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Natural killer cell activity and function in chronic HCV-infected patients during peg interferon and ribavirin: Early effects of active substance use

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ABSTRACT

In Western countries, chronic hepatitis C virus (HCV)-infection mostly affects former and active substance users. The effect of active substance use on interferon (IFN)-responsiveness and therapy efficacy is not well understood. In this study, we compared natural killer (NK) cell activity and function in healthy controls and chronic HCV-infected patients with and without active substance use, as well as the early effects of antiviral therapy with peg-IFN and ribavirin.

No differences were observed between chronic HCV patients and healthy individuals in the number and frequencies of $CD56^{dim}$ and $CD56^{bright}$ NK cells. Also, IL-12/18-induced IFN-gamma production by NK cells was comparable between all groups, whereas the cytotoxic ability of NK cells (granzyme and CD107a levels) was more potent in HCV-infected patients as compared to healthy controls, and highest in non-substance users. Moreover, at baseline, the activation of NK cells was significantly lower in HCV-infected patients who used substances, when compared to healthy individuals. Therapy-induced viral load reduction assessed early at day 7 showed a similar decline in substance users and non-substance use HCV patients, with 25% substance users and 17% non-substance users testing HCV-RNA negative at day 7. Furthermore, early during IFN-based therapy, NK cells from HCV patients remained responsive to IFN, and only a minor decline in the degree of STAT-1 phosphorylation was observed irrespective of substance use. These findings were further supported by comparable *in vitro* p-STAT-1 induction in all three experimental groups.

Despite subtle differences at baseline between healthy individuals and chronic HCV patients, we observed that active substance use in chronic HCV-infected patients did not affect the immune responsiveness to IFN early after start of treatment, and thus, we found no evidence – from an immunological point of view – that antiviral therapy of our cohort of HCV-infected patients with active substance use is less efficient.

1. Introduction

Hepatitis C is a blood-borne virus infection with an estimated 170 million infected individuals worldwide and a prevalence of 3%. Chronic hepatitis C virus (HCV) infection carries an increased risk of developing liver cirrhosis and hepatocellular carcinoma and is currently the leading cause of end-stage liver disease (Kenny-Walsh, 2009; Lim and Kim, 2008). In chronic HCV infection, viral clearance can be achieved by antiviral therapy consisting of pegylated interferon-alpha (peg-IFN) and ribavirin for 24–48 weeks in 41–86% of individuals (Fried et al., 2002; Hadziyannis et al., 2004; Kamal et al., 2007; Khuroo et al., 2004). The HCV genotype and host gene polymorphisms are important factors determining the success of treatment. In combination with the recently introduced protease inhibitors telaprevir or boceprevir, which inhibit specific steps in viral replication, peg-IFN and ribavirin remain the backbone of chronic HCV treatment (Kronenberger and Zeuzem, 2012; Welsch et al., 2012).

In the developed world, injection drug use (IDU) is the most important risk factor for acquiring HCV (Armstrong et al., 2006). Recent studies suggested that the burden of HCV would be lowered if active substance users would be offered antiviral therapy more often (Martin et al., 2012). Substance users have been excluded from antiviral therapy for a long time because of the side effects

Abbreviations: IFN, interferon; PEG, pegylated; HCV, hepatitis C virus; IVDU, intravenous drug use; SVR, sustained virological response; NK, natural killer.

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of peg-IFN and the supposed increased risk of re-infection (Dalgard, 2005; Hatzakis et al., 2011; Tillmann and Thompson, 2008). However, several research groups have demonstrated feasibility of antiviral therapy in former substance users with comparable rates of SVR and low rates of re-infection (Bruggmann et al., 2008; Dore et al., 2010). Unfortunately, some studies excluded active substance users at the initiation of antiviral therapy or included only small numbers (Bruggmann et al., 2008; North et al., 2012). As IDU constitutes the major mode of transmission, it is of clinical importance to know if active substance use affects the outcome of antiviral therapy. Dore et al. found that drug dependency was independently associated with a lower rate of SVR (Dore et al., 2010). Grebely et al. studied the outcome of antiviral treatment in former and current drug users and observed a significantly lower rate of SVR in a small subset of frequent injectors (Grebely et al., 2007). To treat active substance users effectively, more knowledge is needed on the potential effects of concomitant substance use on the effects of peg-IFN and ribavirin.

Peg-IFN exerts its antiviral effects through the induction of numerous products encoded by IFN-stimulated genes (ISG), which possess direct antiviral activity. Apart from the direct effects, the impact of IFN-based treatment on immune cells may also be important in determining the treatment response (Boonstra et al., 2008), and it was only recently reported that the induction of cytotoxic natural killer (NK) cell function by IFN-α correlates with virologic response to therapy (Ahlenstiel et al., 2011). This is highly relevant since NK cells can recognize virus-infected cells, and eliminate them via cytolytic (e.g. via perforins and granzymes) and non-cytolytic (e.g. via IFN-γ or TNF) pathways (Mondelli et al., 2010; Norris et al., 1998; Vivier et al., 2011).

