

High frequencies of CD158b⁺ NK cells are associated with persistent hepatitis C virus infections

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ABSTRACT

Background. During the early phases of a hepatitis C virus (HCV) infection, NK cell activation appears to be critical to the induction of adaptive immune responses that have the potential of clearing the infection. This study aimed to investigate the phenotype and function of NK cells in chronic HCV (CHC) patients, particularly patients who cleared HCV infections spontaneously (SR-HCV). **Material and methods.** Peripheral blood NK cells were compared between 36 CHC patients, 12 SR-HCV patients, and 14 healthy controls (HC). The phenotype and function of NK cells were characterized by flow cytometry. In addition, the potential associations between the frequency of NK cell subsets and ALT, AST and HCV viral loads were also analyzed. **Results.** Our data revealed that the population of CD3-CD56⁺ NK cells was significantly decreased in CHC and SR-HCV patients compared to levels in HC (P = 0.031, P = 0.014). Interestingly, we found that the levels of the CD158b inhibitory receptor were higher in CHC patients compared to levels observed in HC and SR-HCV subjects (P = 0.018, P = 0.036). In addition, the percentages of the activation receptors NKp30 and NKp46 were significantly decreased in CHC and SR-HCV patients compared to their expression levels in HC (P < 0.05). Moreover, the frequencies of inducible CD107a (but not IFN- γ -secreting) NK cells from both CHC and SR-HCV patients were significantly lower than frequencies observed in controls (P = 0.018, P = 0.027). **Conclusion.** Our data indicated that the higher frequency of inhibitory NK cells combined with fewer activated NK cells may be associated with HCV-related chronic inflammation involved in CHC pathogenesis.

Key words. Hepatitis C virus (HCV). Chronic HCV infection. NK cells. Activation markers. CD107a.

INTRODUCTION

Hepatitis C virus (HCV) is one of the major causes of chronic liver disease in the world. More importantly, many patients with chronic hepatitis C will eventually develop cirrhosis and hepatocellular carcinoma.¹ Following infection with HCV, about 15% of patients clear the virus and the remaining go on to develop persistent chronic infections.^{2,3} This difference in disease progression can be attributed in part to different immune responses between individuals

that determine the outcome of the virus/host interaction.¹⁻⁴

During the early phases of an HCV infection, activation of the innate immune system appears to be an important factor in determining the subsequent elicitation of adaptive immunity that can ultimately clear the HCV infection.⁵ Natural killer (CD3-CD56⁺) cells are important components of the innate system with the ability to both directly kill target cells and interact with both antigen-presenting cells and T cells.^{6,7} Human NK cells primarily populate the liver and peripheral blood. NK cells are generally activated during the early phases of viral infection, and induction of an adequate innate response results in the eradication of hepatotropic viruses.⁸ Activated NK cells express activating receptors such as NKG2D in addition to the natural cytotoxicity receptors NKp30, NKp44, and NKp46 among others.⁹ In addition, activated NK cells can produce inflammatory cytokines (such as IFN- γ), granzymes,

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and other cytotoxic factors that mediate cytotoxic responses directed against target cells. As a means of preventing unwanted cytotoxicity, NK cells are equipped with various inhibitory receptors such as the lectin-like CD94-NKG2A receptor and the killer cell immunoglobulin-like receptors (KIRs) such as CD158a (KIR2DL1), CD158b (KIR2DL3), and CD158e (KIR3DL1) that allow NK cells to recognize self from non-self thereby preventing them from damaging normal, healthy cells.^{10,11}

It is known that NK cells play important role in clearing HCV infections.^{2,12} However, HCV seems to have evolved multiple strategies to counter NK cell mediated responses and several studies have demonstrated that CHC patients possess functionally impaired NK cell-mediated responses.¹³⁻¹⁵

There is limited data available regarding the Natural Killer Receptor (NKR) in NK cells from patients that have spontaneously cleared their HCV (SR-HCV) infections. This report describes alterations in the NK activation receptors (NKG2C, NKG2D, NKp30, NKp44, and NKp46) and in the inhibitory NKRs (NKG2A, KIR3DL1, CD158a, CD158b). Changes were also observed in the production of IFN- γ and CD107a by NK cells from CHC and SR-HCV patients at baseline, in addition we describe a potential association between the frequency of different NK cell subpopulations and different clinical measures.

