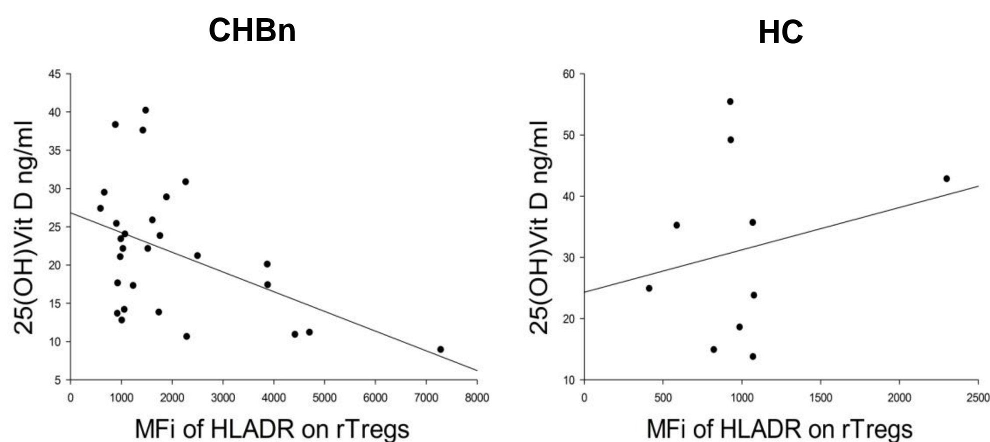


Figure 3.14. Correlation analysis of 25(OH)D serum concentration with costimulatory molecules on Tregs and its subsets in PBMCs of CHBn patients. Serum 25(OH)D levels are negatively correlated with HLA-DR as MFI within rTregs in CHBn cohort (r= -0.47, p=0.012), but not in HC (r=0.24, p=0.507). rTregs resting regulatory T cells.

3.8. No correlation was found between 25(OH)D serum concentrations and serum ALT, HBV DNA levels in peripheral blood of CHB treatment naïve patients.

Farnik et al[176] demostated, in both uni- and multivariate analyses, that there was a strong inverse correlation between 25(OH)D3 serum levels and HBV DNA viral loads in peripheral blood in treatment naïve CHB patients. However, our study did not display such a correlation. Serum 25(OH)D concentrations associated neither with HBV DNA serum levels (r=0.07, p=0.776) nor with ALT levels (r= -0.05, p=0.836) See **Figure 3.15**.

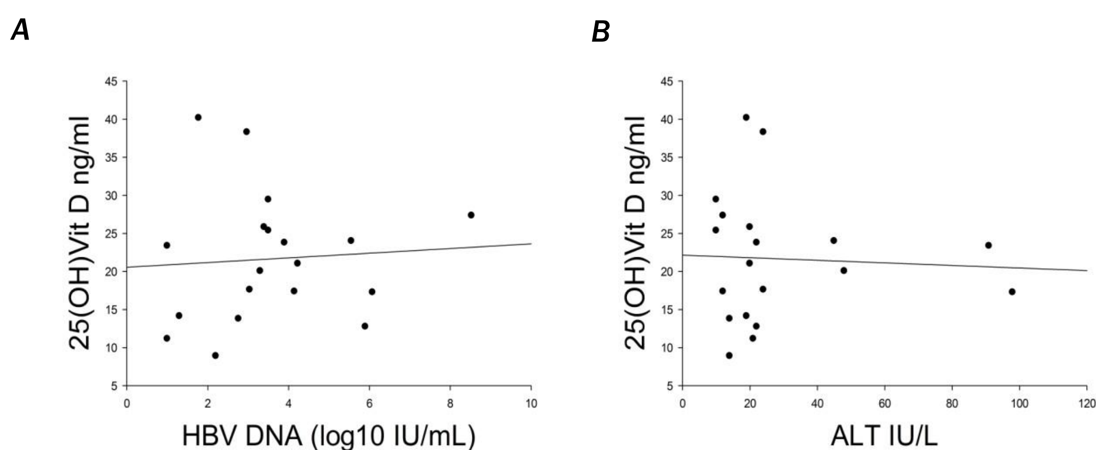


Figure 3.15. Correlation analysis of 25(OH)D serum concentration with serum HBV viral loads and ALT levels of CHBn patients. No correlation between serum 25(OH)D levels and HBV viral loads (r=0.07, p=0.776), between serum 25(OH)D levels and ALT levels (r= -0.05, p=0.836) in CHBn cohort.