It is generally accepted that heroin deregulates the function of T-cells, B-cells and NK cells *in vitro* and *in vivo* (Govitrapong et al., 1998; Roy et al., 2006; Wang et al., 2012), as well as *in vitro* HCV replication in cell culture (Li et al., 2007; Li et al., 2003; Zhang et al., 2006). However, little is known on the effects of substance use during IFN-based therapy on responsiveness of immune cells, such as NK cells. Cocaine appears to negatively affect human $CD4^+$ T-cell activation (Chiappelli et al., 1994), and inhibitory effects have been observed on the functions of all subsets of lymphocytes and monocytes/macrophages in mice (Xu et al., 1999). In contrast, in HCV-infected patients, the use of the combination of both cocaine and heroin led to augmented levels of both Th1 and Th2-associated cytokines, while lymphocyte proliferation was reduced (Rios-Olivares et al., 2006).

In several industrialized countries, heroin is prescribed as maintenance therapy. When treating patients who receive maintenance therapy, it is important to understand the clinical and biological outcome of the drug interactions. In addition, heroin use under supervision provides a controlled setting to study the effects of ongoing heroin use during antiviral therapy. Therefore, we aimed to study the effect of heroin as well as cocaine use on NK cell frequency and function before and during antiviral therapy with peg-IFN and ribavirin.

2. Methods

2.1. Patients and antiviral therapy

Twenty-three patients with chronic HCV infections and 12 healthy individuals were included in the study (Table 1). Patients were seen at our outpatient clinic and at the local addiction treatment center. For the exact type, way and frequency of substance use, we refer to Supplementary Table 1, which also presents information on the use of nicotine, alcohol and medication. Data on illicit substance use as well as nicotine and alcohol use were reported by the patient to the physician and/or nurse of the addiction care unit, and is considered reliable since they receive maintenance therapy and are being seen at the addiction treatment center for many years. Also, the absence of substance use was reported by the HCV-infected patients themselves at the outpatient clinic. HCV-infected patients without substance use and healthy individuals were included only when they were not treated by the addiction treatment center and when there was no suspicion of any recent substance use. The physician questioned all patients for their habits related to substance use prior to inclusion and during antiviral therapy. According to standard clinical care and the guidelines for antiviral treatment of chronic HCV patients of the AASLD, (chronic) infection with the hepatitis B virus and/or human immunodeficiency virus was a contra-indication and hence tested for before initiation of therapy. Antiviral therapy consisted of peg-IFN-2b and ribavirin for 24–48 weeks. Peg-IFN-2b was given subcutaneously at 1.5 mg/kg/week; ribavirin was dosed

Table 1
Characteristics of chronic HCV patients and healthy individuals.

	HCV-infected patients without substance use $N = 11$	HCV-infected patients with substance use $N = 12$	Healthy controls $N = 12$
Gender, *n* (%)			
Male	9 (82)	9 (75)	7 (58)
Age, years			
Median (range)	51 (26–64)	53 (45–64)	50 (38–61)
Viral load, baseline IU/mL			
Median (range)	291000 (1420–9460000)	925000 (106000–3570000)	
Alanine aminotransferase (ALT), baseline, U/L			
Median (range)	84 (31–472)	58 (18–123)	
Cirrhosis			
n (%)	2 (18)	2 (17)	
IL28 rs12979860 genotype, *n* (%)			
CC	3 (27)	7 (58)	
CT	5 (45)	4 (33)	
TT	3 (27)	1 (8)	
Genotype, *n* (%)			
1	3 (27)	4 (33)	
2	1 (9)	0	
3	6 (55)	6 (50)	
4	1 (9)	1 (8)	
6	0	1 (8)	

800–1200 mg (for all genotypes, and depending on weight). Blood was collected before the injection of peg-IFN-2b at baseline and at day 7 of antiviral therapy. Use of peg-IFN-2b and heroin were well controlled; at both treatment sites peg-IFN-2b was administered by a nurse, and heroin was prescribed and used at the addiction treatment center. HCV-RNA levels were monitored using the Ampliprep/Cobas Taqman HCV/HPS assay (Roche Molecular Systems; detection limit: 15 IU/mL). Genotyping of the IL28B-associated SNPs rs12979860 was performed using competitive allele-specific PCR in blood (KASP; kBioscience). The institutional review board of the Erasmus MC approved the protocols, and informed consent was obtained from all individuals.

2.2. Enumeration of leukocyte populations in whole blood, and quantitation of lymphocyte subpopulations

Absolute numbers of leukocytes, lymphocytes, monocytes and granulocytes in whole blood were measured by an automated impedance hematology analyzer (ABX Micros-60, Horiba Medical). To determine the frequency of distinct leukocyte subpopulations, PBMC were stained for CD3, CD56, and CD14. NK cells were defined as CD3-negative lymphocytes that expressed CD56, including $CD56^{dim}$ and $CD56^{bright}$ NK cells. All flow cytometric measurements were evaluated using a FACS Canto-II, and analyzed using Diva software (BD). Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by ficoll separation (Ficoll-Paque™ plus, Amersham).