MATERIAL AND METHODS

Study population

Patients with HCV infections (n = 48) were enrolled sequentially at the inpatient services division at the First Hospital of Jilin University during 2010-2011. From this group, 36 CHC (23 persistently nor-

mal alanine aminotransferase [PNALT] and 13 presenting with chronic hepatitis) and 12 SR-HCV patients were enrolled and 14 healthy controls that were matched for gender, age, and ethnicity with no history of liver diseases or evidence of HBV, HCV, or HDV infections were also recruited. Individuals with positive anti-HCV antibody responses and detectable serum HCV RNA for at least 6 months were categorized as CHC.¹⁶ Individuals with SR-HCV were defined as those subjects who had prior HCV RNA detection (HCV-Ab⁺) but were HCV RNA negative for 12 weeks after enrollment in the absence of treatment.¹⁷ HCV genotyping demonstrated that 12 CHC patients were infected with genotype 2a, 18 with genotype 1b, and 6 were infected with an unclassified genotype.

Individuals with a history of or had current hepatitis B or D virus or HIV infections, autoimmune hepatitis, or metabolic liver disease, or individuals who had received immuno-suppressive therapy or antiviral therapy during the past 12 months before the enrollment date were excluded. All patients denied drug use or exposure to hepatotoxin. The demographic and clinical characteristics of enrolled subjects are described in table 1.

CHC patients were treated with subcutaneous injections of 5 x 10⁶ IU of short-acting recombinant human interferon alpha (Kexing Biological, Shenzhen, China) every 2 days for 12 weeks plus 15 mg/kg/day of ribavirin for 3 months. Written informed consent was obtained from participants, and the study protocol was approved by the First Hospital Ethical Committee of Jilin University.

Serologic analysis

The concentrations of serum antibodies against HCV were detected using an ELISA II kit (Abbott

Table 1. Demographic characteristics and clinical features of participants.

Parameters	SR-HCV	CHC	Healthy controls
Number	12	36	14
Age (years)	48 (33-59)	46 (29-69)	36 (24-54)
Male (%)	7 (58.3)	16 (44.4)	9 (64.3)
HCV RNA (log ₁₀ copies/mL)	NA	6.4 (1.2-7.5)*	NA
Anti-HCV	Positive	Positive	Negative
ALT (U/L)	27.9 (18.7-78.4)	32.1 (11.2-274.90)*	17 (3-22)
AST (U/L)	30.5 (18.7-58.3)	29.5 (13.9-262)*	15 (6-30)
White Blood Cells (x 10 ⁹ /L)	6.16 (5.62-7.54)	6.08 (5.57-7.51)	6.19 (6.65-7.45)
Lymphocytes (x 10 ⁹ /L)	2.15 (1.25-2.85)	2.11 (1.08-2.65)	2.18 (1.16-2.81)

The data were described by median and rang. Normal values: ALT \leq 40 IU/L. AST \leq 40 IU/L. HCV RNA \leq 3log₁₀ copies/mL. *P < 0.05 HCV vs. healthy controls. NA: normal.

Laboratories, Abbott Park, USA).¹⁶ The levels of serum ALT and AST were detected using a Biochemistry Automatic Analyzer (Roche Diagnostics, Branchburg, USA).¹⁸ The amounts of serum HCV RNA were measured by quantitative PCR assay using a luciferase quantification kit following the manufacturers instructions (Roche Amplicor, Basel, Switzerland). The detection limit of viral RNA was 300 copies/mL.¹⁸

Sample preparation

Venous blood was collected after an overnight fast and analyzed on the day of collection. Routine laboratory analyses were performed using standard techniques. Venous blood (10 mL) was collected in heparinized tubes for lymphocyte profiling. Peripheral blood mononuclear cells (PBMCs) were purified using density-gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, UK).

Flow cytometric analysis

The phenotype of NK cells was analyzed by flow cytometry using antibodies specific for respective markers. Briefly, 100 μ L of a blood sample was incubated with a mixture of fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (SK7), allophycocyanin (APC)-conjugated anti-CD56 (B159), peridinin chlorophyll protein (PerCP)-conjugated anti-CD16 (3G8), and phycoerythrin (PE)-conjugated anti-NKG2D (1D11), anti-NKp30 (p30-15), anti-NKp44 (p44-8.1), anti-NKp46 (9E2/NKP46), anti-CD158a (HP-3E4), anti-CD158b (CH-L) (BD PharMingen, San Diego, USA), or anti-NKG2C (134591), anti-NKG2A (131411) (R&D Systems, Abingdon, UK) at 4 °C in the dark for 20 min respectively. Mouse isotype matched controls (FITC-IgG1, PE-IgG1, APC-IgG1, PE-IgG2a, and PE-IgG2b) (BD PharMingen, San Diego, USA) were used as negative controls. Erythrocytes were lysed using BD FACS lysing Solution (BD Bioscience) and the frequency of different subsets of NK cells determined by flow cytometry using a FACSCalibur (BD Bioscience) flow cytometer and the data analyzed using FlowJo software (v5.7.2) (TreeStar, Ashland, OR, USA).