4. Discussion

HBV infection is one of the most common public health concerns worldwide. Immune tolerance to HBV are the major host factors leading to HBV persistence. Tregs, costimulatory and coinhibitory receptors that are directly expressed on T cell may be a crucial mechanism responsible for the hyporesponsiveness of an antiviral immune system.

Recently Vitamin D has received particular attention as it has an unexpected and crucial interaction with both innate and adaptive immune responses. Previous studies displayed emerging roles of Vitamin D in Chronic hepatitis C both in vivo and in vitro, especially as a complementary medication for its antiviral and immunomodulatory perspectives. In contrast, data of vitamin D in HBV infection are scanty, not to mention researches on the relationship between vitamin D and immune responses in CHB.

4.1. Vitamin D insufficiency in patients with chronic HBV infection.

Vitamin D deficiency and insufficiency has recieved more and more attention as a healthy risk worldwide[216]. The critical function of vitamin D3 in bone health through modulating calcium and phosphorus metabolism is already well understood[217,218]. However, concerning the immunomodulatory effects of vitamin D, it is not surprising that more attention has been paied to vitamin D and autoimmune diseases, infectious diseases, cancer, diabetes and viral hepatitis[218–220]. Because of the important role of the liver in vitamin D metabolism, liver disease may, consequently, induce a series of vitamin D deficiency related syndromes by reducing vitamin D levels. Vitamin D related abnormal bone metabolism has also been reported in viral hepatitis[221–223] and liver cirrhosis[224–226]. Deficiency of vitamin D is reported common among patients with chronic hepatitis C[164].

The results of this study suggest that patients with chronic HBV infection are prone to vitamin D insufficiency. This is consistent with a past research by Farnik et al, where vitamin D deficiency was identified in hepatitis B patients, but not correlated with liver fibrosis[176]. Varying degrees of lower serum 25(OH)D levels were observed in hepatitis B patients with or without cirrhosis

in northeastern China[175]. These may suggest that the reason of low level 25(OH)D in CHB patients is more than just liver dysfunction. Moreover, no correlation was found between 25(OH)D serum concentrations and ALT levels in the CHBn cohort in our study. Therefore, we consider that, except for liver injury, there could be some other mechanisms leading to decreased vitamin D levels in HBV infection. In contrast to Farnik et al., our data did not show a significant correlation between 25(OH)D serum levels and HBV DNA replication. Instead we found relationships between 25(OH)D serum levels and adaptive immune cells as well as costimulatory molecules in the CHBn cohort, which did not appear in the HC group. This may suggest that viral replication does not directly affect the vitamin D metabolism, but acts through some kind of immune-mediated mechanism upon vitamin D levels.

4.2. Subpopulations of Tregs separated by the expression of FOXP3, CD45RA and CD62L.

Tregs, as major contributors to immune tolerance, play an important role in persistance of HBV infection. Therefore, an accurate systemic identification of Treg subpopulations and a comprehensive comparision of both their quantities and qualities might be usefull for a better understanding of their effects in HBV infection. CTLA-4, and HLA-DR are usually used as the phenotype markers for Treg cells. But in our study the expression of CTLA-4 on Tregs was too low to be analized. Therefore, we compared the expression of HLA-DR and CD39, which can be used as a marker of functional Tregs[210], on all Treg subpopulations to identify their different characteristics.

As shown in **Figure 3.3** and **3.4**, CD4 T cells were selected and further sorted by FOXP3, CD25, CD62L and CD45RA surface markers into $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs and its subpopulations, TregN, TregCM, TregPreEM and TregTerEM, and by FOXP3, CD45RA markers in PBMCs into rTregs, aTregs and non-Tregs. As shown in **Figure 3.3. C**, aTregs expressed the most HLA-DR and CD39 molecules, whereas rTregs and non-Tregs poorly expressed HLA-DR and CD39. These three $CD4^+$ $FOXP3^+$ T cell subsets were also identified by Miyara et al.[227]. aTregs were identified to be activated/effector Tregs, which play the main role in immune suppression and tolerance. However these died rapidly, whereas rTregs proliferated and

