



Original Article

# Direct-acting Antiviral-induced Transient Recovery of NK Cells in Early-stage Treatment of Chronic Hepatitis C Patients



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## Abstract

**Background and Aims:** The rapid clearance of hepatitis C virus induced by direct-acting antivirals (DAAs) affects natural killer (NK) cells, but the reported results are not consistent, and the relative mechanism was unclear. This study focused on the dynamic changes of NK cells during and after DAA treatment and analyzed the reasons. **Methods:** Peripheral blood from 35 chronic hepatitis C patients who were treated with DAAs were collected at baseline and weeks 1, 2, 4, 12, and post-treatment week-12. The frequency, subset, and phenotype of NK cells were assayed by flow cytometry. Lactate dehydrogenase assays were used to evaluate the cytotoxicity of NK cells. Cytokine concentrations were measured with Luminex kits. **Results:** All patients achieved a sustained viral response (SVR), and the NK cell frequencies were not changed significantly during DAA therapy. However, the cytotoxicity of NK cells recovered significantly early in week 1, and then continuously decreased below normal levels. The changes of genotypes including Nkp30<sup>+</sup>, Nkp46<sup>+</sup>, and NKG2A<sup>+</sup> NK cells were parallel to NK function. The subset of CD56<sup>dim</sup> NK cells continuously increased and did not return to normal even at

12 weeks after treatment. Interleukin (IL)-2, IL10, IL15, interferon-gamma, and tumor necrosis factor-alpha all increased after week 4, peaked at the end of therapy, and then exhibited varying degrees of reduction with time. **Conclusions:** DAA treatment led to transient functional recovery of NK cells in the early stage of treatment, and then continuously decreased to below normal levels. Alterations of NK subsets, phenotypes, and the microenvironment may be involved in the changes.

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## Introduction

Approximately 170 million people worldwide suffer from chronic infection with the hepatitis C virus (HCV), which is a major cause of liver cirrhosis and hepatocellular carcinoma (HCC).<sup>1,2</sup> The recent development of highly potent, interferon (IFN)-free direct-acting antivirals (DAAs) has achieved sustained viral response (SVR) rates of more than 90% after standard treatment.<sup>3,4</sup> The high efficiency and shortening of the course of treatment have resulted in great convenience, but a few patients fail to respond to DAA regimens. DAAs also offer the chance to investigate the immune response to HCV.

It is well known that HCV infection leads to chronic activation of innate immunity and a failure of adaptive immunity.<sup>5,6</sup> Natural killer (NK) cells are a major element of the innate immune system of the liver and represent a critical component of infection control and the elimination of virally infected cells.<sup>7,8</sup> HCV proteins exhaust NK cells and suppress their function in chronic HCV infection.<sup>9</sup> Rapid viral clearance following treatment with DAAs affects the innate

**Keywords:** Hepatitis C; Natural killer cell; Cytokines; Phenotype.

**Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHC, chronic hepatitis C; DAA, direct-acting antiviral; DC, dendritic cell; GGT, gamma-glutamyl transpeptidase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN-γ, interferon-gamma; IFN, interferon; IL10, interleukin10; IL12, interleukin12; IL15, interleukin15; IL2, interleukin2; LDH, lactate dehydrogenase; NK, natural killer; NKR, NK cell receptor; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; SVR, sustained viral response; TBIL, total bilirubin; TNF-α, tumor necrosis factor-alpha.

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**Table 1. Subject characteristics**

Characteristic	CHC patients (n=35)	Healthy donors (n=24)	P-value
Sex (M/F)	16/19	10/14	0.758
Age (year)	44.1±13.4	43.2±12.9	0.794
ALT (U/L)	39.0 (227.1)	14.3 (43.8)	<0.001
AST (U/L)	33 (176.5)	18.5 (21.5)	<0.001
TBIL (μmol/L)	14 (26)	9.3 (23.3)	0.005
ALB (g/L)	45.9±2.9	44.5±3.3	0.096
GGT (U/L)	34 (142.0)	15.9 (66.4)	0.004
Genotype	1b (29) +2a (6)	–	–
HCV RNA (10 <sup>6</sup> IU/mL)	3.68 (30.4)	–	–

Data are means ± standard deviation or median (range). CHC, chronic hepatitis C; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; ALB, albumin; GGT, gamma-glutamyl transpeptidase; HCV, hepatitis C virus.

immune condition, including NK cells. However, reports of changes in NK cells that occur during treatment with DAAs are not consistent.<sup>10–13</sup> Moreover, a high risk of recurrence or *de novo* occurrence of HCC shortly after DAA treatment in patients with cirrhosis was reported in some case studies, which some investigators believe to be caused by the abrupt change in the immune microenvironment of the liver.<sup>14</sup> It is essential to evaluate the change of innate immune system, which is predominated by NK cells, in studies of the mechanism of HCC after DAA-induced HCV clearance. In this study, we investigated the dynamic changes in NK cell frequency, subsets, phenotype, function, and the cytokines associated with NK cells during and after DAA therapy in Chinese chronic hepatitis C (CHC) patients. The aim was to elucidate the influence of DAA-induced HCV clearance on NK cells and the possible reasons.