Detection of intracellular IFN- γ

PBMCs (10⁶ cells/well) were stimulated in the presence or absence of 50 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, MO) and 1.0

mg/mL of ionomycin (Sigma-Aldrich) in complete RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) for 2 h at 37 °C in 5% CO₂ and then incubated with Brefeldin A (GolgiPlug; BD Biosciences) for an additional 5 h. Cells were then harvested, washed with cold phosphate-buffered saline (PBS), stained with FITC-anti-CD56 and APC-anti-CD3, fixed with 4% paraformaldehyde, and then permeabilized with 0.5% saponin, 10% fetal bovine serum in PBS. After washing with cold PBS, the cells were stained with or without PE-anti-IFN- γ (45B3) (BD PharMingen) and subjected to flow cytometric analysis.

NK cell degranulation

Measurement of CD107a degranulation is widely used for evaluating NK cell cytotoxicity.¹⁹ Isolated PBMCs (10⁶ cells/well) were co-cultured in duplicate with K562 cells at a an effector:target (E:T) cell ratio of 10:1 in RPMI-1640 medium for 1 h in the presence of anti-CD107a(H4A3) (BD PharMingen) or an isotype control IgG2a (BD PharMingen) and then exposed to 2 mM monensin (GolgiStop; BD Biosciences) for an additional 5 h. Unstimulated cells were treated in similar fashion and used as a negative control. Subsequently, cells (10⁶/tube) were stained in duplicate with FITC-anti-CD3 and APC-anti-CD56 at 4 °C in the dark for 20 min, respectively. After washing with PBS (containing 1% FCS and 2.5% paraformaldehyde), the frequency of CD107⁺CD3⁺CD56⁺NK cells was determined by flow cytometric analysis using a FACS Calibur flow cytometer. At least 50,000 events per sample were analyzed.

Statistical analysis

The data were expressed as median and range. Differences between groups were analyzed by the Mann-Whitney U nonparametric test using SPSS 19.0 software. The relationship between variables was evaluated using the Spearman's rank correlation test. A two-side P value < 0.05 was considered statistically significant.

RESULTS

Correlation of ALT and AST levels with infection status

There were no significant differences in the distribution of age and gender between participants in the CHC, SR-HCV, and HC groups, but serum ALT and

AST concentrations in CHC patients were significantly higher than levels observed in SR-HCV patients and in the HC subjects (Table 1). High levels of viral RNA were detected in CHC patients but no detectable viremia was observed in either the SR-HCV patients or HC subjects. In addition, anti-HCV antibodies were detected in CHC patients and individuals with SR-HCV but not in HC subjects.

NK cell numbers are decreased in the blood of HCV infected patients

The CD3⁺CD56⁺ and CD3⁺CD16⁺ NK cell profile from the different patient populations was examined by flow cytometry. The percentage of blood NK cells was significantly decreased in CHC and SR-CHC patients compared to NK cell levels observed in HC subjects. No differences in the percentage of NK cells were evident between CHC and SR-HCV patients ($P = 0.014$ and $P = 0.033$, respectively) (Figure 1B). The frequencies of CD3⁺CD16⁺ NK cells

were similar between the 3 groups ($P > 0.05$). These results suggested that NK cell function was impaired in CHC and SR-CHC patients.

The frequency of inhibitory receptor CD158b expression on NK cells was elevated in chronic HCV infection patients

To further define the NK receptor expression profile between the HCV populations studied, analysis of the NK activation and inhibitory receptor profiles were performed (Figure 2). The inhibitory NK receptor CD158b was up-regulated in NK cells harvested from CHC patients compared to CD158b expression levels found on NK cells from HC subjects ($P = 0.014$) (Figure 2). Interestingly, CD158b expression levels were significantly decreased in SR-HCV patients compared to CHC patients ($P = 0.033$) (Figure 2) and there were no differences in expression levels between SR-HCV patients and HC subjects. The percentage of NK cells expressing the

