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Rapid Decrease in Hepatitis C Viremia by Direct Acting Antivirals Improves the Natural Killer Cell Response to IFNα

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Abstract

Objective: Chronic hepatitis C virus (HCV) infection is characterized by innate immune activation with increased interferon-stimulated genes (ISG) expression and by an altered phenotype of interferon-responsive natural killer (NK) cells. Here, we asked whether a rapid reduction in viremia by daclatasvir (DCV) and asunaprevir (ASV) improves the response to pegylated interferon (PegIFN) in patients who had previously failed a standard course of PegIFN/ribavirin (RBV) therapy.

Design: Twenty-two HCV-infected non-responders to previous PegIFN/RBV therapy were studied for IFN-responsiveness of NK cells during quadruple (QUAD) therapy with DCV, ASV, PegIFN and RBV. A direct comparison of early NK cell responses in PegIFN/RBV therapy and QUAD therapy was performed for 7 patients using paired cryopreserved PBMC from both treatment courses. As a validation cohort, 9 DCV/ASV-treated patients were studied for their NK cell response to *in vitro* stimulation with IFNα.

Results: The 24h-virological response to QUAD therapy correlated with an increase in STAT1, pSTAT1 and TRAIL expression in NK cells, and the STAT1/pSTAT1/TRAIL induction was greater during QUAD therapy than during previous PegIFN/RBV therapy. Successful QUAD therapy as well as successful IFN-free DCV/ASV regimen resulted in an improved functional NK cell response (degranulation and TRAIL expression) to *in vitro* stimulation with IFNα.

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CONTRIBUTORS

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The authors do not have a commercial or other association that might pose a conflict of interest.

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Conclusion: IFN-responsiveness can be improved by inhibiting HCV replication and by reducing the HCV-induced activation of the innate immune response. This may provide a rationale for clinical trials of a brief period of DAA therapy followed by PegIFN/RBV therapy to reduce the overall treatment costs in low and middle-income countries.

Keywords

Natural killer cells; direct acting antiviral; therapy; interferon; hepatitis C virus

INTRODUCTION

The introduction of potent direct acting antivirals (DAA) has significantly improved the treatment response of patients with chronic hepatitis C virus (HCV) infection. However, at present, these interferon (IFN)-free treatment regimens are not available for most patients worldwide, partly due to the high costs. In its 2014 guidelines on screening, care and treatment of HCV infected patients in low- and middle-income countries [1], the World Health Organization (WHO) has therefore provided recommendations for all currently licensed treatments for HCV infection, i.e. for IFN-based regimens (pegylated IFN [PegIFN]/ribavirin [RBV]), for combination regimens of PegIFN/RBV and DAAs, and for IFN-free DAA regimens. The WHO conceded in its report that 'it is likely that in many countries PegIFN and RBV will be the only available medicines for the next several years' [1].

PegIFN also remains important for the treatment of HCV genotype 3-infected patients, because the addition of PegIFN to a sofosbuvir(SOF)/RBV regimen increased the sustained virological response rate in patients with cirrhosis from 47% to 83% and in those without cirrhosis from 76% to 94% [2]. These data are even more remarkable when one considers the length of treatment. The length of the PegIFN/SOF/RBV regimen was 12 weeks, whereas the length of the SOF/RBV regimen was 16 weeks. Even an extension of the SOF/RBV regimen to 24 weeks did not yield the SVR rates of the 12-week PegIFN/SOF/RBV regimen [2].

Responsiveness to IFN-based therapy is determined by the pre-treatment activation state of the innate immune response [3]. HCV infection is known to induce endogenous type I and III IFNs, which increase the expression of IFN-stimulated genes (ISGs) [3] and activate IFN-sensitive innate immune cells such as NK cells [4–6]. Chronic HCV patients with a highly activated innate immune state do not respond well to IFN-based therapy compared to patients with weaker activation [3]. Likewise, a strong activation of NK cells in chronic HCV infection prior to treatment has been associated with nonresponse to IFN-based therapy [7–9]. The level of innate immune activation is greater, thus the responsivness to exogenous IFN is lower, in the presence than in the absence of the unfavorable IL28B variants rs12979860-T [10, 11] and rs368234815 ΔG [12]. At present, it is not known whether the IFN-responsiveness of individual patients can be improved.

Here we asked whether the rapid decrease in HCV titer by DAAs reduces the activation state of the innate immune system and thereby improves the responsiveness to PegIFN/RBV. For this purpose we compared IFN-responsiveness in a cohort of HCV genotype 1a-infected

patients with unfavorable IL28B genotype during a failed course of PegIFN/RBV treatment, and during a subsequent successful combination therapy of PegIFN/RBV and the direct acting antivirals daclatasvir (DCV) and asunaprevir (ASV) (hereafter called QUAD therapy). HCV genotype 1a-infected patients are an ideal cohort to study the synergistic effect of DCV/ASV and PegIFN/RBV in a QUAD regimen because in contrast to HCV genotype 1b-infected patients [13–16], HCV genotype 1a infected patients have a very low SVR rate when treated with DCV/ASV alone and require the addition PegIFN and RBV [17, 18]. A similar synergistic effect is observed when other DAAs are combined with PegIFN/RBV for treatment of HCV genotype 1a-infected patients [19]. As a readout for IFN-responsiveness we studied NK cells, because they express the IFNα/β receptor and are highly sensitive to type I IFN [20]. We and others have previously shown quantifiable changes in the phenotype and function of NK cells in response to both endogenous, HCV-induced IFN [4] and to exogenous, therapeutically administered PegIFN [6, 21]. Specifically, IFNα induces STAT1 and pSTAT1 levels in NK cells [5, 6] and enhances NK cell cytotoxicity, as evidenced by increased degranulation and by increased production of the cytotoxic molecule TNF-related apoptosis-inducing ligand (TRAIL), which itself is an ISG [4, 21, 22]. In contrast, pSTAT4 levels and cytokine production decrease upon stimulation with type I IFN [4, 6, 22]. Based on an analysis of *vivo* pSTAT1, STAT1 and TRAIL levels in peripheral blood NK cells we now demonstrate that the rapid reduction in HCV viremia during the first 24h of successful QUAD therapy improves NK cell responsiveness to PegIFN in comparison to a previous unsuccessful course of PegIFN/RBV without DAAs. We validate these findings in a separate cohort of HCV genotype 1b infected patients who were treated with the same DAAs (i.e. with DCV/ASV) without PegIFN/RBV and show that the NK cell response to *in vitro* stimulation with IFNα improves after the DCV/ASV-mediated decrease in HCV titer.

These results imply that IFN-responsiveness is not solely genetically determined but that it can be improved by rapidly reducing HCV RNA levels. This may explain the benefit of combining PegIFN with SOF/RBV therapy in difficult-to-cure patient populations as shown in the BOSON study [2]. The results may also provide a rationale for clinical trials of a brief period of DAA therapy followed by PegIFN/RBV therapy to reduce the overall costs of treatment. Finally, improved NK cell responsiveness may be relevant for improved immune surveillance and prevention of viral relapse, which is an interesting area for future research.

MATERIALS AND METHODS

Cohorts for NK cell studies.

NK cells were studied from 22 HCV genotype 1a infected non-responders to previous PegIFN/RBV therapy (Table 1) prior to and at day 0, day 1, and week 4 of a 24-week QUAD treatment course with DCV (60 mg, once daily, Bristol-Myers Squibb, New York, NY), ASV (100 mg, twice daily, Bristol-Myers Squibb), pegylated IFN alfa-2a (PegIFN, 180 μg/week s.c.) and weight-based RBV (RBV, 1000 mg for <75 kg bodyweight and 1200 mg for ≥75 kg bodyweight p.o daily; clinicaltrials.gov registration NCT01888900). Nineteen patients were sustained virological responders with no detectable HCV RNA at week 12 post therapy. One patient stopped treatment at week 8 due to IFN intolerance, one patient experienced a virological breakthrough at week 8, and one patient relapsed after the end of

treatment. Based on the availability of cryopreserved PBMC seven responders to QUAD therapy were also studied for NK cell responses during a previous course of PegIFN/RBV therapy, to which they were non-responders (clinicaltrials.gov registration NCT00718172). An additional group of 9 non-responders to past PegIFN/RBV therapy was studied at day 0, day 1 and week 24 of an IFN-free therapy with DCV (60 mg, once daily) and ASV (100 mg, twice daily), which they responded to (clinicaltrials.gov registration NCT01888900). NK cell responses of 15 uninfected subjects were studied as controls. All patients gave written informed consent for research testing under protocols by the institutional review board of NIDDK/NIAMS. The human experimentation guidelines of the United States Department of Health and Human Services were followed.

Genotyping.

Genomic DNAs were used to identify the single nucleotide polymorphisms (SNP) associated with rs12979860 [10, 11], rs368234815 (previously designated as ss469415590) [12], and rs117648444 [12, 23]. SNP analysis of rs12979860 was carried out with primers and probes from Life Technologies (Carlsbad, CA) and SNP analysis of rs368234815 was performed as previously reported [12]. SNP analysis of rs117648444 was carried out using forward primer `5′ GTCCCGCAGGAGGAAGAG3′`, reverse primer `5′ GGGCCTCACGGATGGC 3′`, and the probes `5′ CGGAGGATCCCTCC (VIC) 3′` and `5′ CGGAGAATCCCTCC (FAM) 3′`. To validate the primer and probe set for the rs117648444 SNP analysis, the PCR product was subcloned into the PCR®4 TOPO vector (Life Technologies) followed by sequencing.

