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# BASIC AND TRANSLATIONAL—LIVER

## Successful Interferon-Free Therapy of Chronic Hepatitis C Virus Infection Normalizes Natural Killer Cell Function

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**BACKGROUND & AIMS:** Chronic hepatitis C virus infection activates an intrahepatic immune response, leading to increased expression of interferon (IFN)-stimulated genes and activation of natural killer (NK) cells—the most prevalent innate immune cell in the liver. We investigated whether the elimination of hepatitis C virus with direct-acting antiviral normalizes expression of IFN-stimulated genes and NK cell function. **METHODS:** We used multicolor flow cytometry to analyze NK cells from the liver and blood of 13 HCV-infected patients who did not respond to treatment with pegylated interferon and ribavirin. Samples were collected before and during IFN-free treatment with daclatasvir and asunaprevir and compared with samples from the blood of 13 healthy individuals (controls). Serum levels of chemokine C-X-C motif ligand (CXCL) 10 or CXCL11 were measured by enzyme-linked immunosorbent assay. **RESULTS:** Before treatment, all patients had increased levels of CXCL10 or CXCL11 and a different NK cell phenotype from controls, characterized by increased expression of HLA-DR, NKp46, NKG2A, CD85j, signal transducer and activator of transcription 1 (STAT1), phosphorylated STAT1, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). NK cells from patients also had increased degranulation and decreased production of IFN$\gamma$ and tumor necrosis factor $\alpha$ compared with NK cells from controls. Nine patients had an end-of-treatment response (undetectable virus) and 4 had virologic breakthrough between weeks 4 and 12 of therapy. A rapid decrease in viremia and level of inflammatory cytokines in all patients was associated with decreased activation of intrahepatic and blood NK cells; it was followed by restoration of a normal NK cell phenotype and function by week 8 in patients with undetectable viremia. This normalized NK cell phenotype was maintained until week 24 (end of treatment). **CONCLUSIONS:** Direct-acting antiviral–mediated clearance of HCV is associated with loss of intrahepatic immune activation by IFN$\alpha$, which is indicated by decreased levels of CXCL10 and CXCL11 and normalization of NK cell phenotype and function.

*Keywords:* Immune Regulation; NS5A Inhibitor; NS3 Inhibitor; ISG.

Chronic hepatitis C virus (HCV) infection is a major cause of cirrhosis and hepatocellular carcinoma owing to the large number of up to 170 million infected people worldwide. The progression of liver disease from hepatitis to fibrosis and cirrhosis may take decades and is caused by a low-level inflammatory response. An interferon-driven innate immune activation is thought to contribute to this process.[1]

A characteristic of the innate response in HCV infection is the up-regulation of interferon-stimulated genes (ISGs) that readily are detectable in liver biopsy specimens of chronically infected patients.[2] The products of some ISGs, such as CXCL10, also are detectable at increased levels in the systemic circulation.[3] This increase in ISG levels is consistent with increased activation of innate immune cells that respond to type I interferon (IFN).

Natural killer (NK) cells are an important part of this IFN-responsive innate population because they are enriched among lymphocytes in the liver (30%) compared with the blood (5%–20%), and their percentage increases further in viral hepatitis.[4] NK cells of patients with chronic HCV infection express higher levels of signal transducer and activator of transcription 1 (STAT1) and phosphorylated STAT1 (pSTAT1) than NK cells of healthy controls,[5,6] suggesting that they are activated by type I IFN. STAT1 itself is the product of an ISG and its phosphorylation is an essential part of signaling downstream of the IFN$\alpha/\beta$ receptor. NK cells of chronically infected patients also show altered expression of activating and inhibitory receptors compared with those of healthy uninfected controls.[7,8] The integration of all these signals results in activation of blood and liver NK cells in HCV infection[7] and altered functional phenotype with increased cytotoxicity and decreased production of antiviral cytokines.[7,9] It currently is unknown whether this altered functional profile of NK cells is reversible.

The development of highly effective IFN-free regimens against HCV infection[10,11] provides a unique opportunity to analyze whether and how fast NK cell activation and liver

*Abbreviations used in this paper:* APC, allophycocyanin; ASV, asunaprevir; CXCL, chemokine C-X-C motif ligand; DAA, direct-acting antiviral; DCV, daclatasvir; EMA, ethidium monoazide; EOT, end of treatment; FBS, fetal bovine serum; FITC, Fluorescein isothiocyanate; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; IQR, interquartile range; ISG, interferon-stimulated gene; MFI, mean fluorescence intensity; NK, natural killer; PBMC, peripheral mononuclear cell; pSTAT, phosphorylated signal transducer and activator of transcription; RBV, ribavirin; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; TRAIL, tumor-necrosis factor-related apoptosis-inducing ligand.


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inflammation resolve when HCV replication is blocked. A normalization of NK cell effector functions also would be of interest in the context of adaptive immune responses because HCV-specific T cells are dysfunctional as a result of chronic antigen stimulation in HCV infection.[12,13] Although a recovery of T-cell proliferation recently was reported during treatment of HCV-infected patients with direct-acting antivirals (DAAs),[14] it remains unknown whether this translates into a full recovery of effector functions. Past IFN-based treatment regimens were not suitable to answer these questions because IFN not only has an antiviral, but also an immunomodulatory, function.[15] IFN-based therapies activate the innate immune response followed by the induction of a refractory state.[16] They also exacerbate the functional dichotomy of NK cells toward increased cytotoxic effector functions and reduced IFN$\gamma$ production.[17,18]

Here, we ask whether an IFN-free treatment regimen of daclatasvir (DCV) and asunaprevir (ASV) normalizes innate immune activation and NK cell function. DCV is a potent NS5A replication complex inhibitor with broad genotypic coverage; ASV is an NS3 protease inhibitor active against HCV genotypes 1a and 1b.[19] HCV genotype 1b nonresponders to previous pegylated IFN (PegIFN)/ribavirin (RBV) therapy were treated with DCV/ASV for 24 weeks. We show that successful treatment with the DCV/ASV regimen decreases serum levels of the ISG products CXCL10 and CXCL11, and that it decreases STAT1 expression and STAT1 phosphorylation in NK cells. This is associated with changes in the expression of activating and inhibitory receptors on NK cells and a normalization of NK cell function by week 8 of therapy. The results were verified for end-of-treatment (EOT) responders at week 24, indicating that the normalization of NK cell activation and function are maintained in patients who clear HCV.

## Materials and Methods

### Study Cohort

NK cells were studied in 13 HCV-infected nonresponders to previous PegIFN/RBV therapy before treatment, at days 0 and 1 of treatment, and then at weeks 2, 4, 8, and 24 of a 24-week treatment course with 60 mg DCV once daily and 100 mg ASV twice daily (Bristol-Myers Squibb, New York, NY; ClinicalTrials.gov: NCT01888900). NK cells were studied in 13 uninfected subjects for comparison. Patients underwent paired liver biopsies pretreatment and at either week 2 ($n = 5$) or week 4 ($n = 8$) of therapy. Three patients who later experienced virologic breakthrough had another biopsy at week 2 of therapy, the biopsy specimen of the fourth patient with a virologic breakthrough was not studied for NK cell responses. Patients provided written informed consent for research testing under protocols by the Institutional Review Board of National Institute of Diabetes and Digestive and Kidney Diseases/National Institute of Arthritis and Musculoskeletal and Skin Diseases.

### Serologic Analyses

Serum HCV-RNA level was quantitated using the Cobas TaqMan real-time polymerase chain reaction (Roche Molecular Diagnostics, Branchburg NJ) with a lower limit of detection of 10 IU/mL and a lower limit of quantification of 25 IU/mL. Serum chemokine C-X-C motif ligand (CXCL) 10 and CXCL11 levels were quantitated using the enzyme-linked immunosorbent assay MAX kit (Biolegend, San Diego, CA) and the Quantikine enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN), respectively.

### Lymphocyte Isolation

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 (Mediatech), and cryopreserved in 70% fetal bovine serum (FBS; Serum Source International, Charlotte, NC), 20% RPMI1640 (Mediatech) and 10% dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO). Liver biopsy specimens were homogenized, washed with phosphate-buffered saline, and studied the same day as described later.

### NK Cell Analysis

For each patient, cryopreserved PBMCs from week 0 to week 8 were thawed and tested on the same day. A second experiment was performed with cryopreserved samples from week 0 and week 24. Healthy donor PBMCs were included in each experiment. Because the flow cell of the LSRII flow cytometer was replaced between weeks 8 and 24 of the study protocol; the mean fluorescence intensity (MFI) data from the week 24 (EOT) time point cannot be compared with those from the earlier experiment.