2.3. Flow cytometric analysis of pSTAT-1 staining

For determination of the phosphorylation of STAT-1, frozen PBMC were thawed and rested for 1 h by incubation at 37 °C in culture medium (RPMI1640 supplemented with L-glutamin, penicillin, streptomycin, HEPES, and 5% human serum (Lonza). 2×10^6 PBMC were stimulated in 200 μl with 10,000 U/ml IFNα-2b (Intron A, Schering-Plough). After 30 min, the cells were washed with PBS, incubated for 20 min with 2% formaldehyde and stained with CD3-Pacific Blue (UCHT1, BD Pharmingen), and CD56-APC (N901, Beckman). Stimulated cells were incubated with BD Phosflow Perm III buffer (BD) for 12 min on ice. After washing, cells were stained for 15 min with pSTAT-1-Alexa-Fluor-488 (4a, BD), and the phosphorylation state of STAT-1 was measured by flow cytometry. The medium condition was used to set the threshold for the IFN-α condition.

2.4. Expression of intracellular and cell surface molecules by flow cytometry

Cytokine production by NK cells in PBMC was determined upon stimulation with 100 ng/mL IL-18 (MBL International Corporation) plus 10 ng/mL IL-12 (Miltenyi Biotec) for 24 h in 48-well plates (2×10^6 cells/250 μl). Brefeldin A (10 μg/mL; Sigma Aldrich) was added for the last 3 h, before fixation with 2% formaldehyde. After washing, cells were permeabilised with 0.5% saponin (VWR), and stained with CD56-PE (MY31), CD3-PerCp-Cy5.5 (UCHT1), CD69-APC (L78) and IFNy-FITC (25723.11, all BD) for 15 min. The medium condition was used to set the threshold for the IL-12/IL-18 condition. Results are expressed as percentage of cytokine producing cells within the NK cell population.

For perforin and granzyme B staining, frozen PBMC were thawed, rested and fixed with 2% formaldehyde. After washing, the cells were permeabilized with 0.5% saponin for 10 min and then incubated with perforin-PerCp-Cy5.5 (dG9, eBioscience), granzyme B-PE (GB11, eBioscience), CD56-APC (N901, Beckman), CD3-Pacific Blue (UCHT1, BD Pharmingen), and CD69-PECy7 (TP1.55.3, eBioscience). Stained cells were analyzed by flow cytometry.

2.5. CD107a degranulation assay

To measure degranulation of NK cells upon stimulation with K562 target cells, frozen PBMC were thawed and rested overnight in culture medium at 37 °C. PBMC (250,000/200 μl) were then seeded with K562 cells in a 96 wells plate in a 10:1 ratio. CD107a-PE (H4A3, BD Pharmingen) was added to the culture. After 90 min, monensin (BD) was added, and the cells were incubated for an additional 3.5 h under the same conditions. Samples were further stained using CD3-PerCp-Cy5.5 (UCHT1, BD) and CD56-APC (N901, Beckman). The medium condition was used to set the threshold for the K562 condition.

2.6. Statistical analysis

Among the three groups of participants (patients with and with substance use and healthy controls) mean values were compared and tested for statistically relevant differences using the Kruskal–Wallis test. Among patients with and without substance use, mean values were compared and tested for statistically relevant differences using Mann–Whitney.

3. Results

3.1. Circulating leukocyte numbers are not affected in chronic HCV patients regardless of active substance use

We first examined whether the absolute numbers of leukocyte populations in peripheral blood of chronic HCV patients differed from healthy controls and whether substance use affected their numbers. As shown in Fig. 1, we observed that the absolute number of monocytes and lymphocytes, but not leukocytes and granulocytes, differed among healthy individuals and both groups of chronic HCV patients.

Recently, the induction of NK cell function by IFN-α has been shown to correlate with virologic response to therapy (Ahlenstiel et al., 2011). To examine possible differences between healthy individuals and chronic HCV patients with and without substance use, we focussed on NK cells, and observed no differences between the three groups in the absolute numbers of circulating $CD3^{-}CD56^{+}$ NK cells in blood (Fig. 2). Within the NK cell compartment, also no significant differences were observed in the ratio between $CD56^{dim}$ and $CD56^{bright}$ NK cells between healthy controls and chronic HCV patients, irrespective of their substance use.