converted into aTregs to maintain the Treg pool. Similarly, higher expression of HLA-DR and CD39 were observed in $CD45RA^-$ Tregs (TregCM, TregPreEM) than $CD45RA^+$ Tregs (TregN, TregTerEM). $CD45RA^+$ Tregs, considered naïve Tregs, are more prevalent in early life, whereas CD45RO is expressed on 90-95% of all Tregs in the perifheral blood of adults, which were highly differentiated primed Tregs[228,229]. However, activated $CD45RA^-$ Tregs are sensitive to apoptosis and have the limitation of self-renewal, meanwhile, $CD45RA^+$ Tregs expand readily to converte to CD45RO expression $CD45RA^-$ Tregs in adults after activation, suggesting that $CD45RA^+$ Tregs, similar to rTregs, might contribute to maintening the Treg pool in adults[230,231]. Booth et al.[232] confirmed that Tregs expressing $CD45RA^+$ or $CD45RO^+$ display different preferntial migration to different tissues and considered that they might be radically different cells, but not the same cells at different stages of differentiation, by ascertaining there very distinct gene expression patterns. Together with a previous study, which has shown that only activated Tregs, but not naïve Tregs, can migrate to inflamed tissue and exert antigen-specific suppressive action effectively[233], we conclude that aTregs and $CD45RA^-$ Tregs represent the major immune suppressor.

The above results established a solid basis to classify Tregs into concrete Treg subtypes. Different from previous studies, our research was not conducted in general terms of the function of Tregs in chronic HBV infection, but based on a precise classification of Tregs to further clarify the changes in Treg subtypes in the chronicity of hepatitis B.

4.3. Dynamic of T lymphocytes in peripheral blood may not reflect the immunological mechanisms in persistence of HBV infection.

Whether the peripheral CD8 and CD4 T lymphocytes are decreased and Tregs are increased in chronic HBV infection remains controversial. In HBV infection, it is widely accepted that CD8 T cells are primarily responsible for the removal of viruses, whereas HLA-class II restricted CD4 helper-T cells are required for the development of functional efficient CD8 cytotoxic T lymphocytes and B cells[62]. Numerous previous researches found that both HBV-specific CD4 and CD8 T cells have not only declined in quantity but also in quality in chronic HBV infection compared to acute infection and non-infected individuls[234–237]. A

significant decrease of CD3, CD4 T cells, CD4:CD8 ratio and increase of CD8 T cells in peripheral blood in CHB patients was also reported[200]. In contrast, another study found, that CD8 T cells were sifnificantly decreased, and CD4:CD8 ratio was higher in CHB patients[238]. More controversial results regarding Tregs changes in chronic hepatitis B have been reported[89,239,240]. A likely reason for these contradictory results may be the determination of regulatory T cells.

There is no significant differences in circulating CD8, CD4 T cells and $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs frequencies among CHBn, CHBt and HC groups in our analysis. Similar results were obtained in another study by our group, which found no increase of Tregs in peripheral blood but in liver tissue of patients with chronic hepatitis C. A possible explaination for this discrepancy is that the kinetics of peripheral lymphocytes may not reflect the immune activities at the site of liver[241]. Since the liver is highly immunotolerant and the hepatic enviroment is quite different to that found in the circulatory system[242]. It is possible, that the frequencies of CD4, CD8 and Treg populations are not different in the circulation. Another underlying reason for the indistinct frequencies of CD8, CD4 T cells and $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs in the blood in hepatitis B patients and healthy controls might be that the quantitative variation of T cells in peripheral blood could not reflect the populations of HBV-specific T cells in HBV infection. As for the experiment itself, there were some defects in the selection of patients. Firstly, 7 patients showed viral loads lower than 1×10^3 IU/ml in the CHBn group. Secondly the clinical data of CHBt cohort were incomplete, for example the stage of HBV infection and HBV DNA levels before treatment were missing. This high variance of HBV viral load and missing of comparable clinical data may explain why we have shown neither a viral load associated T cell function failure in the CHBn cohort [197,200] nor an antiviral therapy related T cell restoration in the CHBt cohort[243].

In summary, the immnological mechanisms of HBV persistence are still not fully clear. The dynamic of T lymphocytes in peripheral blood may not explain the persistence of HBV infection. More focus should be placed on HBV-specific T cells and intrahepatic immunity.

4.4. Imbalanced expression of costimulatory/coinhibitory molecules on T cells may play a role in the persistence of HBV infection .

In addition to Tregs, the activation and tolerance of T lymphocytes are strictly regulated by APCs and costimulatory/coinhibitory molecules to restrict autoimmunity while foreign antigens are eliminated effectively[244,245]. Variances in the levels of some costimulatory/coinhibitory molecules were observed during different stages of CHB. As inhibitory molecules, PD1 and CTLA-4 were better understood both in phenotype and in different expression between intrahepatic T cells and peripheral T cells in HCV infection[246–248] than in HBV.