Serological analyses.

Serum HCV RNA levels were quantitated using the Cobas TaqMan real-time PCR assay (Roche Diagnostics, Palo Alto, CA), with a lower limit of detection of 10 IU/ml and lower limit of quantification of 25–43 IU/ml (Fig. 1). Serum CXCL10 was quantitated using the ELISA MAX kit (Biolegend, San Diego, CA) following the manufacturer's protocol.

Extraction of RNA, synthesis of cDNA and quantitative PCR (qPCR) of CXCL10.

Total RNA was isolated from snap-frozen, mechanically homogenized liver biopsies in RNAlater (Life Technologies, Carlsbad, CA) using the Picopure RNA Isolation Kit (Life Technologies) with on-column DNase digestion. A cDNA equivalent to 1–2 ng total RNA, generated with Superscript III Reverse Transcriptase (Life Technologies), was used to determine *CXCL10* mRNA levels with pre-designed human TaqMan Gene Expression Assay (Life Technologies). The mRNA levels were normalized to GAPDH and PSMB4 and presented as $2^{\Delta CT}$ in figure 1.

NK cell analysis.

Peripheral blood mononuclear cells (PBMCs) were separated from heparin-anticoagulated blood on Ficoll Histopaque (Mediatech, Manassas, VA) density gradients, washed 3 times with phosphate-buffered saline (PBS, Mediatech) and cryopreserved in 70% fetal bovine serum (FBS, Serum Source International, Charlotte, NC), 20% RPMI1640 (Mediatech) and 10% DMSO (Sigma Aldrich, St. Louis, MO). Thawed PBMCs were processed and stained as described below prior to analysis on an LSRII flow cytometer using FacsDiva Version

6.1.3 (BD Biosciences, San Jose, CA) and FlowJo Version 8.8.2 software (Tree Star, Ashland, OR).

(i) TRAIL and STAT1 staining: Three million thawed PBMCs were incubated with or without 300 ng/mL consensus sequence IFNα (IFNα con1; InterMune Inc., Brisbane, CA) in 0.5 ml RPMI1640 with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine (Mediatech) for 10h at 37°C 5% CO_2 (Fig. 2–4). For staining, the cell suspension was split into 2 tubes. Cells in the first tube (TRAIL analysis) were stained with ethidium monoazide (EMA) for 10 min on ice followed by staining with anti-CD3-AlexaFluor700, anti-CD19-PeCy5, anti-CD16-V500, anti-CD56-PeCy7 and anti-TRAIL-PE (all from BD Biosciences), and with anti-CD14-PECy5 (AbD, Raleigh, NC) for 25 min at 4°C. Cells in the second tube (STAT1 analysis) were fixed with BD Cytofix buffer for 10 min at 37°C and 5% CO_2, centrifuged, permeabilized with BD Perm buffer III for 20 min on ice, washed twice and resuspended in BD Phosflow Buffer (all from BD Biosciences). Cells in this tube were stained with anti-CD56-PE (Beckman Coulter, Brea, CA), anti-CD3-FITC, anti-CD20-PerCP/Cy5.5 and anti-STAT1-Alexa647 (all from BD Biosciences) for 20 min at room temperature.

(ii) pSTAT1 staining: Thawed PBMCs (1.5×10^6) were rested in 0.5 ml RPMI1640 with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine (Mediatech) either overnight (Fig. 2–5) or for 2h (Fig. 6) at 37°C, 5% CO_2 prior to stimulation with or without 300 ng/mL consensus sequence IFNα for 5 min at 37°C. Thereafter, cells were fixed and permeabilized following the same procedure as for STAT1 analysis and stained with anti-CD56-PE (Beckman Coulter), anti-CD3-APC, anti-CD20-PerCP/Cy5.5 and anti-pSTAT1-Alexa488 (all from BD Biosciences) for 20 min at room temperature.

(iii) Response to in vitro stimulation with IFN-α: To assess TRAIL induction one million thawed PBMCs were incubated without or with 30, 3, 0.3, 03, 0.01, 0.003, 0.001, 0.0003, 0.0001 ng/mL consensus sequence IFNα in 1 ml RPMI1640 with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine for 18–20h at 37°C 5% CO_2. To assess NK cell degranulation one million thawed PBMCs were incubated without or with 3 ng/mL consensus sequence IFNα in 1 ml RPMI1640 with 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine and anti-CD107-PE (BD Biosciences) for 7h at 37°C 5% CO_2. In contrast to previous degranulation experiments [4] cells were not rested overnight, which resulted in a higher baseline expression of CD107a. Cells were stained with EMA for 10 min on ice and with anti-CD3-AlexaFluor700, anti-CD19-PeCy5, anti-CD16-V500, anti-CD56-PeCy7, anti-TRAIL-APC (all from BD Biosciences) and anti-CD14-PECy5 (AbD Serotec) for 25 min at 4°C.

Statistical analysis.

Depending on data distribution based on D'Agostino & Pearson omnibus normality tests, Wilcoxon-signed-rank test, Mann-Whitney test, unpaired t-test and linear regression analysis were performed with GraphPad Prism 5.0a (GraphPad Software, La Jolla, CA). Two-sided p-values <0.05 were considered significant. IFN dose-response stimulation curves (non

linear regression, variable slope) and the corresponding 50% activity concentrations (EC50) were calculated with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

RESULTS

The rapid decrease in viremia during QUAD therapy is associated with a decrease in intrahepatic inflammation

To answer the question whether a rapid DAA-mediated reduction of HCV viremia improves the innate immune response to IFN we studied 22 non-responders to PegIFN/RBV during a subsequent course of QUAD therapy. The patient characteristics are described in table 1. Of note, 19/22 patients had the unfavorable rs12979860-T and rs368234815-ΔG / rs117648444-G genetic variants associated with non-responsiveness to IFN-based therapy. All 22 patients experienced a rapid decline in HCV RNA levels within 24h of QUAD therapy and had undetectable viremia by week 4 of QUAD therapy (Fig. 1A). This decrease in HCV RNA level was associated with a significant reduction in liver inflammation as demonstrated by a decrease in ALT levels ($P<0.0001$, Fig. 1B) and in serum protein and liver mRNA levels of the inflammatory cytokine CXCL10 ($P=0.0004$ and $P=0.0002$, Fig. 1C and D, respectively). Nineteen patients were sustained virological responders with no detectable HCV RNA at week 12 post therapy. One patient stopped treatment at week 8 due to IFN intolerance, one patient experienced a virological breakthrough at week 8, and one patient relapsed after the end of treatment.

NK cells express increased STAT1, pSTAT1 and TRAIL levels in HCV infection with further increase during QUAD therapy

NK cells express the IFNα/β receptor, but not the IFNλ receptor chain 1 [24] and thus can be used to monitor immune activation by endogenous [5, 6] and exogenous type I IFN [6, 21]. Engagement of the IFNα/β receptor on NK cells results in Jak-STAT signaling, which includes STAT1 phosphorylation and increased transcription of ISGs. STAT1 and the cytotoxic effector molecule TRAIL are themselves ISGs and can therefore be used in addition to pSTAT1 to monitor NK cell activation by endogenous and exogenous IFNα.

Using multicolor flow cytometry, NK cells were identified as CD3-CD56+ cells in the PBMC population by sequential gating on single cells, lymphocytes and CD14-CD19- live cells (Fig. 2A). As illustrated by representative dot plots (Fig. 2 B, C) and by data from all studied subjects (Fig. 3), the frequencies of STAT1+ and TRAIL+ NK cells were significantly higher in HCV-infected patients than in uninfected subjects ($P<0.0001$ and $P=0.037$, respectively, Fig. 3A, B, left graphs). Likewise, the expression level (mean fluorescence intensity, MFI) of STAT1 and TRAIL per cell were significantly higher for NK cells of HCV-infected patients than of uninfected subjects ($P=0.0007$ and $P=0.022$, respectively, Fig. 3A, B, right graphs), reflecting a response to HCV-induced endogenous IFNα. Importantly, all HCV-infected patients were studied at two time points in chronic HCV infection, i.e. at a time point prior to therapy (‘pre’) and at day 0 of therapy with consistent results. Within the first 24h of QUAD therapy the percentage of STAT1+ and TRAIL+ NK cells ($P=0.0002$ and $P=0.004$, respectively) and the expression level of these markers per NK cell ($P<0.0001$ and $P=0.0008$, respectively) increased further (Fig. 3A, B).

The frequency of pSTAT1+ NK cells and the pSTAT1 level per NK cell (representative dot plots shown in Fig. 2D) increased simultaneously ($P<0.0001$ and $P<0.0001$, respectively, Fig. 3C), consistent with a response to the injected PegIFN. NK cell subset analysis revealed a differential response of $CD56^{dim}$ and $CD56^{bright}$ NK cells. While pSTAT1 expression was equal in $CD56^{dim}$ and $CD56^{bright}$ NK cells subsets, STAT1 expression was greater in the $CD56^{dim}$ NK cell subset and TRAIL expression was greater in the $CD56^{bright}$ subset (Suppl. Fig. 1, 2). All parameters remained increased during during follow up due to the weekly injection of PegIFN. These results indicate that NK cells are activated by both endogenous and exogenous type I IFN in HCV infection.

The first phase virological response correlates with the increase in STAT1 and TRAIL levels in NK cells during QUAD therapy.

