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Altered effector functions of NK cells in chronic hepatitis C are associated with *IFNL3* polymorphism

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RECEIVED OCTOBER 31, 2014; REVISED MARCH 11, 2015; ACCEPTED APRIL 12, 2015. DOI: 10.1189/jlb.4A1014-520R

ABSTRACT

Interferon α-mediated effector functions of NK cells may contribute to the control of HCV replication and the pathogenesis of liver disease. The single-nucleotide polymorphism rs12979860 near *IFNL3* (previously known as *IL28B*) is important in response to IFN-α treatment and in spontaneous resolution of acute hepatitis C. The role of the *IFNL3* polymorphism in NK cell function is unclear. Thus, we investigated the role of *IFNL3* polymorphism in type I IFN-dependent regulation of NK cell functions in patients with cHC and healthy control subjects. We demonstrated a marked polarization of NK cells toward cytotoxicity in response to IFN-α stimulation in patients with hepatitis C. That TRAIL up-regulation was present, particularly in patients with the *IFNL3*-TT allele, was supported by a shift in the pSTAT-1:pSTAT-4 ratios toward pSTAT-1. In patients bearing the *IFNL3*-TT allele, NK cell effector function correlated with liver disease activity. In contrast, higher cytokine production of NK cells was observed in healthy individuals with the *IFNL3*-CC genotype, which may support spontaneous HCV clearance in acute infection. Overall, these findings show that the role of NK cells may differ in chronic infection vs. early antiviral defense and that the *IFNL3* genotype differentially influences NK cell function. *J. Leukoc. Biol.* 98: 283–294; 2015.

Introduction

HCV infection is a major global health problem. Available estimates indicate that more than 160 million people are persistently infected with HCV worldwide [1]. The infection can cause development of liver cirrhosis and liver cancer and leads to almost 500,000 deaths every year [1, 2]. Despite the sharp reduction in HCV transmission by blood products, the disease continues to be one of the leading blood-borne infections in Europe [3]. No vaccine is currently available for HCV [4]. Treatment of cHC infection has been based on the administration of IFN-α for more than 2 decades [5]. Even after the introduction of novel direct antivirals, IFN-α remains the backbone of antiviral treatment of HCV in many countries [6]. Restoration of the type I endogenous IFN response also seems to play a role in sustained responses to IFN-free treatments for cHC [7].

Genome-wide association studies identified the SNP rs12979860 on chromosome 19 located upstream from *IFNL3* (previously known as *IL28B*) as highly predictive of a virological response during peginterferon alfa (IFN-α) and ribavirin treatment of cHC [8–11]. In addition, distinct *IFNL3* genotypes are associated with resolution of acute HCV infection [11–15]. However, the detailed mode of action how *IFNL3* SNPs mediate spontaneous HCV clearance and response to IFN-α-based therapies is only partially understood [16]. In particular, the function of immune cells and their response to type I IFN stimulation are not well characterized in the context of the different *IFNL3* genotypes.

NK cells as part of innate immunity contribute to defense mechanisms that control infections and tumor establishment. In humans, they are characterized by expression of CD56, CD16, or both in the absence of CD3 [17]. NK cell action is based on cytotoxicity through release of $CD107^+$ granules containing perforin and granzymes and induction of apoptosis by TRAIL or Fas ligand. Moreover, NK cells orchestrate action of other immune cells by IFN-γ and TNF-α secretion [18] and may directly interact with APCs and T cells [19]. In HCV infection, NK cells have been suggested to be of importance during

Abbreviations: ALT = alanine transaminase, CD = cluster of differentiation, cHC = chronic hepatitis C, HCV = hepatitis C virus, *IFNL3* = interferon λ-3, *IL28B* = interleukin 28B, MFI = mean fluorescence intensity, NKG2A = killer cell lectin-like receptor subfamily C, member 1, Peg = polyethylene glycol, PBMC = peripheral blood mononuclear cell, SNP = single nucleotide polymorphism

The online version of this paper, found at www.jleukbio.org, *includes supplemental information.*

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different phases of the infection [20, 21], and acute and chronic hepatitis C are associated with altered phenotype and function of NK cells [22–24]. Specifically, NK cells may be involved in protection against HCV infection [25, 26], spontaneous resolution of acute hepatitis C [27–29], fibrosis progression in cHC [30, 31], and response to IFN-α-based therapies both in acute and chronic hepatitis C [32–35]. We have shown that up-regulation of TRAIL on NK cells after IFN-α stimulation correlated inversely with a decline in HCV RNA during the early phases of IFN-α therapy [36].

The *IFNL3* SNP rs12979860 TT genotype has been associated with higher NKG2A expression and reduced expression of TRAIL on NK cells [37], whereas, in contrast, the cytolytic activity of NK cells was higher in patients bearing the *IFNL3*-TT genotype in another recent report [38]. Two other groups did not find an association between NK cell phenotype and function and *IFNL3* polymorphism with respect to treatment response [35, 39]. Thus, considering these partially conflicting data, the purpose of our study was to investigate the role of the *IFNL3* SNP rs12979860 genotype in type-I-IFN-dependent regulation of NK cell functions in patients with cHC. NK cells from patients with cHC, particularly those with the *IFNL3*-TT genotype, were highly susceptible to IFN-α stimulation, which may contribute to liver disease in persistent infection. However, higher cytokine production of NK cells in *IFNL3*-CC-bearing healthy individuals could support spontaneous HCV clearance in acute infection.

MATERIALS AND METHODS

Study cohort

Peripheral blood samples were drawn from Caucasian patients with cHC ($n = 33$; including 10 bearing the CC, 12 the CT, and 11 the TT genotype of *IFNL3* SNP rs12979860) and from age-range-matching (21–78 and 22–63 years, respectively) uninfected healthy volunteers as a control group ($n = 23$; including 10 bearing the CC, 7 the CT, and 6 the TT genotype of the *IFNL3* gene). Demographic, biochemical, and virological characteristics of the patients are listed in **Table 1**. None of the patients with cHC was receiving antiviral treatment at the time of blood collection. Nineteen patients had been treated with IFN-based therapies, whereas 14 were treatment naïve. In all the patients enrolled in the study, IFN-based therapy had been discontinued for at least 6 mo. Subsequent treatment approaches were diverse and included different strategies. All patients were recruited from the outpatient clinic of the Department of Gastroenterology, Hepatology, and Endocrinology at Hannover Medical School in Germany. All patients gave written informed consent for participation. The study was part of a protocol approved by the ethics committee of the Hannover Medical School.

All patients tested positive for anti-HCV with a third-generation ELISA (Architect; Abbott Diagnostics, Wiesbaden, Germany). HCV RNA was quantified with the Cobas *Taq*Man Assay (Roche Diagnostics, Grenzach-Whylen, Germany), with a lower limit of quantification of 15 IU/ml.

