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Phenotypic Characteristics of Natural Killer Cells in Acute Hepatitis

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Natural killer (NK) cells are the principal effector cell population in innate immune defense against many types of infections. These cells are enriched in the liver, where they comprise approximately 40% to 60% of the intrahepatic lymphocyte pool compared to the peripheral blood compartment. In chronic HBV and HCV infection, NK cells were reported to be partially dysfunctional due to impaired IFN-γ secretion. Few studies have examined phenotypic features of NK cells in acute hepatitis. We identified NK (CD56+CD3-) cell populations in patients with acute hepatitis by examining the expressions of phenotypic NK cell markers (CD16, NKG2A, and NKG2D). Peripheral blood mononuclear cells were isolated from patients with acute hepatitis A (7) and patients with non-viral acute toxic hepatitis (6) during the symptomatic and convalescent phases. Expressions of NK (CD56+CD3-) cell markers, CD16, NKG2A, and NKG2D, were measured by flow cytometry. Symptomatic acute hepatitis including non-viral hepatitis and HAV infection showed significant increases of NKG2A expression compared to healthy controls. Interestingly, there was a direct correlation between the proportion of NK cell populations and liver function parameters (AST, ALT) in HAV infection. The strong correlation was also observed between the expression of NKG2A+NK cells and ALT, which suggests that most of NK cells in severe phase of disease express high level of NKG2A on their surface. In addition, decreased number of NK cells (CD56+CD3-) in symptomatic phase began to increase in the convalescent phase of acute hepatitis A. However, the expression of NKG2A tended to be reduced, which indicates that NKG2A, the inhibitory receptor on NK cells, can be a severity parameter in acute hepatitis.

Keywords: acute hepatitis, hepatitis A infection, natural killer (NK) cells, NKG2A

Introduction

Acute hepatitis is a liver disease with a typical duration of less than 6 months. Etiologies of acute hepatitis include toxins and certain chemicals, but the most common cause is hepatitis A viral infection (Kang *et al*., 2007). Host defense against viral hepatitis depends on the interplay between the innate and adaptive immune responses. The latter is known to play an essential role in the complete control of viral infection. Neutralizing antibodies are responsible for the elimination of circulating virus, and virus-specific T-cells eliminate intracellular viral infections (Protzer *et al*., 2012). However, very little information is available on the role of the innate immune response in viral infection.

Natural killer (NK) cells are an important component of innate immunity against viral hepatitis. NK cells comprise 5–15% of the total lymphocyte population, and are morphologically described as large granular lymphocytes (Doherty and O'Farrelly, 2000). They contain perforin and granzyme granules, which exert cytotoxic antiviral activity (Gregoire *et al*., 2007). NK cells can be identified with flow cytometry by the expression of CD56 and absence of CD3, a typical T-cell surface marker. Most NK (CD56+CD3-) cells express the FcγIII receptor (CD16) which mediates antibody-dependent cell cytotoxicity. NK effector function is tightly regulated by several inhibitory (NKG2A, NKR-P1A) and activating (NKp44, NKG2D) receptors (Lanier, 2008).

Recently, increased number of NK cells was reported in the initial stage of HBV infection, suggesting that NK cells play a principal role against early viral infection (Webster *et al*., 2000). Decreased cytotoxic effects of NK cells have been observed in the liver in chronic HCV infection (Tripathy *et al*., 2008). Mondelli *et al*. (2010) reported impaired function of NK cells in chronic HBV and HCV infection. However, the role of NK cells in acute hepatitis remains poorly understood.

In this study, the NK cell population in the setting of acute hepatitis was identified by flow cytometry through the expression of CD56 and CD16 and the absence of CD3. The percentages of NK cells were compared among 3 groups including patients with non-viral acute hepatitis, HAV infection, and a healthy control group. In addition, expressions of inhibitory (NKG2A) and activating (NKG2D) receptors were monitored during symptomatic and convalescent phases of acute hepatitis. The expression of the inhibitory receptor, NKG2A in NK (CD56+CD3-) cells from patients with acute hepatitis, is significantly increased compared to those from healthy controls. In addition, there is a direct correlation between the number of NKG2A+NK cells and ALT in symptomatic phase of acute hepatitis.

