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Recovery of NK(CD56+CD3-) Cells after One Year of Tenofovir Therapy for Chronic Hepatitis B Infection

Hwan Hee Lee[1,2†], Hyojeung Kang[3†], and Hyosun Cho[1,2*]

[1]*Department of Pharmacy, College of Pharmacy,* [2]*Innovative Drug Center, Duksung Women's University, Seoul 01369, Republic of Korea*
[3]*College of Pharmacy, Research Institute of Pharmaceutical Sciences and Institute for Microorganisms, Kyungpook National University, Daegu 41566, Republic of Korea*

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*Corresponding authors
Phone: +82-2-901-8678;
Fax: +82-2-901-8386;
E-mail: hyosun1102@gmail.com

†These authors contributed equally to this work.

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Natural killer (NK) cells have been reported to be dysfunctional in chronic hepatitis B (CHB) infection. However, the functional recovery of NK cells under antiviral therapeutic agents in CHB was not clearly understood. In this study, we investigated the phenotypic changes of NK(CD56+CD3-) cells in terms of their functional markers (CD16, NKG2A, NKG2D) during tenofovir therapy in CHB. The frequency of NK(CD56+CD3-) cells in CHB patients was significantly increased after 12 months of tenofovir therapy when compared with baseline. The expression levels of CD16+/CD56+CD3- and NKG2A+/CD56+CD3- cells were also affected by tenofovir treatment. In addition, there was a positive correlation between the proportion of NK(CD56+CD3-) cells and HBV DNA (log copies/ml) in CHB patients.

Keywords: Chronic hepatitis B, natural killer (NK) cells, tenofovir, CD16, NKG2A

Chronic hepatitis B (CHB) infection is associated with liver cirrhosis and hepatocellular carcinoma, which result in end-stage liver failure [1]. Even though many studies have reported that adaptive immunity seems to have more critical roles in the control of hepatitis B virus (HBV) infection [2–4], natural killer (NK) cells are the major effector cells in the innate immune defense against HBV infection [5]. Specifically, NK cells are known to compose about 40–60% of the intrahepatic lymphocytes in the liver, which is a main target of HBV [6].

NK cells contain granules that are capable of applying cytotoxic, antiviral activity through the release of perforin and granzyme [6]. Therefore, NK cells can target and eliminate virus-infected cells efficiently [7, 8]. The function of NK cells can be controlled by the expression of cell surface markers such as CD16, NKG2A, and NKG2D. CD16 is known to be an Fc receptor (FcγRIIIa or FcγRIIIb). CD16 initiates antibody-dependent cell-mediated cytotoxicity in NK cells by binding to the Fc portion of IgG antibodies. NKG2A is a C-type lectin receptor that mediates inhibition of NK cell activity. NKG2D is one of the CD94/NKG2 family proteins, which perform cytotoxic activities by recognizing MHC class I chain-related protein or RAET1/ULBP on the surface of transformed or infected host cells [9].

In HBV infection, NK cells play an important role in the initial stage of viral infection [10]. In addition, the impairment of NK cell function in chronic HBV infection has been reported [11]. Recently, numerous research groups have described an immunomodulatory function of NK cells, which suggests that NK cells may have a regulatory role on other immune cells [12–14].

Tenofovir (tenofovir disoproxil fumarate) is an adenosine nucleotide analog that specifically inhibits HBV reverse transcriptase. Tenofovir has been reported to be very effective against HBV and HIV-HBV co-infection [15, 16]. However, in the context of chronic HBV infection, only a few studies have investigated the effect of nucleotide analog treatment on the antiviral function of NK cells [17–20]. It is important to elucidate the mechanisms of NK cell effector functions in host innate immunity while under antiviral therapeutic treatment for the control of HBV infection. In this study, we investigated the phenotypic

profiles of NK cell populations in CHB patients during the first year of tenofovir therapy, and examined the relationship between the functional characteristics of NK cells and clinical parameters in tenofovir-treated CHB patients.