Next, we asked whether the rapid decline in viremia at the start of QUAD therapy was associated with the change in NK cell phenotype. As seen in figure 4, the $\log_{10}$ decrease in HCV RNA level during the first 6h of therapy correlated with the 24h-increase in STAT1 and TRAIL expression in NK cells in a linear regression ($P=0.04$, $R^2=0.19$ for STAT1 and $P=0.04$, $R^2=0.20$ for TRAIL, Fig. 4A, B). A stronger correlation was observed for the 24h decrease in HCV RNA level and the increase in STAT1 and TRAIL expression during the same period ($P=0.02$, $R^2=0.24$, respectively, Fig. 4C, D).

The NK cell response to PegIFN is greater during QUAD therapy than during PegIFN/RBV therapy.

To assess the effect of the rapid DCV/ASV-mediated decrease in viremia on the NK cell response to the injected PegIFN we performed a paired analysis of the early NK cell response of 7 patients during QUAD therapy and during previous PegIFN/RBV therapy. While QUAD therapy resulted in an average 3 $\log_{10}$ decrease in HCV viremia within 24h, the previous course of PegIFN/RBV therapy did not significantly reduce HCV viremia in these patients (Fig. 5A). For each patient, cryopreserved PBMC from both treatment courses were thawed at the same time and tested for STAT1, pSTAT1 and TRAIL expression. As shown in figures 5B-C, the 24h-increase in STAT1 and pSTAT1 expression in NK cells in response to the first dose of injected PegIFN was significantly greater during QUAD therapy than during PegIFN/RBV therapy. This was consistent with a trend towards a greater increase in the frequency of pSTAT1+ NK cells during the first 24h of QUAD therapy than during PegIFN/RBV therapy (Fig. 5D). Likewise, the increase in TRAIL expression level per NK cell and the increase in the percentage of TRAIL+ NK cells was significantly greater in response to the first dose of PegIFN during QUAD therapy then during PegIFN/RBV therapy (Fig. 6A, B). Because the smaller $CD56^{bright}$ NK cell subpopulation expresses higher TRAIL levels than the bulk NK cell population (Suppl. Fig. 2B compared to Fig. 3B) we confirmed that the differential increase in TRAIL expression in both treatment courses extended to $CD56^{bright}$ NK cell subset (Fig. 6C, D). These results demonstrate that the addition of DCV and ASV to the PegIFN/RBV treatment regimen improves the NK cell response to the first dose of injected PegIFN.

Successful QUAD therapy normalizes the IFNα-induced NK cell response for at least 24 weeks after the end of therapy.

To evaluate whether successful QUAD therapy results in a long-term improvement of NK cell function in response to IFNα, we studied NK cells prior to and week 24 after the end of QUAD therapy. Using cell surface expression of the degranulation marker CD107a as a readout for NK cell cytotoxicity we show that the *ex vivo* frequency of CD107a+ NK cells decreased significantly from pretreatment to week 24 after the end of QUAD therapy (Fig. 7a, left graph), and that this is associated with a concomitant increase in the NK cells' ability to respond to *in vitro* stimulation with IFNα (Fig. 7a, right graph). These results extended to the CD56 ^{dim} NK cell population (Fig. 7B), which is regarded as more cytotoxic than the CD56^{bright} population [25]. Moreover, while total NK cells and CD56^{dim} NK cells from HCV patients prior to QUAD therapy had a significantly lower ability to degranulate in response to IFNα than those of healthy controls, their response was completely normalized at week 24 after the end of QUAD therapy (Fig. 7A, B). In fact, there was a direct correlation between the decrease in the frequency of CD107a+ CD56^{dim} NK cells during the course of QUAD therapy and their responsiveness to *in vitro* stimulation with IFNα in a linear regression analysis (Fig. 7C). Collectively, these results show that the early DAA-mediated decrease in viremia at the beginning of QUAD therapy improves the NK cell response to the first dose of injected PegIFN and that complete HCV clearance over the course of therapy results in a long-term improvement of NK cell function in response to IFNα.

IFN-free therapy with DCV/ASV improves IFNα-signaling in NK cells.

As a validation cohort, we studied 9 PegIFN/RBV non-responders who were treated with an IFN-free regimen of DCV/ASV. Similar to the course of viremia during QUAD therapy (Fig. 5A), viremia decreased by more than 2 $\log_{10}$ within 24h of IFN-free DCV/ASV therapy (Fig. 8A) and was undetectable by week 2–4 of therapy [15]. While NK cells of the QUAD cohort were exposed to PegIFNα *in vivo*, NK cells of the IFN-free cohort were subjected to *in vitro* stimulation with recombinant IFNα. The fold-increase in the frequency and mean fluorescence intensity of pSTAT1+ NK cells after *in vitro* stimulation with IFNα was used as a read-out for IFN-responsiveness of NK cells. As shown in figure 8 B-E, the DAA-mediated decrease in HCV viremia during successful DAA therapy improved the pSTAT1 response of NK cells to *in vitro* stimulation with recombinant IFNα. This improved IFN responsiveness was observed within the first 24h of treatment initiation (Fig. 8B, C) and was more prominent when treatment start and end date were compared (Fig. 8D, E).

IFN-free therapy with DCV/ASV improves the effector response of NK cells.

The concept of improved IFN responsiveness was further validated by determining the IFN-sensitivity of NK cells before and after successful DCV/ASV therapy in a titration experiment using IFN-induced expression of the cytotoxic molecule TRAIL as a read-out. As shown in figure 9, PBMC were *in vitro* stimulated with the indicated concentration of IFNα and the percentage of TRAIL+ cells in the CD56^{bright} NK cell population was determined. Because TRAIL expression is greater in the CD56^{bright} than in the CD56^{dim} NK cell subset (Suppl. Fig. 1, 2) and to be consistent with the literature [26–28] we gated on

CD56^{bright} NK cells in this analysis. For each of the indicated patients (patient numbers refer to Table 1), the EC50, i.e. the IFNα concentration that stimulated 50% of the maximal TRAIL response of CD56^{bright} NK cells, decreased significantly during the course of DCV/ASV therapy (P=0.031 comparing the mean EC50 values prior to and at the end of successful DCV/ASV therapy). These results indicate that HCV clearance improves the NK cell sensitivity to IFNα.

DISCUSSION

The development of potent DAAs has opened a new era of HCV treatment and cure [29]. It has also offered a unique opportunity to investigate the homeostasis of the intrahepatic innate immune system. IFNα is an important part of the intrahepatic immune system because it stimulates antiviral effector functions of infected hepatocytes via induction of ISGs [30] and it recruits and activates innate immune cells such as NK cells [20]. However, both clinical and experimental data demonstrate that HCV persists despite increased ISG expression [31, 32]. The presence of high ISG levels in the liver and a highly activated NK cell phenotype in the blood [7–9] are associated with a reduced response to IFN-based therapy [3]. Because non-responsiveness to IFN-based therapy is also associated with an unfavorable host genotype [10–12], it is unknown whether IFN responsiveness can be modulated.

The current study demonstrates that IFN-responsiveness is not solely determined by genetics. It was prompted by our recent demonstration that patients with a less than 2 $\log_{10}$ first-phase HCV RNA decline during PegIFN/RBV therapy exhibit a significantly lower increase in NK cell pSTAT1 induction in response to PegIFN than patients with a greater than 2 $\log_{10}$ first-phase HCV RNA decline [6]. Remarkably, NK cells of the slow virological responders retained their *in vitro* responsiveness to IFNα in that study [6]. These results suggested that the suboptimal PegIFN response was not due to an irreversible NK cell intrinsic or genetic factor, but due to a factor that was present *in vivo* but not in the *in vitro* experiment. We now show that this factor is the virus itself because rapid DAA-mediated inhibition of viral replication improves the response to PegIFN. Importantly, while all patients in the study by Edlich et al. responded to PegIFN/RBV therapy (albeit with different viral kinetics), the current study was conducted in previous PegIFN/RBV nonresponders. We show here that a reduction in HCV viremia improves the IFNα response of patients even if they have an unfavorable genetic background, and they provide a mechanistic rationale for treatment combination of DAA with PegIFN. This is consistent with a recent study on mathematical modeling, which suggested that DAAs may reset the altered steady state of the intrahepatic IFN signaling network in the chronically HCV infected liver [33].

As a readout for IFN-responsiveness, we quantified the frequency of pSTAT1, STAT1 and TRAIL+ NK cells and the respective expression level of these markers. Phosphorylation of STAT1 represents one of the earliest events after engagement of the IFNα/β receptor. Of note, NK cells do not express the IFNλ receptor chain 1 [24], which excludes the possibility that type III IFNs contribute to this effect. pSTAT1 forms heterodimers with pSTAT2, which then interact with the IFN regulatory factor 9 to form the IFN-stimulated gene factor 3 transcription complex. Nuclear translocation and binding of this complex to the IFN-

stimulated response element induces the transcription of ISGs. TRAIL is one of the most responsive ISGs in human NK cells as shown by transcriptional profiling of IFNα-stimulated NK cells from healthy controls [26] and it is also a relevant effector molecule against HCV [26]. Studying NK cell degranulation as readout for cytotoxicity we also confirmed that successful QUAD therapy resulted in a long-term improvement of NK cell function in response to IFNα.