PBMCs were separated by the standard density centrifugation method with Biocoll (Biochrom AG, Berlin, Germany) and directly cryopreserved for long-term storage in liquid nitrogen. The frozen PBMCs were thawed and washed twice with PBS, resuspended in AB medium (composition in the Supplemental Data), and seeded ($0.3–0.5 \times 10^6$ cells per well) in a 96-well round-bottomed plate. The expression of TRAIL and CD107a, as well as IFN-γ and TNF-α, were assessed in short-term cultures stimulated with recombinant pegylated IFN-α (Peginterferon alfa-2b; Bayer Schering Pharma AG, Berlin-Wedding, Germany).

Functional NK cell assays

NK cell phenotype and effector function were determined as described elsewhere [36]. After 6 h of stimulation with different doses of IFN-α (0, 0.5, 1, 2.5, 5, and 10 ng/ml), the PBMCs were washed, fluorochrome-labeled antibodies (CD3, CD56, CD16, and anti-TRAIL) were added for 20 min of incubation at 4°C in the dark, and the cells were again washed.

For intracellular cytokine production and degranulation, the PBMCs were stimulated overnight with 10 or 100 ng/ml IFN-α or left untreated in medium and then cocultured with Huh-7.5 target cells in an effector:target ratio (E:T) ratio of 10:1. After 1 h of coculture, Brefeldin A was added, and the cells were cultured for another 5 h. The cells were then stained for surface antigens (CD3, CD56, CD16, and CD107a). Intracellular cytokine staining (anti-IFN-γ and -TNF-α) was performed after fixation and permeabilization with Cytofix/Cytoperm Solution (BD Biosciences, Heidelberg, Germany), according to the manufacturer's instructions.

Expression of STAT-1, pSTAT-1 (p-Y701), and pSTAT-4 (p-Y693) was studied after in vitro stimulation of PBMCs with 0, 2.5, 10, or 100 ng/ml IFN-α for 30 min at 37°C, as previously described [40].

The data were acquired on FACS Canto II with Diva software (BD Biosciences) and analyzed using FlowJo Software (TreeStar, San Carlos, CA, USA). NK cells were identified in PBMCs as $CD3^-/CD56^+/CD16^{+/-}$ cells. The gating strategy used to identify that NK cells is shown (see Fig. 1A). Detailed information regarding antibodies used can be found in the Supplemental Data.

IFNL3 genotyping

IFNL3 SNP rs12979860 genotyping was performed in healthy controls and patients with cHC by real-time PCR, followed by melting curve analysis on the Light Cycler 480 II System (Roche, Mannheim, Germany). DNA was extracted from whole blood samples with the DNeasy purification Kit (Qiagen, Hilden,

TABLE 1. Demographic, biochemical, and virological characteristics of patients with cHC

	IFNL3-CC	*IFNL3*-CT	*IFNL3*-TT	
Patients, *n*	10	12	11	*P*
Age, y, median (range)	63 (41–70)	52 (21–70)	51 (28–78)	0.33[a]
Sex, M/F	6/4	9/3	6/5	
Fibroscan, median (range)[b]	5.90 (3.40–21.80)	8.30 (5.90–14.30)	9.65 (3.50–29.90)	0.77[c]
METAVIR score, *n* (F1/F2/F3/F4)[d]	5/1/2/2	4/4/2/2	3/4/3/1	0.0001[e]
ALT IU/ml, median (range)	49 (17–239)	94 (37–288)	58.3 (29–224)	0.07[c]
HCV RNA log 10, IU/ml, median (range)	5.46 (1.18–6.59)	6.51 (5.50–6.85)	6.07 (5.00–6.81)	0.01[c]
HCV genotype, *n* (1/2/3/4)	7/0/0/0	4/1/0/0	6/0/2/0	
Treatment in the past/naïve, *n*	4/6	8/4	7/4	

[a]One-way ANOVA. [b]FibroScan (Echosens, Paris, France), a technique for measuring liver stiffness in staging fibrosis of the liver. [c]Kruskal-Wallis 1-way ANOVA. [d]METAVIR score, quantifies the degree of inflammation and fibrosis in a liver biopsy. [e]χ^2 test.