## Methods

### Study subjects

NK cells were obtained from 35 CHC patients, including twenty-nine genotype 1b and six genotype 2a patients who were enrolled between June 2015 and July 2017 at the Beijing YouAn Hospital, Capital Medical University. CHC was diagnosed by the guidelines for diagnosis and treatment of hepatitis C in China.<sup>15</sup> All patients were treatment naïve. They received elbasvir (50 mg) + grazoprevir (100 mg), ledipasvir (90 mg) + sofosbuvir (400 mg), or paritaprevir (150 mg)/ritonavir (100 mg)/ombitasvir (25 mg) + dasabuvir (250 mg) once/twice a day for 12 weeks. Patients who had signs of coinfection with hepatitis A virus, hepatitis B virus (HBV), hepatitis D virus, hepatitis E virus, or human immunodeficiency virus were excluded. Pregnant patients, liver cirrhosis, or HCC patients were also excluded. The characteristics of the study population are shown in Table 1. Peripheral blood was obtained at baseline, week 1 (W1), week 2 (W2), week 4 (W4), week 12 (W12) and week 12 post-treatment (PTW12). Twenty-four age- and sex-matched healthy donors were used as controls.

### Patient consent statement

All study participants provided written consent; the study was approved by the Medical Ethics Review Committee of Beijing YouAn Hospital, Capital Medical University.

### Serological analysis

All samples used for assay of serum HCV RNA and biochemical indicators, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were processed at the central laboratory of Beijing YouAn Hospital, Capital Medical University. Serum HCV RNA was assayed by Cobas TaqMan real-time PCR (Roche Molecular Diagnostics, Branchburg, NJ, USA) with a lower limit of quantification of 15 IU/mL.

### Peripheral blood mononuclear cell (PBMC) isolation

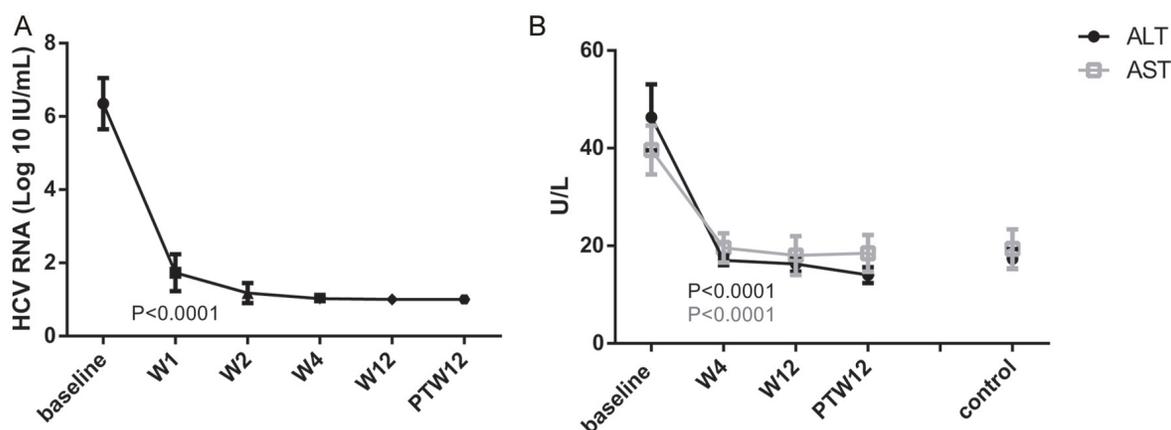
PBMCs were obtained by Ficoll gradient centrifugation (Haoyang Biological Manufacture Co., Ltd, Tianjin, China). After washing three times with phosphate-buffered saline (PBS), the fresh PBMCs were assayed flow cytometry or cryopreserved in 90% fetal bovine serum (FBS) and 10% DMSO at –80°C.

### Flow cytometry

PBMCs were suspended in PBS containing 2% FBS and stained for cell surface antigen expression in the dark after uniform mixing. After washing three times with PBS, the cells were assayed flow cytometry with a BD FACS Canto-II device (Becton Dickinson, Fairlawn, NJ, USA). Isotype-matched control antibodies were used to determine the background levels of staining. Fluorochrome-labeled monoclonal antibodies specific for CD3 and CD56 (BD Biosciences, San Jose, CA) were used to identify NK (CD3<sup>+</sup>CD56<sup>+</sup>) cells within the overall lymphocyte population. Anti-CD335-PE-CY7 (NKp46) and CD337-APC (NKp30) were purchased from BD Biosciences. Anti-CD159A-PE (NKG2A) was purchased from Beckman Coulter (Indianapolis, IN, USA). A Live/Dead Fixable Aqua Dead Cell Staining kit (Invitrogen, Eugene, OR, USA) was used for dead cell exclusion. Data were analyzed by FlowJo version 7.6.1 software (Tree Star, Ashland, OR, USA).

### Killing function of NK Cells (cytotoxicity of NK cells)

Thawed PBMCs were cultured overnight in RPMI 1640 with 10% FBS and 1% penicillin/streptomycin in an incubator. The NK cells were isolated from patient PBMCs with Dynabeads following the manufacturer's instructions (Dynabeads untouched human NK cell kit, Invitrogen, Norway), and cultured in medium with IL2. The purification rate was >80%.