Materials and Methods

Study sample

Patients were recruited from the Department of Internal Medicine of Uijeongbu St. Mary's Hospital and all patients

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Table 1. Clinical characteristics of acute hepatitis patients

	Non-viral toxic acute hepatitis n=6	Hepatitis A virus infection n=7
Sex (M:F)	5:1	4:3
Age (years)[a]	36	34
Peak AST (IU/L)[a]	898	1714
Peak ALT (IU/L)[a]	1733	2263
Total Bilirubin (mg/dL)[a]	2.85	4.08
IgM-HAV	-	+

[a]Median values

provided informed consent. The institutional review board approved the study protocol. The 3 groups defined in our sample included patients with symptomatic acute hepatitis due to HAV infection (7 patients) or non-viral toxic hepatitis (6 patients) and healthy controls (5 patients). Exclusion criteria included heavy alcohol consumption, HBV, HCV, or HIV infection, autoimmune liver disease, Wilson's disease, or other significant medical comorbidities. Acute hepatitis A was diagnosed by the presence of serum IgM anti-HAV. The following blood tests were obtained at the time of admission: hemoglobin, platelet count, prothrombin time, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), and serum total bilirubin. Clinical characteristics of acute hepatitis patients are shown in Table 1. Peripheral blood mononuclear cells (PBMCs) analyzed at the time of symptomatic phase of acute hepatitis were obtained at the day of admission, and PBMCs of convalescent phase of acute hepatitis were obtained in 5 to 6 weeks.

Isolation of PBMCs

PBMCs were isolated by Ficoll-Histopaque (Sigma Chemical Co, USA) density centrifugation and cryopreserved as previously described (Sugimoto *et al.*, 2003).

Fluorescent antibodies

All monoclonal antibodies including Anti-CD56 PE CY7, Anti-CD3 APC CY7, Anti-CD16 FITC, and Anti-NKG2D APC were purchased from BD Bioscience (USA). Anti-NKG2A PE was obtained from R&D Systems (USA). Dead cells were excluded with 7-AAD (7-Amino-actinomycin D).

Cell surface antigen staining by flow cytometry

Cells were stained by fluorescent antibodies per manufacturer instructions. Briefly, thawed PBMCs (1×10^6) were mixed with a fluorescent antibody master mix and incubated at 4°C for 30 min in the dark. After staining, cells were washed three times with staining buffer consisting of 1% BSA and 0.09% sodium azide in PBS. Cells were fixed with 2% paraformaldehyde (Sigma-Aldrich, USA). Events were acquired with a FACSCanto (Becton Dickinson, USA) and analyzed with FlowJo (Tree Star Inc., USA), gating on live lymphoid cells based on forward and side scatter profiles and dead cells were excluded with 7-AAD staining. Compensations were established using single color controls. The positivity of NK cells was defined using fluorescent anti-CD3 and anti-CD56 monoclonal antibodies within the overall lymphocyte population. Unstained controls were used to determine background staining levels.

Statistical analysis

Clinical and immunological parameters were compared between patient groups by nonparametric Mann Whitney U or Wilcoxon signed-ranks tests depending on the relationships of the variables. Correlations were tested for statistical significance by Spearman rank correlation. *P*-values of <0.05 were considered significant.