While an NK cell-mediated antiviral effect has been shown in co-cultures with hepatoma cells that were either infected with HCV [34, 35] or harbored subgenomic HCV replicons [35, 36], it is important that we are not concluding that the antiviral effect of PegIFN-based therapy is mediated by NK cells. Rather NK cells are used as a read-out for IFN responsiveness in this study. Intrahepatic *ISG* induction in the early phase of IFN-based therapy has been validated as an indicator of IFN-responsiveness and predictor of treatment response [3]. We argue that there are parallels between IFN-responsiveness (ISG induction) of the liver as described by Dill et al [37] and IFN-responsiveness (pSTAT1, STAT1 and TRAIL induction) of NK cells as described in this study. The high response rate in our cohort requires PegIFN/RBV and is not due to DCV/ASV alone, because HCV genotype 1a infected patients have a low SVR rate when treated with an IFN-free DCV/ASV regimen [17, 18]. Collectively, the results may explain the benefit of combining PegIFN with DAA/RBV therapy in some patient populations as shown in the BOSON study [2], and may also provide a rationale for clinical trials of a brief period of DAA therapy followed by PegIFN/RBV therapy to reduce the overall costs of treatment in middle- and low-income countries. Whether improved NK cell responsiveness to IFN may also be relevant for immune surveillance and prevention of viral relapse is an interesting area for future research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ASV	asunaprevir
DAA	direct acting antiviral
DCV	daclatasvir
EMA	ethidium monoazide
FBS	fetal bovine serum

HCV	hepatitis C virus
IFN	interferon
ISG	interferon-stimulated gene
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Peg	pegylated
QUAD	quadruple therapy with PegIFN, RBV, DCV and ASV
RBV	ribavirin
SNP	single nucleotide polymorphism
SOF	sofosbuvir
STAT	signal transducer and activator of transcription
SVR	sustained virological response
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand.

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SUMMARY BOX

What is already known about this subject?

- Hepatitis C virus (HCV) induces endogenous type I and III IFNs which stimulate high expression levels of interferon-induced genes (ISGs) and activate IFN-sensitive innate immune cells such as NK cells.
- The degree of innate immune activation and the patient's IFN-λ genotype predict the response to IFN-based therapy.
- Due to the limited availability and high costs of direct acting antivirals, the WHO has estimated in its treatment guidelines that IFN-based therapy will be the sole available therapy in low and middle-income countries for the next several years.
- In addition, IFN remains important in combination with direct acting antivirals in difficult-to-cure patients, such as patients with HCV genotype 3 infection.

What are the new findings?

- Interferon-responsiveness is not solely genetically determined but can be improved by inhibiting viral replication and reducing the virus-induced activation of the innate immune response.
- HCV genotype 1a-infected non-responders to past PegIFN/RBV responded to combination therapy with PegIFN, RBV, daclatasvir (DCV) and asunaprevir (ASV).
- The rapid DCV/ASV-induced reduction in viremia increased the innate response to PegIFN as evidenced by greater induction of STAT1, pSTAT1 and TRAIL than during past PegIFN/RBV therapy. A response to QUAD therapy was associated with improved NK cell degranulation upon in vitro stimulation with IFNα.
- Rapid reduction of viremia by an IFN-free DCV/ASV regimen also improved IFN-responsiveness of NK cells, as shown by in vitro stimulation with IFNα.

How might it impact on clinical practice in the foreseeable future?

- The results may explain the benefit of combining PegIFN with DAA/RBV therapy in some patient cohorts as shown in the BOSON study.
- They may also provide a rationale for clinical trials of a brief period of DAA therapy followed by PegIFN/RBV therapy to reduce the overall costs of treatment in low and middle-income countries.
- The improved NK cell responsiveness may enhance innate immune surveillance, possibly preventing viral breakthrough and relapse.

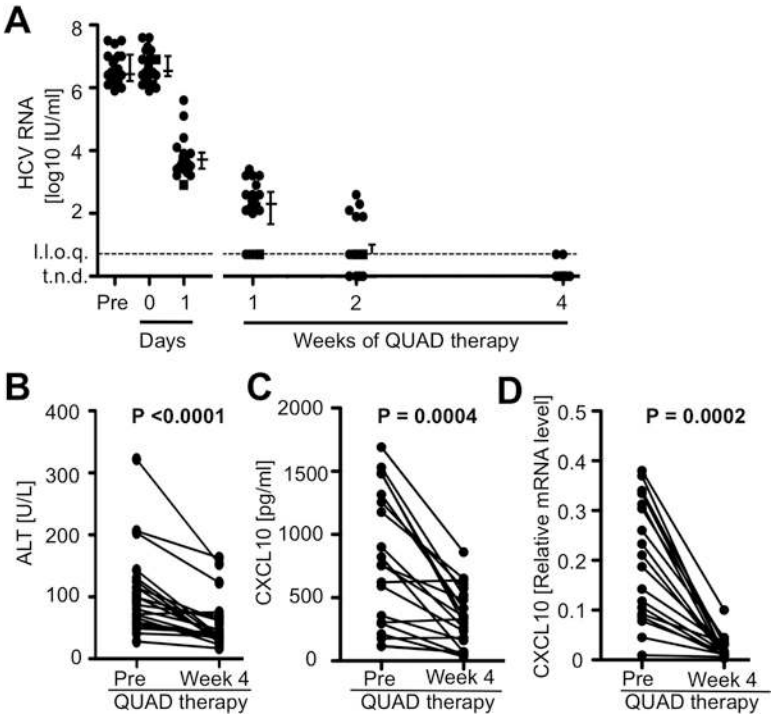

Figure 1. The rapid decrease in viremia during QUAD therapy is associated with a decrease in intrahepatic inflammation.

(A) Serum HCV RNA levels at the time of the pretreatment liver biopsy (pre) and at the start (day 0) and during the first 4 weeks of QUAD therapy. Filled squares represent data from patients who developed a virological breakthrough at week 8 (n=1) or relapsed after the end of therapy (n=1). L.l.o.q., lower level of quantitation; t.n.d., target not detected.

(B) Serum ALT levels prior to and at week 4 of QUAD therapy.

(C-D) CXCL10 protein concentration in the serum (C) and CXCL10 relative mRNA level in the liver (D) prior to and at week 4 of therapy. 'Pre' indicates the time point of the pre-treatment liver biopsy (up to 4 weeks prior to therapy). Statistical analysis: non-parametric paired Wilcoxon-signed-rank test.

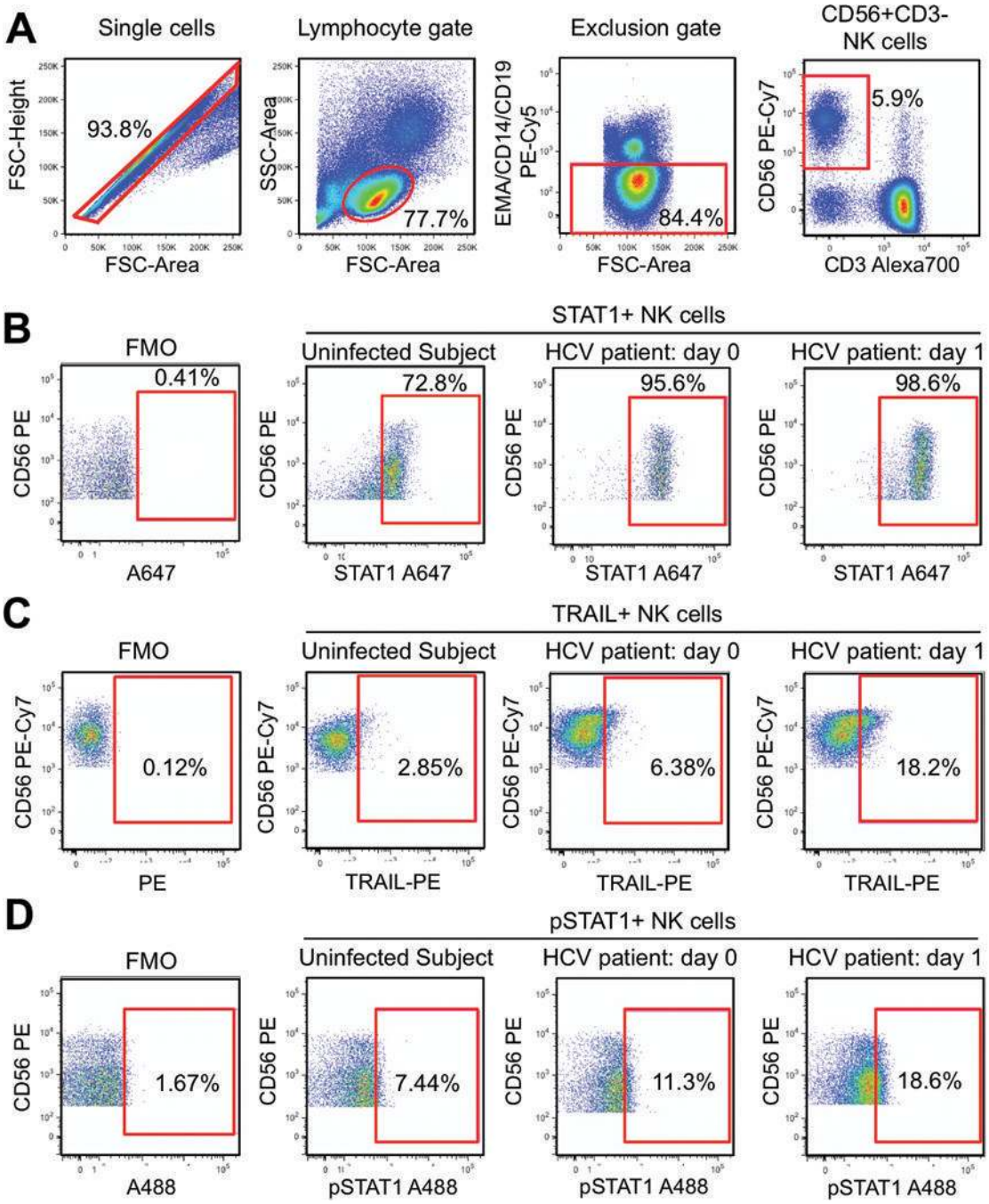

Figure 2. Detection of STAT1, TRAIL and pSTAT1-expressing NK cells of uninfected subjects and HCV-infected patients by flow cytometry.