**Fig. 1. Changes in (A) hepatitis C virus RNA and (B) alanine aminotransferase, and aspartate aminotransferase at baseline, on week 12, at 12 weeks after treatment with direct-acting antiviral therapy and in health donors.** W1, week 1; W2, week 2; W4, week 4; W12, week 12; PTW12, week 12 post-treatment.

K562 cells were cocultured with patient NK cells at a ratio of 1:20 in 96-well plates for 24 h at 37°C, and then the supernatant was collected for lactate dehydrogenase (LDH) assay following the manufacturer's instructions (CyQuant LDH cytotoxicity assay kit, Invitrogen). The absorbance was measured at 490 nm and 680 nm via microplate. The killing function of NK cells was reported as percentage (%) cytotoxicity.

#### Luminex-based multiplex assay

Cytokines were measured in each serum sample using a 6-multiplex array on a Bio-Plex 200 system following the manufacturer's instructions. Samples were analyzed for IL2, IL10, IL12, IL15, interferon-gamma (IFN- $\gamma$ ), and tumor necrosis factor-alpha (TNF- $\alpha$ ). Serum samples were incubated with antibody beads with shaking in the dark. After washing, the samples were incubated with biotinylated detection antibodies for 1 h and a streptavidin-phycoerythrin fluorescent conjugate for 30 min. After the final wash, the plates were placed on a Bio-Plex 200 instrument (Bio-Rad Laboratories, Inc.) to read the fluorescence signal intensity of each microsphere added to the samples. The protein concentration of each sample was calculated by standard curve regression.

#### Statistical analysis

Normal distribution was determined by the Kolmogorov-Smirnov test. Comparisons of normally distributed values including NK cell frequency, subset, phenotype, cytotoxicity, and cytokine expression in HCV patients at different times were analyzed by paired *t*-tests or Wilcoxon matched pair tests. Unpaired *t*-test or Mann-Whitney tests was used to compare differences between patients and healthy controls. The statistical analysis was conducted with SPSS 22.0 (IBM Corp., Armonk, NY, USA) or GraphPad Prism 6.0. Significant differences were defined at  $p < 0.05$ .

## Results

### DAA treatment induced rapid HCV elimination in CHC patients

Characteristics of patients and healthy donors are shown in Table 1. All CHC patients experienced a rapid decrease in se-

rum HCV RNA levels within the first week of DAA treatment ( $p < 0.0001$ ), and it remained low until W12 and PTW12. None of the 35 patients had virological breakthrough before or at PTW12 (Fig. 1A). Serum ALT and AST levels both decreased significantly within 4 weeks of DAA treatment ( $p < 0.0001$ ) and reached normal ranges at W12 and PTW12 (Fig. 1B), indicating that hepatocyte inflammation was alleviated during and after DAA therapy.

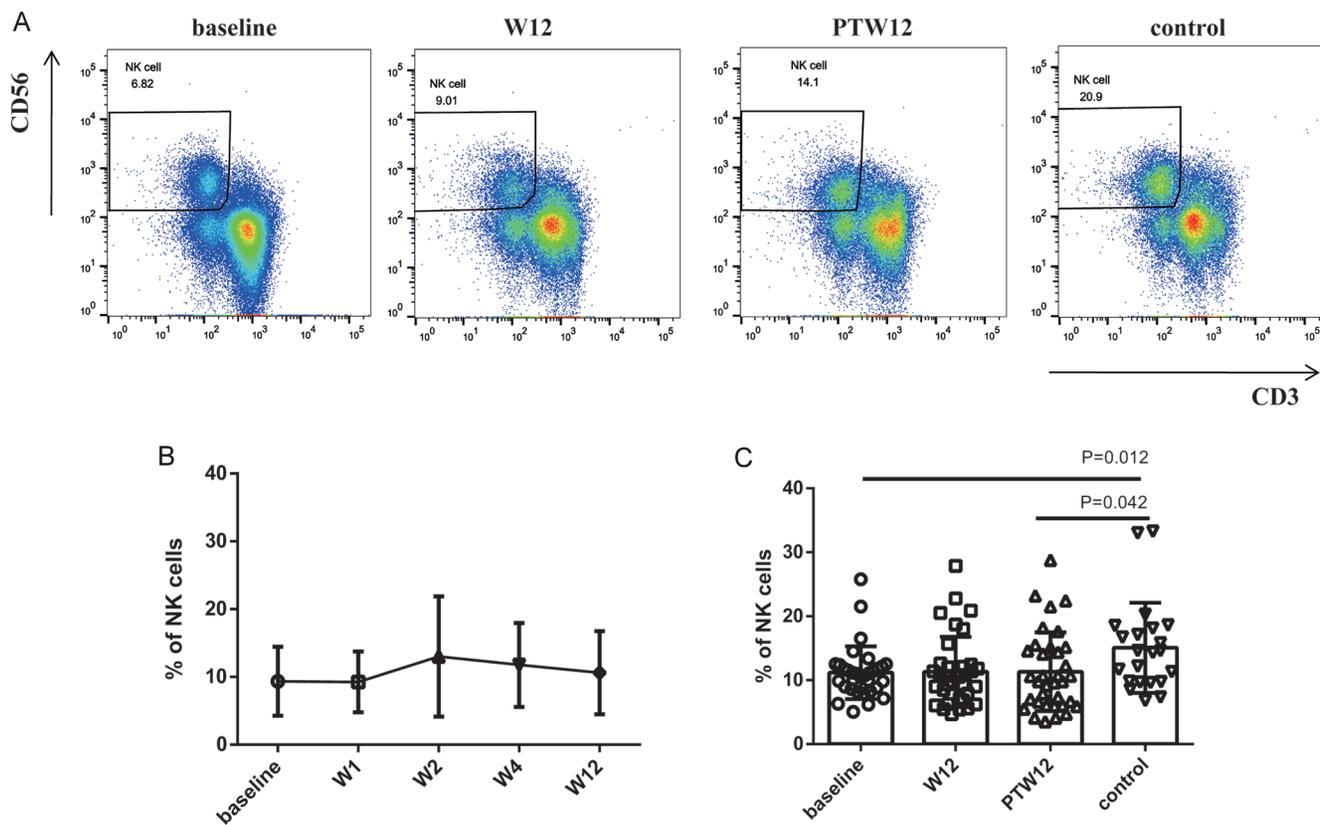
### Effect of DAAs treatment on the NK cell population