**NK cell frequency and phenotype.** Thawed PBMCs were stained with ethidium monoazide (EMA), anti-CD19–PeCy5, anti-CD3–AlexaFluor700 (both from BD Biosciences, San Jose, CA), and with either anti-CD14–V711 (Biolegend) or anti-CD14–PeCy5 (AbD Serotec, Raleigh, NC) to exclude dead cells, T cells, B cells, and monocytes. NK cells were identified using anti-CD56–PeCy7 (BD Biosciences). Fluorescein isothiocyanate (FITC)–conjugated antibodies against CD122, CXCR3 (R&D Systems), CD69 and HLA-DR (BD Biosciences), PE-conjugated antibodies against tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL), CD300 (BD Biosciences), NKG2A, NKG2D, and NKp44 (Beckman Coulter, Brea, CA), and allophycocyanin (APC)-conjugated antibodies against CD85j (eBiosciences, San Diego, CA), CCR5 (BD Biosciences), NKG2C (R&D Systems), NKp30 and NKp46 (Miltenyi Biotec, Auburn, CA) were added. Liver-infiltrating lymphocytes were stained with anti-TRAIL–PE, anti-CD69–APC/Cy7 (BD Biosciences), and anti-NKp46–APC (Miltenyi Biotec) in addition to EMA and the lineage-specific antibodies described earlier.

**NK cell degranulation.** Thawed PBMCs were cultured overnight in RPMI1640 with 10% FBS (Serum Source International), 1% penicillin/streptomycin, 2 mmol/L L-glutamine, and 10 mmol/L HEPES (Mediatech). The next day, PBMCs were counted and cultured in the presence of anti-CD107–PE (BD Biosciences) with or without K562 cells (ATCC, Manassas, VA) as described[7] without addition of cytokines, and then stained with EMA and lineage-specific antibodies as described earlier.

**Cytokine production.** Thawed PBMCs were incubated with or without interleukin (IL)12 (0.5 ng/mL; R&D Systems) and IL15 (20 ng/mL; R&D Systems) for 14 hours, followed by the addition of brefeldin A (BD Biosciences) for 4 hours as described.[7] Cells then were washed and stained with EMA and the lineage-specific antibodies as described earlier. Cells were washed again, fixed, and permeabilized with the Cytofix/

Cytoperm Kit and stained with anti-IFN$\gamma$–PE and anti–tumor necrosis factor (TNF)$\alpha$–APC (all from BD Biosciences).

**STAT1/pSTAT1 staining.** Thawed PBMCs were rested for 2 hours at 37°C, 5% $CO_2$ in RPMI1640 with 10% FBS (Serum Source International), 1% penicillin/streptomycin, and 2 mmol/L L-glutamine (Mediatech). After fixation with Cytofix (BD Biosciences) for 10 minutes at 37°C and 5% $CO_2$ and subsequent centrifugation, cells were permeabilized with BD Phosflow III (BD Biosciences) for 20 minutes on ice, then washed twice and resuspended in BD Phosflow Buffer (BD Biosciences). All samples were stained with anti-CD56–PE (Beckman Coulter), anti-CD20–PerCP/Cy5.5, anti-CD3–fluorescein isothiocyanate or anti-CD3–APC, and anti-pSTAT1–Alexa488 or anti-STAT1–Alexa647 (all from Biosciences) for 20 minutes at room temperature.

Samples were analyzed on an LSRII flow cytometer using FacsDiva Version 6.1.3 (BD Biosciences) and FlowJo Version 8.8.2 software (Tree Star, Ashland, OR).

### Statistical Analysis

D'Agostino and Pearson omnibus normality tests, Wilcoxon signed-rank tests, Mann–Whitney tests, or linear regression analyses were performed with GraphPad Prism 5.0a (GraphPad Software, La Jolla, CA). Two-sided *P* values less than .05 were considered significant.

## Results

### Effect of DCV/ASV Therapy on HCV Viremia and Liver Inflammation

All 13 HCV-infected nonresponders to PegIFN/RBV (Supplementary Table 1) experienced a rapid decrease in serum HCV-RNA levels within the first 2 weeks of DCV/ASV therapy ($P = .0038$) (Figure 1*A*). Nine patients developed an EOT response, whereas 4 patients had a virologic breakthrough (week 4, n = 1; week 6, n = 2; and week 12, n = 1). Seven of 9 EOT responders achieved a sustained virologic response at 24 weeks after treatment; the remaining had not yet reached week 24 post-treatment. Serum alanine aminotransferase, CXCL10, and CXCL11 levels decreased significantly within the first 8 weeks of DCV/ASV therapy ($P = .008$, $P = .0005$, and $P = .0007$, respectively) (Figure 1*B–D*).

### Effect of DCV/ASV Therapy on NK Cell Activation

The effect of the rapid DAA-mediated decrease in HCV titers on the activation status and function of NK cells was studied by multicolor flow cytometry. Expression of the activation marker HLA-DR, the activating receptor NKp46, and the inhibitory receptors CD85j and NKG2A were higher on NK cells of chronically HCV-infected patients before DAA

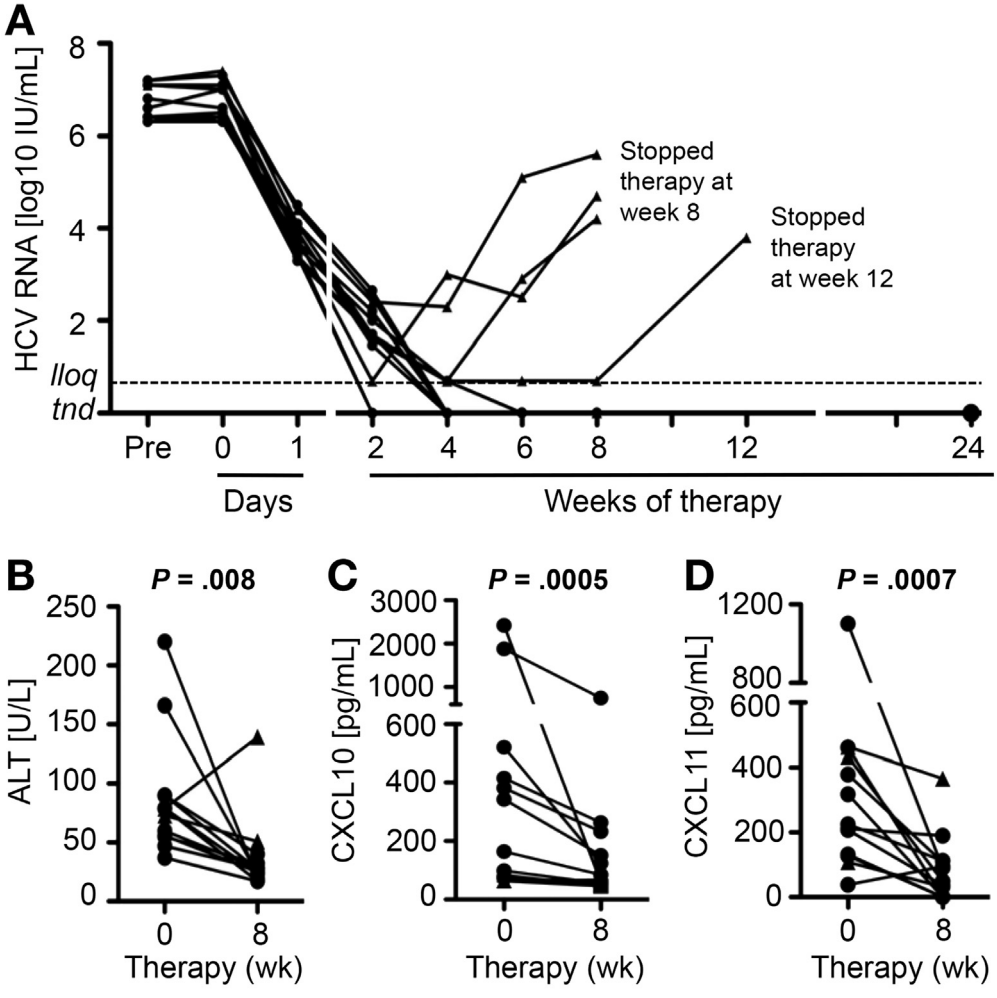


**Figure 1.** Serum HCV-RNA level and liver inflammation rapidly decrease with DCV/ASV therapy. (*A*) Serum HCV-RNA levels of patients who responded to therapy (n = 9, *solid circles*) or had a viral breakthrough and subsequently stopped therapy (n = 4, *solid triangles*). A response to DCV/ASV therapy was defined as undetectable viremia at EOT (week 24). Pre, time point of the pretreatment liver biopsy (1–4 weeks before therapy); Lloq, lower limit of quantitation; tnd, target not detected. (*B*) Serum alanine aminotransferase (ALT), (*C*) CXCL10, and (*D*) CXCL11 levels of all patients (n = 13) at week 0 and at week 8 of therapy. *Solid circles*, EOT responders; *solid triangles*, patients with virologic breakthrough. The patient with an increased ALT value is patient 12 in Supplementary Table 1.