3.2. At baseline, NK cells from chronic HCV patients who are substance users show reduced expression of activation and cytotoxicity markers compared to non-substance users

Next, we examined the functionality of circulating NK cells in more detail by evaluating if their activation status and function was affected as a consequence of chronic HCV infection and substance use. As shown in Fig. 3A, flowcytometric analysis showed that the activation status of $CD56^{dim}$ NK cells, as demonstrated by CD69 expression, was lower only in patients who used substances, but not in HCV-infected non-users, as compared to healthy individuals. No differences in the frequency of CD69-expressing $CD56^{bright}$ NK cells were observed between the three groups. NK cells are known to produce high levels of IFN-γ upon viral infection, which may lead to priming or activation of innate cells, and to modulate adaptive immune responses (Vivier et al., 2011). We now show that upon exposure to IL-12 and IL-18, similar frequencies of IFN-γ producing NK cells were observed when

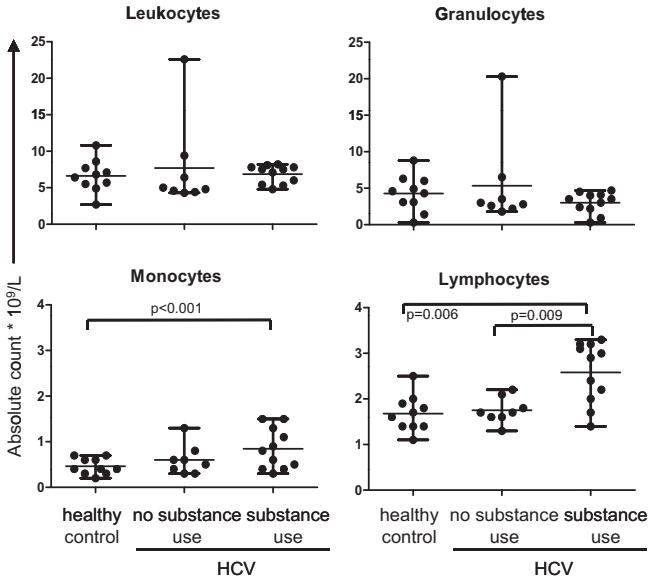

Fig. 1. Circulating leukocyte numbers are not affected in chronic HCV patients who are active substance users. The individual absolute cell counts ($\times 10^9$ cells/liter) in blood of healthy individuals and chronic HCV patients are depicted with median and range. Some data points are not presented since the leukocount was not determined. The comparison of three groups and two groups have been performed and tested for statistical significance using Kruskall-Wallis test and Mann–Whitney test, respectively.

comparing patients and healthy individuals (Fig. 3A). Also, substance use did not affect the frequencies of NK cells that produced IFN-γ.

NK cells are capable of direct cytolysis of virus-infected cells, which is dependent on release of granules containing perforins and granzymes (Cooper et al., 2001). Degranulation of these granules, as an indication of cytotoxic potential, can be determined by assessing CD107a expression upon co-culture with K562 target cells. As shown in Fig. 3B, the percentage of NK cells in PBMC that stained positive for perforin was similar between all three groups. In contrast, the frequency of granzyme B-positive $CD56^{dim}$ NK cells, as well as the frequency of CD107a-expressing $CD56^{dim}$ and $CD56^{bright}$ NK cells was significantly increased in chronic HCV patients as compared to healthy individuals. Interestingly, patients who did not use substances had higher levels of $CD56^{dim}$ cells expressing granzyme B and CD107 as compared to chronic HCV patients who were substance users (Fig. 3B).

3.3. In vitro, IFN-α induced pSTAT-1 levels are similar between substance user and non-user patients

Next, we examined, *in vitro*, the responsiveness of NK cells to IFN-α in PBMC from chronic HCV patients to determine whether this was affected by ongoing heroin use. To study this, phosphorylation of STAT-1 was measured by flowcytometry upon exposure to IFN-α *in vitro*. As shown in Fig. 4, no significant differences in pSTAT-1 levels were detected in the three experimental groups studied (Kruskall-Wallis test). These findings suggest that in our cohort of heroin and cocaine users the responsiveness of NK cells to IFN-α *in vitro* did not differ from NK cells obtained from non-user chronic HCV patients prior to therapy.

3.4. Therapy-induced modulation of pSTAT-1 levels by NK cells of substance user and non-user chronic HCV patients

To study the consequences of heroin and cocaine use in more detail, we assessed if the efficacy of antiviral IFN-based therapy of chronic HCV patients with no substance use differed from pa-