Patients were recruited from the Department of Internal Medicine of Uijeongbu St. Mary's Hospital (Korea). Informed consent was obtained from all study participants and the study was approved by the hospital's Institutional Review Board (IRB: UC13TISI0036). Two groups of study participants were evaluated: those with chronic hepatitis due to HBV infection (n = 19) and five healthy controls (n = 5). CHB was diagnosed on the basis of the presence of anti-HBV antibodies and hepatitis B surface antigen (HBsAg). All CHB patients received tenofovir for 48 weeks. Clinical characteristics of the CHB patients at T0 are presented in Table 1. The blood from CHB patients was drawn at baseline (T0), and at 1 (T1), 3 (T3), 6 (T6), and 12 (T12) months during the tenofovir therapy. PBMCs were isolated by Ficoll-Histopaque (Sigma Chemical, USA) density centrifugation and cryopreserved as described previously [21]. All monoclonal antibodies were purchased from BD Biosciences (USA) except for anti-NKG2A-PerCP from R&D Systems (USA) and anti-NKG2D (CD314)-APC from eBioscience (USA). PBMCs (1×10^6) from the patients were incubated with the fluorescent antibodies, washed three times, and then fixed with 2% paraformaldehyde (Sigma-Aldrich). The cells were then subjected to flow cytometry on a FACSCanto (Becton Dickinson, USA) and analyzed using FlowJo (Tree Star, USA), gating on live lymphoid cells based on forward and side scatter profiles, and 7-amino-actinomycin D staining. Compensations were

Table 1. Clinical characteristics of patients with chronic hepatitis B.

	Chronic hepatitis B (n = 19)	Healthy (n = 5)
Sex (M:F)	9:10	3:2
Age (years)[a]	51 (33-67)	32
AST*	62 (14-412)	20
ALT*	113 (14-829)	24
Total bilirubin[a]	0.89 (0.44-1.42)	0.8
HBV DNA (log copies/ml)[a]	7.2 (3.2-10.1)	NA
eAg status (positive:negative)	13:6	NA

[a]Median values (range).
AST, serum aspartate aminotransferase; ALT, serum alanine aminotransferase; HBV, hepatitis B virus.
NA, not applicable.

