Clinical and Experimental Medicine (2019) 19:299–308
https://doi.org/10.1007/s10238-019-00564-9

ORIGINAL ARTICLE

Direct antiviral agents upregulate natural killer cell potential activity in chronic hepatitis C patients

Han-ji Jiang[1] · Xiao-xiao Wang[1] · Bi-fen Luo[1] · Xu Cong[1] · Qian Jin[1] · Hong Qin[1] · Hai-ying Zhang[1] · Xiang-sha Kong[1] · Lai Wei[1] · Bo Feng[1]

Received: 23 December 2018 / Accepted: 12 June 2019 / Published online: 20 June 2019

Abstract

Direct antiviral agents (DAAs) can eliminate hepatitis C virus rapidly and make chronic hepatitis C (CHC) curable. The changes in the innate immune system during treatment with DAAs are still in dispute. To investigate how the functions of natural killer (NK) cells change during and after treatment with DAAs in each NK cell subset. Thirteen CHC patients were treated with sofosbuvir/ledipasvir, and the expression levels of NKp46 and NKG2A were tested via flow cytometry at baseline, at 2, 4, 8 and 12 weeks during the therapy and 12 and 24 weeks after the end of treatment; expression levels were compared between CHC patients and 13 healthy controls. A redirected killing assay was used to detect the cytotoxicity of NK cells. After coculturing NK cells with JFH-Huh7 cells for 72 h, HCV RNA was tested to analyze the inhibition ability of NK cells. All patients achieved sustained virologic response. The expression of the activating receptor NKp46 was decreased first at week 8 during therapy with DAAs and then increased and normalized to levels in healthy controls after treatment with DAAs. The expression of the inhibitory receptor NKG2A was decreased during and after treatment with DAAs. Each NK cell subset has a similar changing trend during and after treatment with DAAs, although some differences can be found earlier and later. The ratio of NKp46 and NKG2A was upregulated after treatment with DAAs. $CD56^{bright}$ NK cells have less amplitude in the frequency ratio changes after treatment with DAAs. The coculture results showed that both the specific lysis and the inhibition of HCV replication were significantly upregulated after treatment with DAAs. DAA treatments can affect patients' NK cell function. After DAA treatments, the expression of functional markers is downregulated, but the potential activity of NK cells is upregulated. The function of NK cells is normalized to levels in healthy controls. $CD56^{bright}$ NK cells play an important role in this process.

Keywords Direct antiviral agents · Hepatitis C virus · Innate immune system · Natural killer cells · Function

Introduction

Chronic hepatitis C virus (HCV) infection is a major cause of liver cirrhosis and hepatocellular carcinoma, affecting approximately 71 million people worldwide. [1] Because of the particularly higher sustained virologic response (SVR) rate (> 95%) and less toxicity, direct antiviral agents (DAAs) have been a first-line treatment option for patients with chronic hepatitis C (CHC) and are recommended by international guidelines, instead of the traditional CHC treatment by pegylated-interferon (PEG-IFN)/ribavirin (RBV). [2, 3] IFN-α can induce immunomodulatory effects by acting on both the innate and the adaptive immune systems. [4, 5] However, DAAs inhibit the replication of HCV by inhibiting the NS3 protease, the NS5A replication complex or the NS5B polymerase activity, which means DAAs do not directly affect the immune system. [6] Thus, treatment with DAAs is a convenient model to study the reaction between HCV and the immune system in humans.

Natural killer (NK) cells play an important role in the human antiviral and antitumor immune responses. [7, 8] NK cells degranulation of cytotoxic granules can secrete perforin and granzymes to lyse virus-infected cells. [9] NK cells are able to induce apoptosis of target cells through tumor necrosis factor-related apoptosis inducing ligand (TRAIL) to exert

✉ Bo Feng
fengbo@pkuph.edu.cn

[1] Beijing Key Laboratory of Hepatitis C and Immunotherapy for Liver Diseases, Peking University Hepatology Institute, Peking University People's Hospital, No. 11 Xizhimen South Street, Beijing 100044, People's Republic of China

cytotoxicity. [10] In addition to cytotoxicity, NK cells can also produce and release various antiviral cytokines, such as IFN-γ and tumor necrosis factor-α (TNF-α). [11] NK cells are characterized by $CD56^{+}CD3^{-}$ lymphocytes and can be divided into three subsets, including $CD56^{bright}$ NK cells, $CD56^{dim}$ NK cells and $CD56^{neg}$ NK cells, which are detected in chronic viral infections and are characterized by $CD56^{-}CD3^{-}CD16^{+}$ lymphocytes. [12] The activity of NK cells is regulated by several NK cell receptors, including natural killer group 2C (NKG2C), NKG2D, natural cytotoxicity receptors (NCRs) NKp30, NKp44, NKp46 and inhibitory receptors, such as NKG2A and killer cell immunoglobulin-like receptors (KIRs). [13–15] During chronic HCV infection, the population of both NK cell subsets and their receptors is still under dispute. [16–21].

NKp46 is one of the NCRs family members, and its expression is upregulated on both peripheral blood NK cells and intrahepatic NK cells in CHC patients. [16] $NKp46^{high}$ NK cells are a special subgroup of NK cells with a high expression of NKp46. $NKp46^{high}$ NK cells have enhanced cytotoxicity and increased IFN-γ secretion. [22] It has been reported that NKp46 expression is much higher in CHC patients than in healthy controls and decreases during treatment with DAAs. [23] However, no research has reported dynamic changes in NKp46 expression during treatment with DAAs or after the end of treatment. From a general perspective, NKp46 expression may maintain a low level after the end of the treatment with DAAs. However, we found different results in our study.

Because of the same transmission mode of HCV and hepatitis B virus (HBV), coinfected patients are not unusual. [24] Similarly, in IFN therapy, previous reports indicate that HBV may reactivate in CHC patients coinfected with HBV after treatment with DAAs, resulting in different severities of hepatitis ranging from HBV reactivation without hepatitis to fulminant hepatic failure, requiring liver transplantation. [25–29] In patients coinfected with HBV and HCV, HCV infection usually dominates with a high level of HCV viral load and a low or undetectable HBV viral load. After HCV is cured by treatment with DAAs, the competition between these viruses is broken, and HBV plays a leading role in the infection. Then, HBV is able to replicate and break through. [30] An additional theory is that CHC patients usually have a very active immune system. When HCV is cured, the patient's immune system is less active, and HBV may supersede the patient's immune control. [31] However, the mechanism of HBV reactivation in HBV/HCV coinfected patients after treatment with DAAs is still unknown.