Results

The frequency of NK (CD56+CD3-) cells in symptomatic HAV acute hepatitis patients was decreased compared to healthy controls

First, we examined the expression of NK cells from 3 patient

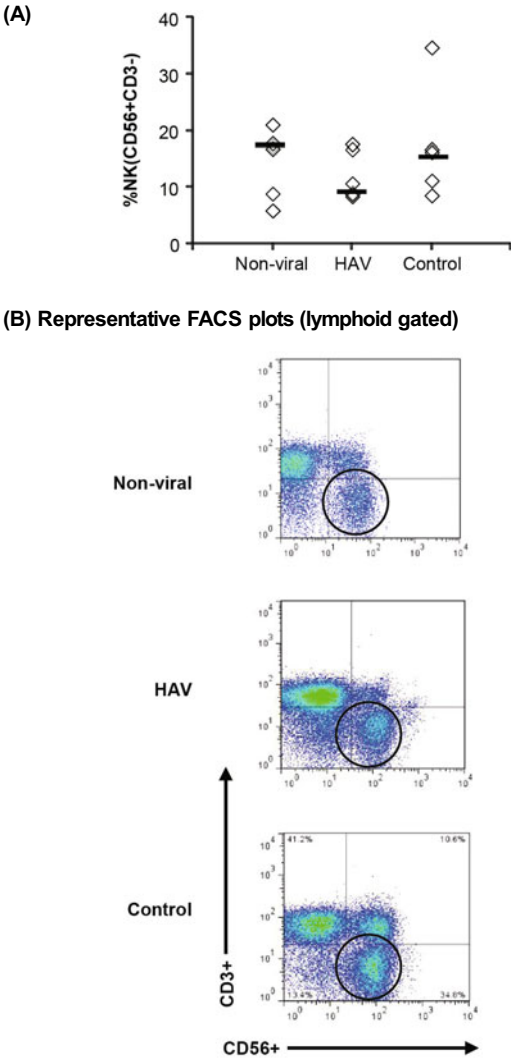

Fig. 1. The frequency of NK(CD56+CD3-) cells from acute hepatitis patients. (A) % NK cells (CD56+CD3-) (median: non-viral 17.1; HAV 9.9; healthy 16.2) from 6 patients with non-viral acute hepatitis infection, 7 patients with HAV infection, and 5 healthy controls. (B) Representative FACS plots showing CD56/CD3 expression in live lymphoid- gated cells.