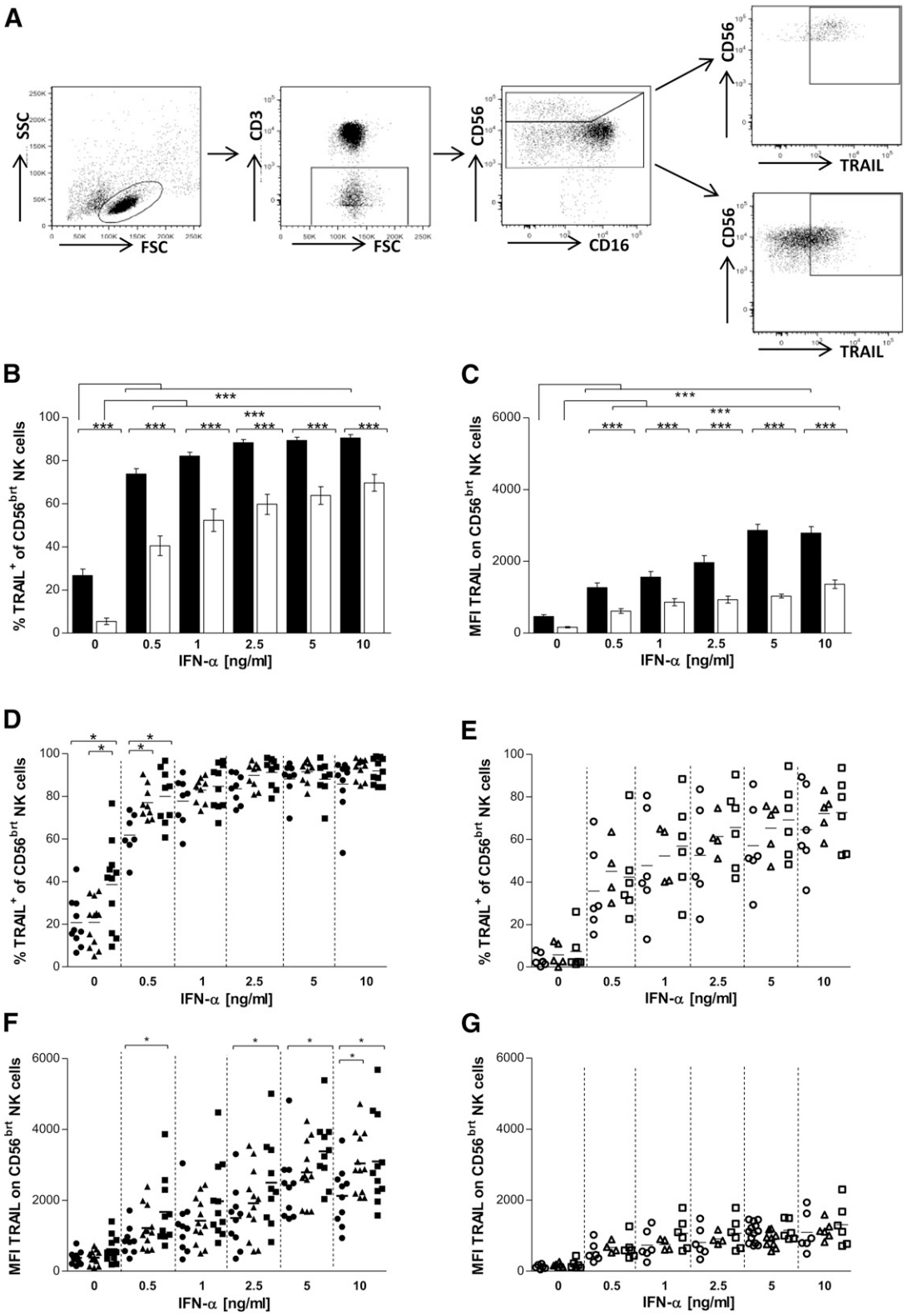

Figure 1. TRAIL expression on CD56^{bright} NK cells in response to IFN-α stimulation. PBMCs from patients with cHC and healthy controls were stimulated in vitro with the indicated different doses of IFN-α for 6 h or were left untreated. (A) Representative flow cytometry plots showing gating strategy for TRAIL$^+$ NK cells. (B) Percentage and (C) MFI of TRAIL expression on CD56^{bright} NK cells of patients with cHC (n = 33,■) compared with healthy controls (n = 18, □) (mean ± SEM). (D–G) Percentages of TRAIL$^+$CD56^{bright} NK cells and MFI of TRAIL expression on CD56^{bright} NK cells in relation to *IFNL3*-CC (circles), -CT (triangles), and -TT (squares) genotypes among patients with cHC (filled symbols) (D, F, respectively) and healthy controls (open symbols) (E, G respectively). Horizontal lines: means. *$P < 0.05$; ***$P < 0.001$.

Germany). Primers and hybridization probes were purchased from TIB MOLBIOL (Berlin, Germany).

Statistical Analyses

Data are presented as means ± SEM. The Shapiro-Wilk test was used to test the normal distribution of quantitative variables. Qualitative variables were summarized as counts and percentages, and patient groups were compared by the χ^2 or Fisher's exact test, as appropriate. Comparisons between groups were evaluated by parametric (Student's *t* test with the Welsh correction if needed and 1-way ANOVA with the Bonferroni *post hoc* test) or nonparametric (Mann-Whitney *U* test and Kruskal-Wallis test) tests, depending on the data distribution. Spearman's or Pearson's correlation coefficients were used to test correlations between two variables. A 2-sided $P < 0.05$ was considered to be statistically significant. Prism 5.0 and 6.0 (GraphPad , La Jolla, CA) were used for all computations.

RESULTS

IFN-α-induced TRAIL expression on NK cells was partially dependent on the *IFNL3* genotype in patients with cHC

To investigate the role of the *IFNL3* genotype in type I IFN-dependent regulation of NK cell functions in patients with cHC, we studied the percentage of TRAIL$^+$ CD56^{bright} NK cells after stimulation with different doses of IFN-α in vitro (**Fig. 1**). A marked polarization of NK cells toward a cytotoxic phenotype was found in patients with cHC, indicated by a significant up-regulation of TRAIL on both CD56^{bright} and CD56^{dim} NK cells in comparison to that in healthy controls, regardless of *IFNL3* genotype (Fig. 1B and C and Supplemental Fig. 1). These significant differences were detected ex vivo and after stimulation with IFN-α, even in a low concentration (0.5 ng/ml; $P < 0.001$). A significantly higher percentage of TRAIL$^+$ CD56^{bright} NK cells was observed in patients with the *IFNL3*-TT genotype who had cHC in comparison to those with the *IFNL3*-CC genotype, with or without 0.5 ng/ml IFN-α stimulation (Fig. 1D). Similarly, the differences were obvious, not only in the frequency of positive cells but also in the MFI of TRAIL expression with a wide range of IFN-α doses (Fig. 1F). In contrast, in healthy individuals with any the 3 *IFNL3* genotypes, no significant differences were found in the percentage or MFI of TRAIL expression on CD56^{bright} NK cells in response to IFN-α stimulation (Fig. 1E and G).

To summarize, the IFN-α-induced TRAIL expression differed between the *IFNL3* genotypes, and NK cells from patients with cHC were polarized toward a TRAIL-expressing phenotype.

Opposing effects of *IFNL3* genotype on IFN-γ expression by NK cells after IFN-α stimulation in patients with cHC and healthy control subjects

We next sought to investigate whether the frequency of cytokine-producing NK cells before and after IFN-α stimulation was also related to the *IFNL3* genotype. Therefore, NK cells, before coculture with Huh-7.5 target cells, were stimulated with 10 or 100 ng/ml IFN-α or left untreated in AB medium (**Fig. 2**). Significantly increased frequencies of IFN-γ$^+$ and CD107a$^+$ CD56^{bright} and CD56^{dim} NK cells were detected after stimulation with IFN-α compared with unstimulated levels in both the cHC and healthy control groups (Figs. 2A and B, **3A–D**). The same was true of TNF-α$^+$ CD56^{dim}NK cells (Supplemental Fig. 2B). However, there were no significant differences in cytokine production, displayed as the percentage of IFN-γ$^+$ CD56^{bright} and CD56^{dim} NK cells, as well as TNF-α$^+$ CD56^{dim} NK cells, between patients with cHC and healthy controls (Fig. 2A and B and Supplemental Fig. 2B). CD107a expression (MFI) on CD56^{bright} and CD56^{dim} NK cells was significantly increased in patients with cHC in comparison to healthy subjects with or without IFN-α stimulation (Fig. 3B and D).

Analysis of cytokine production by NK cells in regard to the *IFNL3* genotype revealed distinct expression patterns in patients with cHC and healthy control subjects (Fig. 2C–F; Supplemental Fig. 2C and D). Patients with cHC who have the genotype *IFNL3*-TT showed significantly higher percentages of IFN-γ$^+$ and CD107a$^+$ CD56^{bright} and CD56^{dim} NK cells and of TNF-α$^+$ CD56^{dim} cells, compared with those bearing the *IFNL3*-CC genotype (Figs. 2C and E; 3E and G; Supplemental Fig. 2C). In contrast, healthy subjects of the *IFNL3*-TT genotype had a lower IFN-γ response than subjects bearing the *IFNL3*-CC genotype (Fig. 2D), which was not the case for TNF-α production (Supplemental Fig. 2D).

Moreover, cytokine production and degranulation (CD107a) of NK cells after IFN-α stimulation were more prominent in healthy individuals of the *IFNL3*-CC genotype compared to patients with cHC who are of the same genotype ($P < 0.05$) (Figs. 2C–F, 3E–H and Supplemental Fig. 2C and D).

Overall, these data indicate that the cytokine response and degranulation of NK cells after IFN-α stimulation in healthy individuals and patients with cHC differ according to *IFNL3* genotype.