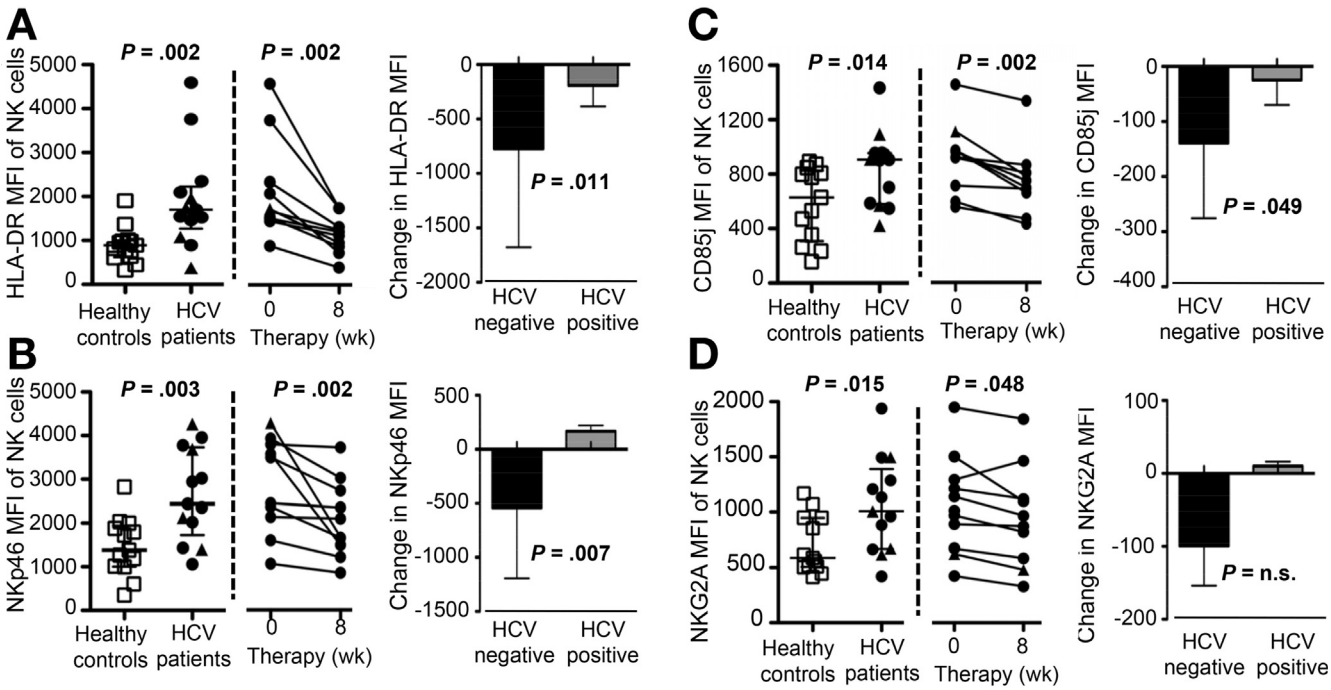


**Figure 2.** NK cell activation decreases within 8 weeks of successful DCV/ASV therapy. Expression of the activation markers (*A*) HLA-DR, (*B*) NKp46, and the inhibitory receptors (*C*) CD85j and (*D*) NKG2A on peripheral blood NK cells of chronic HCV patients before treatment (n = 13) compared with NK cells of healthy controls (*white squares, left graphs*). Change in the expression of these NK cell markers in 10 patients who had undetectable viremia (*middle graphs*) compared with 3 patients who were viremic at week 8 (*right graphs*). Statistical analysis: nonparametric paired Wilcoxon signed-rank test or unpaired Mann–Whitney test. Median and IQR are shown. *Solid circles*, EOT responders; *solid triangles*, patients with breakthrough after the study time points shown in this figure.

therapy than on those of uninfected controls (Figure 2). The expression level of HLA-DR, NKp46, CD85j, and NKG2A normalized in patients with undetectable viremia by week 8 of therapy, reaching levels similar to those of NK cells of uninfected controls, and the decrease in expression was greater than in the 3 patients who were viremic at week 8 (Figure 2). Because the latter were offered a rescue protocol with DCV/ASV/PegIFN/RBV, week 8 was the last time point at which to compare patients with and without detectable viremia in this study. Week 8 therefore was chosen to document the effect of a DAA-mediated decrease in viremia on NK cell phenotype and function.

NKp30, NKp44, CD69, NKG2C, NKG2D, CCR5, CD300, and CD122 expression also were assessed. There was a trend of higher NKp30 expression on NK cells of HCV patients compared with those of healthy controls ($P = .08$), and NKp30 MFI decreased significantly within 8 weeks of DAA therapy ($P = .014$, not shown). In contrast, the expression level of NKp44, CD69, NKG2C, NKG2D, CCR5, CD300, and CD122 did not differ on NK cells of chronically HCV-infected patients and those of healthy controls and did not change during DCV/ASV therapy (not shown).

### *Effect of DCV/ASV Therapy on NK Cell Cytotoxicity*

NK cells typically show increased cytotoxicity and TRAIL expression but decreased IFNγ production in chronic HCV infection.[7,9] To examine whether the DAA-induced decrease in HCV viremia modulated NK cell effector functions, PBMCs were incubated with major histocompatibility complex class I–negative K562 target cells, and the CD56dim NK cell population was assessed for cell surface expression of CD107a as a read-out for degranulation and cytotoxicity. CD56dim NK cells account for 90% of all NK cells in the circulation and represent the fully mature highly cytotoxic NK cell subset.[20] The frequency of CD56dim and CD56bright NK cells did not differ between HCV-infected patients and healthy controls (not shown).

The frequency of CD107a+ cells within the CD56dim NK cell population was higher in chronic HCV patients before therapy than in uninfected subjects (Figure 3*A* and *B*, left graphs). A significant decrease in the frequency and expression level of CD107a+ CD56dim NK cells was observed by week 8 of therapy in patients with undetectable viremia ($P = .031$ and $P = .011$, respectively) (Figure 3*A* and *B*, middle graphs), and the decrease in the percentage of CD107a+ CD56dim NK cells was greater in patients with undetectable viremia than in those who were viremic at week 8 ($P = .014$) (Figure 3*A*, right graph). The same pattern was observed in the total CD56+ NK cell population (Supplementary Figure 1*A* and *B*).

The frequency of TRAIL+ cells and the TRAIL expression level in the CD56dim NK cell subset also decreased significantly during the first 8 weeks of therapy ($P = .012$ and $P = .004$, respectively) (Figure 3*C* and *D*, left graphs), and the