Our results displayed higher expression of PD1 on $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs and lower expression of CD39 on CD8 and its subtypes CD8PreEM/TerEM T cells in peripheral blood of CHB patients. These may indicate that the imbalanced expression of costimulatory/coinhibitory molecules on T cells may play a role in the persistence of HBV infection. In contrast to the inhibitory effect on virus-specific T cells, expression of PD1 on Tregs is required for maintenance and enhances the suppressive effect of Tregs[249,250], thereby contributing to immune tolerance. However, we did not see a increased expression of PD1 but an increased MFI in the CHB cohort. This could be explained by the different expression of costimulatory/coinhibitory molecules among intrahepatic T cells, virus-specific T cells and general peripheral T cells. Previous studies have shown higher PD1 expression on virus-specific T cells and intrahepatic HBV-specific CD8 cells[122,251]. Another study confirmed that the intrahepatic HBV-specific CD8 cells and their counterparts in peripheral blood are different phenotypes[252]. This could also explain why the increased levels of PD1 were not observed in total CD8 cytotoxic T cells in peripheral blood of the CHB cohort. Similarly, we have not observed an increase of CD39 levels on Tregs, even though $CD39^+$ Tregs were recently reported to be expanded in peripheral blood of asymptomatic HBV carriers but reduced in chronic active hepatitis B, which correlated positively with HBV DNA replication[210]. However, reduced CD39 expression in circulating CD8 T cells and their subtypes CD8PreEM/TerEM T cells in peripheral blood of the CHB cohort suggests that CD8 T cells are less activited in HBV infected patients compared to uninfected individuals. As

shown in **Figure 3.7**, no significant difference of CD39 expression on $CD45RA^-$ functional effector memory CD8 T cells was observed between the CHBt group and healthy controls suggest that the activity of $CD45RA^-$ functional effector memory CD8 T cells has been restored after treatment, but the result could also be distorted by individual samples that have higher CD39 MFI value.

Collectively, our study suggests probable changes of $PD1^+$ Tregs in the peripheral blood of patients with hepatitis B. We speculate that the variations in the MFI levels of PD1 and CD39 while the frequencies remain unchanged probably point to the fact that the major changes may take place on virus-specific T cells (including Tregs), which we will analyse further.

4.5. CD226 might be an indicator of immune response restoration.

CD226, also known as DNAM-1, was confirmed as an adhesion molecule implicated in CTL-mediated cytotocicity and lymphokine secretion[253]. An increased CD226 expression polarizes differentiation of CD4 T cells towards Th1 and enhances their activation and effector functions, while Th2 cells are down regulated[105]. However, in a study of DNAM-1 gene deficient mice with lymphocytic choriomeningitis virus (LCMV) infection, Welch et al. demonstrated that virus-specific CD4 T cells, in contrast to CD8 T cells, displayed significant elevations in expression of TNF-α and IL-2[107]. Therefore, the role of CD226 positive CD4 T cells in viral infection is still uncertain. CD226 is involved in differentiation and proliferation of naïve T cells, but previous studies focused only on CD4 T cells, whereas the effect of CD226 molecules on other T cell subsets was not investigated in chronic HBV infection.

In this study, we observed a significant increase in CD226 levels on CD4 nonTreg cells and CD8 T cells and its $CD45RA^+$ subsets (CD8N and CD8TerEM) in the CHBt group compared to healthy controls. Since all patients in the CHBt group were treated with NAs, the above results may indicate that CD226 might be an efficient indicator of immune restoration effected by NAs treatment. However, we also saw, as shown in **Figure 3.6**, a slightly elevated frequency of CD226 in the CHB treatment naïve group compared to the healthy cohort, even though these differences were not statistically significant. Since the role of CD226 in HBV infection poorly investiagted, we compared our

results with research on HIV infection, demonstrating significantly higher expression of CD226 on CD3, CD4, and CD8 T cells of HIV-infected patients than that of normal controls[254]. Another study on HIV and LCMV in humans and mice revealed that DNAM-1 did not express on PD1 high positive CD8 T cells and demonstrated a positive correlation between DNAM-1^{-}/PD1^{high} CD8 T cells and HIV-1 viral loads in both humans and mice[108]. However, a slightly increase of DNAM-1 expression on PD1^{+} CD8 T cells was reported in mice, meanwhile the levels of DNAM-1 expression on this T cell subset reduced in humans[108]. A possible reason for the discrepancies of the above reports, could be that under the stimulation of virus, the virus non-specific T cells are also activated and start to proliferate and differentiate, so that the levels of CD226 expression are increased in general T cells, but the absolute variance of CD226 on virus-specific T cells is unclear. Taken together, we concern that CD226 may play a role in the immune response to HBV infection and further investigation is needed to characterise CD226 in chronic HBV infection.