In this study, we analyzed the inhibition ability of CHC patients' NK cells by both detecting the surface marker levels in each NK cell subset and coculturing NK cells with HCV-infected cells, trying to observe the true activity changes of NK cells during and after treatment with DAAs.

Materials and methods

Study cohort

All patients included in this study were based on our past study. [32] Thirteen HCV-infected genotype 1b patients received ledipasvir (90 mg, orally, once daily) and sofosbuvir (400 mg, orally, once daily) for 12 weeks. Thirteen healthy subjects were enrolled in this study for comparison. Informed consent was obtained from all participants. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Peking University People's Hospital. Patients had no signs of coinfection with hepatitis A virus (HAV), HBV, hepatitis D virus (HDV), hepatitis E virus (HEV) or human immunodeficiency virus (HIV). Patients with evidence of hepatocellular carcinoma or cirrhosis were excluded from this study. Pregnant patients or patients with psychiatric disorders were also excluded. Baseline characteristics of patients and healthy controls are shown in Table 1. Patients' blood samples were obtained at baseline; 2, 4, 8 and 12 weeks (2 W, 4 W, 8 W and 12 W, respectively) after the start of therapy and 12 and 24 weeks after the end of therapy (Pt-12 W and Pt-24 W, respectively). There were six experienced-treated patients who relapsed to PEG-IFN/RBV and seven naïve-treated patients in our study.

NK cell surface markers

For each patient, cryopreserved peripheral blood mononuclear cells (PBMCs) from baseline to Pt-24 W were thawed and tested. PBMCs of healthy donors were included in this experiment. Thawed PBMCs were stained with anti-CD45-APC-H7, anti-CD3-PerCP-Cy5.5, anti-CD56-APC, anti-CD16-BV510, anti-CD335(NKp46)-PE-Cy7 and anti-CD159a(NKG2A)-PE (all from BD Bioscience) for 15 min and with 7-aminoactinomycin D (7-AAD; BD Bioscience) for 10 min before detection by flow cytometry (FCM; BD FACS Aria II, BD Bioscience). Data were analyzed by BD FACSDiva Software v7.0.

Huh7 HCV replication cells

Huh7 cells were infected with HCV JFH-1 strain (genotype 2a) at a multiplicity of infection (MOI) of 1 for 16 h (JFH-Huh7 HCV replication cells). These cells were grown in high-glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Gaithersburg, MD, USA) supplemented with glutamine, 10%

Table 1 Baseline characteristics of CHC patients and healthy controls

	CHC patients	Healthy controls	*P* value
Number	13	13	
Age (years)	30 (24–63)	39 (27–55)	0.063
Gender (male/female)	6/7	3/10	< 0.001
BMI (kg/m2)	23.62 (15.85–29.33)	23.24 (17.58–29.38)	0.372
Pretreatment Condition (Native/Experienced)	7/6	–	
ALT (U/L)	30 (11–85)	21 (10–44)	0.079
AST (U/L)	25 (20–75)	20 (14–25)	0.072
HCV RNA (log)	6.51 (4.60-6.98)	NT	
Response to treatment	SVR24	–	

CHC chronic hepatitis C; *BMI* body mass index; *ALT* alanine aminotransferase; *AST* aspartate aminotransferase; *HCV* hepatitis C virus; *SVR24* sustained virologic response at 24 weeks post treatment; *NT* not detected

fetal bovine serum and 1% penicillin/streptomycin (Gibco Life Technologies, Gaithersburg, MD, USA).

NK cell separation and coculture with virus replication cells

NK cells were isolated from PBMCs by magnetic bead separation kits (Miltenyi Biotech, Bergisch Gladbach, Germany) and cultured in RPMI 1640 medium (Gibco Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Gaithersburg, MD, USA) and 1% penicillin/streptomycin at 37 °C and 5% CO_2 for 5 h.

JFH-Huh7 HCV replication cells (2×10^4/well) were seeded in 96-well plates. After 5 h, the medium was removed, and purified NK cells were cocultured with JFH-Huh7 cells at a 2:1 effector/target (E:T) ratio at 37 °C for 72 h. The supernatant was separated after coculturing NK and JFH-Huh7 cells, and HCV RNA was detected by the Cobas TaqMan automated real-time PCR platform reaction (Roche Molecular Systems, Pleasanton, CA, USA). The inhibition rate of HCV RNA was determined as (HCV RNA of a blank well - HCV RNA of a detection well)/(HCV RNA of a blank well).

Redirected killing assay

NK cells were cocultured with JFH-Huh7 cells labeled with carboxyfluorescein succinimidyl ester (CFSE, BD Bioscience). After incubating for 4 h, the 7-AAD antibody (BD Bioscience) was added prior to FCM analysis. The rate of specific lysis of target cells was determined as the number of $7\text{-AAD}^+\text{CFSE}^+$ cells/number of CFSE^+ cells.