First, we investigated whether the DAA-mediated decrease in HCV RNA affected the number of NK cells in peripheral blood. Flow cytometry showed a significant reduction of NK cells in CHC patients at baseline compared with healthy donors ( $p = 0.012$ ; Fig. 2A, C). However, the number of NK cells in CHC patients did not change significantly at each time point during therapy ( $p = 0.220$ ; Fig. 2B). Similarly, after treatment (PTW12) the number of NK cells remained low compared with healthy donors ( $p = 0.042$ ; Fig. 2C).

Second, we detected whether the DAA-mediated decrease in HCV RNA affected the subsets of NK cells in peripheral blood. The numbers of CD56<sup>dim</sup> NK cells at baseline in CHC patients were significantly lower than those in healthy donors ( $p < 0.001$ ; Fig. 3A, B). However, there was a significant increase at the end of DAA treatment (W12) compared with baseline ( $p < 0.001$ ; Fig. 3B). The increase in CD56<sup>dim</sup> NK cells continued to PTW12 but was still lower than the number seen in healthy donors ( $p = 0.004$ ; Fig. 3B). The changes of CD56<sup>bright</sup> NK cells were opposite to those observed in CD56<sup>dim</sup> NK cells (Fig. 3B).

### Effect of DAA treatment on the killing function of NK cells

To investigate the dynamic change in NK cell function, we cocultured patient NK cells with K562 cells and then assayed the cytotoxicity of NK cells by LDH assay. The cytotoxicity of NK cells at baseline in CHC patients was less than half that of healthy donors (28% vs. 64%,  $p < 0.0001$ ; Fig. 3C), but in the first week, it was significantly increased compared with baseline values (46% vs. 28%,  $p = 0.0038$ ; Fig. 3C). After that, the cytotoxicity of NK cells decreased with time, and at PTW12, the cytotoxicity of NK cells was close to the baseline level and significantly lower than it was in healthy donors ( $p < 0.0001$ ; Fig. 3C).



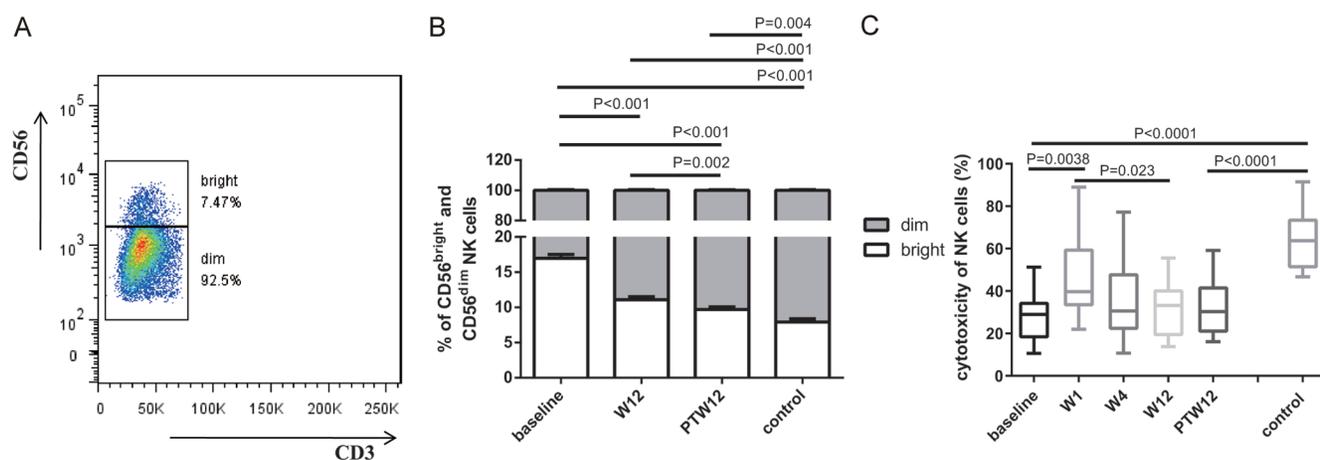
**Fig. 2. Number of NK cells in the peripheral blood of chronic hepatitis C patients during and after direct-acting antiviral therapy.** (A) Representative flow cytometry scatter plot of NK cells (CD3-CD56<sup>+</sup>) and (B, C) Dynamic change in the NK cell numbers at different times. Healthy donors were the controls. NK, natural killer; W1, week 1; W2, week 2; W4, week 4; W12, week 12; PTW12, week 12 post-treatment.