(A) Flow cytometry gating strategy. Dot plots from left to right show gating on single cells in forward scatter (FSC) area versus FSC height plots, gating on lymphocytes in FSC versus side scatter (SSC) plots, exclusion of EMA+ dead cells, CD14+ monocytes and CD19+ B cells, and gating on CD56+CD3- negative NK cells. Percentages indicate the frequency of events in the red gate relative to total number of events in the respective plot.

(B) Frequency of STAT1+ cells in the peripheral blood CD3-CD56+ NK cell population of a representative uninfected subject and a representative HCV-infected patient on day 0 and day 1 of QUAD therapy. FMO, fluorescence minus one control using all antibodies in the panel except for the anti-STAT1 A647 antibody to define the threshold of positivity. A647, alexa647.

(C) Frequency of TRAIL+ cells in the peripheral blood CD3-CD56+ NK cell population of a representative uninfected subject and a representative HCV-infected patient on day 0 and day 1 of QUAD therapy. FMO, fluorescence minus one control using all antibodies in the panel except for the anti-TRAIL PE antibody to define the threshold of positivity. PE, phycoerythrin.

(D) Frequency of pSTAT1+ cells in the peripheral blood CD3-CD56+ NK cell population of a representative uninfected subject and a representative HCV-infected patient on day 0 and day 1 of QUAD therapy. FMO, fluorescence minus one control using all antibodies in the panel except for the anti-pSTAT1 A488 antibody to define the threshold of positivity. A488, alexa488. Note that the CD56 dot plots in panels B/D (co-staining for STAT1/pSTAT1) differ from those in panel C (staining for TRAIL) because two different CD56 antibodies with different fluorochromes were used, and because of differential processing of the PBMC (fixation, permeabilisation and intranuclear staining for panels B/D; cell surface staining in panel C).

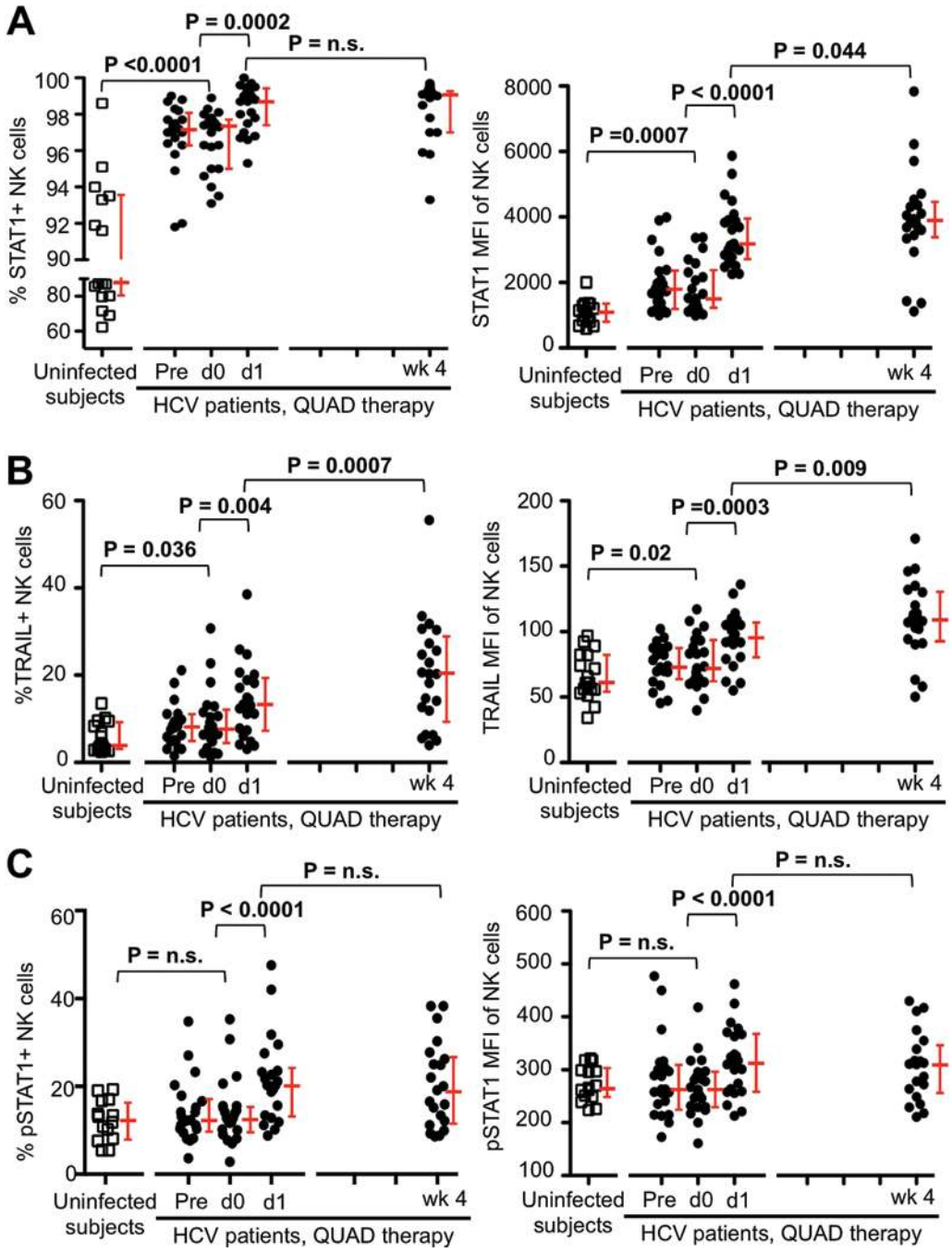

Figure 3. NK cells express increased STAT1, TRAIL and pSTAT1 levels in HCV infection with further increase during QUAD therapy.

Frequency of **(A)** STAT1+, **(B)** TRAIL+ and **(C)** pSTAT1+ NK cells and mean fluorescence intensity (MFI) of these markers per NK cell prior to (pre, day 0) and during (day 1, week 4) of QUAD therapy (filled circles). Immunological read-outs are shown for the first 4 weeks of QUAD therapy, because HCV is already undetectable at week 4, and values of the immunological read-outs plateau with continued weekly PegIFN injections. NK cells of uninfected subjects are shown for comparison (open squares). Statistical analysis: non-

parametric paired Wilcoxon-signed-rank test or unpaired Mann-Whitney test. Median and IQR are shown.

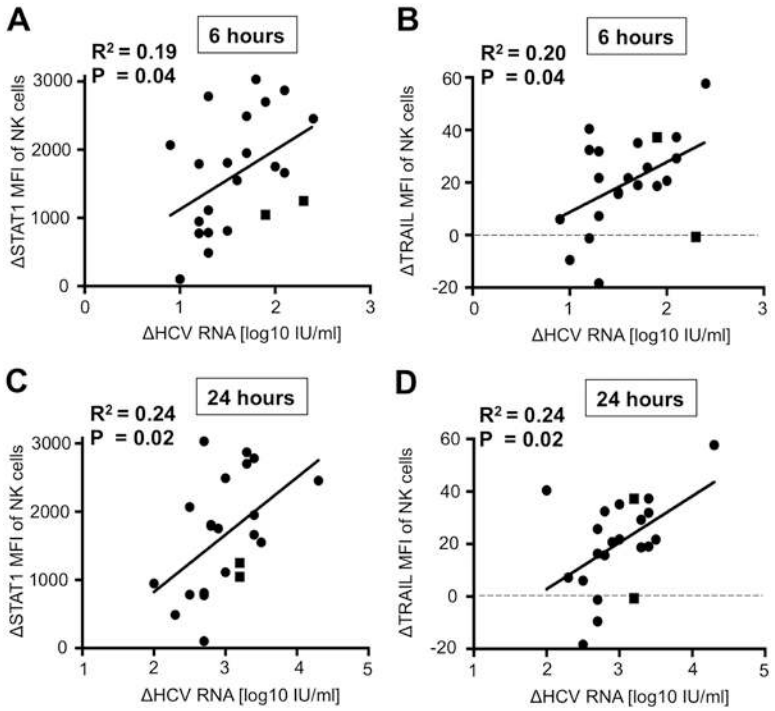

Figure 4. The first-phase virological response correlates with the increase in STAT1 and TRAIL levels in NK cells during QUAD therapy.

(A, B) Linear regression analysis of the decrease in viremia during the first 6h of QUAD therapy and the 24h-increase in STAT1 (A) and TRAIL (B) mean fluorescence intensity (MFI) on NK cells.

(C, D) Linear regression analysis of the decrease in viremia during the first 24h of QUAD therapy and the increase in STAT1 (C) and TRAIL (D) MFI on NK cells during the same period. R2: Coefficient of determination. Filled squares represent data from patients who developed a virological breakthrough at week 8 (n=1) or relapsed after the end of therapy (n=1).