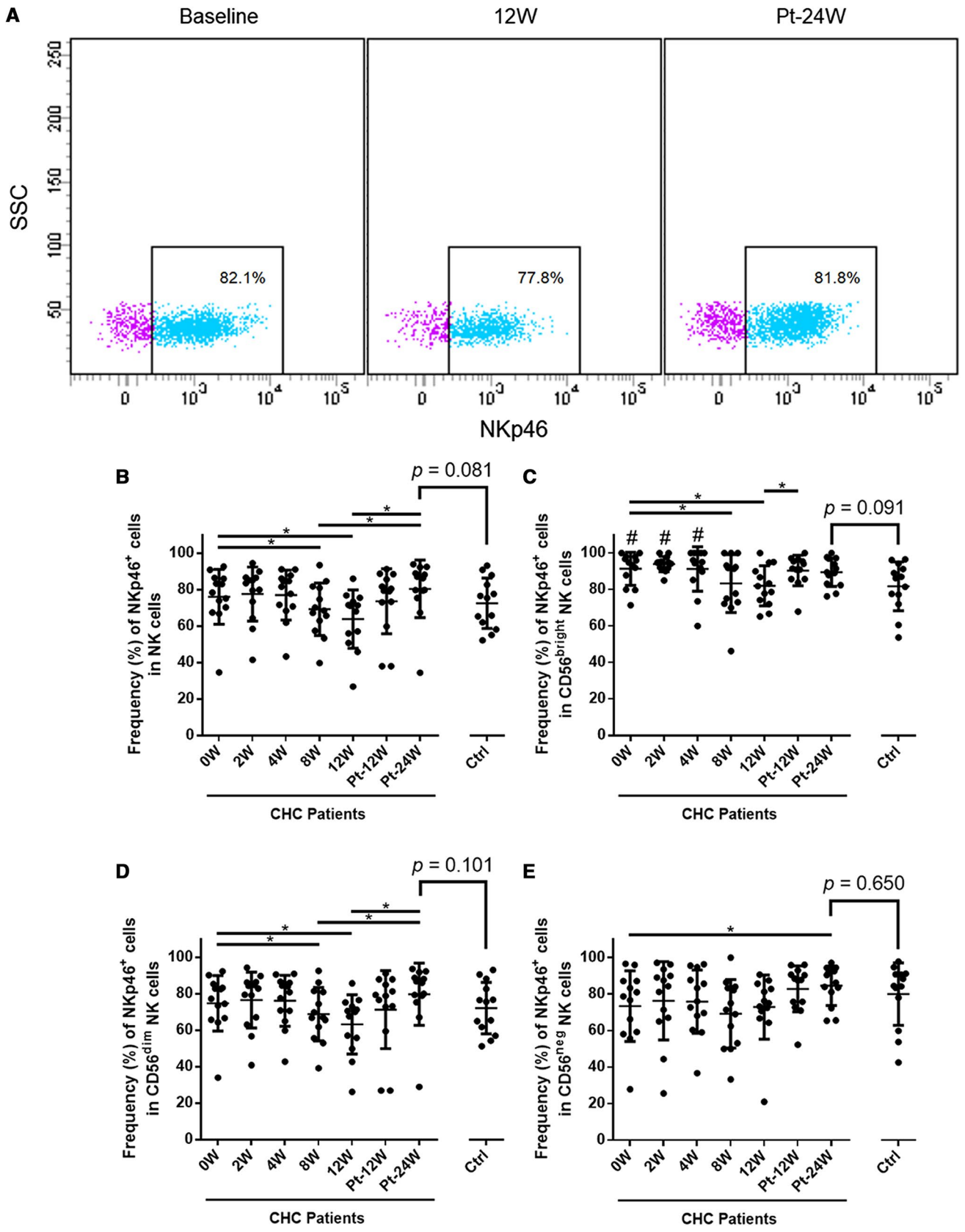
Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 5.0a (GraphPad Software Inc., San Diego, CA, USA) and SPSS 16.0 (SPSS, Chicago, IL, USA). Normal distribution was tested with the Kolmogorov–Smirnov test. Values in our study were not normally distributed, and comparisons of expression levels among different time points in CHC patients were analyzed by Wilcoxon matched pairs test. Comparisons of expression levels between the CHC patients and healthy controls were analyzed by the Mann–Whitney test. Two-sided *P* values less than 0.05 were considered significant.

Results

1. Changes of NKp46 during and after treatment with DAAs

   NKp46 expression was analyzed on each NK cell subset by FCM. Its expression is shown at baseline, 12 W and Pt-24 W in Fig. 1a. At baseline, there were no significant differences between CHC patients and healthy controls. The frequency of NKp46 showed a significant decline at 8 W and 12 W and increased after the end of treatment at Pt-12 W and Pt-24 W (Fig. 1b). In the $\text{CD56}^{\text{bright}}$ NK cell subset, the frequency of NKp46 at baseline and before 4 W was significantly higher in CHC patients than in healthy controls. The expression of NKp46 also declined first at 8 W and increased after the end of treatment with DAAs, but no significant increase was found at Pt-24 W (Figure 1c). In the CD56^{dim} NK cell subset, the change in NKp46 was the same as that in whole NK cells (Figure 1d). However, in CD56^{neg} subsets, the expression of NKp46 was upregulated at


A
Baseline
12W
Pt-24W
SSC
NKp46
82.1%
77.8%
81.8%
B
p = 0.081
Frequency (%) of NKp46+ cells in NK cells
C
p = 0.091
Frequency (%) of NKp46+ cells in CD56bright NK cells
D
p = 0.101
Frequency (%) of NKp46+ cells in CD56dim NK cells
E
p = 0.650
Frequency (%) of NKp46+ cells in CD56neg NK cells
0W
2W
4W
8W
12W
Pt-12W
Pt-24W
Ctrl
CHC Patients

◂**Fig. 1** Changes in NKp46 expression of NK cells from peripheral blood during and after treatment with DAAs. **a** The expression of NKp46 in one CHC patient at baseline, 12 weeks (12 W) and 24 weeks post treatment (Pt-24 W). **b** Changes in the frequency of NKp46^{+} natural killer (NK) cells in whole NK cells during and after treatment with direct antiviral agents (DAAs). **c** Changes in the frequency of NKp46^{+} NK cells in CD56^{bright} NK cells during and after treatment with DAAs. **d** Changes in the frequency of NKp46^{+} NK cells in CD56^{dim} NK cells during and after treatment with DAAs. **e** Changes in the frequency of NKp46^{+} NK cells in CD56^{neg} NK cells during and after treatment with DAAs. $^{*}P<0.05$ between different time points. $^{\#}P<0.05$ between each time point and healthy controls

Pt-24 W, while no significant changes were found at 8 W or 12 W (Figure 1e). At Pt-24 W, the expression of NKp46 showed no significant differences between healthy controls and both the whole NK cells and each NK cell subset.