**Effect of DAA treatment on the phenotype of NK cells**

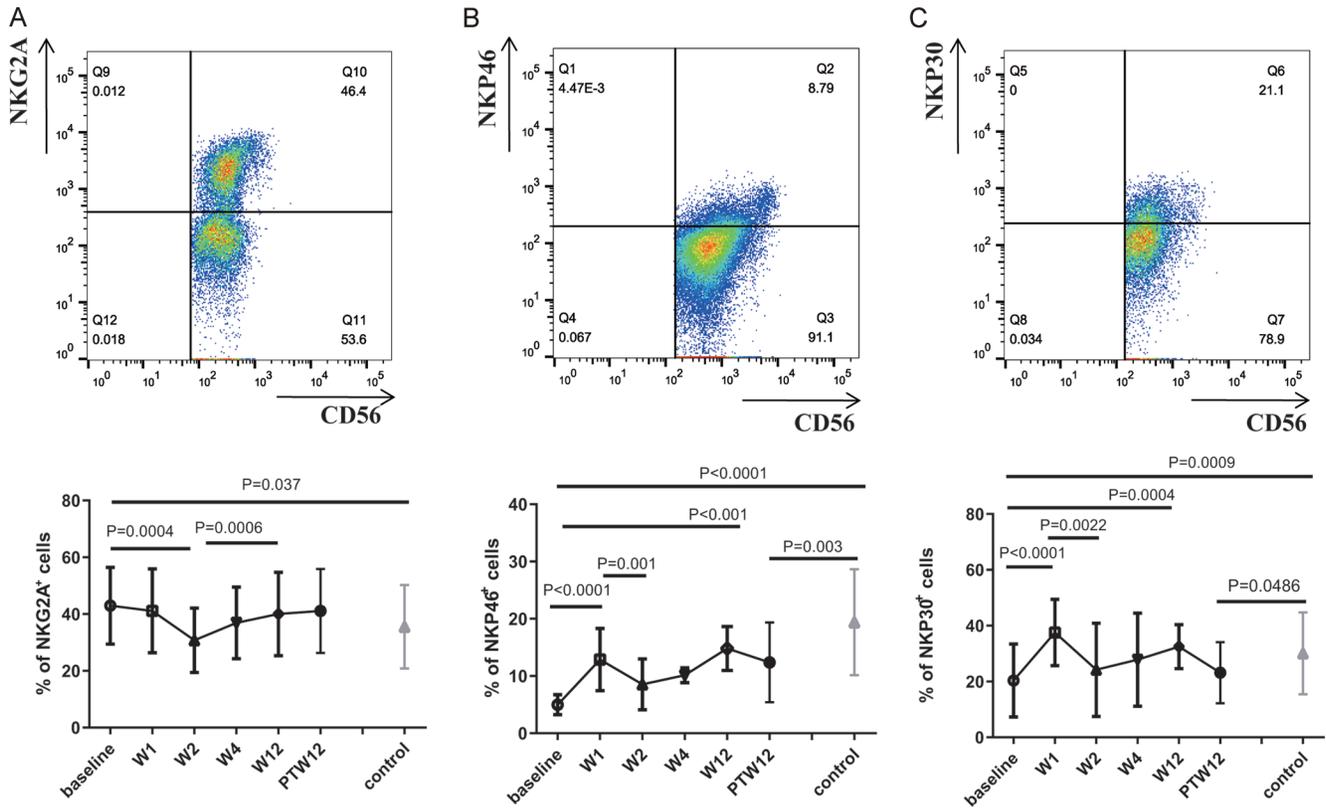
We detected activating (NKp30, NKp46) and inhibitory receptors (NKG2A) on the surface of NK cells. Compared with healthy donors, the proportion of inhibitory NKG2A<sup>+</sup> NK cells in CHC patients was higher at baseline ( $p=0.037$ ; Fig.

4A). During DAA therapy, NKG2A<sup>+</sup> NK cells were reduced at W2 compared with baseline (43% vs. 31%,  $p=0.0004$ ); and then gradually increased and were close to baseline at W12 (Fig. 4A, B).

The proportion of activating NKp46<sup>+</sup> NK cells in CHC patients at baseline was lower than that in healthy donors (7% vs. 19%,  $p<0.0001$ ; Fig. 4C, D). DAA treatment induced a



**Fig. 3. Subsets and cytotoxicity of NK cells in chronic hepatitis C patients during and after direct-acting antiviral therapy.** (A) Representative flow cytometry scatter plot of CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells; (B) Percentages of CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells in patients and healthy donors at different times; (C) Functional capacity of NK cells during and after direct-acting antiviral therapy. NK cells were cocultured with K562 cells and the cytotoxicity was determined in patients and healthy donors by a lactate dehydrogenase assay. NK, natural killer; W1, week 1; W4, week 4; W12, week 12; PTW12, week 12 post-treatment.