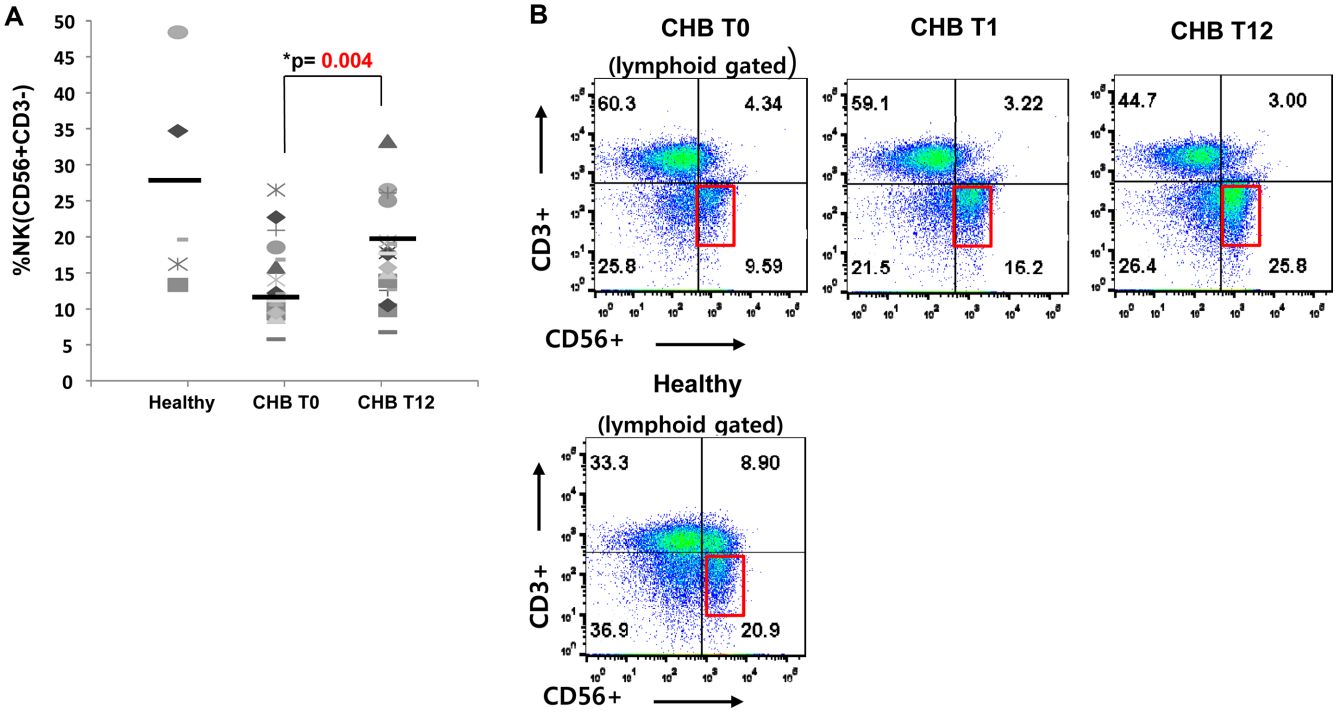

Fig. 1. Recovered frequency of NK(CD56+CD3-) cells from chronic hepatitis B (CHB) patients after 12 months of tenofovir therapy. (**A**) Percentage of NK(CD56+CD3-) cells from 5 healthy controls and 19 CHB patients. (**B**) Representative FACS plots showing CD56/CD3 expression in lymphoid-gated cells from a healthy control and a CHB patient at T0, T1, and T12.

established using single-color controls [21]. Clinical and immunological parameters in the different patient groups were compared using the nonparametric Mann–Whitney U-test. Correlations were tested for statistical significance using the Spearman rank correlation. A p value of <0.05 was considered to indicate significance.

First, we identified the proportion of NK cells in CHB patients and compared it with that from healthy controls. Fig. 1A shows that the frequency of NK cells from CHB patients was decreased at baseline (T0, 11.2%) compared with healthy controls (27.2%) but this proportion recovered substantially in CHB patients after 12 months of tenofovir therapy (T12, 17.7%) (Fig. 1A). Fig. 1B shows representative FACS plots for NK cell populations from healthy controls (20.9%) and CHB patients at baseline (T0, 9.59%), T1(16.2%), and T12(25.8%), where NK cells are identified based on the presence of CD56 and absence of CD3. Several studies have reported a reduced frequency of NK(CD56+CD3-) cells in chronic viral infections, including HIV and HCV [22]. Interestingly, current anti-HBV reagents such as combined IFNα-2a with adefovir, entecavir, or telbivudine have been shown to increase the numbers of NK cells in CHB patients [17–19], which clearly agrees with our results in Fig. 1.

As mentioned earlier, the functions of NK cells are tightly regulated by activating and inhibitory receptors expressed on their cell surface. Therefore, we examined whether or not the expression levels of receptors CD16, NKG2A, or NKG2D were changed during 12 months of tenofovir treatment. In Fig. 2, we show that the expression of CD16 was down-regulated in NK cells from CHB patients (T0, 64.8%) when compared with NK cells from healthy controls (71.2%). Of note, the expression of NKG2A, an inhibitory receptor, seemed to be up-regulated in NK cells from CHB patients (T0, 11.5%) when compared with NK cells from healthy controls (4.7%) (Fig. 2B). Interestingly, the expression of CD16 recovered after 12