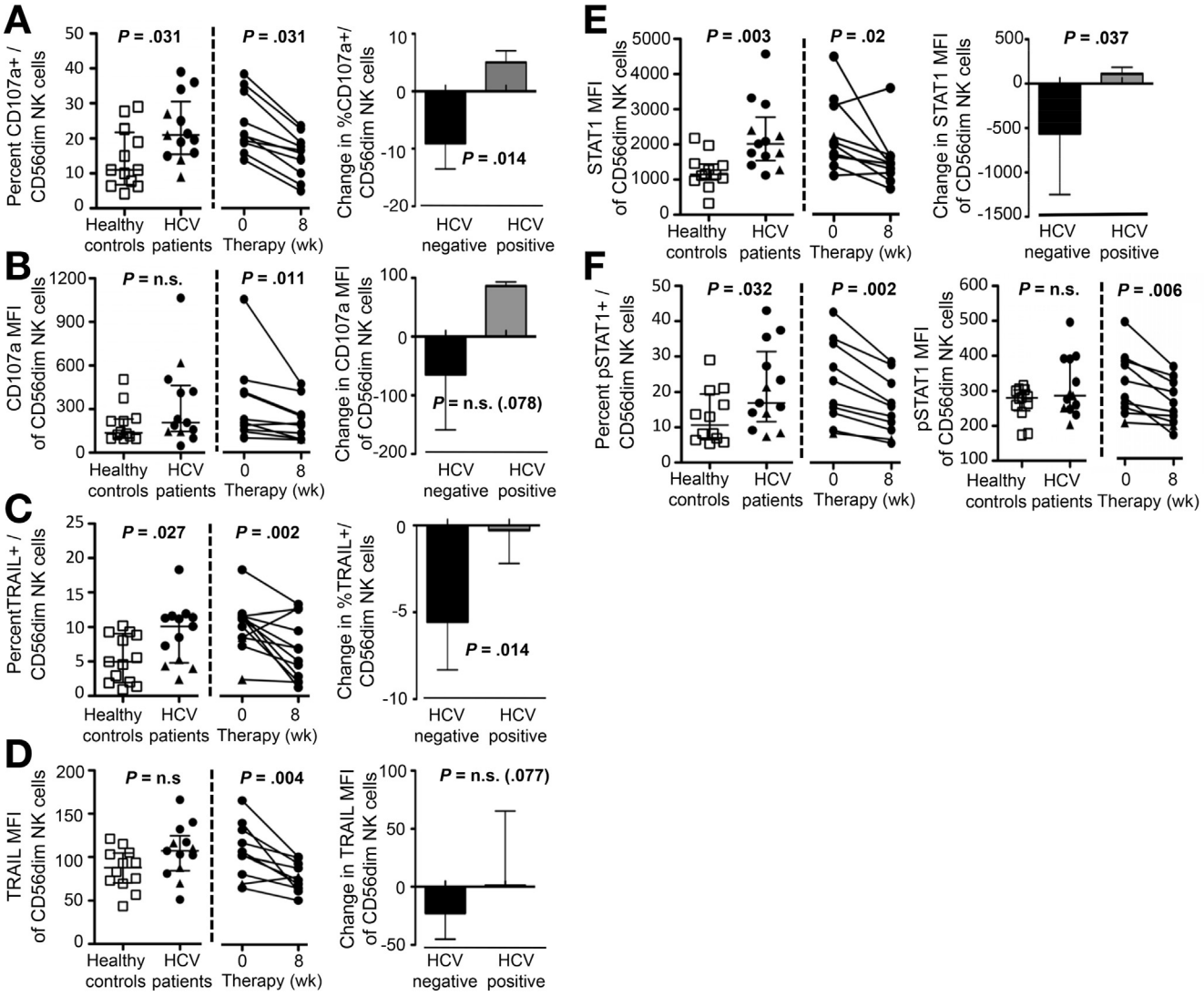


**Figure 3.** Increased NK cell cytotoxicity normalizes within 8 weeks of successful DCV/ASV therapy. (*A*) Frequency of CD107a+ cells, (*B*) CD107a expression level, (*C*) frequency of TRAIL+ cells, (*D*) TRAIL expression level, and (*E*) STAT1 expression level in the CD56dim NK cell population of HCV-infected patients before treatment (n = 13) compared with NK cells of healthy controls (*white squares, left graphs*). Changes in these parameters in 10 patients who had undetectable viremia (*middle graphs*) compared with 3 patients who were viremic at week 8 (*right graphs*). (*F*) Frequency of pSTAT1+ cells and pSTAT1 expression level in the CD56dim NK cell population of HCV-infected patients before treatment (n = 13) compared with NK cells of healthy controls (*white squares, left graph*). Changes in these parameters in 10 patients who had undetectable viremia at week 8 (*right graph*). Statistical analysis: nonparametric paired Wilcoxon signed-rank test or unpaired Mann–Whitney test. Median and IQR are shown. *Solid circles*, EOT responders; *solid triangles*, patients with breakthrough after the study time points shown in this figure.

decrease in the frequency of TRAIL+ cells was greater in patients with undetectable viremia at week 8 than in those who had experienced virologic breakthrough ($P = .014$) (Figure 3*C*, right graph). The decrease in TRAIL expression followed this trend ($P = .077$) (Figure 3*D*, right graph). Similar results were observed for the CD56bright NK cell subset (not shown), in which TRAIL is highly expressed.[21] Specifically, the median frequency of TRAIL+ cells in the CD56bright NK cell subset decreased from 45.7% (interquartile range [IQR], 23.2%–58.5%) before therapy to 22.2% (IQR, 11.4%–47%; $P = .027$), and median TRAIL expression in CD56bright NK cells decreased from 220 (IQR, 166–233) to 165 (IQR, 32–210; $P = .002$) during the first 8 weeks of therapy in patients with undetectable viremia (not shown).

Increased NK cell cytotoxicity in chronic HCV infection is thought to be driven by chronic exposure to virus-induced endogenous type I IFN.[6,7,22] We therefore examined whether the DCV/ASV-induced decrease in HCV titer resulted in decreased expression of STAT1 and pSTAT1. The expression level of STAT1, which itself is the product of an ISG, was significantly higher in CD56dim NK cells of chronic HCV patients than in those of uninfected subjects ($P = .003$), but decreased by week 8 of therapy ($P = .02$) (Figure 3*E*). Again, the absolute decrease in STAT1 expression was greater in NK cells of patients with

undetectable viremia than in those with viremia at week 8 of therapy ($P = .037$) (Figure 3*E*). Likewise, the frequency of pSTAT1-expressing cells in the CD56dim NK cell subset and the pSTAT1 expression level per cell decreased significantly in patients who had undetectable viremia at week 8 of therapy ($P = .002$ and $P = .006$, respectively) (Figure 3*F*).

### *Effect of DCV/ASV Therapy on NK Cell Cytokine Production*

To assess the capacity of NK cells to produce IFNγ and TNFα, PBMCs were stimulated with IL12 and IL15 in vitro and the CD56bright NK cell subset, which constitutes the main source of NK cell–derived cytokines,[22] was studied by flow cytometry. As shown in Figure 4*A* the percentage of IFNγ-producing cells in the CD56bright NK cell subset and the IFNγ expression level were significantly lower in chronic HCV patients compared with uninfected subjects ($P = .007$ and $P = .008$, respectively) and increased within the first 8 weeks of therapy in patients with undetectable viremia ($P = .008$ and $P = .002$, respectively) (Figure 5*A* and *B*). These observations were consistent with changes in the frequency of TNFα+ cells and IFNγ+/TNFα+ cells in the CD56bright NK cell subset (Figure 4*C* and *D*), and were confirmed in the total NK population (Supplementary Figure 1*C*–*F*).

### *Effect of DCV/ASV Therapy on Intrahepatic NK Cells*

Next, we examined whether these results extended to the liver. NK cells were studied in paired liver biopsy specimens and blood samples before DCV/ASV and, depending on randomization, at week 2 or 4 of DCV/ASV therapy. No patient had experienced a viral breakthrough at the time point of the second biopsy.

The frequency of CD69+ and NKp46+ cells in the CD56dim NK cell population and the MFI of these markers were significantly higher in the liver than in the blood before DCV/ASV therapy (Figure 5*A* and *B*), indicating that NK cells were more activated at the site of infection. In contrast, the frequency of TRAIL+ CD56dim NK cells and the TRAIL expression level per cell did not differ between both compartments (data not shown). The HCV titer before DCV/ASV therapy correlated with the frequency of TRAIL+ CD56bright NK cells in the pretreatment biopsy ($P = .03$, $r = .064$ data not shown). Accordingly, the frequency of TRAIL+ CD56dim NK cells in the liver and the TRAIL expression level per cell were lower in the on-treatment biopsy specimen ($P = .0005$ and $P = .048$, respectively) (Figure 5*C*) that was taken after a significant decrease in viremia had occurred (Figure 1*A*). A similar decrease was observed in the frequency and the expression level of TRAIL+ cells in the intrahepatic CD56bright NK cell population (median frequency, 37% before treatment compared with 13% at weeks 2/4, $P = .004$;