4.6. Both the quantity and quality of Tregs are responsable for the active replication of HBV.

Tregs have been extensively studied in hepatitis B[255–258] and C[259–261] and consistently reported playing an important role in immune tolerance, thereby contributing to viral persistence. We analyzed the correlation of Tregs and HBV viral loads in CHBn cohort, and further investigated the relationship between Treg subphonotypes and HBV viral loads. Our study showed a positive correlation between serum concentrations of HBV and CD4^{+} CD25^{+} FOXP3^{+} Tregs frequencies (among CD4 T cells) in peripheral blood. This result is supported by previous studies in CHB, which reported that both of CD4^{+} CD25^{high} Tregs and CD4^{+} CD25^{+} FOXP3^{+} Treg frequencies are positively correlated with serum viral load[262,263]. Morover, there was signifcant positive correlation between circulating rTreg frequencies and HBV DNA copies. As we discribed above, rTregs are very important in maintaining the Treg pool. This finding indicates, for the first time, that a higher level of HBV replication may induce the expension of rTregs, but not aTregs, which are rapidly dividing and prone to apoptosis[227,232].

To investigate the suppressive function of Tregs in chronic HBV infection in

more depth, acitvation/exhaustion markers (CD38, HLA-DR, CD226, CD39/PD1, CTLA4) were used to evaluate the quality of Tregs. Among all these markers, we found an elevated PD1 expression on aTregs, which also displayed an increased level of MFI in CHB patients, consistent with high levels of HBV replication. PD1, as an inhibitory molecule expressed on the surface of activated T cells, B cells, and macrophages, has a broader function in negatively regulating immune responses. Recent studies concentrated more on the PD1 expression of CD8 and CD4 effector T cells than on Treg PD1 expression in chronic hepatitis B[122,264]. Our results provide new insights into the mechanisms of Tregs in inducing immunotolerance in chronic HBV infection.

In summary, the positive correlations between HBV DNA levels and the frequencies of rTregs, $PD1^+$ aTregs in peripheral blood of CHBn patients suggest that in persistant HBV infection, a sufficient Treg pool ensures the quantity of functional aTregs, meanwhile the quality of aTregs is enhanced by increased PD1 expression improving the suppressive capacity.

4.7. CD38 is a marker of sustained viral replication in CHB.

As shown in **Figure 3.9**, we observed increased frequencies of CD38 on CD8 T cells and Treg subsets concomitant with elevated HBV DNA replications in CHBn patients. As a T cell activation marker, CD38 expresses nonlineage-dependently[265] and serves as a marker of T cell development. Normally, the circulating T cells express less CD38, whereas majority of T cell precursors in thymus and mature T cells in tissue are CD38 positive[266]. CD38 was reported increase in many acute or chronic infections involved in the pathogenesis and progression[267,268]. Based on the above theories, similar results were reported in studies of chronic hepatitis B, in which proportions of CD38+ CD8 T cells were higher in CHB treatment naïve patients, and declined after treated by nucleos(t)ide analogues[269,270]. Even though, in our reasurch, the correlation between HBV viral loads and CD38+ CD8 T cells is not statisticly significant (r=0.42, p=0.0768), a strong positive correlation between serum HBV DNA levels and CD38 frequencies of CD8PreEM was identified in our reasurch (r=0.61, p=0.0055). This result may conclude that the expression of CD38 on $CD45RA^-$ effector CD8 T cell subpopulation in peripheral blood

could reflect more precisely the abnormally activated T cell response in HBV immunity, thereby serving as a marker of progression and be used in prognosis of chronic HBV infection.