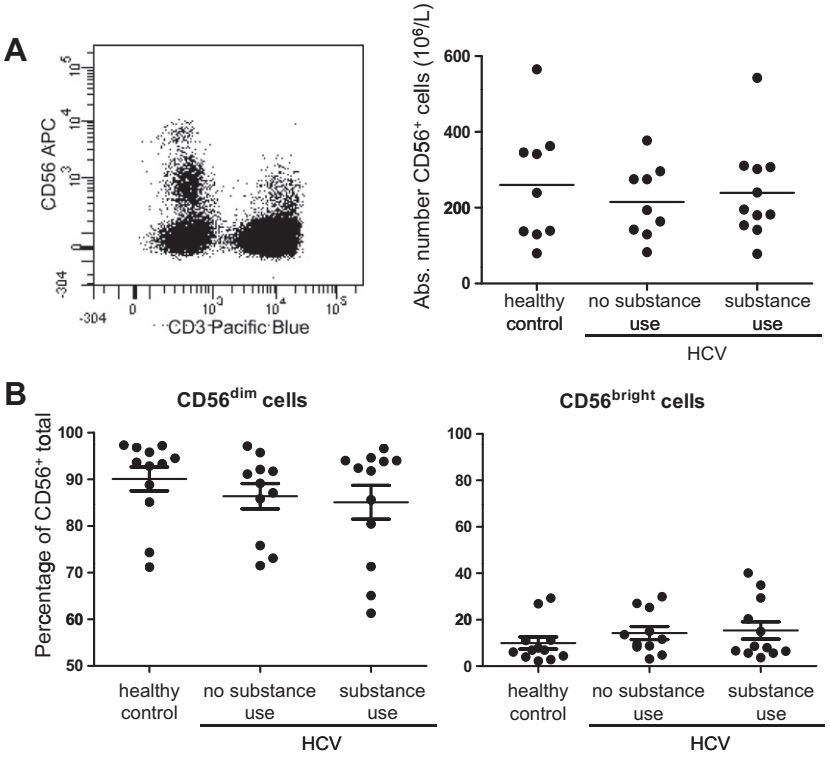

Fig. 2. The numbers and composition of NK cells in blood were not affected by chronic HCV infection, or substance use. (A) The absolute number of $CD3^-CD56^+$ NK cells in peripheral blood of healthy individuals and chronic HCV patients, with and without substance use, is shown. Some data points are not presented since the leukocount was not determined. (B) Lymphocytes were identified on the basis of their FSC/SSC profile, and further characterized by flow cytometry using CD56 and CD3 specific antibodies. The contribution of the specific subpopulation within the total NK cell pool is shown.