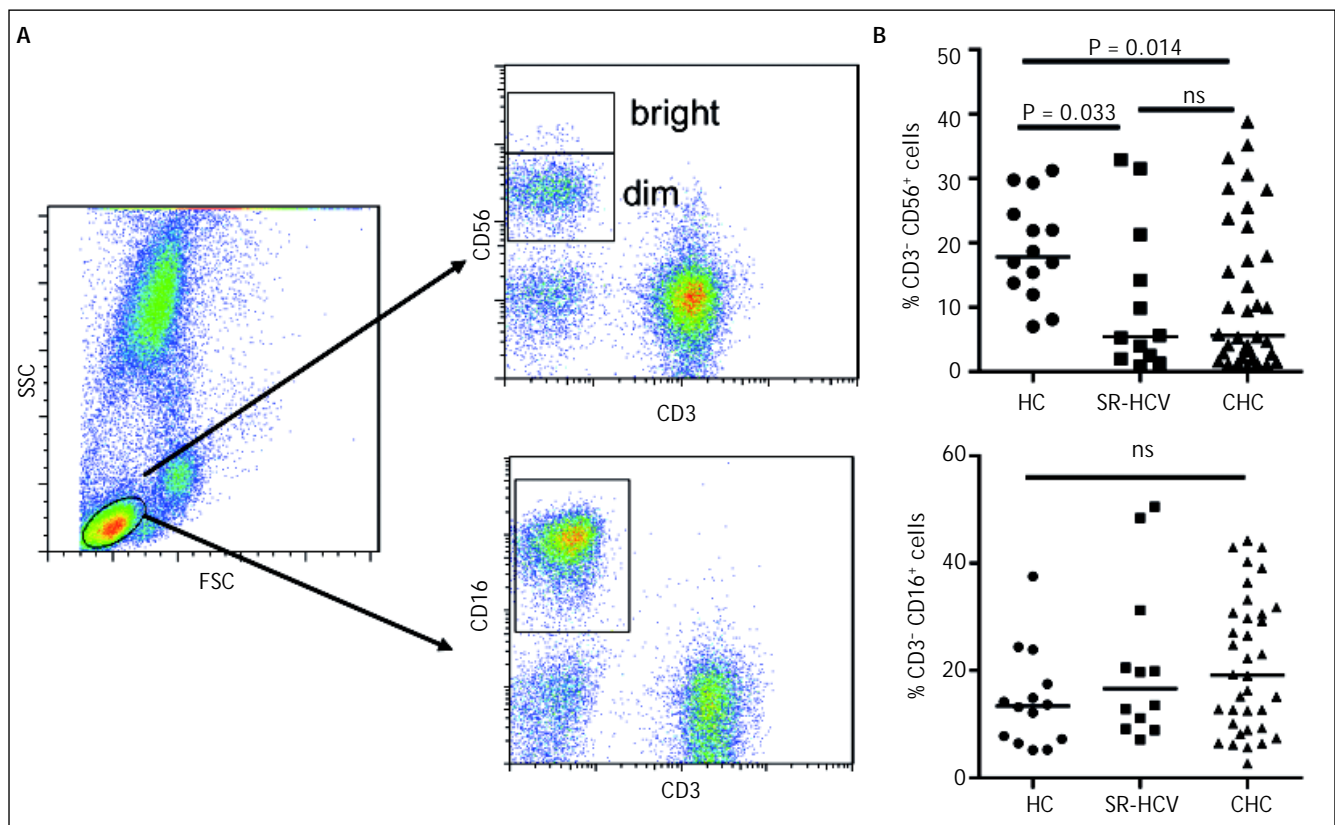


Figure 1. Decreased numbers of NK cells in lymphocytes from HCV infected patients. Flow cytometry was used to identify the CD3⁺CD56⁺ NK cells from PBMCs isolated from healthy controls (HC), patients with spontaneously cleared HCV (SR-HCV), or from patients with chronic HCV infections (CHC). **A.** Harvested cells were gated initially on living lymphocytes and then on CD3⁺CD56⁺ cells. At least 50,000 events were analyzed for each sample. **B.** Data represent the NK cell percentages. The horizontal lines represent the median. Ns = not significant.

NKp30 and NKp46 activation receptors were down-regulated in CHC and SR-HCV patients compared to levels detected in the HC subjects ($P = 0.033$, $P = 0.008$; $P = 0.000$, $P = 0.001$, respectively) (Figure 2). No differences in NKp30 and NKp46 expression levels were found between CHC and SR-HCV patients. The decreased level of activation receptors and the increase in the levels of inhibitory NK receptors found in cells harvested from CHC patients indicated that NK cell activity may be suppressed in these patients. Although the decreased NK cell activity was also observed on NK cells harvested from SR-HCV patients, NK cells from this population had a similar CD158b expression profile to that of HC subjects. No significant changes in the CD3-CD56^{dim} levels were observed among subjects ($P > 0.05$) (Figure 3A) and these cells had a similar receptor ex-

pression profile ($P < 0.05$) (Figure 3B). No changes observed for CD3-CD56^{bright} NK cells (data not shown).