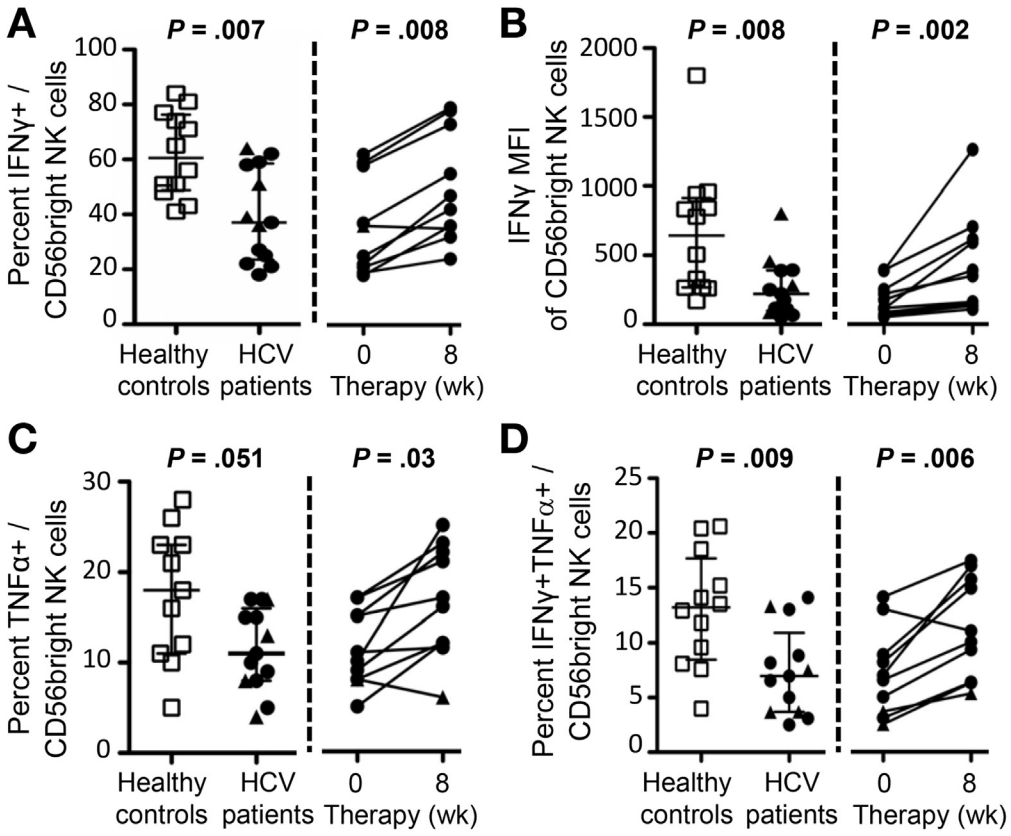


**Figure 4.** Decreased IFNγ and TNFα production by NK cells is restored within 8 weeks of successful DCV/ASV therapy. (*A*) Frequency of IFNγ+ cells, (*B*) IFNγ expression level, and frequency of (*C*) TNFα+ and (*D*) IFNγ+TNFα+ in the CD56bright NK cell population of chronic HCV patients before treatment (n = 13) compared with NK cells of healthy controls (*white squares left graphs*). Changes in these parameters in 10 patients who had undetectable viremia at week 8 (*right graphs*). Statistical analysis: nonparametric paired Wilcoxon signed-rank test or unpaired Mann–Whitney test. Medians and IQR are shown. *Solid circles*, EOT responders; *solid triangles*, patients with breakthrough after the study time points shown in this figure.

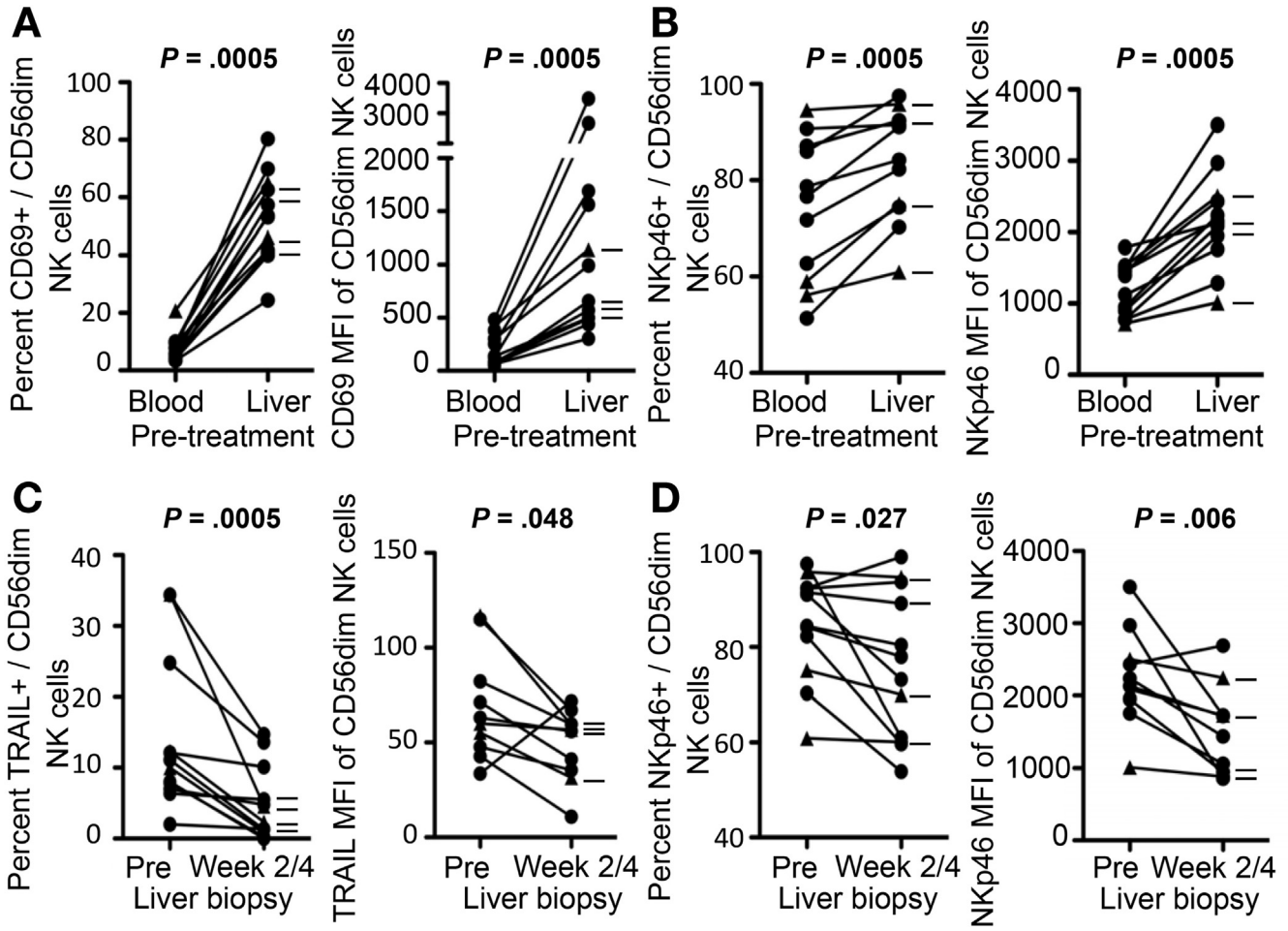


**Figure 5.** Activation and TRAIL expression of intrahepatic CD56dim NK cells decrease within 2–4 weeks of DCV/ASV therapy in parallel to decreasing viremia and liver inflammation. (*A* and *B*) Comparison of frequency and expression level of (*A*) CD69+ and (*B*) NKp46+ cells in peripheral and intrahepatic CD56dim NK cell populations of chronically HCV-infected patients before therapy. (*C* and *D*) Frequency and expression level of (*C*) TRAIL+ and (*D*) NKp46+ CD56dim NK cells in paired liver biopsies before (Pre) and, depending on randomization, either at week 2 or at week 4 of therapy. Horizontal marks identify week 2 biopsies. Statistical analysis: nonparametric paired Wilcoxon signed-rank test. *Solid circles*, EOT responders; *solid triangles*, patient with virologic breakthrough after week 8.



median MFI, 113 before treatment compared with 73 at weeks 2/4, $P = .027$, not shown).

The frequency of NKp46+ cells and the NKp46 expression level in the intrahepatic CD56dim NK cell population decreased as well ($P = .027$ and $P = .006$, respectively) (Figure 5*D*). Collectively, these data indicate a decrease in NK cell activation and normalization of NK cell function in the blood and in the liver during DAA therapy.

### *NK Cell Functions Normalize in a Sequential Manner*

To investigate how early during DCV/ASV therapy the observed changes in NK cell activation and function occur, we tested PBMCs at 24 hours and at 2 weeks of therapy. All patients were included in this analysis because they showed a significant decrease in viral titer during this time period (Figure 1*A*). As shown in Figure 6*A*, expression of the activation marker HLA-DR on the total NK cell population decreased within 24 hours of therapy initiation ($P = .005$). IFN$\gamma$ production did not improve significantly during the first 24 hours, but normalized by week 2 of therapy ($P = .006$) (Figure 6*B*). None of the other cell surface markers (NKp46, NKG2A, and CD85j), that were expressed differentially on NK cells of HCV-infected patients and healthy controls, changed during the first 2 weeks of treatment (not shown). Markers of NK cell cytotoxicity, such as expression of the degranulation marker CD107 (Figure 6*C*) and TRAIL (not shown), did not change during the first 2 weeks of therapy.