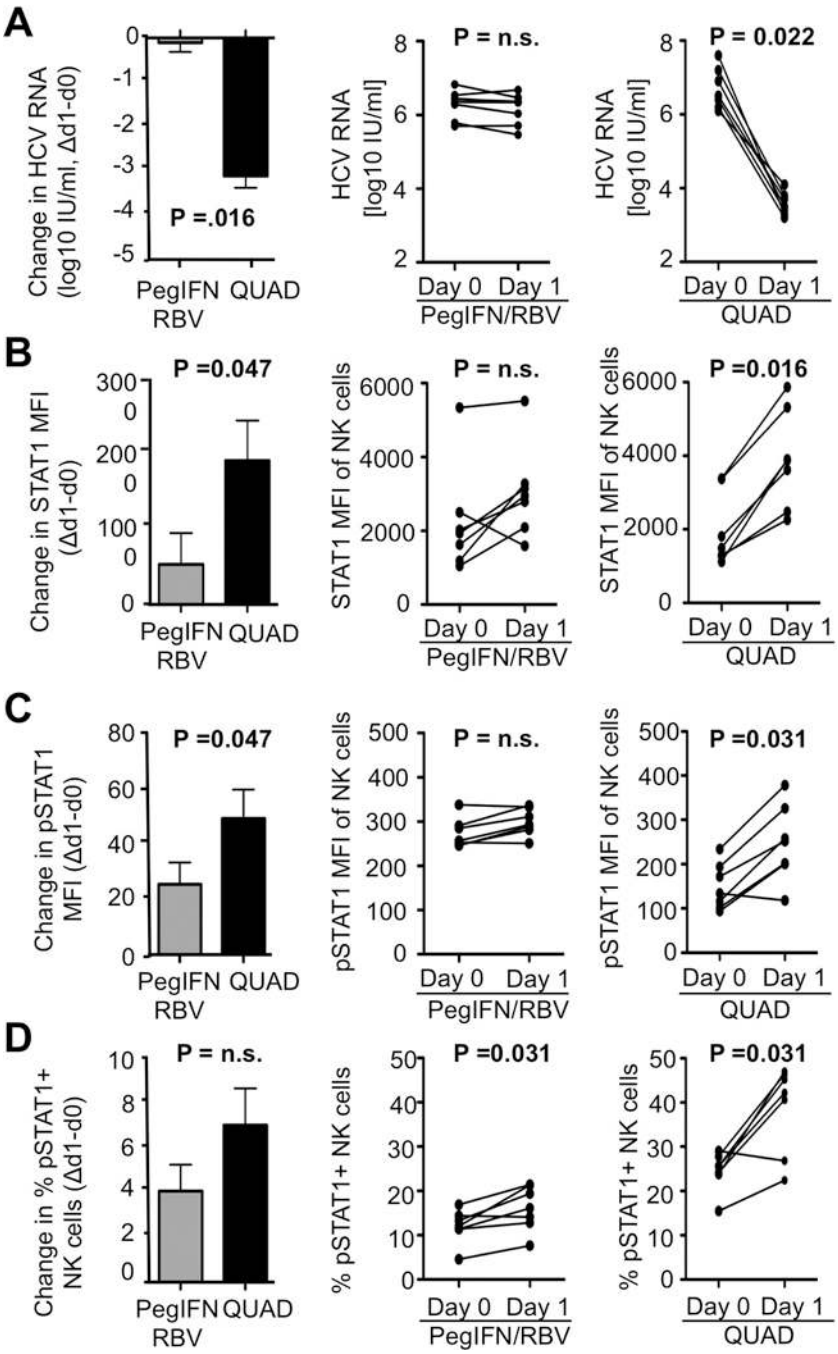

Figure 5. IFN-induced STAT1 and pSTAT expression in NK cells is greater during QUAD therapy than during PegIFN/RBV therapy.

(A) Comparison of the decrease in HCV RNA levels during the first 24 h of therapy in 7 patients who were non-responders to a full course of PegIFN/RBV therapy but had an SVR during a subsequent course of QUAD therapy.

(B, C) Comparison of changes in the expression level of STAT1+ (B) and pSTAT1 (C) NK cells during the first 24 h of therapy in patients who were non-responders to a full course of PegIFN/RBV therapy but had an SVR during a subsequent course of QUAD therapy.

(D) Comparison of changes in the percentage of pSTAT1+ NK cells during the first 24 h of therapy in patients who were non-responders to a full course of PegIFN/RBV therapy but had an SVR during a subsequent course of QUAD therapy. Statistical analysis: non-parametric paired Wilcoxon-signed-rank test. Median and IQR are shown in the left graphs in panels A-D.

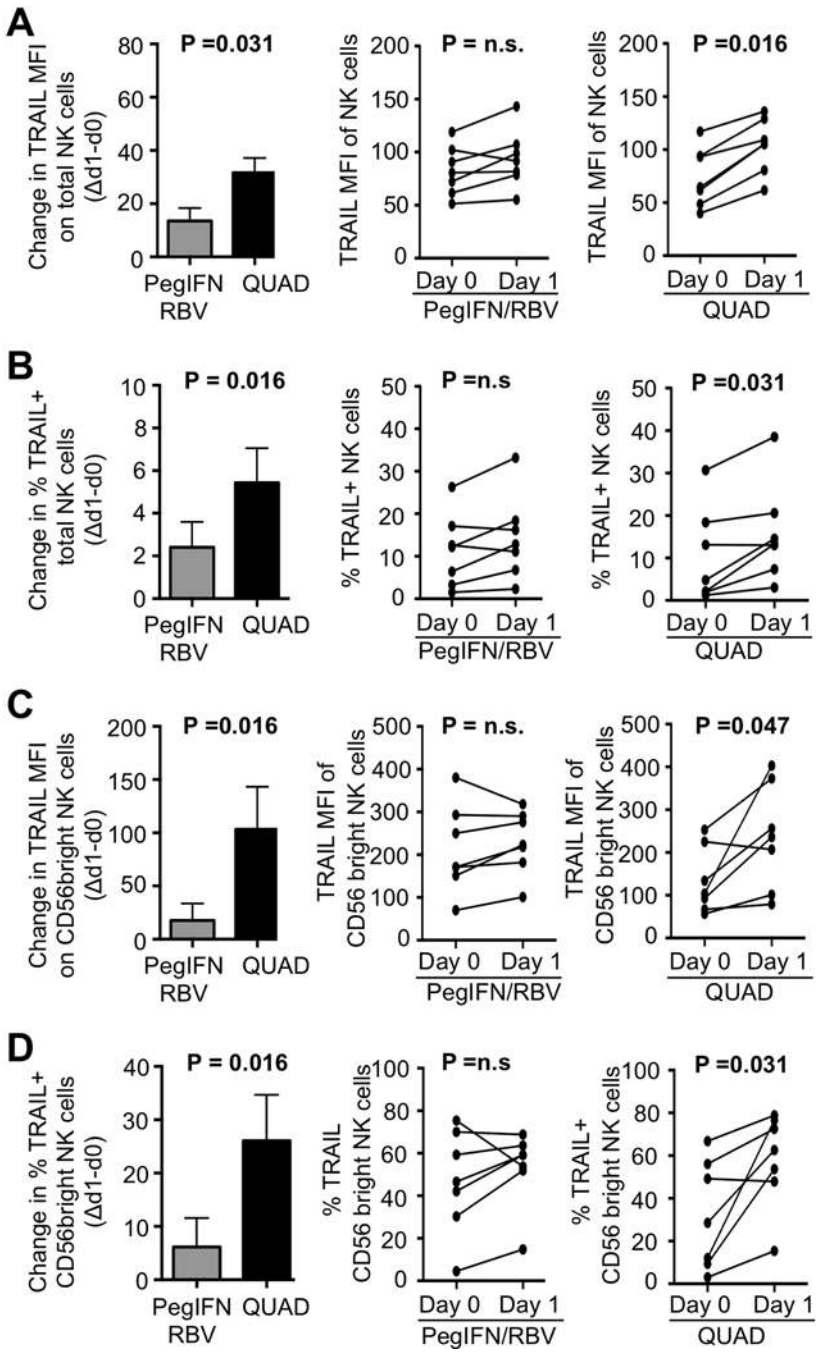

Figure 6. IFN-induced TRAIL expression in NK cells is greater during QUAD therapy than during PegIFN/RBV therapy.

(A, B) Comparison of changes in the expression level (A) and frequency (B) of TRAIL+ NK cells during the first 24 h of therapy in patients who were non-responders to a full course of PegIFN/RBV therapy but had an SVR during a subsequent course of QUAD therapy.

(A, B) Comparison of changes in the expression level (A) and frequency (B) of TRAIL+ $CD56^{dim}$ NK cells during the first 24 h of therapy in patients who were non-responders to a full course of PegIFN/RBV therapy but had an SVR during a subsequent course of QUAD

therapy. Statistical analysis: non-parametric paired Wilcoxon-signed-rank test. Median and IQR are shown in the left graphs in panels A-D.