NK cells from *IFNL3*–TT-bearing patients with cHC were more frequently multifunctional after IFN-α exposure

Multiparameter analysis of NK cell effector functions revealed that patients with cHC with the *IFNL3*-TT genotype had significantly higher percentages of multifunctional (IFN-γ$^+$ TNF-α$^+$ CD107a$^+$) NK cells and of NK cells expressing 2 or 1 function markers followed by IFN-α stimulation in comparison to patients with the *IFNL3*-CC genotype (**Fig. 4A** and **B**). Of note, healthy individuals with the *IFNL3*-CC genotype had higher percentages of NK cells expressing 2 parameters simultaneously or 1, in comparison to the cHC *IFNL3*-CC group after stimulation with 10 or 100 ng/ml IFN-α (Fig. 4A).

IFN-α-mediated STAT-1 and -4 expression

To evaluate potential mechanisms underlying the differential effects of IFN-α on NK cells depending on *IFNL3* polymorphism, we determined levels of STAT-1, pSTAT-1, and pSTAT-4. As shown before [41], a significantly higher expression of STAT-1 was found in patients with cHC than in healthy controls (**Fig. 5A;** $P < 0.001$). As expected based on that study, STAT-1 expression did not change with short-term in vitro IFN-α treatment. Patients with cHC but not healthy controls bearing the *IFNL3*-CC genotype showed considerably higher STAT-1 levels than did

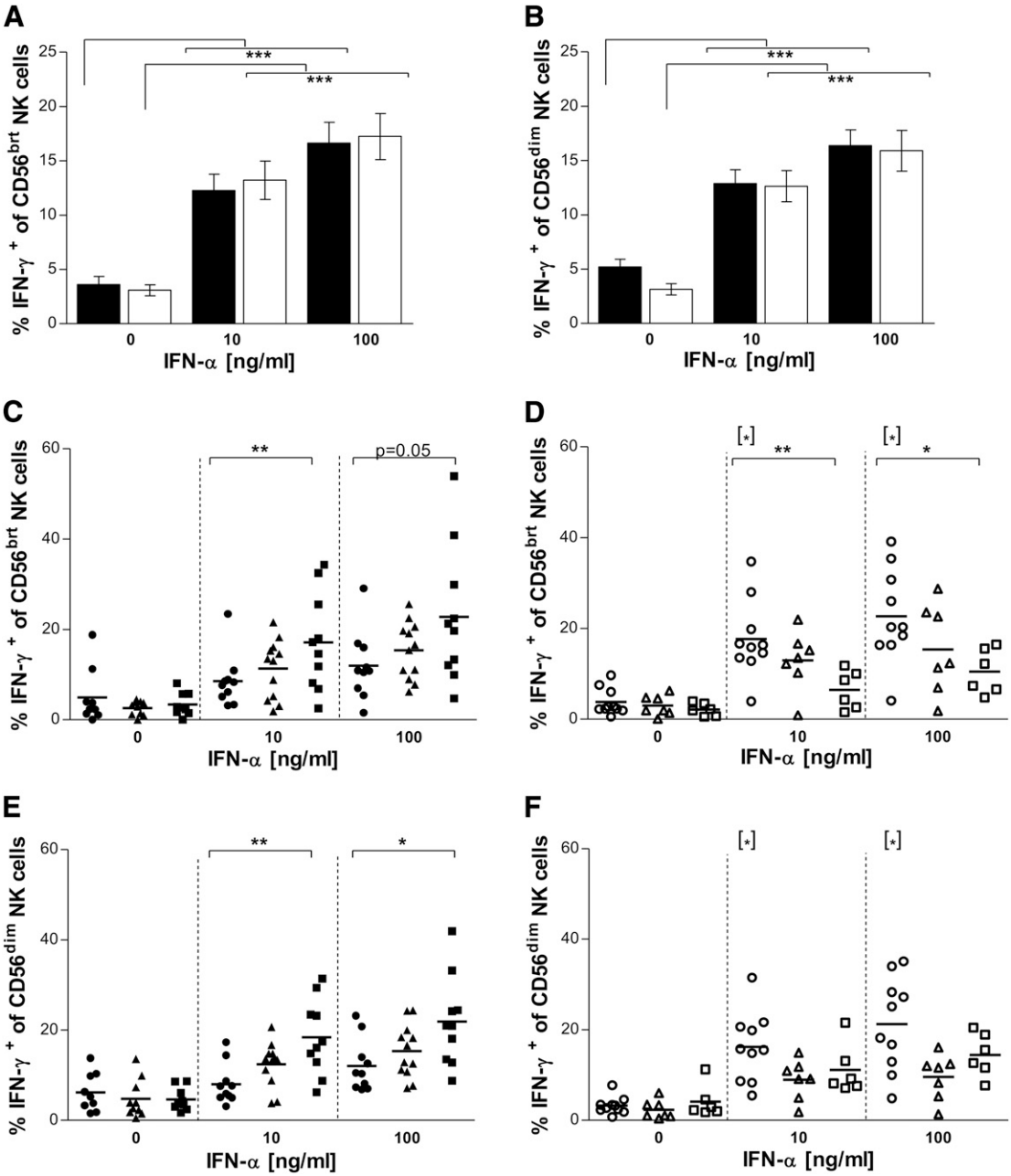

Figure 2. Dose-dependent changes in the functionality of IFN-γ$^+$CD56^{bright} and IFN-γ$^+$CD56^{dim} NK cells in response to IFN-α stimulation. PBMCs were stimulated with 10 or 100 ng/ml IFN-α or left untreated in medium overnight before coculture with Huh-7.5 target cells. Frequency of (A) IFN-γ$^+$CD56^{bright} and (B) IFN-γ$^+$CD56^{dim} NK cells of patients with cHC ($n = 33$, ■) and healthy controls ($n = 23$, □) (mean ± SEM). (C–F) Percentage of IFN-γ$^+$CD56^{bright} and CD56^{dim} NK cells in relation to *IFNL3*-CC (circles), -CT (triangles), and -TT (squares) genotypes among (C, E) patients with untreated cHC (filled symbols) and (D, F) healthy controls (open symbols). Horizontal lines: means. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; [*]$P < 0.05$, cHC vs. healthy controls with the same *IFNL3* genotype and stimulated with the same dose of IFN-α.

IFNL3-TT individuals, irrespective of IFN-α stimulation (Fig. 5B and Supplemental Fig. 3A). STAT-1 phosphorylation did not differ between patients with cHC and healthy subjects in unstimulated cells, and the phosphorylation increased to a similar extent in both groups after IFN-α stimulation (Fig. 5C). *IFNL3*-TT-bearing patients with cHC had a more pronounced relative increase of STAT-1 phosphorylation in response to lower doses of IFN-α than did the *IFNL3*-CC-bearing patients with cHC, which is in line with the observed higher TRAIL and CD107a expression in this group (Fig. 5D). Such differences between genotypes were not observed in the healthy control group (Supplemental Fig. 3B).

pSTAT-4 expression also increased after IFN-α stimulation, in patients with cHC and healthy controls (Fig. 5E). Of note, unstimulated cells showed a significantly higher pSTAT-4 expression in patients with cHC that was maintained after IFN-α