2. Changes of NKp46^{high} NK cells during and after treatment with DAAs

   NKp46^{high} NK cells were a special subgroup of NK cells with high expression of NKp46 with enhanced cytotoxicity and high IFN-γ secretion. The expressions of NKp46^{high} NK cells are shown at baseline, 12 W and Pt-24 W in Fig. 2a. The frequency of NKp46^{high} NK cells showed no significant differences both during and after treatment with DAAs in any NK cell subset (Figure 2b–e). However, the MFI of the NKp46^{high} subset decreased at 12 W and increased at Pt-12 W and Pt-24 W (Fig. 2 F). In CD56^{bright} subsets, no significant decreases in the MFI of the NKp46^{high} subset were found at 8 W or 12 W, while the MFI increased after treatment with DAAs compared with the MFI at 8 W and 12 W (Figure 2g). The changing trend of the MFI in NKp46^{high} NK cells in the CD56^{dim} subset was similar to that in the whole NK cells but significantly lower than that at 12 W for healthy controls (Fig. 2h). There were no significant changes in CD56^{neg} NK cells (Fig. 2i). At Pt-24 W, the expression of NKp46^{high} NK cells showed no significant differences between healthy controls and both the whole NK cells and each NK cell subsets.

3. Changes in NKG2A during and after treatment with DAAs

   NKG2A expression was analyzed at each time point during and after treatment with DAAs, and its expression is shown at baseline, 12 W and Pt-24 W in Fig. 3a. At baseline, the expression of NKG2A was significantly higher in both the whole NK cells and each NK cell subset than in healthy controls (Fig. 3b–e). The frequency of NKG2A decreased at 12 W and further decreased after the end of treatment with DAAs at Pt-12 W and Pt-24 W in the whole NK cells and in the CD56^{neg} NK cell subsets (Fig. 3b, e). However, the frequency of NKG2A decreased only after treatment with DAAs in CD56^{bright} and CD56^{dim} NK cell subsets (Fig. 3c, d). At Pt-24 W, the expression of NKG2A showed no significant differences between healthy controls and both the whole NK cells and each NK cell subset.

4. Changes in the ratio of NKp46 and NKG2A during and after treatment with DAAs

   The ratio of NKp46 and NKG2A (NKp46/NKG2A) was calculated to analyze the potential inhibition ability of NK cells. At baseline, this ratio was significantly higher in both the whole NK cells and each NK cell subset than in healthy controls (Fig. 4a–d). The ratio increased at 12 W and further increased after the end of treatment with DAAs at Pt-12 W and Pt-24 W, showing no significant differences in whole NK cells from healthy controls after treatment with DAAs (Fig. 4a). In the CD56^{bright} and CD56^{dim} cell subsets, the increase in the frequency ratio appeared only after treatment with DAAs (Fig. 4b, c). However, the increase in the frequency ratio appeared at Pt-24 W in CD56^{neg} subsets (Fig. 4d). At Pt-24 W, there were no significant differences between healthy controls and both the whole NK cells and each NK cell subset.

   The amplitude of the potential inhibition ability of NK cell changes after treatment with DAAs was calculated as the difference between the ratio of NKp46 and NKG2A at baseline and Pt-24 W. The amplitude of this ratio in CD56^{bright} subsets was significantly lower than that in whole NK cells and CD56^{dim} NK cells (Fig. 4e).

5. Specific lysis of HCV-infected cells and inhibition of HCV replication after coculturing JFH-Huh7 cells with patients' NK cells before and after treatment with DAAs

   We then detected the specific lysis ability and inhibition ability of patients' NK cells by coculturing these cells with JFH-Huh7 cells. Because of the sample consumption, only 9 of the 13 patients' samples and six healthy controls were analyzed. The specific lysis of JFH-Huh7 cells cocultured with NK cells after treatment with DAAs was much higher than that before treatment with DAAs, showing no significant differences between healthy controls (Fig. 5a). The inhibition ratio was significantly higher after treatment with DAAs as well, but it is still lower than healthy controls (Figure 5b).

Discussion

In our study, all 13 CHC patients treated with sofosbuvir/ledipasvir achieved SVR24, and no virologic breakthrough occurred. [32] The main surface markers and inhibition functions of NK cells were analyzed by FCM and in coculture systems. We showed that the amount of NKp46 and NKG2A decreased within the first 12 weeks of treatment with DAAs in NK cells. However, after the end of treatment with DAAs, the expression of NKp46 increased to