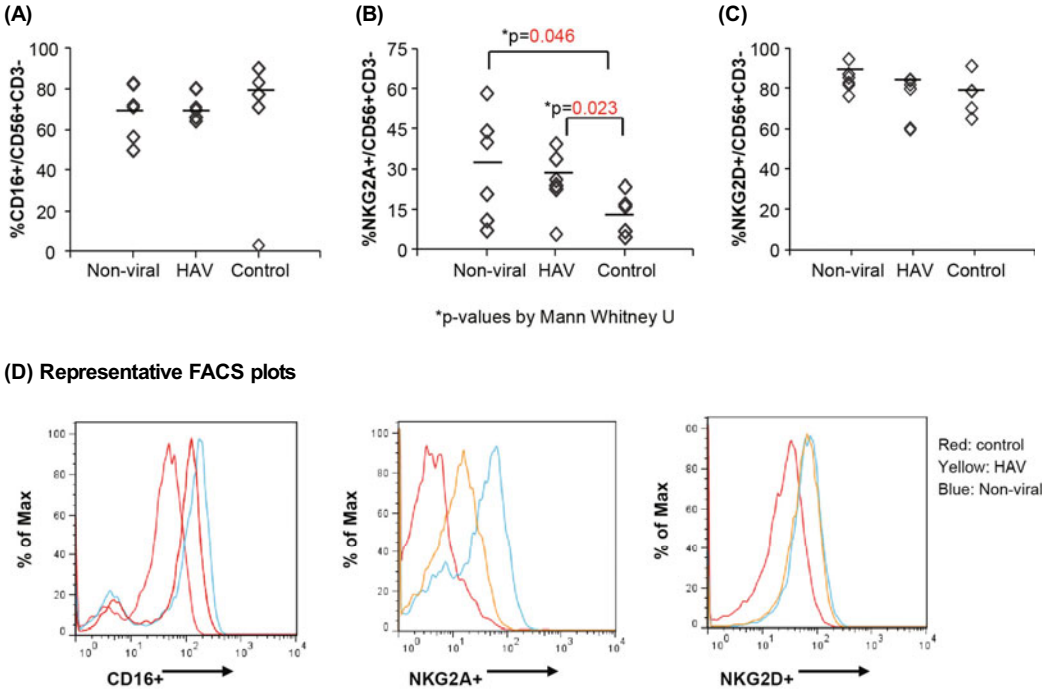

Fig. 2. The frequencies of CD16+, NKG2A+ and NKG2D+ in NK (CD56+CD3-) cells from acute hepatitis patients. (A) %CD16+ (median: non-viral 71.7; HAV 70.5; healthy 77.6). (B) %NKG2A+ (median: non-viral 30.4; HAV 25; healthy 16.2). (C) %NKG2D+ (median: non-viral 84.7; HAV 81.4; healthy 79.1) from 6 patients with non-viral acute hepatitis infection, 7 patients with HAV infection, and 5 healthy controls. (D) Representative FACS plots showing CD16, NKG2A, and NKG2D expression in NK(CD56+CD3-) gated cells in each group.

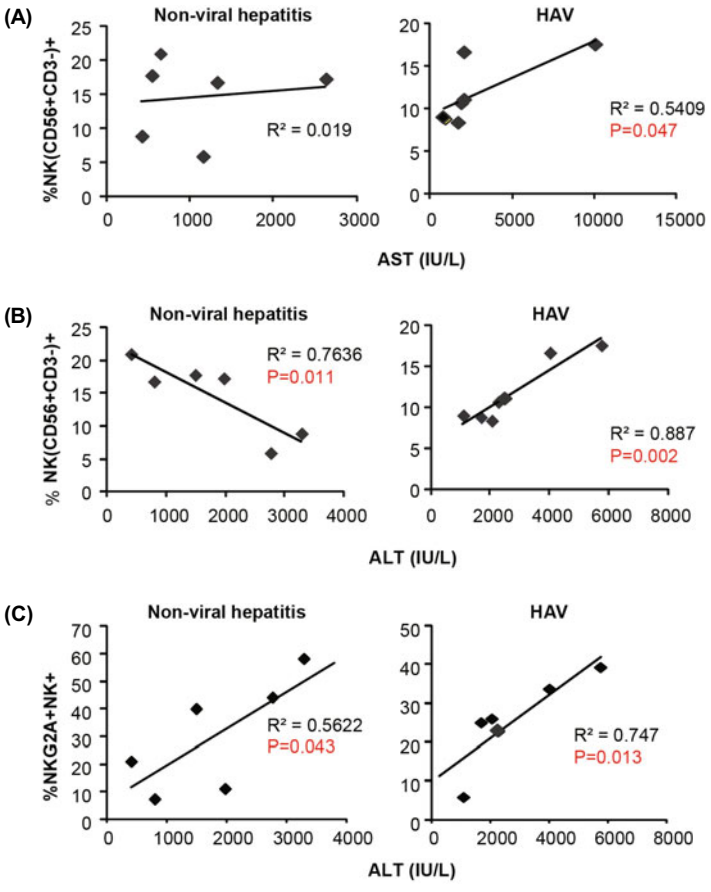

Fig. 3. Correlation between NK cells, NKG2A+NK cells and clinical liver parameters in acute hepatitis infection. (A) Correlation between %NK (CD56+CD3-) cells and AST (IU/L) in non-viral hepatitis or HAV infection. (B) Correlation between %NK (CD56+CD3-) cells and ALT (IU/L) in non-viral hepatitis or HAV infection. (C) Correlation between %NKG2A+NK cells and ALT (IU/L) in non-viral hepatitis or HAV infection.

groups including those with non-viral acute hepatitis, HAV infection, or a healthy control group (Table 1). As shown in Fig. 1, the frequency of NK cells was reduced during the symptomatic phase of HAV infection compared to the healthy control, although the difference was not significant (Fig. 1A). Figure 1B shows representative FACS plots for the NK cell population from all groups.