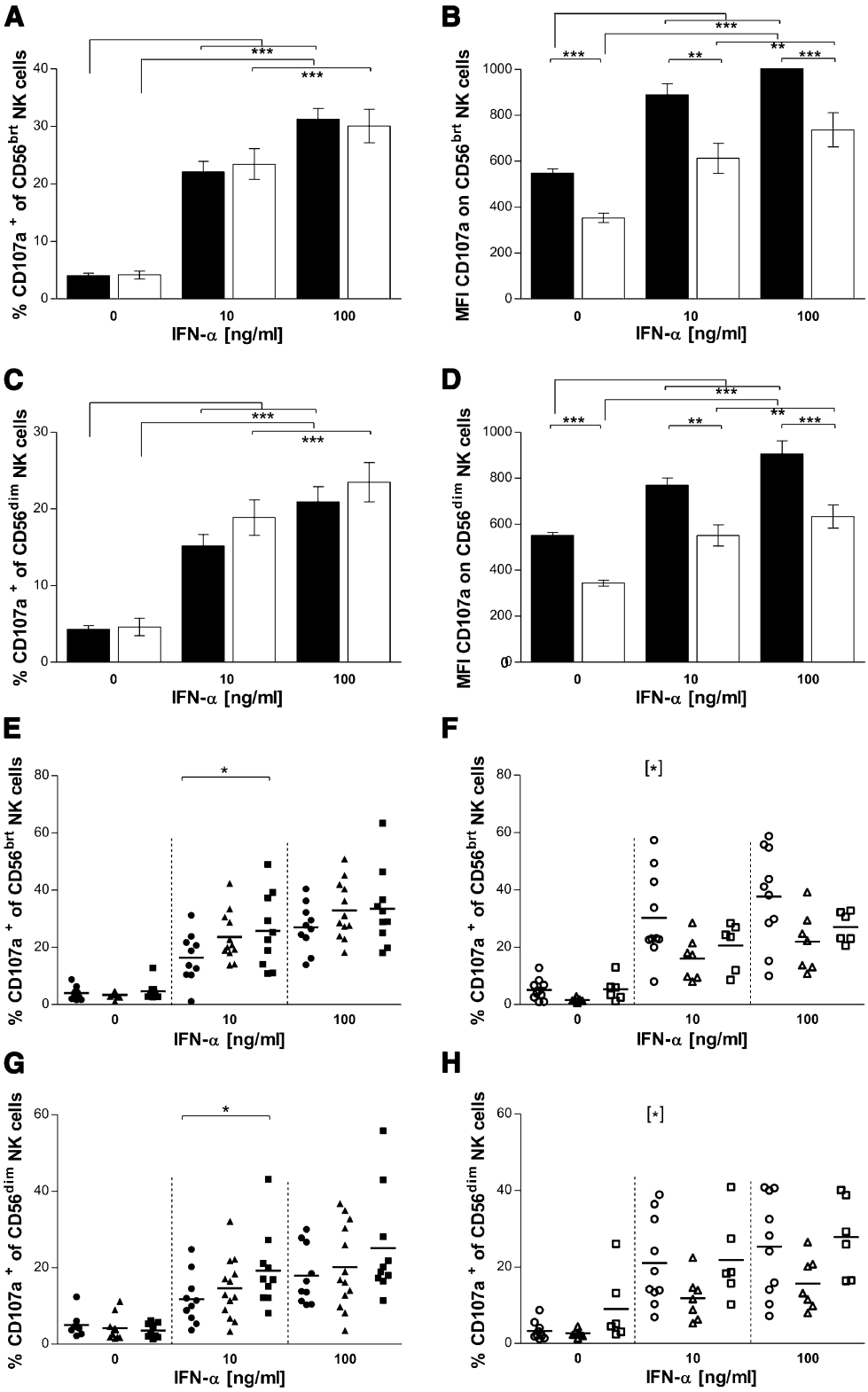

Figure 3. Dose-dependent changes in the frequency of CD107a$^+$CD56^{bright} and CD107a$^+$CD56^{dim} NK cells after IFN-α stimulation. PBMCs were stimulated with 10 or 100 ng/ml IFN-α or left untreated overnight before coculture with Huh-7.5 cells. Percentage of (A) CD107a$^+$CD56^{bright} and (C) CD107a$^+$CD56^{dim} NK cells and the MFI of CD107a expression on (B) CD56^{bright} and (D) CD56^{dim} NK cells of patients with cHC ($n = 33$, ■) compared to healthy controls ($n = 23$, □) (mean ± SEM). Frequency of (E, F) CD107a$^+$ CD56^{bright} and (G, H) CD107a$^+$ CD56^{dim} NK cells in relation to *IFNL3*-CC (circles), -CT (triangles), and -TT (squares) genotypes among (E, G) untreated patients with cHC (filled symbols) and (F, H) healthy controls (open symbols). Horizontal lines: means. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. [*]$P < 0.05$, cHC vs. healthy individuals with the same *IFNL3* genotype and stimulated with the same dose of IFN-α.

stimulation, even though the relative increase in pSTAT-4 was higher in the healthy individuals. Again, *IFNL3*-TT-bearing patients with cHC showed not only a stronger relative increase in pSTAT-1 expression after IFN-α stimulation, but also a significantly higher relative increase in pSTAT-4 than did *IFNL3*-CC-bearing patients with cHC (Fig. 5F). No differences between genotypes were observed in healthy controls (Supplemental Fig. 3C).