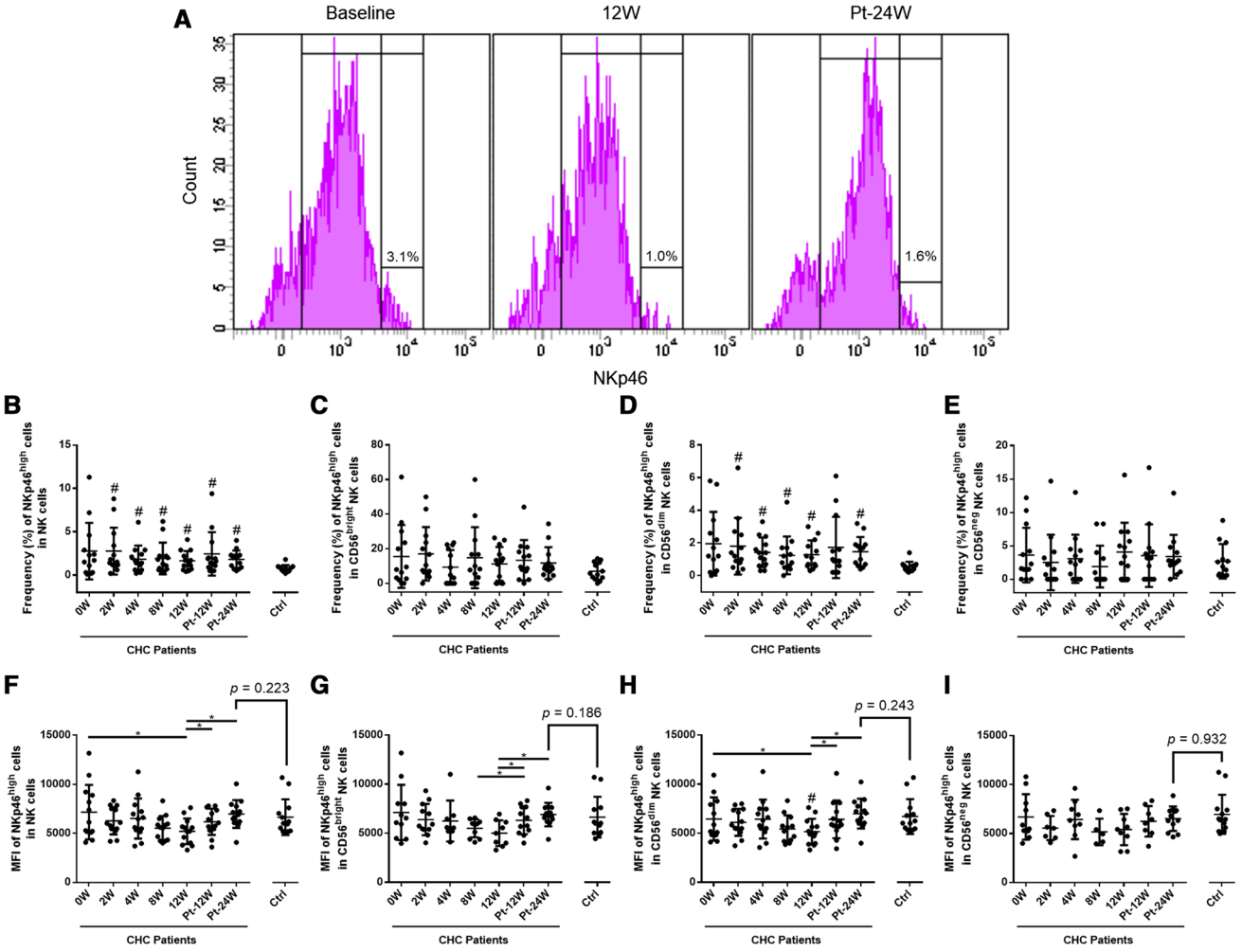


Fig. 2 Changes in NKp46^{high} expression of NK cells from peripheral blood during and after treatment with DAAs. **a** The expression of NKp46^{high} NK cells of one CHC patient at baseline, 12 W and Pt-24 W. **b** Changes in the frequency of NKp46^{high} natural killer (NK) cells in whole NK cells during and after treatment with direct antiviral agents (DAAs). **c** Changes in the frequency of NKp46^{high} NK cells in CD56^{bright} NK cells during and after treatment with DAAs. **d** Changes in the frequency of NKp46^{high} NK cells in CD56^{dim} NK cells during and after treatment with DAAs. **e** Changes in the frequency of NKp46^{high} NK cells in CD56^{neg} NK cells during and after treatment with DAAs. **f** Changes in the mean fluorescence intensity (MFI) of NKp46^{high} NK cells in whole NK cells during and after treatment with DAAs. **g** Changes in the MFI of NKp46^{high} NK cells in CD56^{bright} NK cells during and after treatment with DAAs. **h** Changes in the MFI of NKp46^{high} NK cells in CD56^{dim} NK cells during and after treatment with DAAs. **i** Changes in the MFI of NKp46^{high} NK cells in CD56^{neg} NK cells during and after treatment with DAAs. $^{*}P < 0.05$ between different time points. $^{\#}P < 0.05$ between each time point and healthy controls

a similar level to baseline levels, while the expression of NKG2A remained at a very low level. The ratio of NKp46 and NKG2A increased after treatment with DAAs, which may mean the potential inhibition ability of NK cells increases after treatment with DAAs. After analyzing the data on each NK cell subset, we found that each subset had a similar changing trend during and after treatment with DAAs, although some differences were found earlier and later. Furthermore, after coculturing NK cells with JFH-Huh7 cells, we found that treatment with DAAs intensified both in specific lysis and inhibition ability against HCV-infected cells.

NKp46 is one of the members of the NCR family that activates NK cell receptors. [16] In our study, the expression of NKp46 decreased at 8 W and 12 W but increased after the end of the treatment with DAAs. Other reports have found that NKp46 declines at 8 W as well, [13, 17] but none of these studies have shown dynamic changes in NKp46 expression after the end of treatment with DAAs in each NK cell subset. The frequency of NKp46^{high} NK cells showed no significant changes during and after treatment with DAAs in whole NK cells and every cell subset. However, the MFI of NKp46^{high} NK cells showed a similar changing trend to NKp46 expression.