The expression of the NK cell inhibitory receptor, NKG2A, was increased in symptomatic acute hepatitis compared to healthy controls

Higher expression of NKG2A inhibitory receptors has been associated with treatment failure in chronic HCV infection (Golden-Mason *et al.*, 2011). Therefore, we examined this inhibitory receptor to assess patients with symptomatic acute hepatitis for higher levels. Figure 2B shows that the expression of NKG2A was significantly upregulated in NK cells from patients with acute hepatitis compared to healthy controls (non-viral vs. healthy control P=0.046, HAV vs. healthy control P=0.023 Mann Whitney U). There were no differences, however, between patients with non-viral hepatitis or HAV infection. Figures 2A and 2C shows that the expressions of CD16 and the activating receptor, NKG2D, in NK cells from acute hepatitis were not significantly different from those of healthy controls.

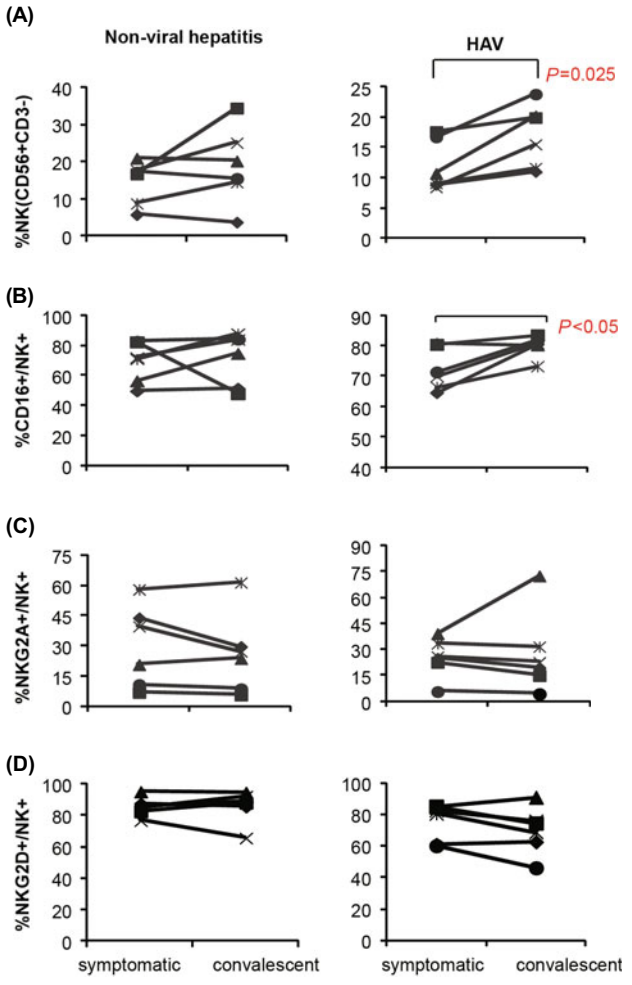

Fig. 4. Increased NK+ cells during the convalescent phase of HAV-infected patients. (A) %NK (CD56+CD3-) (B) %CD16+ in NK (CD56+CD3-) cells (C) %NKG2A+ in NK (CD56+CD3-) cells. (D) %NKG2D+ in NK (CD56+CD3-) cells during symptomatic and convalescent phases of non- viral hepatitis or HAV infection.