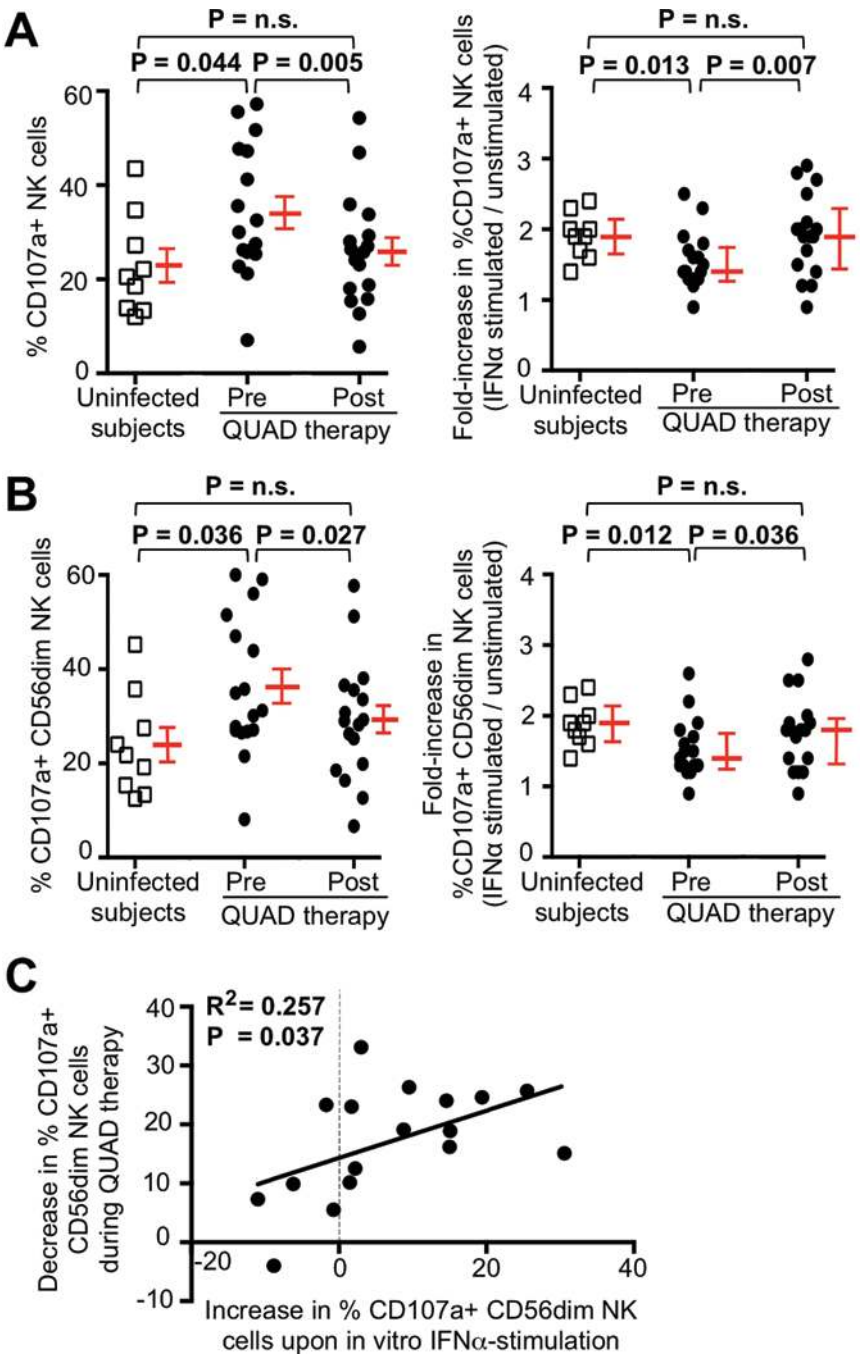

Figure 7. Successful QUAD therapy normalizes NK cell response to *in vitro* IFNα stimulation for at least 24 weeks after the end of therapy.

(A, B) The *ex vivo* frequency of CD107a+ total NK cells (A) and CD107a+ $CD56^{dim}$ NK cells (B) was determined in uninfected controls and in HCV patients prior to and at week 24 after the end of QUAD therapy. Data in the left graphs in panels A and B are normally distributed. Mean and SEM are indicated and parametric paired and unpaired t-tests were used. Data in the right graphs in panels A and B are not normally distributed. Median and

IQR are indicated and non-parametric paired Wilcoxon-signed-rank test and Mann Whitney unpaired t-test are used.

(C) Linear regression analysis of the decrease in the frequency of CD107a+ $CD56^{dim}$ NK cells from prior to QUAD therapy to week 24 after the end of QUAD therapy and the strength of the NK cell response (CD107a expression) to *in vitro* stimulation with IFNα at week 24 after the end of QUAD therapy. R2: Coefficient of determination. One HCV patient was excluded from the analysis in figure 7, because the sole pretreatment blood sample of this patient that was available for the CD107 assays was obtained more than 2 years prior to QUAD therapy.

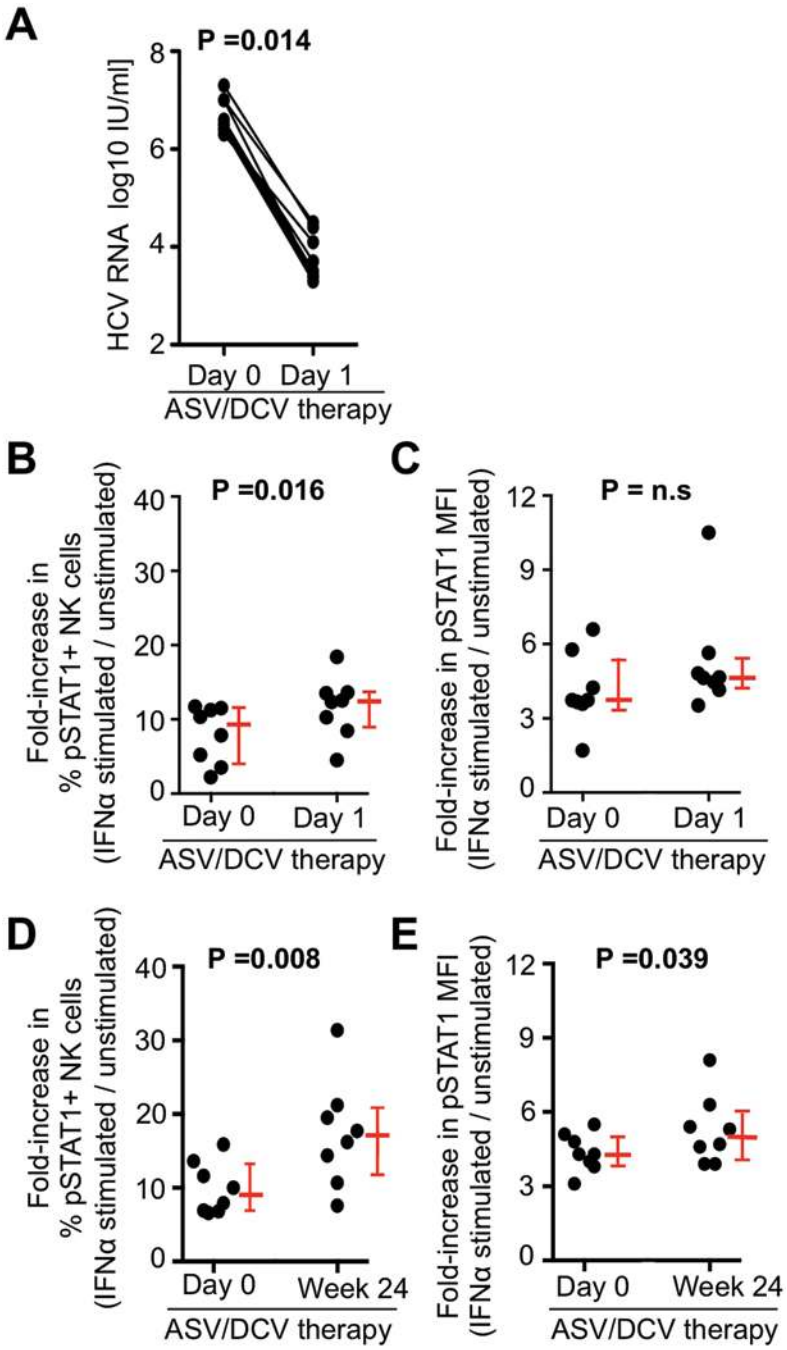

Figure 8. IFN-free therapy with DCV/ASV improves IFNα-signaling in NK cells.

(A) Decrease in HCV RNA levels during the first 24h of IFN-free DCV/ASV therapy in 8 patients with an SVR.

(B, C) Fold-increase in the frequency (C) and mean fluorescence intensity (D) of pSTAT1+ NK cells in response to *in vitro* stimulation of PBMC with IFNα. The *in vitro* response to IFNα was assessed at day 0 and day 1 of DCV/ASV therapy. Statistical analysis: non-parametric paired Wilcoxon-signed-rank test.

(D, E) Fold-increase in the frequency (D) and mean fluorescence intensity (E) of pSTAT1+ NK cells in response to *in vitro* stimulation of PBMC with IFNα. The *in vitro* response to IFNα was assessed at day 0 and week 24 of DCV/ASV therapy. Statistical analysis: non-parametric paired Wilcoxon-signed-rank test.