### *Normalized NK Cell Function Is Maintained in EOT Responders*

Finally, we examined whether the restoration of NK cell phenotype and function were maintained. As shown in Figure 7*A*, HLA-DR, NKp46, and CD85j expression levels on peripheral blood NK cells were lower at week 24 (EOT)

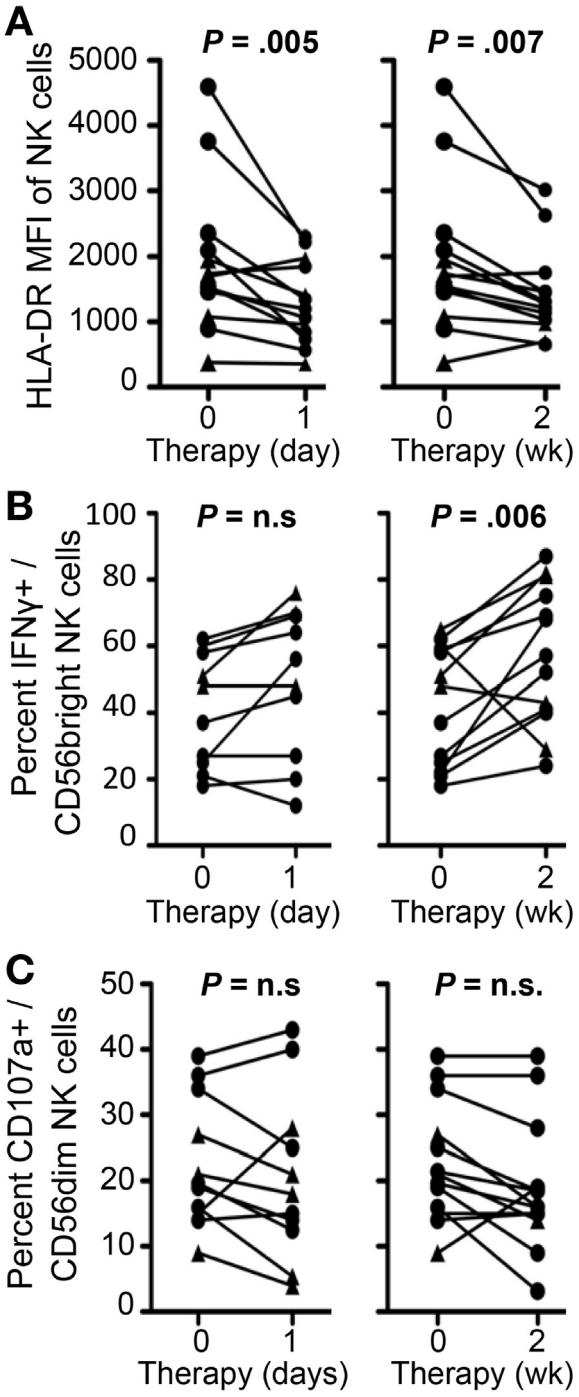


**Figure 6.** NK cell functions normalize in a sequential manner during DCV/ASV therapy. Changes in (*A*) HLA-DR expression in all CD56+ NK cells and in the frequency of (*B*) IFNγ+ CD56bright and (*C*) CD107+ CD56dim NK cells during the first 24 hours (*left graphs*) and the first 2 weeks (*right graphs*) of therapy.

compared with week 0 ($P = .008$, $P = .023$, and $P = .078$, respectively). Interestingly, expression of the chemokine receptor CXCR3 significantly increased from week 0 to week 24 ($P = .023$) (Figure 7*B*). This is consistent with a lack of CXCR3 stimulation because the serum concentration of the CXCR3 ligands CXCL10 and CXCL11 had decreased by week 8 of therapy (Figure 1*C* and *D*).

Confirming the week 8 data (Figure 3), the frequency of CD107+ and TRAIL+ cells in the CD56dim NK cell subset ($P = .022$ and $P = .023$, respectively) (Figure 7*C*) and in the total NK cell population ($P = .016$ and $P = .039$, respectively) (Supplementary Figure 2*A*) were significantly lower at week 24 than before therapy. Likewise, the frequency of pSTAT1-expressing cells and the expression level of the ISG STAT1 in the CD56dim NK cell subset decreased during the 24-week treatment course ($P = .016$ and $P = .023$, respectively) (Figure 7*D*).

The normalization of NK cell cytokine production was confirmed at the week 24 time point, with significantly increased frequencies of IFNγ, TNFα, and IFNγ/TNFα-producing cells and IFNγ MFI in both the CD56bright (Figure 7*E*) and the total NK cell populations (Supplementary Figure 2*C*). Collectively, these results indicate that effective removal of HCV by an IFN-free DAA regimen normalizes both phenotype and function of NK cells.

## Discussion

NK cells constitute the main innate immune cell population in the liver. Their activation in chronic HCV infection is associated with increased cytotoxic functions, as assessed by TRAIL expression and degranulation, and with decreased production of the antiviral cytokine IFNγ.[7,9] It previously has been suggested that prolonged exposure to low levels of HCV-induced IFNα is responsible for this phenotype.[7] However, IFNα protein has rarely and only at exceedingly low levels been detected in patients[23] and chimpanzees[24] with HCV infection. Moreover, a similar NK cell phenotype also has been reported in patients with chronic hepatitis B virus infection,[9] a disease that does not induce many ISGs in the liver.[25] IFN-free therapy regimens for HCV infection provide a unique opportunity to study the interaction between HCV and the intrahepatic immune system because these regimens rapidly decrease viremia to undetectable levels.

The current study shows that DCV/ASV-mediated HCV clearance is associated with a decrease in NK cell activation and a normalization of NK cell cytotoxic effector functions to levels observed in uninfected subjects. The rapid normalization of the NK cell phenotype is associated with a decrease in the percentage of pSTAT1-expressing NK cells and a decrease in the expression level of the ISG STAT1 in NK cells. Collectively, these results confirm that type I IFN-mediated NK cell activation via the IFNα/β receptor is indeed responsible for the alteration of NK cell function in chronic HCV infection. Thus, the phenotypic and functional NK cell profile that is observed in chronic HCV infection and exacerbated during IFN-based therapy is normalized in IFN-free DAA therapy. This is of interest in the context of recent findings in a mouse model of lymphocytic choriomeningitis virus (LCMV) induced chronic hepatitis. Blocking of the IFNα/β receptor in this model increased the number of NK cells and virus-specific CD4 T cells and restored systemic IFNγ levels, thereby inducing LCMV clearance.[26,27]

Overall, normalization of NK cell phenotype and function followed a hierarchy. Although a decrease in the expression