Positive correlation between CD158b⁺NK cells and serum ALT levels

We further analyzed the role of altered NKp30, NKp46, and CD158b expression patterns on NK cells in relation to CHC pathogenesis. This analysis indicated that high frequency of CD158b⁺ NK cells was higher in the high serum ALT levels group than low group ($P = 0.021$) (Figure 4A). Spearman's correlation analysis revealed that the percentage of CD158b⁺ NK cells significantly positively correlated with serum ALT levels in CHC patients ($P = 0.04$, $R = 0.34$) (Figure 4B). We also evaluated the

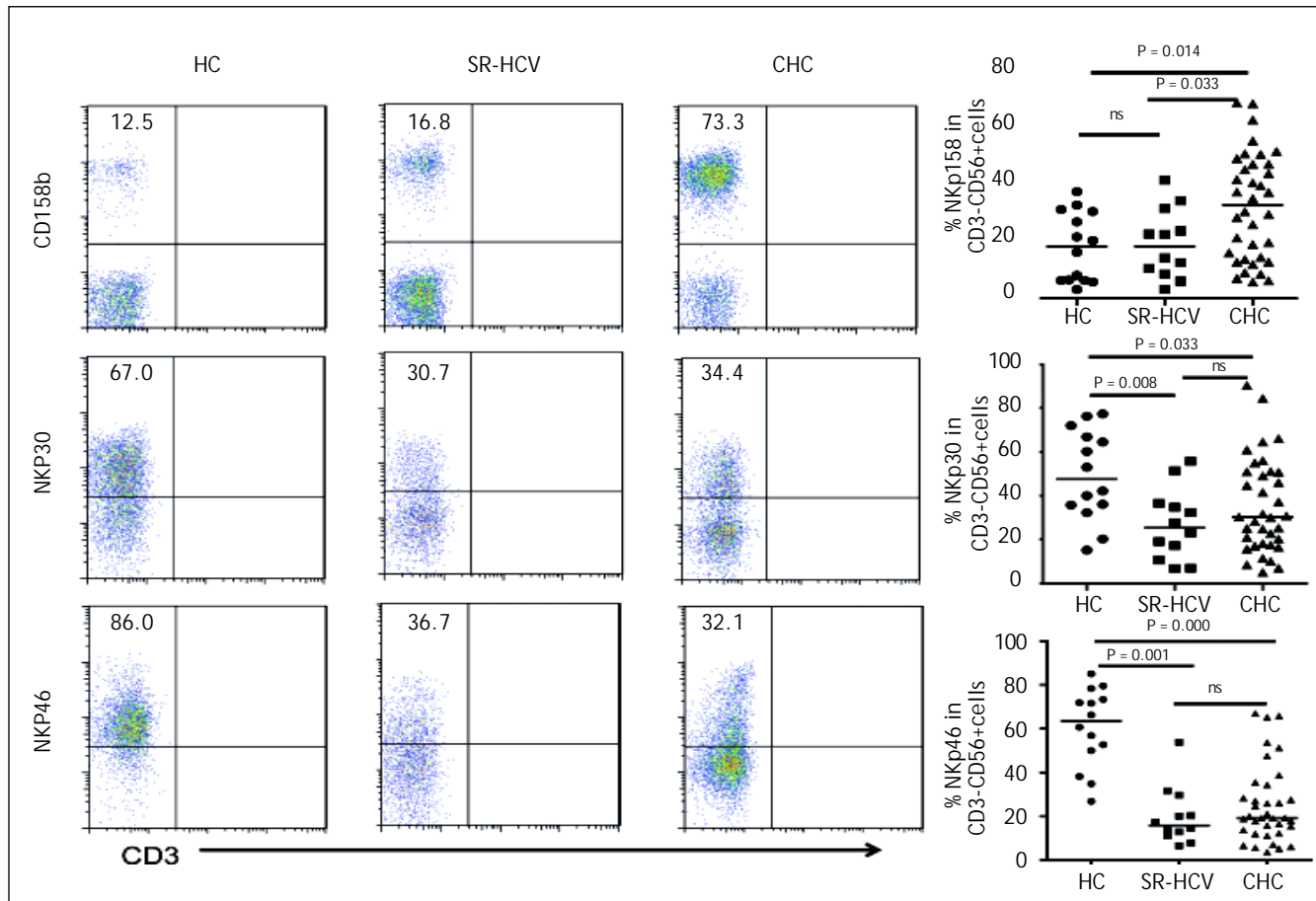


Figure 2. NK cell subset frequencies. Activation receptors expressed by NK cells are down-regulated in HCV infected patients. **A.** Harvested cells were gated initially on living lymphocytes and then on CD3⁺CD56⁺ cells. At least 50,000 events were analyzed for each sample. Data shown are representative results from different groups of subjects and the percentages of specific NK cell subsets from individual subjects are shown. **B.** Summarized data show the percentage of peripheral blood NK cells expressing different NK receptors. Data are expressed as the mean percent of individual samples from at least 2 separate experiments. The horizontal lines represent the median.