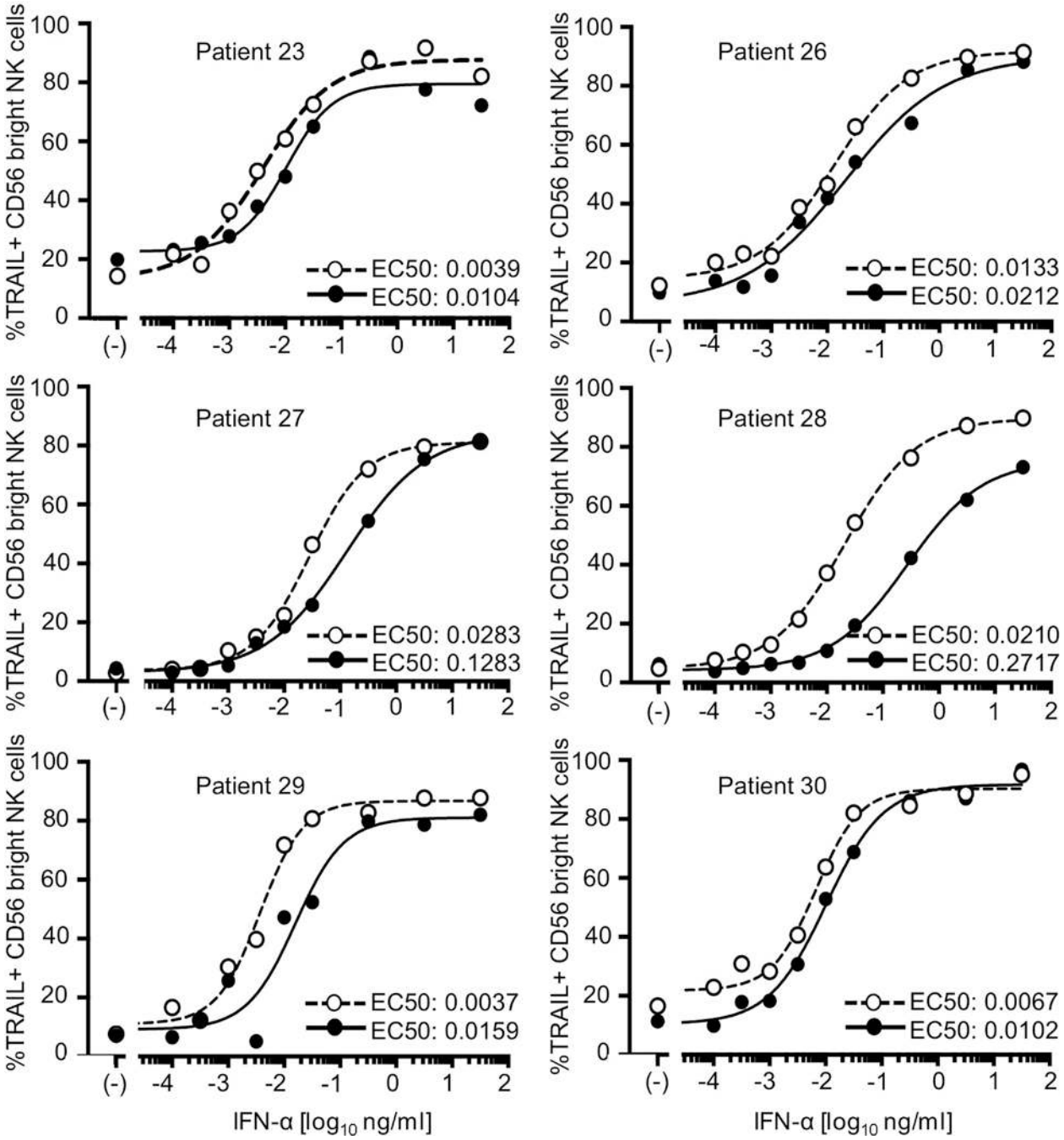

Figure 9. IFN-free therapy with DCV/ASV improves the effector response of NK cells.

PBMC were stimulated *in vitro* with the indicated concentration of IFNα and the percentage of TRAIL+ cells in the $CD56^{bright}$ NK cell population was determined. For each of the indicated patients (patient numbers refer to Table 1), the NK cell response in PBMC sample prior to DCV/ASV therapy (open circles, dotted line) was compared to the NK cell response in a PBMC sample at the end of successful DCV/ASV therapy (filled circles, continuous

line). EC50, IFNα concentration resulting in 50% of the maximal TRAIL response of $CD56^{bright}$ NK cells.

Table 1:

Patient characteristics

Patient[1]	Age	Sex	Response to to Previous PegIFN/RBV[2] treatment	Patient Genotype (Single Nucleotide Polymorphism)			HCV genotype	Baseline HCV RNA [log_{10} IU/ml]	Baseline ALT [U/L]	Liver cirrhosis[3]	Treatment in current study	
				rs 12979860	rs 368234815	rs 117648444					Regimen	Outcome[4]
1	59	M	No (null)[5]	TT	ΔG/ΔG	GG	1a	7.2	139	yes	QUAD	SVR
2	60	M	No (null)	TT	ΔG/ΔG	GG	1a	6.4	42	no	QUAD	SVR
3	58	M	No (null)	CT	TT/ΔG	GG	1a	7.6	59	no	QUAD	SVR
4	60	M	No (null)	CT	TT/ΔG	GG	1a	6.1	82	yes	QUAD	SVR
5	64	M	No (null)	CT	TT/ΔG	GG	1a	6.9	25	no	QUAD	SVR
6	53	F	No (null)	CT	TT/ΔG	GG	1a	6.5	48	yes	QUAD	SVR
7	28	F	No (null)	TT	ΔG/ΔG	GG	1a	6.2	57	yes	QUAD	SVR
8	60	M	No (null)	TT	ΔG/ΔG	GG	1a	6.9	62	no	QUAD	SVR
9	46	M	No (null)	CT	TT/ΔG	GA	1a	6.7	74	no	QUAD	SVR
10	57	M	No (null)	CT	TT/ΔG	GG	1a	6.9	123	yes	QUAD	SVR
11	67	M	No	TT	ΔG/ΔG	GG	1a	7.6	60	no	QUAD	relapsed[6]
12	59	M	No (null)	TT	ΔG/ΔG	GA	1a	6.9	104	yes	QUAD	stopped[7]
13	65	M	No (null)	CT	ΔG/ΔG	GG	1a	6.5	113	no	QUAD	SVR
14	56	M	No (null)	CT	TT/ΔG	GG	1a	6.4	184	yes	QUAD	SVR
15	49	F	No (null)	TT	ΔG/ΔG	GA	1a	7.3	70	no	QUAD	SVR
16	59	M	No (null)	TT	ΔG/ΔG	GG	1a	7.2	160	no	QUAD	SVR
17	58	M	No (null)	CT	TT/ΔG	GA	1a	6	159	yes	QUAD	SVR
18	58	F	No (null)	TT	ΔG/ΔG	GG	1a	6.5	68	yes	QUAD	SVR
19	54	M	No (null)	CC	TT/TT	GG	1a	6.5	93	yes	QUAD	SVR
20	54	M	No (null)	CC	TT/TT	GG	1a	6.4	245	no	QUAD	SVR
21	55	M	No (null)	CC	TT/TT	GG	1a	5.9	105	yes	QUAD	SVR
22	62	F	No (null)	TT	ΔG/ΔG	GG	1a	6.1	114	no	QUAD	Break-through[8]
23	69	M	No (null)	CC	TT/TT	GG	1b	7	66	no	DCV/ASV	SVR
24	75	F	No (null)	TT	ΔG/ΔG	GG	1b	6.6	80	no	DCV/ASV	SVR
25	41	M	No (null)	CT	TT/ΔG	GA	1b	7.3	56	no	DCV/ASV	SVR

Patient[1]	Age	Sex	Response to to Previous PegIFN/RBV[2] treatment	Patient Genotype (Single Nucleotide Polymorphism)			HCV genotype	Baseline HCV RNA [log_{10} IU/ml]	Baseline ALT [U/L]	Liver cirrhosis[3]	Treatment in current study	
				rs 12979860	rs 368234815	rs 117648444					Regimen	Outcome[4]
26	63	F	No (null)	CT	TT/ΔG	GG	1b	6.6	172	no	DCV/ASV	SVR
27	61	M	No (null)	CT	TT/ΔG	GG	1b	7	146	no	DCV/ASV	SVR
28	53	M	No (null)	CT	TT/ΔG	GA	1b	6.5	64	no	DCV/ASV	SVR
29	65	F	No (null)	CT	TT/ΔG	GG	1b	6.5	38	yes	DCV/ASV	SVR
30	37	M	No (null)	CT	TT/ΔG	GG	1b	6.4	55	no	DCV/ASV	SVR
31	50	M	No (null)	CT	TT/ΔG	GG	1b	6.3	47	yes	DCV/ASV	SVR

[1] NK cells from patients 1–7 were also studied during a past course with PegIFN/RBV (Fig. 5).

[2] PegIFN/RBV, combination therapy with pegylated IFN-α and ribavirin.

[3] Liver biopsies were performed 1–4 weeks prior to QUAD therapy. Cirrhosis is based on an Ishak fibrosis score of 5–6, absence of cirrhosis is based on an Ishak fibrosis score <5

[4] SVR, sustained virological response defined as HCV RNA negative at week 12 post treatment.

[5] All patients were nonresponders to previous PegIFN/RBV treatment. A null response is defined as a decrease of HCV-RNA titer <2 log10 IU/ml during the first 12 weeks of PegIFN/RBV.

[6] Relapsed one month after end-of-treatment.

[7] Stopped treatment at week 8 because of IFN intolerance

[8] Viral breakthrough detected at week 8 of treatment.