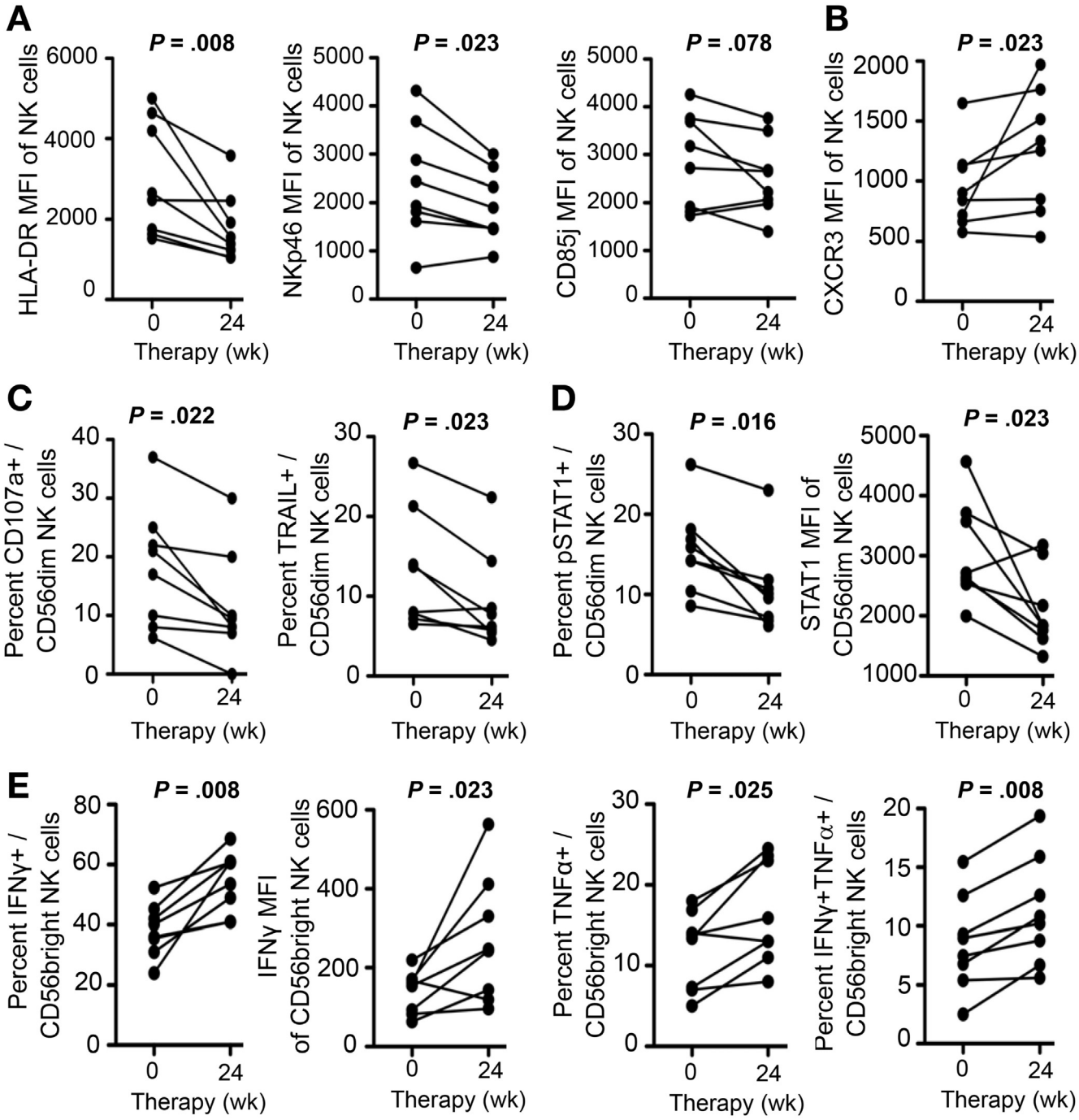


**Figure 7.** Normalized NK cell phenotype and function are maintained in EOT responders to DCV/ASV therapy. Expression of (*A*) the activation marker HLA-DR, the activating receptor NKp46, (*A*) the inhibitory receptor CD85j and (*B*) the chemokine receptor CXCR3 on peripheral blood CD56+ NK cells of EOT responders at weeks 0 and 24 of therapy. (*C*) Frequency of CD107a+ and TRAIL+ cells and (*D*) frequency of pSTAT1+ cells and STAT1 expression level in the CD56dim NK cell population of EOT responders at weeks 0 and 24 of therapy. (*E*) Frequency of IFN$\gamma$+, TNF$\alpha$+, and IFN$\gamma$+TNF$\alpha$+ cells and IFN$\gamma$ expression level in the peripheral blood CD56bright NK cell population of EOT responders at weeks 0 and 24 of therapy. Statistical analysis: nonparametric paired Wilcoxon signed-rank test.

level of the activation marker HLA-DR was observed within 24 hours of DCV/ASV therapy in parallel to a significant decrease in HCV titer, changes in NK cell function were not observed during this time period. The frequency of IFN$\gamma$+ NK cells increased significantly by week 2 of therapy, whereas the decrease in the frequency of CD107a+ NK cells became significant only after week 2. Thus, altered cytokine production by NK cells appears to be more readily reversible than altered cytotoxicity. Importantly, all NK cell read-outs were indistinguishable from those of healthy uninfected subjects by week 8 of DCV/ASV therapy and were confirmed at the week 24 time point in EOT responders.

Because we studied a selected group of patients who all were infected with HCV genotype 1b and nonresponders to previous PegIFN/RBV therapy we performed a detailed characterization of this cohort's NK cell phenotype in comparison with published literature. We confirm earlier studies on increased NKp46 and NKG2A expression on NK cells in HCV infection,[7] and in addition report increased expression of the inhibitory receptor CD85j and the activation marker HLA-DR. Increased NKp30 expression in our cohort is consistent with results of De Maria et al,[28] who reported higher NKp30 expression on NK cells of HCV-infected patients than those of uninfected controls. In contrast, Nattermann et al[29] reported lower NKp30 expression, but in a patient cohort with more diverse HCV genotypes. Importantly, the expression of NKp30, NKp46, HLA-DR, NKG2A, and CD85j normalized during successful IFN-free DAA therapy in our study.

NKp44 and NKG2C expression did not differ between NK cells of HCV-infected patients and healthy donors in our study, which is consistent with published literature.[7,29] Likewise, NKG2D expression did not differ between NK cells of HCV patients and healthy controls, and there was no change in expression during DAA therapy. This is consistent with reports by De Maria[28] and Nattermann et al,[29] who found no differential expression of NKG2D on NK cells of HCV patients and healthy controls. In contrast, Oliviero et al[9] reported increased NKG2D levels whereas Dessouki et al[30] reported decreased NKG2D levels in HCV infection. However, the patient cohort studied by Oliviero et al[9] included responders to IFN-based therapy, and the patients were infected with diverse genotypes and had lower HCV viremia and alanine aminotransferase values than in our study. Dessouki et al[30] studied only the frequency of NKG2D+ NK cells and not the NKG2D expression level.

We propose that the restoration of NK cell function is the result of rapid removal of the HCV and its associated IFN signature, also should be seen with other direct-acting antivirals. It also should extend to other HCV genotypes, if they are not associated with an increased incidence of breakthrough or relapse. The restoration of NK cell function, in particular the normalization of suppressed IFN$\gamma$ and TNF$\alpha$ production, may be relevant for studies on adaptive immune responses. Similar to NK cells, HCV-specific T cells show impaired cytokine production in chronic HCV infection.[12,13] Although the proliferation of HCV-specific T cells recently has been reported to improve during antiviral therapy with the DAAs faldaprevir and deleobuvir,[14] it has not yet been reported to what extent cytokine production of HCV-specific T cells can be recovered. The current data on recovery of IFN$\gamma$ and TNF$\alpha$ production by NK cells increase hope that this also may occur for T cells. Restoration of cytokine production may result in better immune surveillance and prevention of virologic relapse, which will be an interesting topic to address in future studies.

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.03.004.

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**Conflicts of interest**
The authors disclose no conflicts.