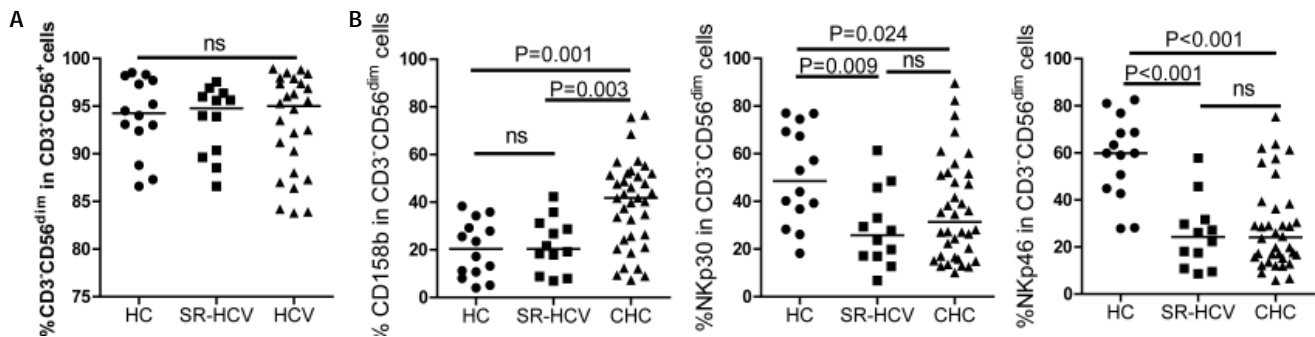


Figure 3. CD3-CD56^{dim} cell subset frequencies. **A.** Percent CD3-CD56^{dim} cells in the CD3-CD56⁺ NK cell compartment. **B.** Percent peripheral CD3-CD56^{dim} cells expressing different NK receptors. Data are expressed as the mean percent of individual samples from at least 2 separate experiments. The horizontal lines represent the median.

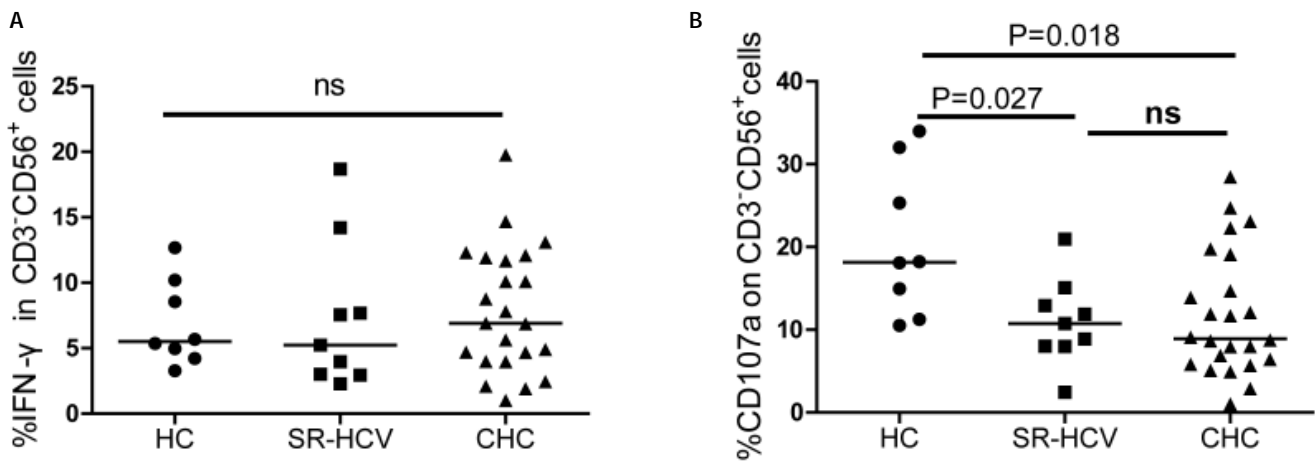


Figure 4. Correlation between serum ALT levels and expression of the CD158b inhibitory NK cell receptor in CHC patients. The frequency of CD158b⁺ NK cells significantly correlated with ALT concentrations. **A.** The frequency of CD158b⁺ NK cells was high in patients with elevated ALT levels. **B)** The levels of CD158b⁺ NK cells positively correlated with serum ALT levels.

relationship between other receptors expressed on NK cells with serum HCV RNA loads and the viral genotypes identified; however, no correlations were found (data not shown).