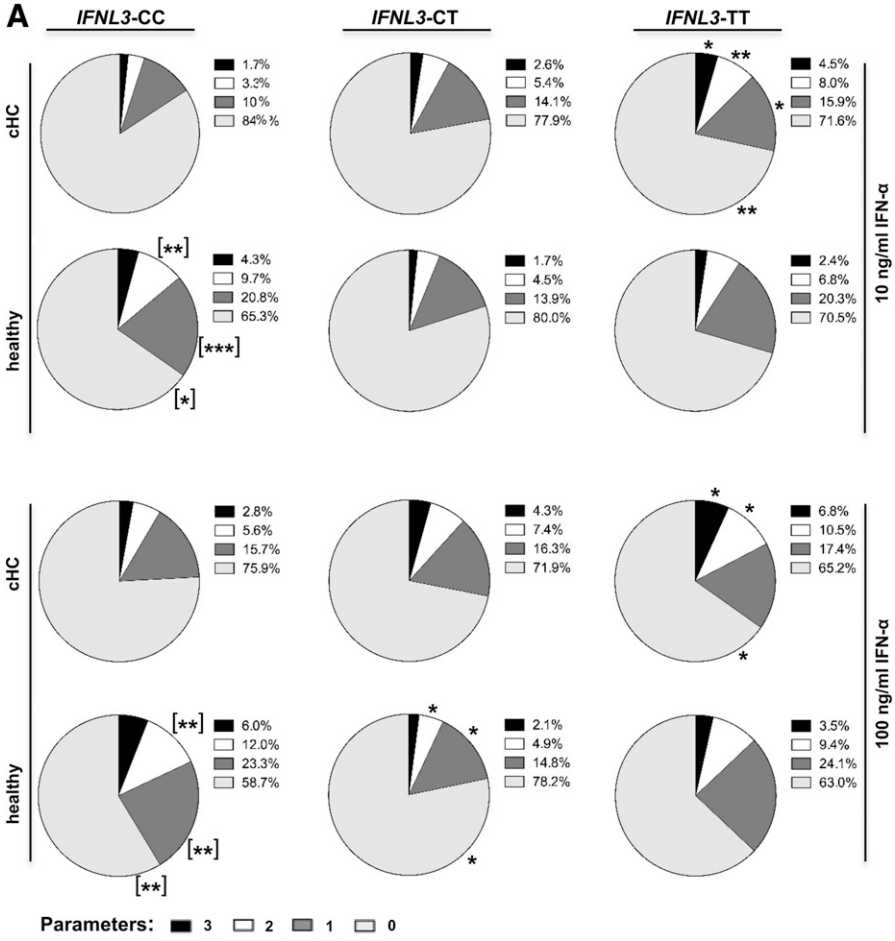

Figure 4. Multiparameter analysis of NK cell effector functions. PBMCs were stimulated with 10 and 100 ng/ml IFN-α overnight. (A) Percentage of NK cells expressing all 3 of these effector function markers (IFN-γ$^+$, TNF-α$^+$, and CD107a$^+$ NK cells), 2 of them simultaneously, or 1 or none. (B) The percentage of NK cells expressing IFN-γ, TNF-α, and CD107a simultaneously in patients with cHC and healthy controls (mean ± SEM), according to *IFNL3* genotype. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, *IFNL3*-CC vs. other genotypes within the cHC and healthy groups stimulated with the same IFN-α dose. [**], [***], patients with cHC vs. healthy individuals with the same *IFNL3* genotype receiving the same dose of IFN-α.

Overall, pSTAT-1 expression intensity in *IFNL3*-TT-bearing patients was higher than that of pSTAT-4 (Fig. 5G), which supports the observed polarization toward cytotoxicity in these patients. In contrast, patients with cHC who have the *IFNL3*-CC genotype showed a higher pSTAT-4 vs. pSTAT-1 expression in NK cells, which shifted toward higher pSTAT-1 after IFN-α stimulation. The differences in functional responses largely confirm effector functions of NK cells in the different *IFNL3* genotypes shown in Figs. 1–4.

Effector functions of NK cells were associated with liver disease activity in *IFNL3*-TT-bearing patients who have cHC

We finally questioned whether NK cell effector functions in patients with cHC correlate with liver disease activity. Indeed, the frequency of IFN-α-induced TRAIL$^+$CD56^{bright} NK cells was associated with ALT levels in patients with cHC (**Fig. 6A**). This investigation was mainly driven by a significant correlation between TRAIL expression and ALT levels in genotype

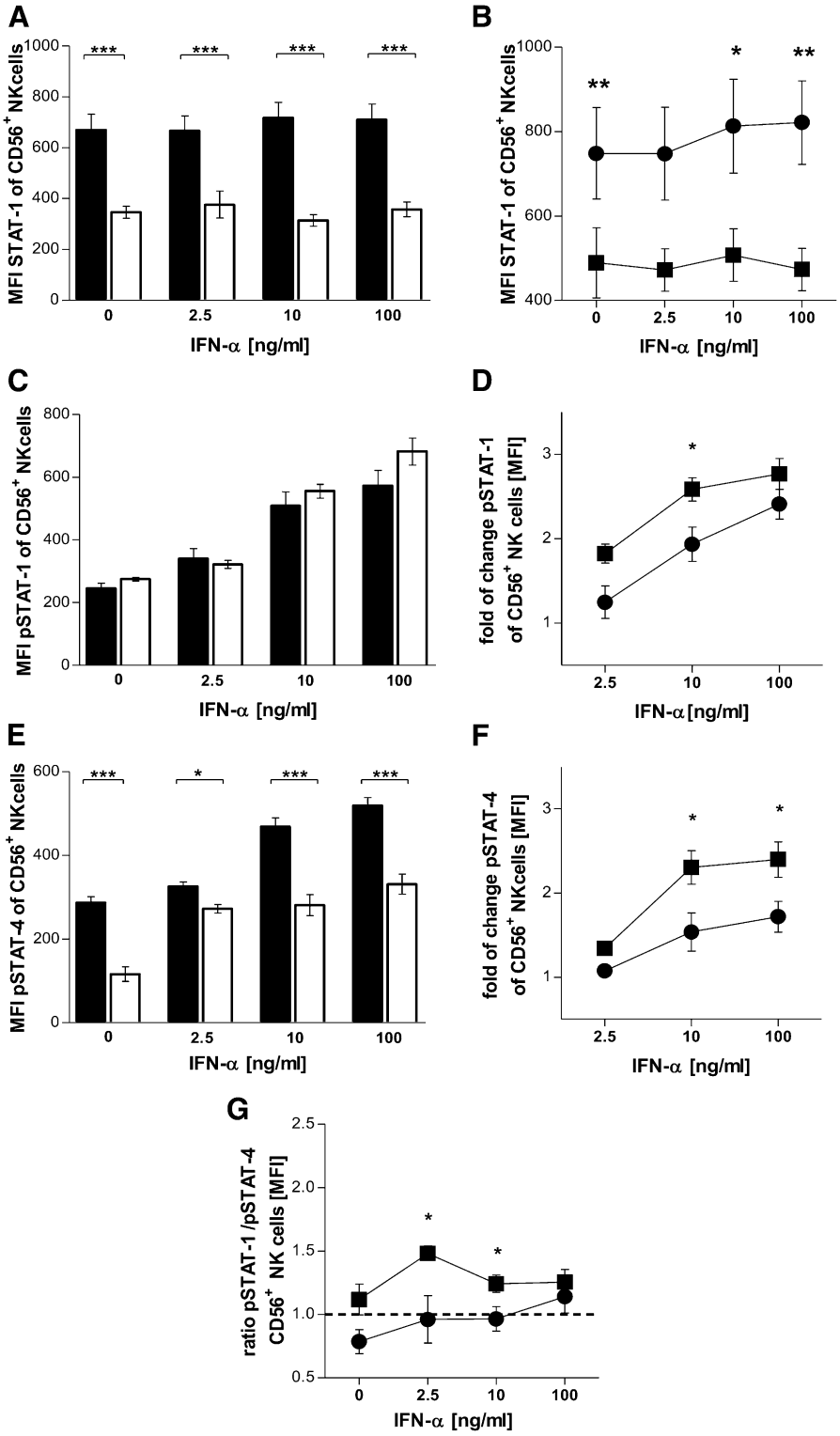

Figure 5. IFN-α mediated the modulation of STAT-1, pSTAT-1, and pSTAT-4 expression in NK cells of patients with cHC and healthy controls. MFI of (A) STAT-1, (C) pSTAT-1, (E) pSTAT-4 expression in NK cells from patients with cHC (■) and uninfected controls (□), with or without IFN-α stimulation (mean ± SEM). MFI of (B) STAT-1 and relative fold changes in (D) pSTAT-1 and (F) pSTAT-4 expression in relation to *IFNL3*-CC (●) and -TT (■) among patients with cHC, with or without IFN-α stimulation (a representative experiment is shown, $n = 5$–6 individuals per genotype). (G) pSTAT-1:pSTAT-4 ratio in relation to *IFNL3*-CC (●) and –TT (■) genotypes among patients with cHC receiving IFN-α treatment. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, *IFNL3*-CC vs. -TT genotypes stimulated with the same IFN-α dose within the cHC group.