**Fig. 4. Alteration of NK cell phenotypes in chronic hepatitis C patients during and after direct-acting antiviral therapy.** Dynamic changes in (A) NKG2A<sup>+</sup> (B) Nkp46<sup>+</sup> and (C) Nkp30<sup>+</sup> NK cell numbers at different times. Healthy donors were the controls. NK, natural killer; W1, week 1; W2, week 2; W4, week 4; W12, week 12; PTW12, week 12 post-treatment.

2.6-fold increase in Nkp46<sup>+</sup> NK cells at W1 ( $p < 0.0001$ ) and then a slight decrease at W2, followed by a gradual increase until W12. At PTW12, the proportion of Nkp46<sup>+</sup> NK cells was still lower than that in healthy donors (12% vs. 19%,  $p = 0.003$ ; Fig. 4C, D). Activating Nkp30<sup>+</sup> NK cells were lower at baseline than in healthy donors (20% vs. 30%,  $p = 0.0009$ ), and underwent a similar alteration as Nkp46<sup>+</sup> NK cells during DAA treatment (Fig. 4E, F). At PTW12, the population of Nkp30<sup>+</sup> NK cells was close to the baseline level, but was smaller than that in healthy donors (23% vs. 30%,  $p = 0.0486$ ; Fig. 4E, F).

**Effect of DAA treatment on cytokines associated with NK cells**

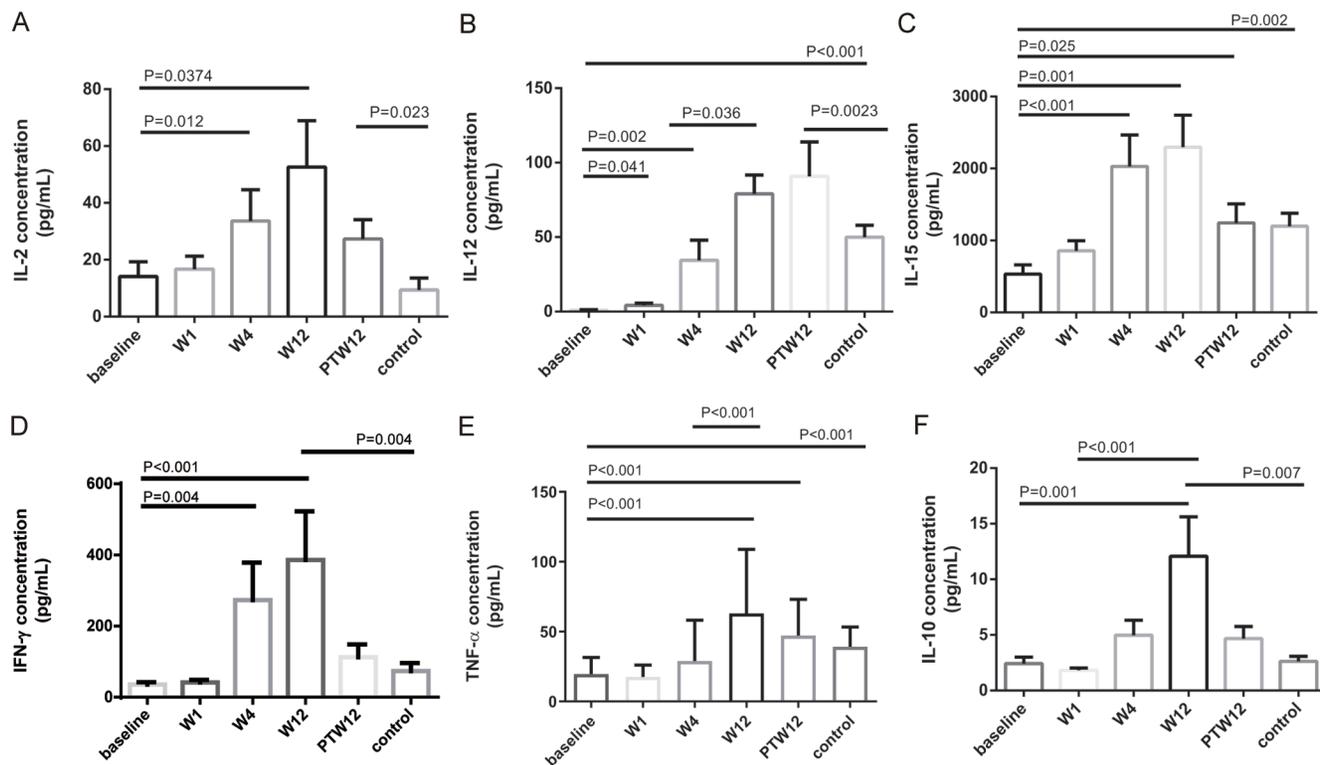
We found that the level of IL2 at baseline was not different from that in healthy donors, but that it began increased significantly after W4 ( $p = 0.012$ ) and reached a peak at W12 when was 2.7-fold higher than the baseline level ( $p = 0.0374$ ). After DAA treatment, the concentration of IL2 at PTW12 was near normal (Fig. 5A). The concentration of IL12 at baseline was too low to be detected, but it peaked at W12 and was detected in nearly all samples (Fig. 5B). The concentration of IL15 at baseline was lower than that in healthy donors ( $p = 0.002$ ), but gradually increased to levels near those in healthy donors (Fig. 5C). IFN- $\gamma$  and TNF- $\alpha$  are the major immunomodulatory cytokines that enhance NK cytotoxicity.<sup>16</sup> The serum concentrations of IFN- $\gamma$  and TNF- $\alpha$  began to increase at W4, peaked at W12, and then decreased at PTW12 (Fig. 5D, E). The change in immunoregulatory cytokine IL10 was similar to that of IFN- $\gamma$  and TNF- $\alpha$  (Fig. 5F).