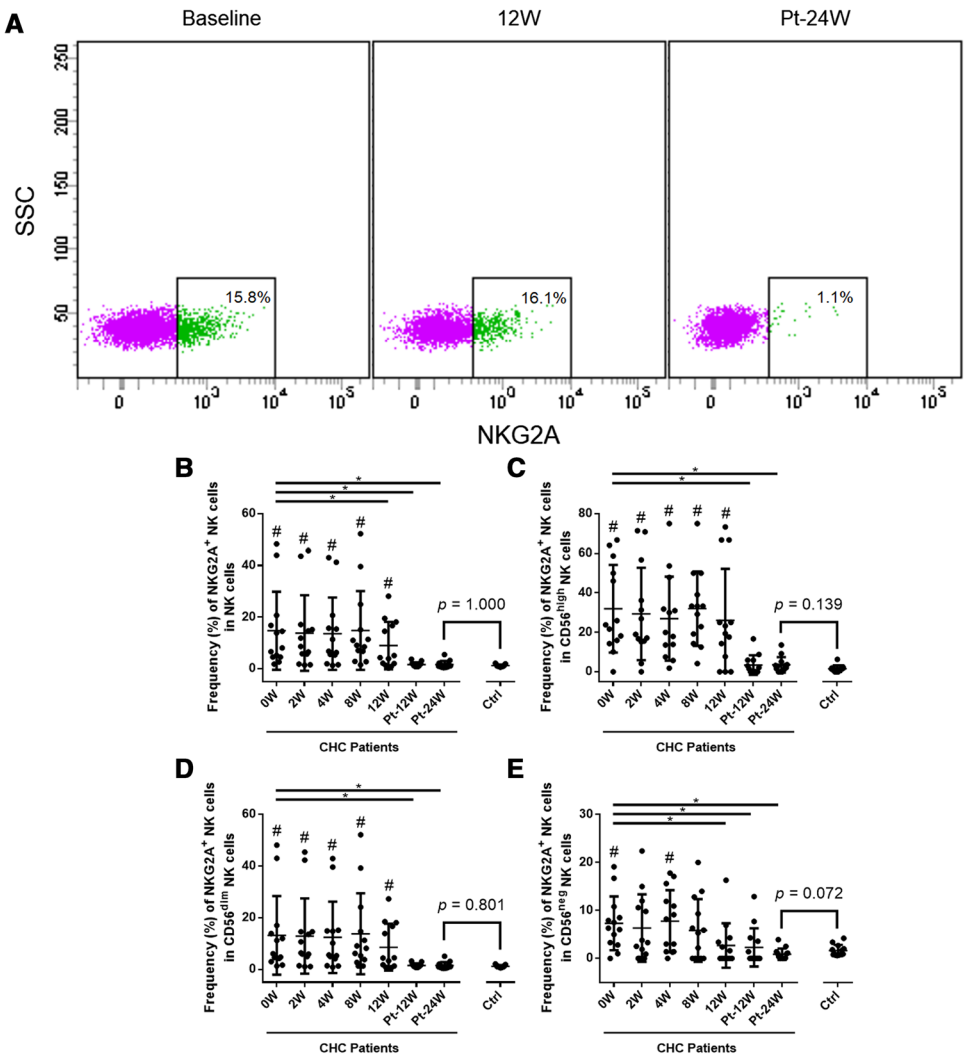


Fig. 3 Changes in NKG2A expression of NK cells from peripheral blood during and after treatment with DAAs. **a** The expression of NKG2A of one CHC patient at baseline, 12 W and Pt-24 W. **b** Changes in the frequency of NKG2A$^+$ natural killer (NK) cells in whole NK cells during and after treatment with direct antiviral agents (DAAs). **c** Changes in the frequency of NKG2A$^+$ NK cells in CD56^{bright} NK cells during and after treatment with DAAs. **d** Changes in the frequency of NKG2A$^+$ NK cells in CD56^{dim} NK cells during and after treatment with DAAs. **e** Changes in the frequency of NKG2A$^+$ NK cells in CD56^{neg} NK cells during and after treatment with DAAs. $^*P < 0.05$ between different time points. $^{\#}P < 0.05$ between each time point and healthy controls

NKG2A is a member of the NKG2 family and a major and prominent inhibitory NK cell receptor. [33] We find that the expression of NKG2A decreased within 12 weeks. Yoshioka et al. found that the expression of NKp46 and NKG2A was positively correlated with chronic HBV patients and that their interaction may contribute to NK cell activation. [34] This result reminds us that the activation of NK cells in CHC patients may also be related to the expression of both NKp46 and NKG2A, and the ratios of NKp46 and NKG2A may approximately show us the activation of NK cells in chronic HCV infection. Although we cannot find a correlation between both the frequency and MFI of NKp46 and NKG2A due to the much smaller capacity than the study of Yoshioka et al., we still calculated and analyzed the ratio of NKp46 and NKG2A during and after treatment with DAAs. The ratio of the frequency of these two markers was significantly lower at baseline in CHC patients than in healthy controls, and this ratio starts to increase at 12 W. In the CD56^{bright} and CD56^{dim} subsets, a similar trend was observed, although the downregulation appeared later after treatment with DAAs. However, in CD56^{neg} NK cells, no such changes were found. This result indicated that the activation of NK cells was upregulated by treatment with DAAs and normalized to the level of healthy controls after the end of treatment in CD56^{bright} and CD56^{dim} subsets but not in CD56^{neg} subsets. However, the expression of many functional markers was downregulated after the end of the treatment with DAAs. [32] This result may be due to the rapid decline in HCV viremia, which decreases the function of NK cells but maintains the potential inhibition of HCV.

Among all three NK cell subsets, the ratio in CD56^{bright} NK cells had less amplitude in frequency changes after treatment with DAAs treatments. CD56^{bright} was identified as a group of young NK cells with high proliferation. [12] During treatment with DAAs, the potential inhibition ability of new CD56^{bright} NK cells is upregulated first, and these cells gradually become CD56^{dim} NK cells. After treatment with DAAs, CD56^{bright} NK cells have the highest potential inhibition ability but are fewer in amount.