IFNL3-TT-bearing patients with cHC, because neither the CC nor CT genotype was associated with ALT (Fig. 6A and data not shown). Likewise, the percentage of IFN-γ^+ NK cells after stimulation with 10 ng/ml IFN-α correlated with ALT levels only in patients with cHC who had the *IFNL3*-TT genotype but not in those with *IFNL3*-CC (Fig. 6B). Percentages of TNF-α^+ or CD107$^+$ CD56^{dim} NK cells did not show any association with ALT levels in patients with cHC (data not shown). In addition, there was no association between NK cell effector functions and viral load (data not shown), and

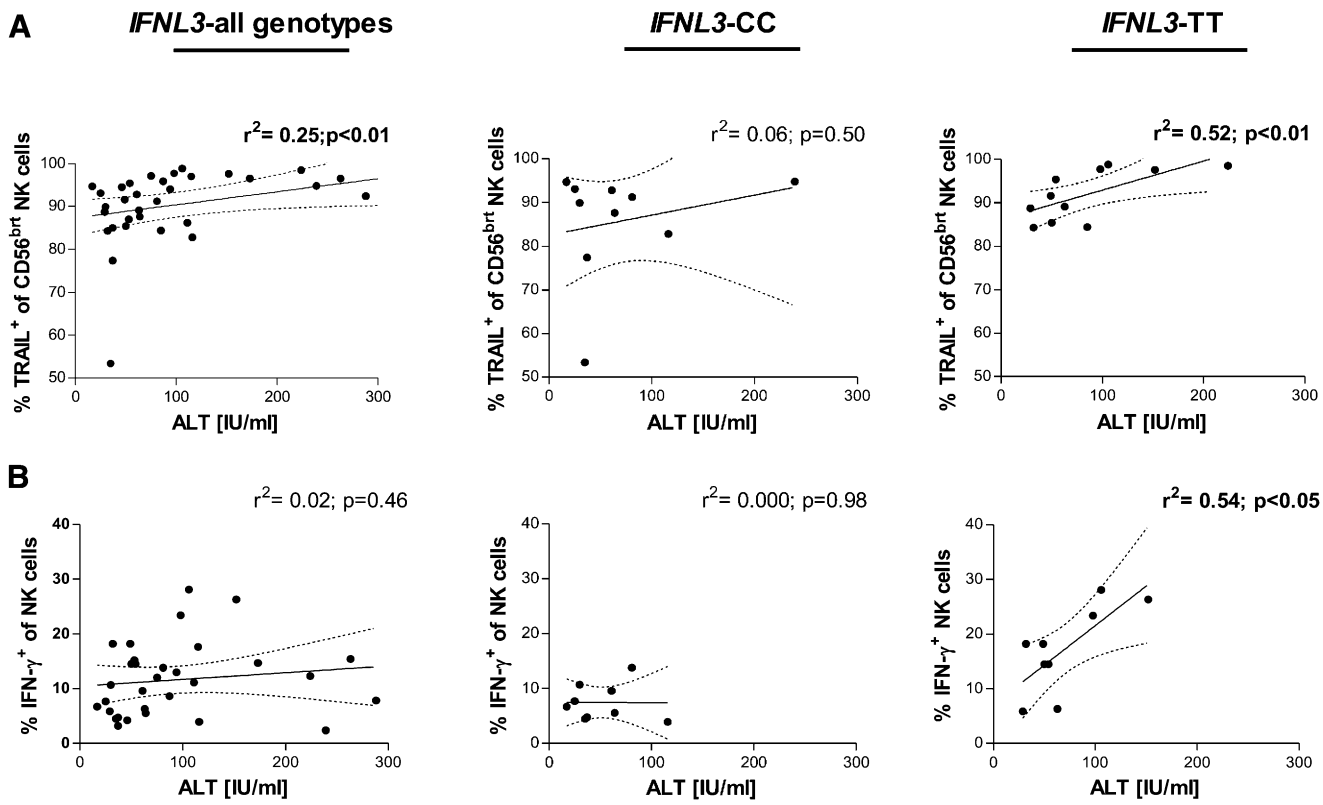

Figure 6. Associations between the clinical marker of liver cell inflammation and the frequency of functional NK cells after IFN-α stimulation in patients with cHC. Associations between ALT and (A) $TRAIL^+CD56^{bright}$ NK cells and (B) IFN-γ^+ NK cells after stimulation with 10 ng/ml IFN-α.

there was no obvious association between NK cell activity and gender or age (data not shown).

Thus, these data indicate that NK cell effector functions may correlate with biochemical disease activity in patients with cHC, particularly in those with the *IFNL3*-TT genotype.

DISCUSSION

The role of NK cells in response to type I IFN-based antiviral therapy in HCV infection and their role in liver pathology is not well defined. We found that 1) NK cells from patients with cHC differed in response to IFN-α stimulation from those of healthy controls with enhanced cytotoxic functions and 2) that the *IFNL3* genotype may have influenced polarization of NK cells toward a cytotoxic phenotype. Furthermore, increased NK cell activity may contribute to liver disease, in particular in patients with the *IFNL3*-TT genotype who have cHC.

It is well established that IFN-α stimulation leads to an up-regulation of TRAIL on NK cells. We recently suggested that this TRAIL up-regulation correlates inversely with the decline in HCV RNA early in antiviral therapy [36]. In the present study, NK cells from patients with cHC were very susceptible to IFN-α stimulation, which is well in line with the study of Ahlenstiel *et al.* [23], who reported a strong increase of TRAIL on both $CD56^{bright}$ and $CD56^{dim}$ cells during IFN-α therapy. In addition, we now provide evidence that this responsiveness of NK cells to IFN-α may differ in patients according to *IFNL3* genotype.

However, the association with the *IFNL3* genotype may be restricted to lower in vitro concentrations of IFN-α, as over-stimulation leads to a "saturation" of TRAIL expression when differences between *IFNL3* genotypes are no longer obvious. In contrast to patients with cHC, healthy controls showed no *IFNL3* genotype-associated differences in TRAIL up-regulation. Patients with the *IFNL3*-TT genotype who had cHC showed not only more TRAIL expression but also a higher frequency of cytokine-producing and multifunctional NK cells than did *IFNL3*-CC-bearing individuals with cHC, suggesting a general role of *IFNL3* genotype in NK cell function. Overall, the observations made in this study are well in line with a recent report demonstrating an association of NK cell activity with race; African-American individuals are more likely to carry the *IFNL3*-TT genotype [38].

On first view, the increased IFN-α responsiveness of *IFNL3*-TT NK cells in patients with cHC may contradict our previous report indicating an association between the second-phase decline in HCV RNA during IFN-α therapy and TRAIL expression on NK cells [36]. However, the *IFNL3* genotype is mainly associated with the first- but not the second-phase decline in HCV RNA during IFN-α treatment of cHC [42]. Thus, taken together, the findings in our study suggest a dual role of IFN-α-activated NK cells in cHC: on the one hand, a potential contribution to the second phase of virological response and, on the other, an involvement in the pathogenesis of chronic liver disease.