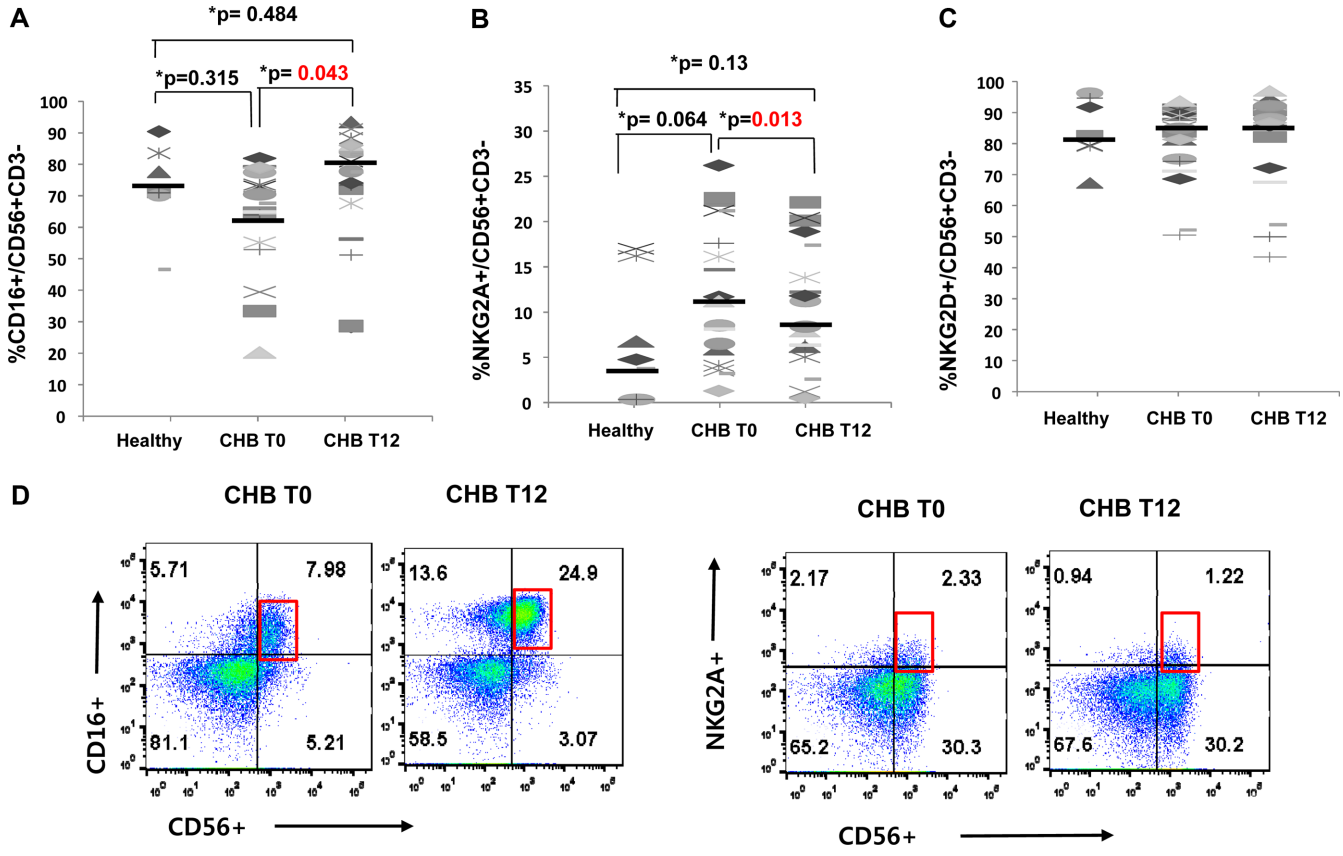

Fig. 2. Differentially regulated frequency of CD16+/CD56+CD3- and NKG2A+/CD56+CD3- cells from chronic hepatitis B (CHB) patients after 12 months of tenofovir therapy.

(**A**) The percentages of CD16+/CD56+CD3-, (**B**) NKG2A+/CD56+CD3-, and (**C**) NKG2D+/CD56+CD3- cells from healthy controls (n = 5) as compared with similar populations in CHB patients (n = 19). (**D**) Representative FACS plots showing CD16 and NKG2A expression in NK(CD56+CD3-) gated cells from a CHB patient at T0 and T12.