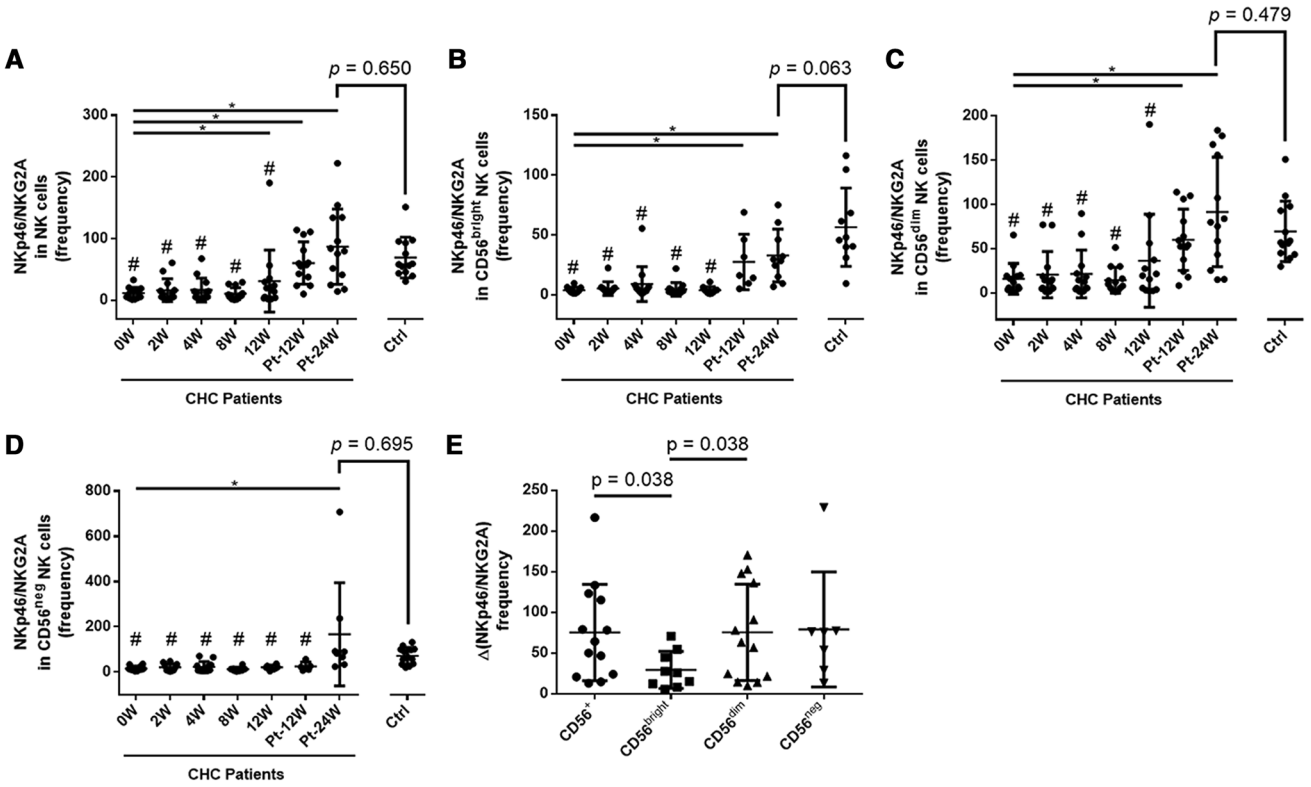


Fig. 4 Changes in the ratio of NKp46 and NKG2A of NK cells from peripheral blood during and after treatment with DAAs. **a** Changes in the ratio of NKp46 and NKG2A from whole natural killer (NK) cells during and after treatment with DAAs. **b** Changes in the ratio of NKp46 and NKG2A from $CD56^{bright}$ NK cells during and after treatment with DAAs. **c** Changes in the ratio of NKp46 and NKG2A from $CD56^{dim}$ NK cells during and after treatment with DAAs. **d** Changes in the ratio of NKp46 and NKG2A in $CD56^{neg}$ NK cells during and after treatment with DAAs. **e** The amplitude of NKp46/NKG2A frequency changes after 24 weeks post treatment (Pt-24 W) in each NK cell subset. $^{*}P<0.05$ between different time points. $^{\#}P<0.05$ between each time point and healthy controls

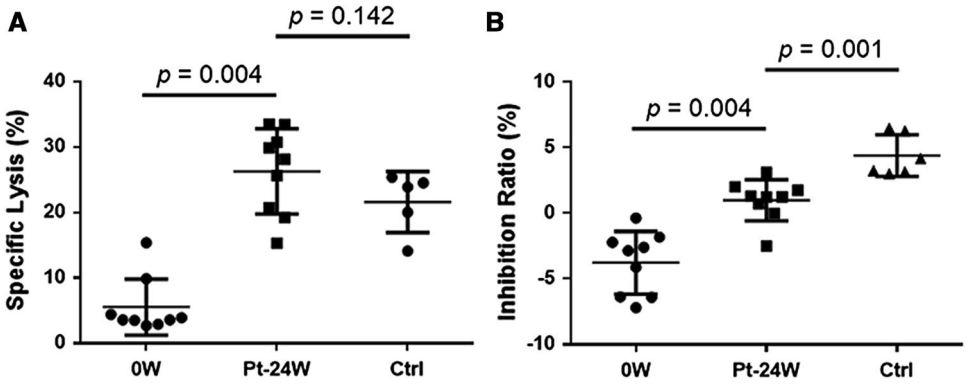


Fig. 5 Specific lysis of HCV-infected cells and inhibition of HCV replication after coculture of patients' NK cells with JFH-Huh7 cells before and after treatment with DAAs. **a** Specific lysis of hepatitis C virus (HCV)-infected cells after coculturing patients' natural killer (NK) cells with JFH-Huh7 cells before and after treatment with direct antiviral agents (DAAs). **b** Inhibition ratio of HCV replication after coculture of patients' NK cells with JFH-Huh7 cells before and after treatment with DAAs. $^{*}P<0.05$ between different time points

Furthermore, both the specific lysis of HCV-infected cells and the inhibition of HCV replication increased after treatment with DAAs. These results further demonstrate that the potential inhibition ability and cytotoxicity of NK cells in CHC patients were upregulated after treatment with DAAs, even if the functional markers were at a very low level. [32] When patients are infected with HCV without being treated with antiviral therapy, their NK cells are overactivated,

which means that these NK cells have a high expression of functional markers, such as IFN-γ and granzyme B but have little cytotoxicity and inhibition ability against additional HCV infection. After using DAAs, the HCV RNA is rapidly eliminated. As functional markers are downregulated, the activity of NK cells is normalized to, or trending to, a level in healthy controls. The potential inhibition ability is upregulated as well but may be later than NK cell normalization; at Pt-24 W, the inhibition ratio of HCV RNA was still significantly less than that of healthy controls. In other studies, some researchers suggest that in HCV/HBV coinfected patients, when HCV is cured by DAAs, the patient's immune system is less active, and HBV may supersede the patient's immune control and reactivate. [31] However, in our study, we found that the potential activity of NK cells was upregulated after treatment with DAAs, which is inconsistent with previous studies.

Our results show that NK cell cytotoxicity and potential inhibition ability were upregulated after treatment with DAAs. The NK cell activity was normalized after treatment with DAAs. $CD56^{bright}$ NK cells play an important role in this process. Our results give an inconsistent opinion about HBV reactivation in HBV/HCV coinfected patients. Further work about patients coinfected with HBV/HCV needs to be performed in the future to explain the mechanism of HBV reactivation in HBV/HCV coinfected patients after treatment with DAAs.

Funding This study was supported by the National S&T Major Projects for Infectious Diseases Control (2017ZX10302201-004-001 and 2017ZX10203202-003-003).

Compliance with ethical standards

Conflicts of interest There were no conflicts of interest in this study.