Functional NK cell analysis of patients with HCV infections

NK cells play a significant role in mediating antiviral defense mechanisms, partly due to their ability to secrete cytokines such as IFN- γ .¹² The ability of NK cells to produce IFN- γ and express CD107a following PMA stimulation in combination with ionomycin and culture in the presence of K562 cells was evaluated. There were no significant differences in the frequencies of inducible IFN- γ -secreting NK cells between CHC or SR-HCV patients and healthy controls ($P > 0.05$) (Figure 5A). Characterization of NK cell degranulation indicated that the frequency

of CD107a⁺ NK cells in response to K562 stimulation was significantly diminished in CHC or SR-HCV patients compared to healthy controls ($P = 0.018$, $P = 0.027$) (Figure 5B).

DISCUSSION

HCV infections represent a global health concern and economic burden. Due to the high polymorphisms among HCV isolates there exists a pressing need to develop both prophylactic vaccines and effective (novel) therapeutic strategies to prevent and treat HCV infections, respectively. To facilitate this goal, a better understanding of the mechanisms involved in the initiation and maintenance of viral persistence and alterations to NK cell function in the context of these infections is urgently required.

The percentage of NK cells was shown to be decreased in SR-HCV and CHC patients compared to

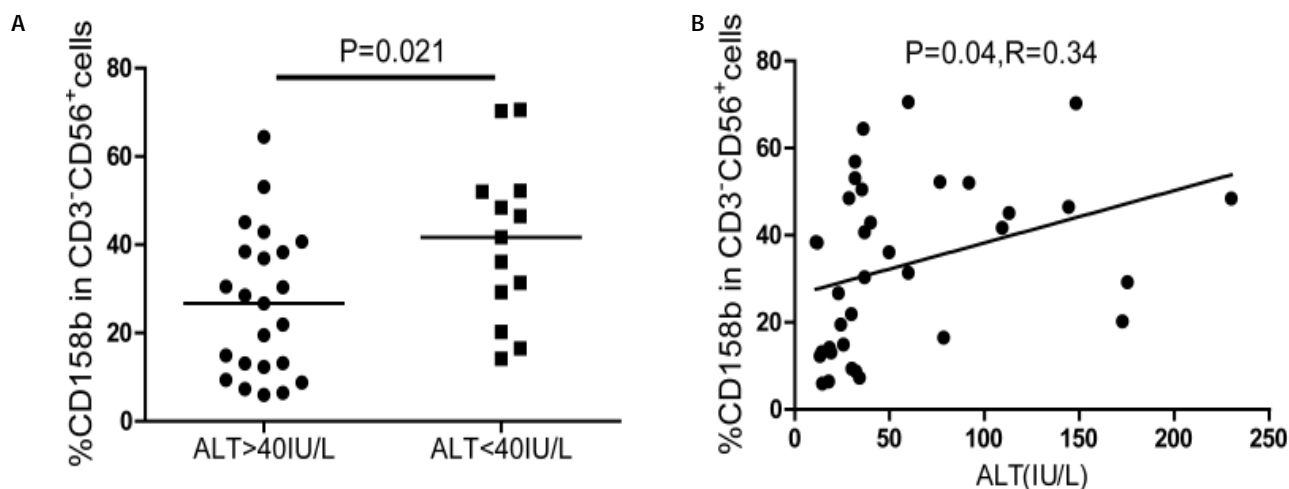


Figure 5. Quantitative analysis of the percentage of IFN- γ and CD107a⁺ NK cells. PBMCs were cultured alone for testing of spontaneous activation or co-cultured in duplicate with PMA+I or K562 cells. Data are expressed as the mean percent of individual samples from at least 2 separate experiments. The horizontal lines represent the median.

the NK cell levels observed in HC subjects, however, no significant changes were found between NK cell levels between SR-HCV and CHC patients. This observation was consistent with work presented by Golden-Mason, *et al.*, that demonstrated that the NK cell numbers were consistently lower in persistently HCV infected patients.²⁰ In addition, previous reports demonstrated that the NK cell frequency was significantly reduced in HCV-infected patients^{21,22} indicating that NK cell development was impaired in HCV infected patients. However, others also demonstrated no differences in NK total cell numbers between HCV infected individuals and healthy controls.²³