Interestingly, apart from identifying increased proportions of $CD38^+$ CD8PreEM T cells, our study has shown positive correlations between CD38 frequencies on Treg subsets, aTregs and CD62L negative Tregs (PreEM and TerEM), and HBV viral loads in peripheral blood of CHBn patients. The relationship between HBV DNA replication and circulating $CD38^+$ Tregs has not been investigated in previous studies. However, in contrast to the expression on CD8 T cells, Arasli et al. reported a significantly low CD38 frequency among CD4 T cells in CHB treatment naïve patients and further decreased percentage after tenofovir treatment[270]. Studies in celiac disease confirmed that CD38 expression was correlated with FOXP3, thereby reflecting a Treg phenotype[271]. Moreover, $CD62L^-$ $CD38^+$ T cells have been detected not only in small intestinal, but also in some other mucosal tissues such as human tonsils.[271] In addition, $CD62L^-$ $CD38^+$ $FoxP3^+$ CD4 Tregs expressed mucosal-philic characteristics in celiac disease[272]. Therefore, it is not surprising that we observed positive correlations between CD38 expression on aTregs and on CD62L negative $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs with HBV viral loads, which may suggest that increased CD38 molecules precipitate $FOXP3^+$ CD4 Tregs homing to the inflamed tissue, futher exacerbate intrahepatic immunotolerance, resulting in unscrupulously HB virus replication.

We have not observed any fluctuations of CD38 neither in frequency nor in MFI on all peripheral T cell subpopulations among the CHBn, CHBt and HC cohorts in our study. This could be an effect of patient selection, there are 7 patients whose HBV viral loads are lower than $1{\times}10^3$ IU/ml, which is sufficient to influence the median of CD38 frequency and MFI. Given the limitation of our study, the immunofunction of CD38 positive CD62L negative T cells in chronic HBV infection requires further confirmation.

4.8. 25(OH)D might influence CD8 T cells more than Tregs in peripheral blood of patients with CHB.

We declared a prevalence of vitamin D insufficiency in patients with chronic

hepatitis B in the present study, which was also observed in chronic hepatitis C. It was demonstrated by many studies on vitamin D in chronic hepatitis C, that vitamin D and/or its metabolites have an antiviral effect, possibly through amplifying the interferon signaling pathway[167–169]. Clinical data has also shown that low 25(OH)D serum levels were correlated with poor SVR achievement, whereas corrected 25(OH)D3 serum levels by supplement of vitamin D3 or calcitriol improved the achieving of SVR in IFN-based antiviral treatment[165,169,273,274]. A recent review analysed the complementary and alternative therapies in HCV infection and concluded that vitamin D could be the best complement of antiviral therapy in patients with vitamin D deficiency[275]. Although clinical and laboratory studies have confirmed the existence of a close relationship between vitamin D and hepatitis C, no explicit correlation between vitamin D serum levels and HCV replication was defined by correlation analysis in vivo. Farnik et al.[176] reported a significant association between 25(OH)D3 serum concentrations and HBV viral loads in treatment naïve patients with chronic hepatitis B. An association between normal vitamin D serum level and spontaneous HBsAg seroclearance was mentioned in a group of chronic inactive hepatitis B patients[177]. Besides these reports, the clinical and laboratory data of vitamin D in chronic hepatitis B is extremely insufficient, compared with that in chronic hepatitis C.

Our ongoing reasearch on the effects of vitamin D and its metabolites on both HBV and HCV replication in vitro showed a dose-dependent suppression of vitamin D and its metabolites in HCV replication, but not in HBV replication (results are not published). In addition, we have not shown correlations between 25(OH)D serum concentrations and HBV viral loads, as well as ALT serum levels. Therefore, we consider that there is no direct correlation between 25(OH)D serum levels and HBV viral replication as well as intrahepatic inflammation. However, we can not completely exclude the existance of an effect of vitamin D on the outcome and prognosis of antiviral treatment in chronic hepatitis B patients with vitamin D deficiency. A possible reason for the discrepancy between the effect of vitamin D in HCV infection and that in HBV may lie in their different structure and replication. As HBV is a DNA virus, the replication of its genome takes place in host cell nucleus, where the virus can complete its replication entirely relying on the host's DNA and

RNA synthesis maschinery. Whereas, HCV is a RNA virus, whose genetic information is stored in RNA. Therefore, the replication of HCV usually occurs in the cytoplasm and is characterized by the usage of its own RNA replicase to replicate the genome. However, it was reported that with respect to the life cycle of HCV the inhibition by 25(OH)D3 took place at the virus assembly step[167].