References

1. World Health Organization. Hepatitis C. http://www.who.int/mediacentre/factsheets/fs164/en/. 19 Dec 2017. Updated October 2017.
2. European Association for Study of Liver. EASL recommendations on treatment of hepatitis C 2015. J Hepatol. 2015;63:199–236.
3. Omata M, Kanda T, Wei L, et al. APASL consensus statements and recommendation on treatment of hepatitis C. Hepatol Int. 2016;10:702–26.
4. Garcia-Sastre A, Biron CA. Type 1 interferons and the virus-host relationship: a lesson in détente. Science. 2006;312:879–82.
5. Markova AA, Mihm U, Schlaphoff V, et al. PEG-IFN alpha but not ribavirin alters NK cell phenotype and function in patients with chronic hepatitis C. PLoS ONE. 2014;9:e94512.
6. Pawlotsky JM. New hepatitis C therapies: the toolbox, strategies, and challenges. Gastroenterology. 2014;146:1176–92.
7. Altfeld M, Fadda L, Frleta D, Bhardwaj N. DCs and NK cells: critical effectors in the immune response to HIV-1. Nat Rev Immunol. 2011;11:176–86.
8. Caligiuri MA. Human natural killer cells. Blood. 2008;112:461–9.
9. Lodoen MB, Lanier LL. Natural killer cells as an initial defense against pathogens. Curr Opin Immunol. 2006;18:391–8.
10. Yoon JC, Yang CM, Song Y, Lee JM. Natural killer cells in hepatitis C: current progress. World J Gastroenterol. 2016;22:1449–60.
11. Lee SH, Miyagi T, Biron CA. Keeping NK cells in highly regulated antiviral warfare. Trends Immunol. 2007;28:252–9.
12. Björkström NK, Ljunggren H-G, Sandberg JK. CD56 negative NK cells: origin, function, and role in chronic viral disease. Trends Immunol. 2010;31:401–6.
13. Spaan M, van Oord G, Kreefft K, et al. Immunological analysis during interferon-free therapy for chronic hepatitis C virus infection reveals modulation of the natural killer cell compartment. J Infect Dis. 2016;213:216–23.
14. Vidal SM, Khakoo SI, Biron CA. Natural killer cell responses during viral infections: flexibility and conditioning of innate immunity by experience. Curr Opin Virol. 2011;1:497–512.
15. de Groen RA, Boltjes A, Hou J, et al. IFNλ-mediated IL-12 production in macrophages induces IFNγ production in human NK cells. Eur J Immunol. 2014;45:250–9.
16. Ahlenstiel G, Titerence RH, Koh C, Edlich B, et al. Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner. Gastroenterology. 2010;138:325–35.
17. Oliviero B, Varchetta S, Paudice E, et al. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. Gastroenterology. 2009;137:1151–60.
18. De Maria A, Fogli M, Mazza S, et al. Increased natural cytotoxicity receptor expression and relevant IL-10 production in NK cells from chronically infected viremic HCV patients. Eur J Immunol. 2007;37:445–55.
19. Sène D, Levasseur F, Abel M, et al. Hepatitis C virus (HCV) evades NKG2Ddependent NK cell responses through NS5A-mediated imbalance of inflammatory cytokines. PLoS Pathog. 2010;6:e1001184.
20. Golden-Mason L, Madrigal-Estebas L, McGrath E, et al. Altered natural killer cell subset distributions in resolved and persistent hepatitis C virus infection following single source exposure. Gut. 2008;57:1121–8.
21. Nattermann J, Feldmann G, Ahlenstiel G, Langhans B, Sauerbruch T, Spengler U. Surface expression and cytolytic function of natural killer cell receptors is altered in chronic hepatitis C. Gut. 2006;55:869–77.
22. Krämer B, Körner C, Kebschull M, et al. Natural killer p46High expression defines a natural killer cell subset that is potentially involved in control of hepatitis C virus replication and modulation of liver fibrosis. Hepatology. 2012;56:1201–13.
23. Serti E, Chepa-Lotrea X, Kim YJ, et al. Successful interferon free therapy of chronic hepatitis C virus infection normalizes natural killer cell function. Gastroenterology. 2015;149:190–200.
24. Stanaway JD, Flaxman AD, Naghavi M, et al. The global burden of viral hepatitis from 1990 to 2013: findings from the Global Burden of Disease Study 2013. Lancet. 2016;388:1081–8.
25. Collins JM, Raphael KL, Terry C, et al. Hepatitis B virus reactivation during successful treatment of hepatitis C virus with sofosbuvir and simeprevir. Clin Infect Dis. 2015;61:1304–6.
26. Ende AR, Kim NH, Yeh MM, Harper J, Landis CS. Fulminant hepatitis B reactivation leading to liver transplantation in a patient with chronic hepatitis C treated with simeprevir and sofosbuvir: a case report. J Med Case Rep. 2015;9:164.
27. Ou PC, Fang ZX, Chen J. Hepatitis B reactivation in a chronichepatitis C patient treated with ledipasvirand sofosbuvir: a case

report. Clin Res Hepatol Gastroenterol. 2016;1:1. https://doi.org/10.1016/j.clinre.2016.08.001.
28. Takayama H, Sato T, Ikeda F, Fujiki S. Reactivation of hepatitis B virus during interferon-free therapy with daclatasvir and asunaprevir in patient with hepatitis B virus/hepatitis C virus coinfection. Hepatol Res. 2016;46:489–91.
29. Wang C, Ji D, Chen J, et al. Hepatitis due to reactivation of HBV in endemic areas among patients with hepatitis C treated with direct-acting antiviral agents. Clin Gastroenterol Hepatol. 2017;15:132–6.
30. Chen G, Wang C, Chen J, et al. Hepatitis B reactivation in hepatitis B and C coinfected patients treated with antiviral agents: a systematic review and meta-analysis. Hepatology. 2017;66:13–26.
31. Gish RG. HBV/HCV Coinfection and Possible Reactivation of HBV Following DAA Use. Gastroenterol Hepatol (N Y). 2017;13:292–5.
32. Wang XX, Luo BF, Jiang HJ, et al. Recovery of natural killer cells is mainly in post-treatment period in chronic hepatitis C patients treated with sofosbuvir plus ledipasvir. World J Gastroenterol. 2018;24:4554–64.
33. Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. J Exp Med. 1998;187:813–8.
34. Yoshioka T, Tatsumi T, Miyagi T, et al. Frequency and role of NKp46 and NKG2A in hepatitis B virus infection. PLoS One. 2017;12:e0174103.