The frequencies of NK cells or NKG2A+NK cells from HAV infection were directly correlated with clinical liver function parameters

The proportion of NK cells (CD56+CD3-) from patients with HAV infection was directly proportional with levels of AST (aspartate aminotransferase) (R^2=0.5409 P=0.047, Fig. 3A right) and ALT (alanine aminotransferase) (R^2=0.887 P=0.002, Fig. 3B right). Interestingly, NK cell levels from patients with non-viral hepatitis were inversely correlated with ALT (R^2=0.7636 P=0.011, Fig. 3B left). The frequencies of NKG2A+NK cells from both non-viral hepatitis (R^2=0.562 P=0.043, Fig. 3C left) and HAV (R^2=0.747 P=0.013, Fig. 3C right) patients positively correlated with ALT.

The expressions of NK (CD56+CD3-) or CD16 in NK cells were increased during the convalescent phase of HAV-infected patients

We examined the expressions of NK (CD56+CD3-), CD16, NKG2A, and NKG2D in NK cells from the symptomatic phase through the convalescent phase of acute hepatitis. The frequency of NK cells was significantly increased during the convalescent phase compared to the symptomatic phase in HAV infection, but not in non-viral hepatitis (Fig. 4A). CD16 expression in NK cells followed a similar trend in HAV infection (Fig. 4B). The expression of NKG2A tended to be decreased in NK cells from HAV infection, but the difference was not statistically significant (Fig. 4C).

Discussion

NK cells are important for the regulation of protective immune responses during viral infection. Several studies have reported reduced frequencies of NK (CD56+CD3-) cells in chronic viral infections including HBV, HCV and HIV although these findings are controversial (Meier *et al.*, 2005; Bonorino *et al.*, 2009). There is little evidence, however, regarding the phenotypic and functional characteristics of NK cells during acute hepatitis. Budarina *et al.* (2003) reported a decrease in the number of NK cells in HAV infected children from Russia. Our study shows a similar trend for NK cells during HAV infection compared to healthy controls but the difference was not significant (Fig. 1A).

The effector function of NK cells is controlled by complicated signals through inhibitory and activating cell surface receptors. In chronic HCV infection, higher expression of the inhibitory receptor (NKG2A) in treatment failure and higher expression of the activating receptor (NKp44) in viral clearance have been reported (Harrison *et al.*, 2010; Golden-Mason *et al.*, 2011) suggesting a close relationship between NK cell activation and disease outcome in HCV infection. Alter *et al.* (2011) reported elevated levels of NKG2A+ and

CD94+ NK cells in acute and chronic HCV infection. Our data show increased expression of NKG2A+ NK cells from symptomatic acute hepatitis compared to healthy controls with little difference in NKG2A between non-viral and HAV-derived acute hepatitis, and we also show a strong correlation between the level of NKG2A in NK cells and ALT. Therefore, the expression of NKG2A on NK cells seems to be associated with worse clinical outcomes in acute hepatitis.

We also investigated whether there is a possible correlation between NK cell proportions and liver function parameters. Interestingly, NK cell levels are directly associated with AST (Fig. 3A) and ALT (Fig. 3B) in HAV infection, but not in non-viral hepatitis. Yilmaz *et al.* reported a positive correlation between NK cell counts and AST, ALT, and aPTT (activated partial thromboplastin time) in Crimean–Congo hemorrhagic fever (CCHF), which is similar to our observation (Yilmaz *et al.*, 2008). The study from Yilmaz *et al.* (2008) suggested high NK cell counts as a severity parameter. However, they did not further examine expressions of other surface markers on NK cells. It is possible that relatively high NK cell counts can be observed in severe patients due to extensive release of cytokines by the activation of other immune cells (e.g., HAV-specific T cells), but this NK cell counts would still be lower compared to that from healthy controls. We suggest that the activation of NK cells can be differentially controlled in symptomatic phase compared to convalescent phase. For example, NK cells express increased level of NKG2A in symptomatic phase, but decreased level of NKG2A in convalescent phase. Actually, we detected that the number of NK cells was increased in the convalescent phase of patients with HAV infection, which indicates that the high NK cell counts in different stages of disease could result in the opposite clinical outcomes based on dynamic changes in their receptor expression.

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