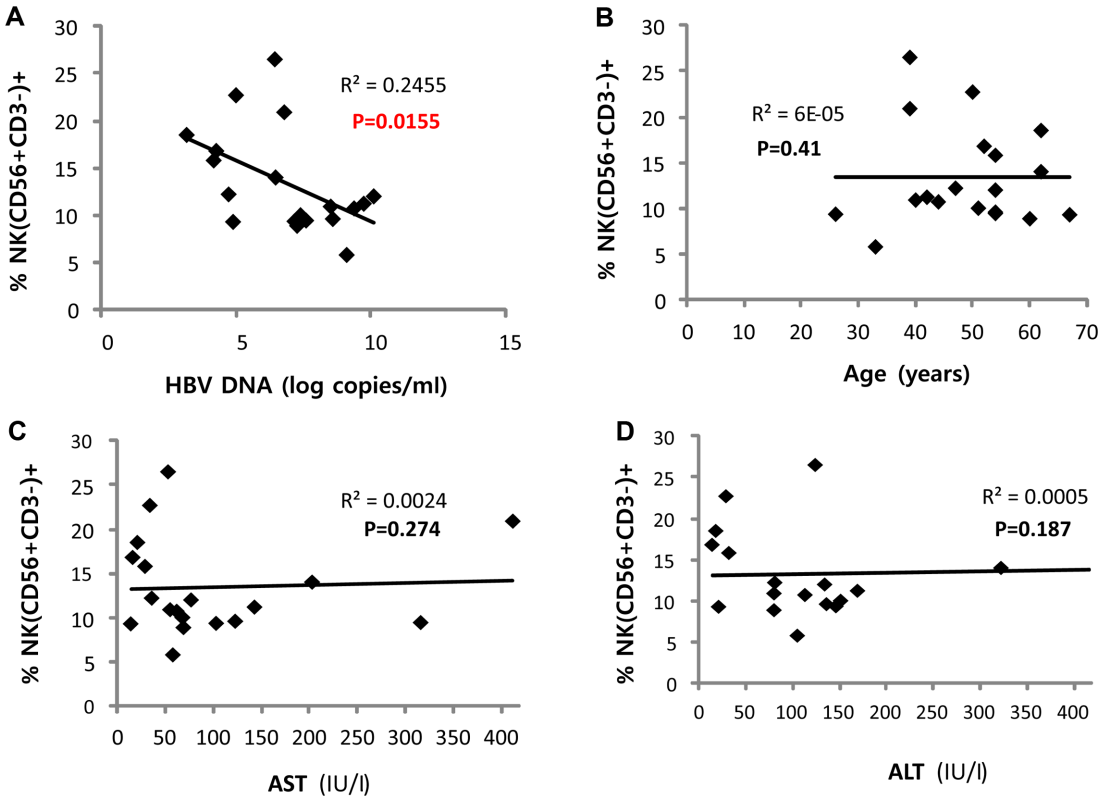

Fig. 3. Correlations between the proportion of NK(CD56+CD3-) cells present and clinical parameters in chronic hepatitis B (CHB) patients.

Correlation between %NK(CD56+CD3-) cells and (**A**) HBV DNA (log copies/ml), (**B**) age (years), (**C**) aspartate aminotransferase (AST; IU/l), and (**D**) alanine aminotransferase (ALT; IU/l) in CHB patients at T0.

months of tenofovir therapy (T12, 77.8%) (Fig. 2A). Moreover, the high expression level of NKG2A was significantly reduced by tenofovir treatment (T12, 9.8%) (Fig. 2B). However, there was no statistical difference in the expression level of NKG2D, an activating receptor, in NK cells during 12 months of tenofovir therapy (Fig. 2C). Fig. 2D shows representative FACS plots for the CD16+ cell populations within NK cells (left) collected from CHB patients at baseline (T0, 7.98%) and T12 (24.9%) as well as the NKG2A+ cell populations within NK cells (right) collected from CHB patients at baseline (T0, 2.33%) and T12 (1.22%). An increased frequency of NKG2A+ and CD94+ NK cells has been reported to be associated with HCV [23]. We also found increased expression of NKG2A+ in NK cells from CHB patients at the baseline (T0), which was reduced after 12 months of tenofovir therapy (Figs. 2B and 2D). In addition, there was a low frequency of CD16+ NK cells recovered in tenofovir-treated patients, which suggests a close relationship between activation of NK cells and disease progress in chronic HBV infection.

Surprisingly, the NK(CD56+CD3-) frequency in CHB patients at baseline (T0) was inversely correlated with HBV DNA (log copies/ml) in the blood of patients (R^2 = 0.2455 p = 0.0155, Fig. 3A), which we report for the first time in CHB infection. Our results indicate that NK cells may have a direct role in controlling viral load in CHB patients. In fact, a recent study has shown that reduced HBV viral load was associated with the activation of NK cells in CHB infection [20]. Therefore, we suggest that maintaining functionally activated NK cells is important for recovery from CHB with a high viral load. However, neither age nor liver parameters such as aspartate aminotransferase and alanine aminotransferase were associated with NK(CD56+CD3-) frequency in CHB patients (Figs. 3B–3D). Our current study provides insight into the phenotypic characteristics of NK cells in CHB patients undergoing tenofovir therapy.

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