Consistent with the immunomodulatory function of 1,25$(OH)_2$D3, our study displayed a reverse correlation between 25(OH)D serum levels and the frequencies of CD8 T cells and CD226+ CD8CM cells. Furthermore we found a positive correlation between 25(OH)D serum levels and the frequencies of CD8N T cells as well as the expression of PD1 on CD8PreEM in peripheral blood of the CHBn group. Whereas, within Tregs, only negative correlation between 25(OH)D serum levels and the proportion of HLADR+ rTregs was found. These findings again confirm the suppressive immunomodulatory effect of adaptive immune responses. However, it is noteworthy that increased 25(OH)D levels are not accompanied by significantly elevated Tregs in treatment naïve patients with CHB. This contradicts the theory that 1,25$(OH)_2$D3 promotes dramatic differentiation of Tregs under normal circumstances, in autoimmune disease or in Graft-versus-host disease. This may suggest that vitamin D might trigger different regulatory mechanisms in immunotolerance diseases and immuno-hyperactive diseases. In hepatitis B, 1,25$(OH)_2$D3 may inhibit the cytotoxic CD8 T lymphocytes more than Tregs, thereby limiting the cytotoxicity on hepatocytes and activation-induced cell death of other immune cells by highly activated CD8 T cells, meanwhile preventing suppression of some innate immune cells, such as NK and APCs, induced by Tregs[249]. Another study suggests that 1,25$(OH)_2$D3 might influence the $CD4^+$ $Foxp3^-$ T cells more than the $CD4^+$ $Foxp3^+$ Tregs in experimental autoimmune encephalomyelitis[276]. Interestingly, Essen et al. reported that vitamin D activates human T cells by controlling T cell antigen receptor signaling[277], which was refuted immediately by Smolders et al.[278].

In conclusion, the relationship of vitamin D and outcome of hepatitis B is still ambiguous. Vitamin D may benefit CHB patients not through regulating adaptive immune responses but through enhancing the innate immune

responses while also protecting the infected tissue against immune attack.

To the best of our knowledge, this study was the frist to investigate the immunomodulatory function of vitamin D on the expression levels of CD38, HLA-DR, CD226, CD39 and PD1, CTLA4 on the T cells of patients with CHB. We obtained some results supported by previous studies, furthermore comprehensively analyzed different activation/exhaustion markers on T-cell subtypes, such as PD1+ Tregs and $CD62L^-$ $CD38^+$ $FoxP3^+$ CD4 Tregs, in chronic HBV infection. Additionally, several limitations in our study should be pointed out. Patient selection and incomplete clinical data may have distorted the results. Additionally, experiments were performed only with samples from peripheral blood but not from liver tissue. However, the description of vitamin D deficency in the CHB cohort and the different influences of vitamin D on effector T cells and Tregs in different diseases may provide a new way of thinking about the relationship between vitamin D and hepatitis B.

5. Summary

To analyze the profile of peripheral blood T lymphocyte subpopulations and serum vitamin D levels in chronic hepatitis B patients, 45 out-patients with CHB (treatment naïve, n=28; under treatment, n=17) 18 healthy individuals were selected aimed at comprehensive analyzing the peripheral blood T lymphocyte subpopulation profile in CHB patients, and to assess their function by measuring their expression of activation/exhaustion markers (CD38, HLA-DR, CD226, CD39/CTLA4, PD1) using flow cytometry. Further investigated serum vitamin D levels and its correlation with the expression of activation/exhaustion markers on T cells in chronic HBV infection. Among all the costimulatory and coinhibitory markers, we demonstrated an increased expression of PD1 on Tregs in CHB cohort, which is also positively correlated with HBV DNA replication, emphasized the important role of Tregs in persistance of HBV infection. Meanwhile, a reduction of costimulatory molecule, CD39, expression on effectory memory CD8 T cells suggested the balance between costimulatory and coinhibitory molecules is broken in CHB. A significant increase in CD226 levels on CD4 nonTreg cells and CD8 T cells and its $CD45RA^{+}$ subsets (CD8N and CD8TerEM) in CHBt group compared to healthy controls, suggested that CD226 might be a marker implies that the immune response start to restore. The increased frequencies of CD38 on CD8 T cells and Treg subsets concomitant with elevated HBV DNA replications in CHBn patients, consistent with the results of previous studies, emphasized that CD38 is a marker of sustained viral replication in CHB.