**Discussion**

NK cells have been implicated in all stages of HCV infection in both genetic and functional studies and are considered as multifaceted players with key roles in hepatitis C immunity.<sup>17</sup> DAAs can eliminate HCV in a short time and disrupt the previous immune homeostasis of HCV. This study investigated the dynamic NK changes associated with DAA-induced HCV clearance and found that DAAs significantly enhanced the killing function of NK cells in only the early stage (W1-W2) of treatment, which then decreased and remained low, but the number of NK cells was not affected. Changes in the NK cell subsets and phenotypes and the microenvironment were all involved in the functional changes of NK cells (Fig. 6).

Chronic infection with HCV results from the reduction and exhaustion of NK cells.<sup>16</sup> Indeed, number of NK cells and the percentage of total lymphocyte population were reduced in CHC patients compared with those in healthy individuals.<sup>18</sup> DAAs specifically inhibit NS3 protease, the NS5A replication complex, or NS5B polymerase activity to reduce the replication of HCV. In theory, the NK frequency should be normalized after HCV clearance. However, the NK cell numbers did not change significantly during the course of DAA treatment, even at PTW12, and were continuously lower than in HCV patients than in healthy donors.

Why did the removal of HCV not restore the NK cell frequencies? We believe that the microenvironment may be a very important factor. The proliferation and development of NK cells depend on the cytokines in the local microenvironment, including IL2, IL3, IL7, IL15, and IL21.<sup>16</sup> We assayed

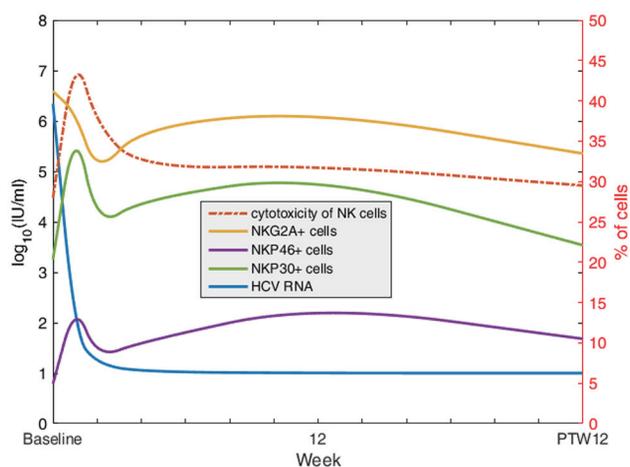


**Fig. 5. Alteration of NK cell-associated cytokines in the serum of chronic hepatitis C patients during and after direct-acting antiviral therapy, including IL2, IL12, IL15, IFN-γ, TNF-α, and IL10.** Healthy donors were the controls. NK, natural killer; IL2, interleukin2; IL12, interleukin12; IL15, interleukin15; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; IL10, interleukin10; W1, week 1; W4, week 4; W12, week 12; PTW12, week 12 post-treatment.

cytokines that promote the proliferation of NK cells, IL2, and IL15, which were elevated at the end of DAA therapy but were reduced at PTW12. Regulatory T cells (Tregs) have been reported to inhibit the proliferation of NK cells.<sup>19,20</sup> The number of Tregs has been reported to decline from baseline to the end of treatment and then increase from the end of treatment to SVR 12 in CHC patients receiving DAA therapy,<sup>21</sup> which means that Treg-induced immune suppression reappears again after HCV clearance. There-

fore, the NK cell population did not normalize after DAA treatment, which might have been caused by a low level of proliferation promotion and a high level of proliferation inhibition in the microenvironment. Some studies have focused on changes of immune diseases after DAA therapy. Previous studies reported that hepatitis C virus-associated cryoglobulinemia vasculitis (HCV-CV) who were treated with DAAs experienced a high rate of remission of vascular inflammation.<sup>22</sup> However, some patients redeveloped vascular disease shortly after DAA treatment ended. The investigators considered that the recurrence of HCV-CV may have been related to the immune microenvironment caused by long-term HCV infection. Given the observations, changes in the microenvironment caused by HCV infection are worth further study.

DAA treatment did not change the number of NK cells, but it did change the subsets and killing function of NK cells. Previous studies reported that the function of NK cells gradually returns to normal after HCV clearance,<sup>10,23</sup> which was not seen in this study. Dynamic investigation of cytotoxicity of NK cells in our study revealed that the killing function of NK cell was partially restored only in the early stage of treatment (W1-W2), and then decreased throughout treatment even at PT12W. How to explain the transient functional recovery of NK cells? We considered the main and direct reason was associated with the early rapid elimination of HCV in the first week. The expression of activating NK receptors (NKp46, NKp30) was enhanced, and that of an inhibitory NK receptor (NKG2A) was reduced, so the cytotoxicity of NK cells was significantly enhanced. The delayed change of the cytokine microenvironment also supports our hypothesis. The function of NK cells in various tissues varies depending on the cellular microenvironment, mainly because of cytokine signals.<sup>24</sup> In this study, the peaks of nearly all



**Fig. 6. Dynamic changes of HCV RNA, cytotoxicity of NK cells, NKG2A+ cells, NKP46+ cells, and NKP30+ cells during and after direct-acting antiviral therapy.** HCV, hepatitis C virus; NK, natural killer; PTW12, week 12 post-treatment.

the measured cytokines associated with NK cells appeared at the end of DAA treatment (W12), which was later than the peak of NK cell cytotoxicity (W1). That indicates that the transient enhanced cytotoxicity of NK cells at an early stage of therapy was dependent on the DAA-induced clearance of HCV over a short time, which removed viral suppression as an NK cell function.