By analyzing the NK cell phenotypes we found that the high-expression levels of CD158b were only present in individuals with chronic HCV infections while the expression of CD158b in SR-HCV patients was similar to expression levels in HC subjects. This data indicated that the increased number of inhibitory receptors present on NK cells suppressed NK cell activation. CD158b is a member of the killer cell immunoglobulin-like receptor family (KIR/CD158) that confers inhibitory signals to NK cells. KIRs have either short or long intracytoplasmic tails that transduce either activated or inhibitory signals, respectively.^{24,25} As an inhibitory receptor, CD158b contains D1 and D2 extracellular immunoglobulin-like domains and a long cytoplasmic tail containing immunoreceptor tyrosine-based inhibition motifs (ITIMs) that recruit and activate SHP-1 and SHP-2 phosphatases for inhibitory signal transduction.^{26,27} In line with results presented in this report, Golden-Mason, *et al.* demonstrated that

the inhibitory CD158b receptor on NK cells was highly expressed in chronically infected, unresponsive patients.²⁸ Khakoo, *et al.* demonstrated that inhibitory receptor KIR2DL3 gene expression correlated with HCV resolution in Caucasians and African Americans exposed to low viral loads but not in individuals exposed to high HCV doses since higher inocula overwhelmed the immune response.¹ These data suggested that inhibitory NK cell interactions were important in defining antiviral immunity and that diminished inhibitory responses conferred protection against HCV.

NKp30 and NKp46 are representative members of the NCR family that are selectively expressed on NK cells and play an important role in immune-surveillance of viral infections.¹³ Analysis of circulating lymphocyte populations demonstrated that NK cells harvested from CHC and SR-HCV patients presented with decreased NKp30 and NKp46 expression levels. Consistent with these results, others have demonstrated that chronic HCV patients with sustained virological response (SVR) had low baseline NKp30 and NKp46 expression levels and had significantly increased activating NKp30 and NKp46 expression levels post treatment.²⁸ These data demonstrated that HCV infections depressed NK cell activity resulting in further impairment of innate immune responses.

The correlation between NCR expression and the clinical index was further analyzed and demonstrated that the CD158b expression levels were highest among patients with elevated ALT levels. Furthermore, a positive correlation was established between CD158b expression and ALT levels. We hypothesized that expression level of the inhibitory receptor

CD158b on NK cells interfered with the development of an immune response associated with HCV immune escape, while persistently weak immune responses resulted in liver cell destruction. It is therefore important that further studies be carried out to understand how inhibitory NK cells participate in the HCV infection and clearance.

We also analyzed the IFN- γ secretion profile of NK cells harvested from the respective treatment groups. The frequency of spontaneous and inducible IFN- γ -secreting NK cells in patients with CHC and SR-HCV was not significantly different from the healthy controls. It is possible that NK cells played a role in the pathology associated with HCV infections by secreting additional pro-inflammatory cytokines such as TNF.²⁹ We found that the frequency of CD107a⁺ NK cells was decreased in SR-HCV and CHC patients. Recently it was found that NK cell degranulation (during acute infections) positively correlated with the magnitude of HCV-specific adaptive T cells response,³⁰ indicating that HCV infections affected the nature of the resulting T cell responses.

In conclusion, data presented in this report demonstrated that HCV infected patients presented with a lower frequency of NKp30⁺ and NKp46⁺ NK cells but with higher frequencies of CD158b⁺ NK cells in CHC patients but not SR-HCV patients. Furthermore, the frequency of CD158b⁺ NK cells correlated with ALT and AST levels in CHC patients. In addition, the lower frequency of activated NK cells was associated with lower levels of NK cell degranulation in HCV-infected patients. These findings suggested that during the chronic HCV infection stage, the function (the antiviral activity of NK cells) was significantly depressed even though in SR-HCV patients the NK cell function was impaired and conferred inhibitory signals. Elucidation of additional mechanisms by which NK cell responses can, in some individuals, facilitate the HCV infection process associated with chronic HCV infections or mediate the clearance of HCV infections observed in SR-HCV patients will need further investigation. The small sample size, lack of longitudinal follow-up, and a limited cytokine profile analysis represent study limitations. Therefore future studies examining the effect of phenotype and function on NK cell function in HCV patients (in a larger population) during the drug treatment stage will be necessary.

ABBREVIATIONS

- **HCV:** hepatitis C virus.
- **SR-HCV:** spontaneously resolved HCV.

- **ALT:** alanine aminotransferase.
- **AST:** aspartate aminotransferase.
- **HC:** healthy controls.
- **KIRs:** killer cell immunoglobulin-like receptors.
- **NKR:** natural killer receptor.
- **PBMCs:** peripheral blood mononuclear cells.
- **PBS:** phosphate-buffered saline.

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CONFLICT OF INTEREST

None.

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