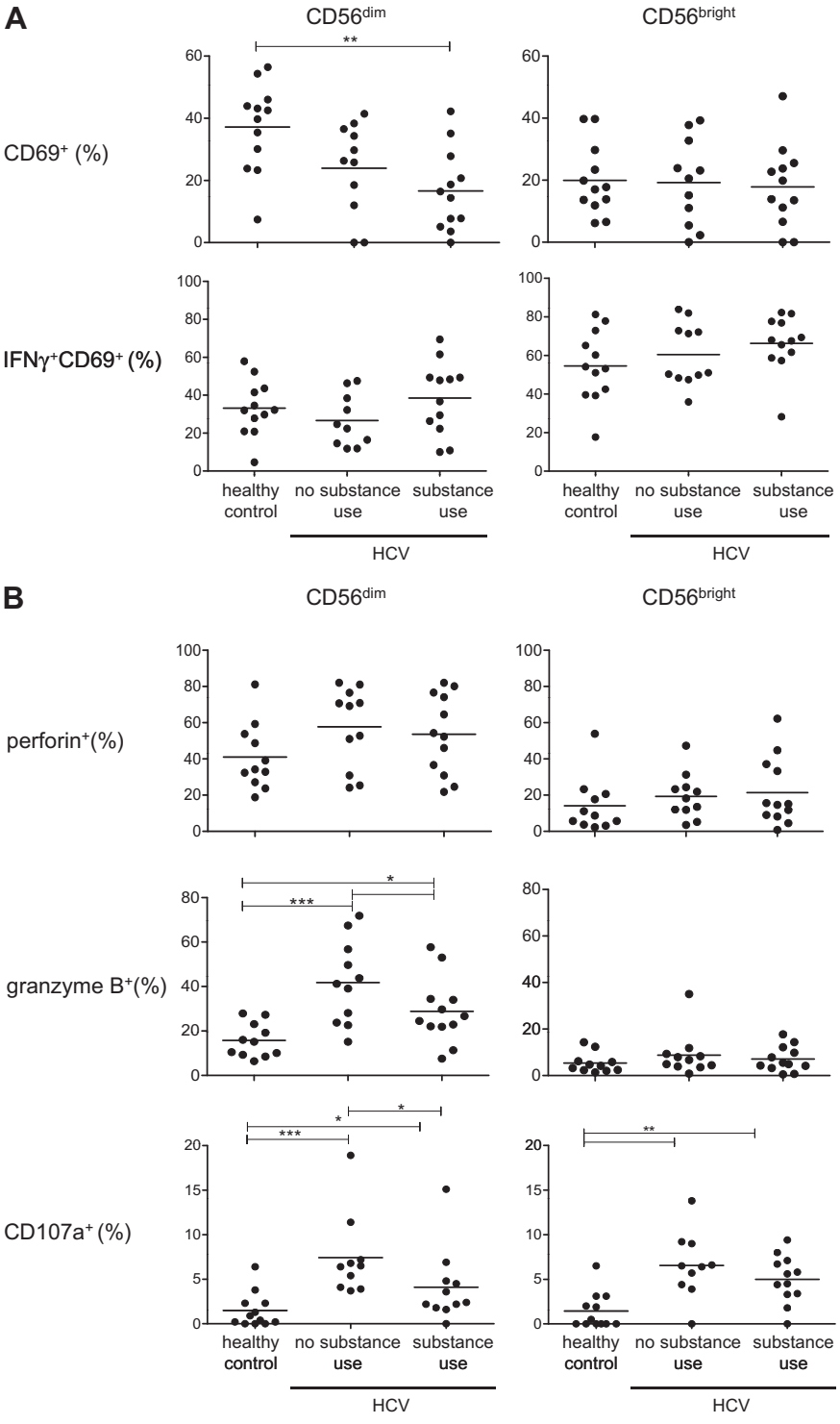

Fig. 3. At baseline, CD56^{dim} NK cells from chronic HCV patients who are substance users show reduced expression of activation and cytotoxicity markers compared to non-substance users. (A) CD69 expression and intracellular IFNy upon stimulation with IL-12 and IL-18 for 24 h were detected in PBMC of healthy controls, HCV patients who are non-users and patients with substance use by flowcytometric analysis. (B) Intracellular perforin, intracellular granzyme B, and CD107a expression were determined in CD56^{dim} and CD56^{bright} NK cells. Some data points are not presented since these samples did not show distinctive CD56^{dim} and CD56^{bright} NK cell populations. Individual data and the mean are depicted.

tients with substance use. At the early stages after start of antiviral treatment no differences were observed in the decline of serum HCV-RNA levels at day 7, and in fact both groups showed non-detectable levels in the majority of patients (Fig. 5). These findings suggest that in our patient cohort, substance use did not adversely affect the viral load decline in patients early after start of treatment (Mann–Whitney).

Additionally, little is known on the effects of substance use during therapy on immune cells, including NK cells, and we therefore also investigated the modulation of IFN-responsiveness by NK cells during IFN-based therapy in chronic HCV patients. Comparison of STAT-1 phosphorylation before and 7 days after start of therapy with peg-IFN-2b and ribavirin resulted in a reduction of pSTAT-1 levels as a consequence of therapy (Fig. 6). Lower pSTAT-1 levels

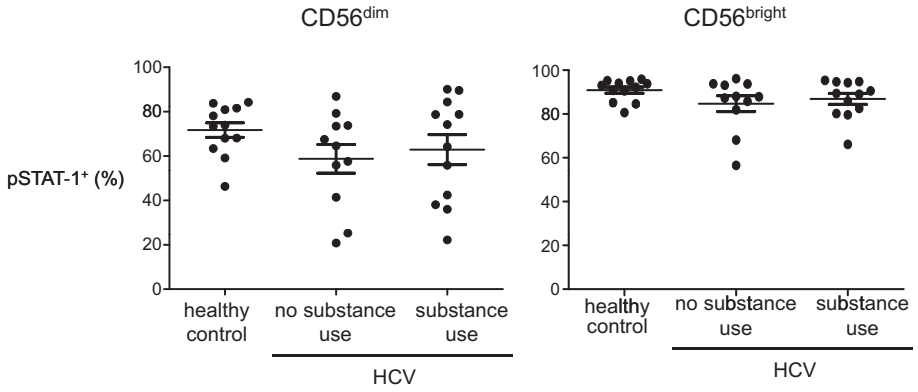

Fig. 4. *In vitro*, IFN-α induced pSTAT-1 levels are similar between substance user and non-user patients. PBMC from healthy controls, HCV patients who are non-users and patients with substance use were stimulated with IFN-α for 30 min. The pSTAT-1 levels were measured by flow cytometry. Data were analyzed by Kruskall-Wallis testing and no significant differences were observed.

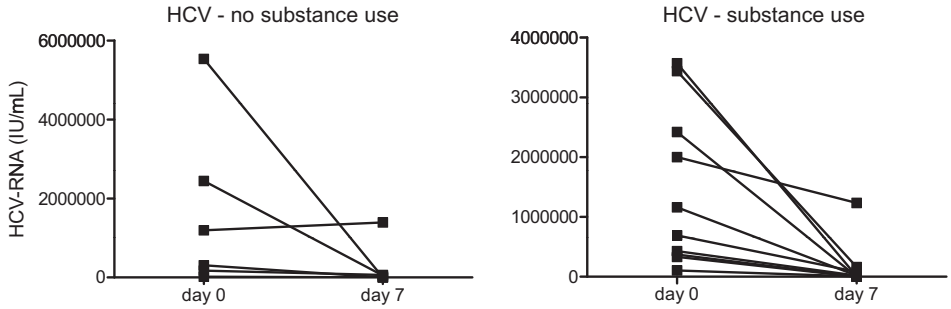

Fig. 5. Therapy-induced viral decline in chronic HCV patients who are substance users is similar as in non-users ($p = 0.233$). HCV-RNA levels were determined in serum before the start of antiviral therapy (peg-IFN and ribavirin) and after 7 days. Individual data of HCV patients who are non-users and patients with substance use are presented. Data were analyzed by non-parametric Mann–Whitney testing and no significant differences were observed.

in CD56^{dim} NK cells *on therapy* as compared to *before therapy* were observed for chronic HCV patients, and this decline was more pronounced in non-users as compared to substance users. In contrast, reduction of the frequency of pSTAT-1-expressing CD56^{bright} NK cells as a consequence of therapy was only observed in patients who did not use substances, but not in chronic HCV patients with ongoing substance use. The early effects of therapy, determined at day 7 after start of treatment, on the frequency of IFN-γ producing NK cells, and on NK cells expressing CD69, granzyme or perforin were modest in both experimental groups. Besides the effect on pSTAT-1 induction, the only significant difference observed was a mildly lower frequency of IFN-γ producing CD56^{bright} cells in substance users versus non-users.