Another issue we need to figure out is, with the clearance of HCV, why the cytotoxicity of NK cells remained low after DAA treatment in our study. Strunz *et al.* also reported that NK cell function did not differ between baseline before the initiation of treatment and at 36 weeks after the patients were off treatment.<sup>25</sup> It is known that NK cells functions are regulated by the balance of signals between inhibitory and activating NK cell receptors (NKR).<sup>26</sup> For example, blocking NKG2A significantly enhances the killing function of NK cells from patients with active CHB<sup>27</sup> and CHC infections.<sup>28</sup> In our dynamic observation of NK cell phenotype, the changes of activating NKP46<sup>+</sup> NK, NKP30<sup>+</sup> NK cells and inhibitory NKG2A<sup>+</sup> NK cells were accompanied by changes in killing function, suggesting they were involved in the changes. The detected cytokines decreased after DAA therapy, which also contributed to the low activity of NK cells. In addition, a previous study reported that the persistence of immunosuppression induced by Tregs continued long-term even after an HCV cure.<sup>29</sup> Therefore, the immune homeostasis after DAA therapy might differ from that in a healthy population. The above results also indicate that DAAs have antiviral activity without producing an immune response to a certain extent.<sup>30</sup>

Our findings have great significance for guiding clinical practice. Although DAA treatment can lead to the clearance of HCV, it does not mean that immune homeostasis is restored to the normal level after therapy. Studies have found that the risk of the occurrence or recurrence remains after DAA-mediated elimination of HCV.<sup>31,32</sup> Our finding that DAA therapy did not improve the killing function of NK cells in the long run, might explain the occurrence or recurrence of HCC to a certain extent. The function of NK cells in DAA-treated patients is defective, and decreased cytotoxicity of NK cells and unrestored NKG2A, NKP46, and NKP30 activity after DAA treatment may be involved in hepatocarcinogenesis. Moreover, Chen *et al.* reported that HBV reactivation occurred much earlier and was generally more severe in patients coinfecting with both HBV and HCV after DAA than after IFN-based treatment.<sup>33</sup> Innate immune mechanisms played a key role in the progression. So, it is important to screen all patients screened for evidence of overt or occult HBV infection during the management of pan-oral DAA therapy. The evidence allows us to conclude that long-term regular follow-up or monitoring while healthy will benefit patients who are off DAA treatment. Doctors should pay attention to whether such patients are at increased risk other diseases, such as tumors and immune system disease.

Our investigation has some limitations. First, it included a relatively small number of patients which may cause us to miss some biologically significant trends. A long-term extensive investigation would have been better. Second, this cohort was limited to investigation of the peripheral immune response, and it was not possible to understand the immune response of liver tissue. Third, our study did not explore the effects of DAAs on adaptive NK cell populations, which were restored to normal after DAAs therapy in previous study.<sup>34</sup> However, that was more pronounced in patients with advanced fibrosis, and indicates that study populations need to be expanded beyond patients with chronic HCV infection.

In addition, although NK cells from cryopreserved PBMC were used in our assay of killing function, their cytotoxicity can be restored by reculturing in media containing IL2.<sup>35</sup> We did, that, and it did not affect our results. Moreover, the trends in the change of the cytotoxicity of NK cells after

thawing were consistent with our preliminary experiments, which used fresh NK cells from HCV-infected patients who were treated with DAA (data not shown). Cryopreserved human specimen, especially those from medium or long-term follow-up, are not the best choice, but are a feasible choice.

In summary, we found that DAA treatment transiently enhanced the killing function of NK cells in the first week of treatment, after that time it was continuously below the normal level. The transient recovery of NK cells might be caused by the early removal of HCV-mediated suppression of NK cell function. The subsequent decrease in the killing function of NK cells might be more related to changes of phenotype and local cytokines. DAA therapy did not affect the number of NK cells. Our study adds to the understanding of the immune consequences of HCV eradication by DAA treatment.

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## Conflict of interest

The authors have no conflict of interests related to this publication.

## Author contributions

XZ designed the study and methodology, drafted and revised the manuscript; YJ collected and analyzed data, performed statistical analysis, and revised the manuscript; SL performed data integration; DB, ML, MK, and YC participated in data collection and study design; ZD provided funding support; SZ participated in the study concept and design, formal analysis, investigation, and critical revision of the manuscript. All authors contributed to manuscript revision, and approved the submitted version.

## Data sharing statement

The data collected for this study have not been made available because the Institutional Ethics Committee required that it should be kept confidential. The statistical analysis plan and the results will be shared.

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