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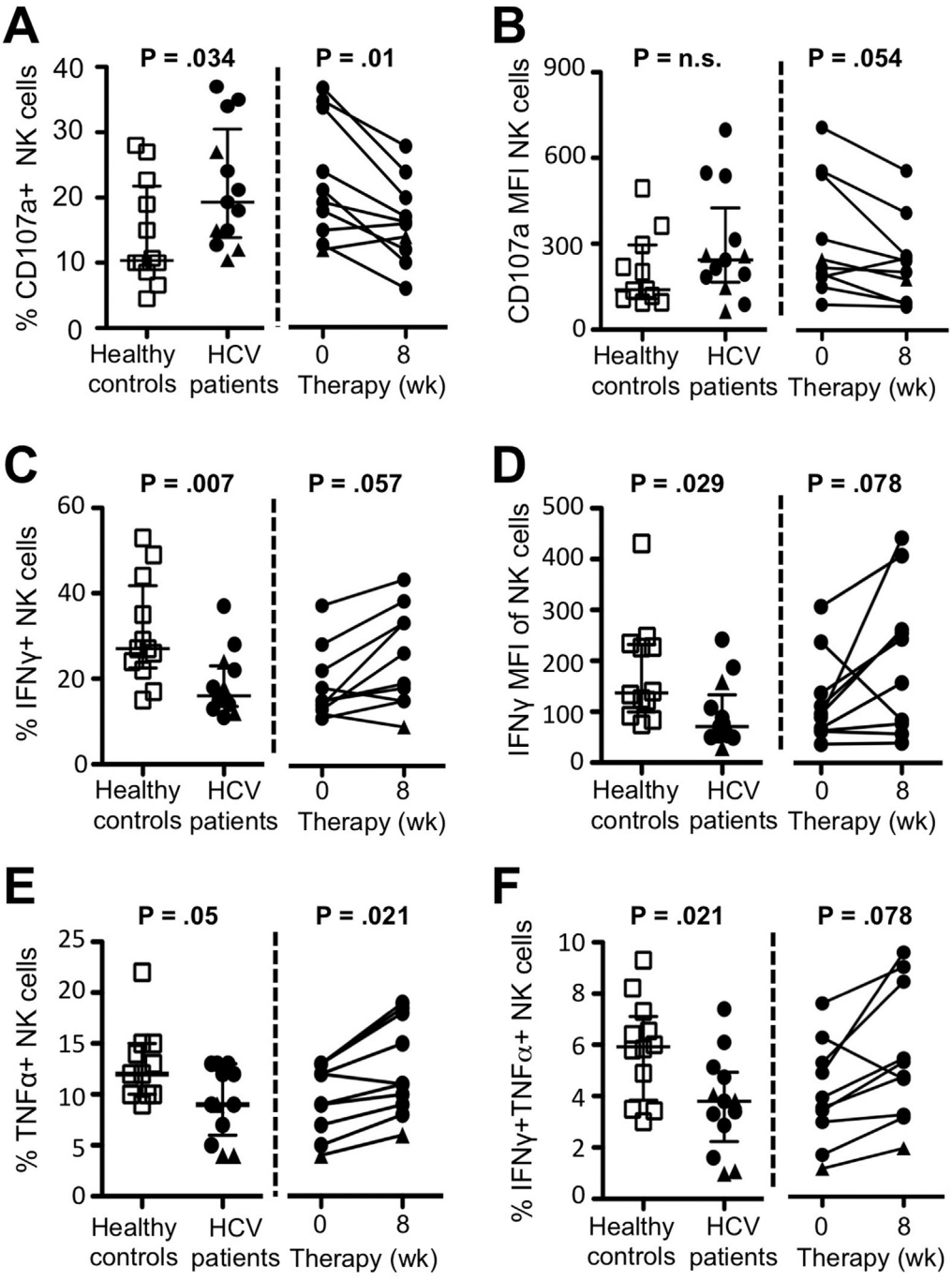
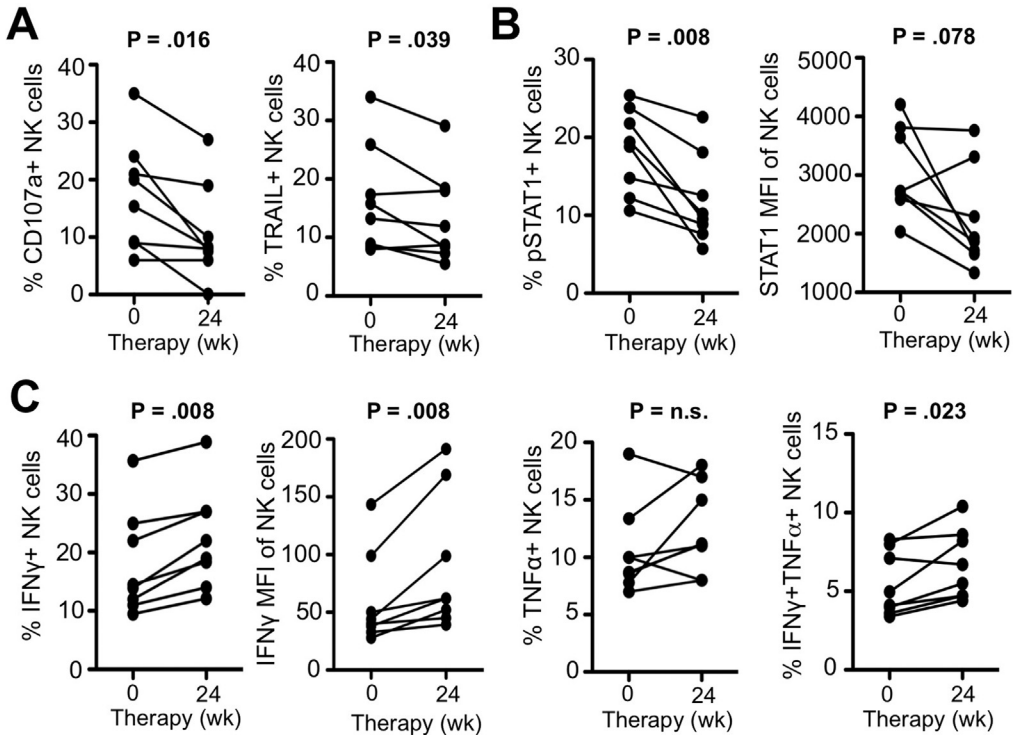
**Supplementary Figure 1.** Cytotoxicity decreases and IFN$\gamma$ and TNF$\alpha$ production increase in the total peripheral blood NK cell population within 8 weeks of successful DCV/ASV therapy. (*A*) Frequency and (*B*) expression level of CD107a+ NK cells of chronic HCV patients (n = 11) compared with NK cells of healthy controls (*white squares, left graphs*). Changes in these parameters in 8 patients with undetectable viremia at week 8 (*right graphs*). (*C*) Frequency and (*D*) expression level of IFN$\gamma$+ NK cells of chronic HCV patients (n = 11) compared with NK cells of healthy controls (*left graphs*). Changes in these parameters in 8 patients who had undetectable viremia at week 8 (*right graphs*). Frequency of (*E*) TNF$\alpha$+ and (*F*) IFN$\gamma$+/TNF$\alpha$+ NK cells of chronic HCV patients (n = 11) compared with NK cells of healthy controls (*left graphs*). Change in these parameters in 8 patients who had undetectable viremia at week 8 (*right graphs*). Statistical analysis: nonparametric paired Wilcoxon signed-rank test or unpaired Mann–Whitney test. Medians and IQR are shown. *Solid circles*, EOT responders; *solid triangles*, patient with virologic breakthrough after the study time points shown in this figure.






**Supplementary Figure 2.** The restoration of function in the total peripheral blood NK cell population is maintained in EOT responders to DCV/ASV therapy. (*A*) Frequency of CD107a+ cells (*left graph*) and TRAIL+ cells (*right graph*) in the total peripheral blood NK cell population of EOT responders at weeks 0 and 24 of DCV/ASV therapy. (*B*) Frequency (*left graph*) and expression level (*right graph*) of pSTAT1+ cells in the total peripheral blood NK cell population of EOT responders at weeks 0 and 24 of therapy. (*C*) Frequency of IFNγ+, TNFα+, and IFNγ+/TNFα+ NK cells in the total peripheral blood NK cell population of EOT responders at weeks 0 and 24 of therapy. Statistical analysis: nonparametric paired Wilcoxon signed-rank test.

**Supplementary Table 1.** Patient Characteristics

| Patient | Age, *y* | Sex | HCV genotype | HCV-RNA, level $\log_{10}$ *IU/mL* | Cirrhosis[a] | ALT level, *U/L* | Response to past PegIFN/RBV therapy | Response to DCV/ASV therapy |
|---|---|---|---|---|---|---|---|---|
| 1 | 69 | M | 1b | 7.0 | No | 66 | Null[b] | SVR24[c] |
| 2 | 75 | F | 1b | 6.6 | No | 80 | Null | SVR24 |
| 3 | 41 | M | 1b | 7.3 | No | 56 | Null | SVR24 |
| 4 | 63 | F | 1b | 6.6 | No | 172 | Null | SVR24 |
| 5 | 61 | M | 1b | 7.0 | No | 146 | Null | SVR24 |
| 6 | 53 | M | 1b | 6.5 | No | 64 | Null | SVR24 |
| 7 | 65 | F | 1b | 6.5 | Yes | 38 | Null | SVR24 |
| 8 | 50 | M | 1b | 6.3 | Yes | 47 | Null | EOT response[d] |
| 9 | 70 | F | 1b | 6.3 | Yes | 47 | Null | EOT response[d] |
| 10 | 70 | F | 1b | 6.3 | Yes | 47 | Null | Breakthrough (week 6)[e] |
| 11 | 68 | F | 1b | 7.1 | Yes | 75 | Partial[f] | Breakthrough (week 12) |
| 12 | 53 | M | 1b | 7.4 | No | 122 | Null | Breakthrough (week 6) |
| 13 | 62 | M | 1b | 7.0 | Yes | 54 | Partial | Breakthrough (week 4) |

ALT, alanine aminotransferase.
[a]Liver biopsies were performed 1–12 weeks before DCV/ASV therapy.
[b]Null response, a decrease of HCV-RNA titer of less than 2 $\log_{10}$ IU/mL during the first 12 weeks of PegIFN/RBV.
[c]SVR24, sustained virologic response at week 24 after treatment (ie, undetectable HCV-RNA level at the end of a 24-week observation period that follows the end-of-treatment).
[d]Undetectable HCV-RNA level at week 24 of treatment. Patients 8 and 9 did not yet complete the 24-week observation period after EOT and thus could not be assessed for an SVR24.
[e]The week indicates the time of viral breakthrough during treatment (Figure 1).
[f]Partial response: a decrease of HCV-RNA titer greater than 2 $\log_{10}$ IU/mL with detectable viremia.