4. Discussion

In this study we examined whether use of morphine-derived products or cocaine affected NK cell function in chronic HCV patients both at baseline and at the early stages of peg-IFN/ribavirin therapy. Evaluation of the early effects of IFN-based treatment demonstrated that there is no indication for weaker responses to IFN-α in chronic HCV patients who are substance users as compared to non-users. Therapy-induced decline in viral load as well as responsiveness to IFN-α *in vitro* were similar between both groups at day 7 of antiviral therapy.

In our study we chose to examine the early response of chronic HCV patients to therapy in order to simultaneously examine the clinical effects (HCV RNA decline) as well as the sensitivity of NK cells to IFN-α. Our findings show that in both groups, at baseline, similar frequencies of pSTAT-1 expressing NK cells were seen. This finding indicates that use of heroin or cocaine does not affect responsiveness of NK cells at the level of IFN-α receptor expression or the direct downstream signalling events (Fig. 4). Further, in both groups of HCV patients, irrespective of the use of heroin or cocaine, HCV RNA load declined strongly immediately after start of therapy, already within the first week. These findings are in agreement with clinical reports showing the high and comparable rates of SVR irrespective of substance use (Jafferbhoy et al., 2012; Sasadeusz et al., 2011; Zanini et al., 2010).

A general feature of cytokine exposure of cells is the initiation of a negative feedback mechanism that prevents long-lasting and excessive activation of cells. Also, negative feedback mechanisms have been described following IFN-α exposure (de Weerd and Nguyen, 2012). Comparison of STAT-1 phosphorylation before start of therapy and on day 7 during treatment showed that in chronic HCV patients who did not use substances, the pSTAT-1 levels were reduced as a consequence of exposure to peg-IFN and ribavirin for 1 week in both the CD56^{dim} and CD56^{bright} NK cells. Chronic HCV patients with substance use showed a milder reduction of pSTAT-1 expressing CD56^{dim} NK cells, and no modulation of the frequency of pSTAT-1-positive CD56^{bright} was observed by comparing day 0 and day 7. These findings demonstrate that the phenomenon of negative regulation to repeated IFN-α exposure does not lead to stronger impairment of responsiveness of NK cells from substance users than NK cells from chronic HCV patients who do not use substances. In fact, the lack of down-regulation of pSTAT-1 frequencies in CD56^{bright} NK cells may even suggest that cells from substance users are weakly more sensitive to IFN as compared to the control patients. Therefore, also on treatment, there are no indications for impaired sensitivity or responsiveness of NK cells, and therefore our immunological study supported the clinical observations that substance use did not adversely affect