Antiviral mechanisms related to type I IFN are based on STATs, which regulate expression of the host genes that play a key role in the defense against pathogens. In line with a previous report

[43], our data suggest that type I IFN-mediated enhancement of NK cell function in patients with cHC is related to STAT-1 and -4 expression, similar to our recent data on hepatitis D virus infection [40]. An increased induction of STAT-1 phosphorylation (pSTAT-1) in NK cells during IFN-α stimulation, with preferential STAT-1 over STAT-4 phosphorylation, was observed in particular in patients who had cHC and had the *IFNL3*-TT genotype. Higher STAT-1 levels in cHC than in uninfected controls were also reported by Miyagi *et al.* [44], and IFN-dependent induction of pSTAT-1 correlated with increased NK cell cytotoxicity (as investigated by TRAIL and CD107a expression) and decreased IFN-γ production in another study [41]. In our study, in patients with the *IFNL3*-TT genotype, low doses of IFN-α stimulation were sufficient to induce pSTAT-1, which may explain the strong up-regulation of TRAIL and CD107a in this subgroup. Overall, the findings suggest that *IFNL3*-TT-bearing patients with cHC may be more susceptible to up-regulation of TRAIL expression on NK cells. Moreover, IFN-α stimulation led to a shift in the pSTAT-1:pSTAT-4 ratio, which differed between patients with the *IFNL3*-TT or -CC genotype (Fig. 5G). However, the differences were overcome by higher doses of IFN-α stimulation, supporting the observed functional changes after IFN-α exposure shown in Figs. 1–4.

The effects of IFN-α stimulation on cytokine production by NK cells in relation to the *IFNL3* genotype were unexpectedly the opposite between patients with cHC and healthy controls. Even though the detailed underlying mechanism is unclear, this observation suggests that NK cells from HCV-naïve, *IFNL3*-CC-bearing, healthy individuals would produce higher amounts of IFN-γ and degranulate upon first contact with HCV. These IFN-γ-producing NK cells then might contribute to clearance of HCV and thus explain in part why *IFNL3*-CC-bearing individuals have a higher likelihood of spontaneous resolution of acute HCV infections, as reported in previous studies [11, 12, 14, 15]. Indeed, NK cells of *IFNL3*-CC-bearing healthy subjects stimulated with IFN-α induced a higher reduction of HCV replication in vitro, when cocultured with Huh-7.5 cells transfected with JFH1 NS3-5B HCV, as compared with those with *IFNL3*-TT (data not shown).

A study performed in healthcare workers who were exposed to small amounts of HCV but remained aviremic revealed early NK cell responses with increased activatory receptors, IFN-γ production, and cytotoxicity reflected by TRAIL and CD107a expression [26]. These findings suggest that first exposure to HCV activates a strong innate response that may contribute to the control of viremia. Indeed, the role of NK cells in clearance of acute hepatitis C has recently been well established in HIV-coinfected patients [45].

An important finding was that the observed association between *IFNL3* genotype and NK cell activity may not reflect a direct differential response of NK cells to IFN-λ. We [unpublished data] and others [38] have not been able to find a consistent effect of IFN-λ on NK cells in vitro, even though one study reported robust expression of IFNLR1 mRNA in NK cells [46] and another suggested a moderate expression of IFNL3 receptor (IFNLR1 and IL10R) on NK cells [47]. Preliminary flow cytometry findings by our group showed IFNLR1 expression on less than 5% of NK cells of patients with viral hepatitis. Thus, the mechanism behind the observed associations between *IFNL3* genotypes and NK-cell function could be either indirect through other cells or by genetic linkage association between the *IFNL3* polymorphism and other molecules involved in the intracellular IFN response pathway. A dinucleotide variant rs368234815 on chromosome 19 (ss469415590, ΔG/TT) was discovered recently that is in high linkage disequilibrium with rs12979860, in particular in Caucasians. rs368234815 [ΔG], is a frameshift variant that creates a novel gene, encoding the IFNL4 protein, which is active in the *IFNL3*-TT genotype and induces STAT1 and -2 phosphorylation and the expression of IFN-stimulated genes in HepG2 cells [48]. The *IFNL4* rs368234815 (ΔG/TT) variant presents an apparent paradox: IFNL4 exhibits clear antiviral activity in vitro, yet its potential for expression correlates with poor outcome of HCV infection. IFNL4 is not efficiently secreted in vitro from primary human hepatocytes and is thought to act in an autocrine manner in the liver [49]. Of note, a recent study analyzing degranulation activity of intrahepatic NK cells showed slightly increased CD107a expression in patients with the *IFNL4*-TT/TT genotype compared with that in patients with the *IFNL4*-ΔG/TT genotype; however, with largely overlapping confidence intervals. Future studies are needed to investigate explanations of the difference in our findings [50].

In summary, in our study, NK cell function was reshaped toward a cytotoxic phenotype in patients with cHC. IFN-α treatment significantly increased the activity of NK cells, in particular in *IFNL3*-TT-bearing patients with cHC, resulting in enhanced cytotoxicity and cytokine production, which could contribute to the severity of liver disease. Moreover, higher IFN-γ production of NK cells in healthy individuals with the *IFNL3*-CC genotype may reflect better early effector functions, supporting spontaneous HCV clearance in acute hepatitis C. Overall, these findings highlight that the role of NK cells may differ in chronic infection vs. early antiviral defense and that the *IFNL3* genotype differentially influences NK cell function.

AUTHORSHIP

M.R.-T. was responsible for the study design, for data acquisition, analysis, and interpretation, and for coauthoring the manuscript; A.T. for data acquisition and critical revision of the draft; A.A.M., A.T., S.L., V.S., R.F., M.P.M., M.C., and A.R.M.K. for administrative, technical, clinical, and material support; A.R.M.K, and V.S. for study review and analysis; H.W. for study design, supervision, and interpretation and for coauthoring the manuscript; and all authors for review and editing of the manuscript.

ACKNOWLEDGMENTS

This work was supported by Federal Ministry of Education and Research (BMBF) Grant 01KI0788 "Host and viral determinants for susceptibility and resistance to hepatitis C virus infection" (to H.W. and M.C.). M.R.-T. was partially supported by the European Social Fund and the national budget under the submeasure 4.1.1 Human Capital Operational Programme, "Higher quality of education is the key to the development of the Medical University of Bialystok, Poland." The authors express their thanks to PD

Dr. Sandra Ciesek and Dr. Sandra Westhaus for introduction to luciferase assay, to all study nurses in the Department of Gastroenterology, Hepatology, and Endocrinology of Hannover Medical School for support in collecting patient samples, and to the patients and blood donors who contributed clinical samples to the study.

DISCLOSURES

The authors declare no competing financial interests.

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KEY WORDS:
TRAIL · cytotoxicity · IFN-α · IFN-γ · STATs · HCV