Moreover, we demonstrated a prevalence of vitamin D insufficiency in CHB patients, but no correlation between HBV viral loads and 25(OH)D serum levels, as well as ALT serum levels. Therefore, we consider that there is no direct correlations between 25(OH)D serum levels and HBV viral replication, intrahepatic inflammation. However, our study displayed a reverse correlation between 25(OH)D serum levels and frequency of CD8 T cells, proportion of CD226+ CD8CM, and a positive correlation between 25(OH)D serum levels and frequency of CD8N T cells, the expression of PD1 on CD8PreEM in peripheral blood in CHBn group. Whereas, within Tregs, only negative correlation between 25(OH)D serum levels and proportion of HLADR+ rTregs

was found. This may demonstrated that, in CHB vitamin D suppresses the activation of CD8 T cells more than increases Tregs, suggests at least vitamin D may perform an unique immunomodulatory function at the pressure of HBV.

For these conclusion, more studies in vivo are needed to investigate the replication of HBV DNA and T cell response incubated with vitamin D and its metabolites, in order to provide a theoretical basis for clinical studies of vitamin D application as a complementary therapy. Meanwhile, attention should also be placed in innate immune response in CHB, while vitamin D may protect infected hepatocytes from cytotoxic T cell induced cell death, whereas inhence the innate immunity to control virus.

6. Abbreviations

1,25$(OH)_2$D3	1,25-dihydroxyvitamin D3
25(OH)D	25-dihydroxyvitamin D
ALT	alanine aminotransferase
AMPs	antimicrobial peptides
APCs	antigen-presenting cells
AST	aspartate aminotransaminase
ATregs	actived Tregs
CccDNA	covalently closed circular DNA
CD8CM	central memory CD8 T cells
CD8N	naïve CD8 T cells
CD8PreEM	preterminally differentiated effector memory CD8 T cells
CD8TerEM	terminally differentiated effector memory CD8 T cells
CHBn	patients with chronic hepatitis B treatment naïve
CHBt	patients with chronic hepatitis B under treatment
CHC	chronic hepatitis C
COX	cyclooxygenase
CTL	cytotoxic T lymphocyte
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CYP	cytochrome P
DCs	dendritic cells
ddH_2O	doble distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

DNAM-1	DNAX accessory molecule-1
EBV	Epstein–Barr virus
ELISA	Enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FOXP3	forkhead box P3
GITR	glucocorticoid-induced TNF receptor
HBcAb	hepatitis B core antibody
HBcAg	hepatitis B core antigen
HBeAb	hepatitis B e antibody
HBeAg	hepatitis B e antigen
HBsAb	hepatitis B surface antibody
HBsAg	hepatitis B surface antigen
HBV	Hepatitis B virus
HBx	hepatitis B X protein
HC	healthy controls
HCC	hepatocellular carcinoma
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IFNγ	interferon gamma
IgG	immunoglobulin G
IL	Interleukin

IsCo	Isotypecontrols
ISGs	IFN-stimulated genes
KCl	Kalium chloride
KH_2PO_4	Kaliumhydrogenphosphat
LCMV	Lymphocytic choriomeningitis virus
MAPK	mitogen activated protein kinase
MFI	mean fluorescent intensity
MHC	major histocompatibility complex
Mrna	messenger ribonucleic acid
MxA	Myxovirus resistance protein A
NA	nucleoside/ nucleotide analogue
$Na_2HPO_4 \cdot 2H_2O$	Dinatriumhydrogenphosphat dihydrate
NaCl	Natriumchlorid
NaN_3	Sodium azide
NK cells	Natural Killer cells
non-Tregs	cytokine-secreting $CD45RA^-FOXP3^{lo}$ non-suppressive T cells
NTCP	sodium taurocholate cotransporting polypeptide
ORF	open reading frame
PBS	phosphate buffered saline
PBS	Phosphate Buffered Saline
PD1	programmed cell death protein 1
PEG-IFN	pegylated interferon
pg mRNA	pregenomic mRNA
PGs	prostaglandins
RBV	Ribavirin

RNA	ribonucleic acid
RPMI	Roswell Park Memoria Institute
rTregs	resting Tregs
Sd	standard deviations
ssDNA	single-strand DNA
SVR	sustained virologic response
TCR	T-cell receptor
TGFβ	transforming growth factor beta
Th	helper T cells
TLR	toll-like receptor
TNF	tumor necrosis factor
Tr1 cells	T regulatory type 1 cells
TregCM	central memory Tregs
TregN	naïve Tregs
TregPreEM	preterminally differentiated effector memory Tregs
Tregs	regulatory T cells
TregTerEM	terminally differentiated effector memory Tregs
TSH	thyroid-stimulating hormone
UV	Ultraviolet
VDR	vitamin D receptor
WHO	World Health Organization

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