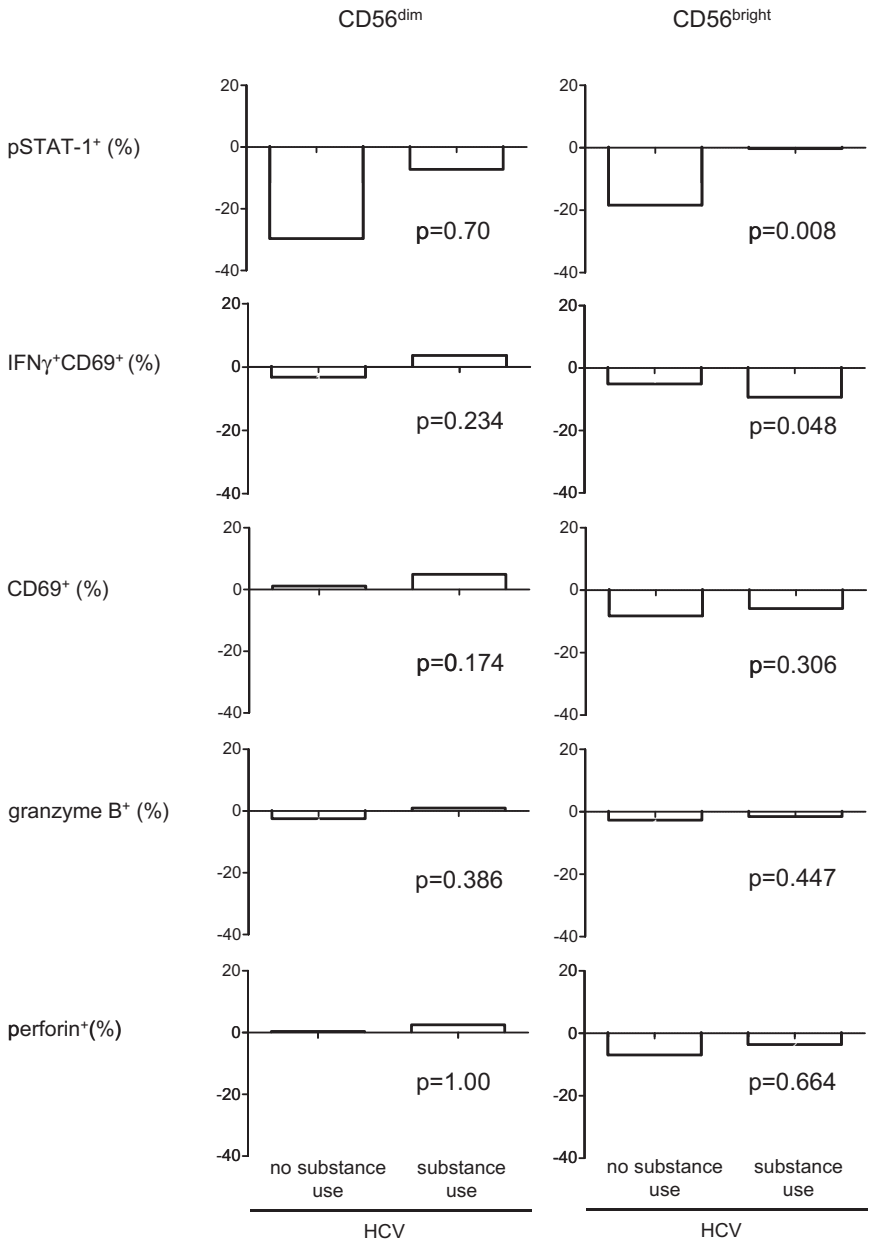

Fig. 6. Therapy-induced modulation of the activity of NK cells of substance user and non-user chronic HCV patients. IFN-α induced pSTAT-1 levels, CD69 expression and intracellular IFNy upon stimulation with IL-12 and IL-18 for 24 h, and intracellular perforin and granzyme B are determined in $CD56^{dim}$ and $CD56^{bright}$ NK cells from HCV patients who are non-users and patients with substance use. The mean change in frequency of the parameters is depicted by comparing the frequencies before treatment and 7 days after start of antiviral therapy. Data were analyzed by non-parametric Mann–Whitney testing.

the efficacy of IFN-based therapy. In line with our findings on pSTAT-1, we also found that therapy-induced modulation of the expression of intracellular perforin and granzyme B by NK cells was similar between substance user and non-user chronic HCV patients (data not shown).

In addition to the examination of their IFN-responsiveness, we also examined the NK cell compartment at baseline of both patient groups to determine if the immune status of substance users differed from non-users. At baseline, $CD56^{dim}$ NK cells from chronic HCV patients who are substance users show lower expression of activation and cytotoxicity markers than non-substance users. The reason for the reduced activation state of NK cells in substance users, as shown by their CD69 expression, is currently unknown, although possible explanations may include a direct effect of the drug metabolites on NK cells as well as indirectly via altered serum cytokine profiles. Functionally, we found no indications that in patients who use heroin or cocaine the frequency of IFN-γ producing NK cells are affected, suggesting that this non-lytic mechanism to control infected cells is intact. In contrast, the frequencies of granzyme B- as well as CD107a positive $CD56^{dim}$ NK cells in substance users were lower as compared to non-users, indicative of a weaker cytotoxic potential of the abundant subpopulation of $CD56^{dim}$ NK cells in substance users. The clinical consequences of these findings are difficult to assess. However, defective NK cells may be relevant for the control of viral load, but also highly relevant for disease progression, since it was reported recently that NK cells play an important role in the development of fibrosis (Eisenhardt et al., 2012; Kramer et al., 2012; Muhanna et al., 2011). Several limitations should be noted. First of all, the number of subjects in this study is quite small. We chose the design of a pilot study because,

to our knowledge, no comparable study has been published so far. Therefore, power calculation based on estimated clinically relevant differences could not be made. Secondly, within our group of substance users, we included both heroin and cocaine users. The effects on NK cell function of both of these compounds may differ, as has been described in the introduction section, since the breakdown products of these illicit drugs are distinct. The patients are therefore a heterogeneous group of individuals, which resembles the actual group of substance users. Thirdly, we cannot exclude that compounds other than heroin or cocaine, such as cannabis, alcohol or nicotine may influence the parameters determined in our study, and that these uncontrolled cofactors in the group of drug users contribute to the observed differences. Finally, it is important to mention that the size of the experimental groups as well as the controlled environment of the addiction treatment center may affect the clinical and immune parameters measured, which may influence the predictive value to all heroin or cocaine users.

In summary, although our study was performed on a relatively small number of patients, our study shows that despite subtle difference in NK cell parameters, we could not provide evidence that the IFN-responsiveness of chronic HCV patients, upon *in vitro* stimulation as well as during the early stages of IFN-based therapy, was affected by use of heroin or cocaine.

5. Conflict of interest

Prof. Dr. H.L.A. Janssen received grants from and is consultant for: Roche, Merck, Abbott, Santaris, Anadys, Medtronic, and Tibotec.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.12.025.

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