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Expansion of Functionally Skewed CD56-Negative NK Cells in Chronic Hepatitis C Virus Infection: Correlation with Outcome of Pegylated IFN-α and Ribavirin Treatment

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NK cells are important innate immune effector cells, normally characterized as CD56+CD3− lymphocytes. In this study, we report that CD56−CD16+ NK cells expand in many patients with chronic hepatitis C virus infection. These CD56− NK cells were functionally impaired with respect to cytokine production upon target cell recognition, in comparison to CD56dim and CD56bright NK cell subsets. In particular, CD56− NK cells were strikingly defective in their polyfunctional response as measured by the coexpression of MIP-1β, IFN-γ, TNF-α, and CD107a degranulation. The ability of these cells to mediate three or four of these functions was poor; expression of MIP-1β alone dominated their response. CD56− NK cells retained expression of receptors such as the natural cytotoxicity receptors and NKG2D, whereas the expression of CD57 and perforin was lower when compared with CD56dim NK cells. Interestingly, pretreatment levels of CD56− NK cells correlated with the outcome of pegylated IFN-α and ribavirin treatment. In patients with CD56− NK cells in the range of healthy subjects, 80% reached a sustained virological response to treatment, whereas only 25% of patients with levels clearly above those in healthy subjects experienced a sustained virological response. Thus, chronic hepatitis C virus infection is associated with an expansion of CD56− NK cells functionally skewed toward MIP-1β production only. Furthermore, high levels of these cells reveal a disturbed in innate cellular immunity that is associated with an impaired ability to respond to antiviral treatment with IFN-α and ribavirin.


NK cells are abundant in the liver and play a significant role in immunity and immunopathology of liver disease (12). A central role for these cells in hepatitis C virus (HCV) infection is supported by the observation that clearance of acute infection is influenced by KIR2DL3 and certain HLA-C alleles (13). Also, NK cells have the capacity to suppress full cycle HCV infection in vitro (14). Furthermore, HCV has been reported to induce changes in NK cell NCR expression and cytolytic function similar to the changes observed in HIV-1 infection (15–18).

HCV infection can be cleared with pegylated IFN-α (peg-IFN-α) and ribavirin treatment (19). However, this treatment is not successful in all patients (20). Viral factors associated with poor treatment outcome are infection with HCV genotype 1 (20), high viral load (20), and high viral sequence diversity (21). High age, male gender, obesity, poor uptake of ribavirin (22), and African American ethnicity are some of a number of host factors that can influence treatment outcome (20, 23). On the molecular level, low amounts of the liver-specific microRNA miR-122 was recently associated with poor virological response to HCV therapy (24). From an immunological viewpoint, it is interesting that pretreatment levels of plasma chemokines can correlate with treatment outcome (25–27). However, direct cellular immunological correlates of treatment outcome are less well described. In this study, in the course of investigating the NK cell compartment in patients with chronic HCV infection, we observed elevated levels of CD56− NK cells in many chronically HCV-infected subjects. This population exhibited limited polyfunctionality with reduced quadruple and triple functionality compared with CD56dim and CD56bright cells. CD56− NK cells retained expression of NKG2D and NCRs, but were mainly negative for CD57. Interestingly, high levels of CD56− NK cells correlated with peg-IFN-α and ribavirin treatment failure. The data are discussed in relation to the biology of CD56− NK
cells, as well as the prospects of using these cells as a biomarker of peg-IFN-α and ribavirin treatment outcome.

Materials and Methods

Patients and samples

Forty-two patients chronically infected with HCV were studied, 10 of which were coinfected with HIV-1 (Table I). The peg-IFN-α-2a (Pegasys; Roche) was given weekly to all patients by s.c. injections at a dose of 180 μg and ribavirin (Copegus; Roche) at a dose of 0.8–1.2 g daily. Patients were treated for 48 wk (genotype 1) or 24 wk (genotype 2 or 3) and followed at the Infectious Diseases Clinic at Karolinska University Hospital in Solna and Huddinge (Stockholm, Sweden). Patients negative for plasma HCV RNA at 24 wk after the end of treatment were considered to have a sustained virological response (SVR). The coinfected subjects were from the Viral Dynamics and Immunology in HCV/HIV-1 Coinfection study, and none of the patients had received prior HCV therapy. The 34 patients before treatment was initiated. PBMC were isolated from heparinized blood samples by Lymphoprep gradient centrifugation (Axis-Shield). Written informed consent was obtained, and the study protocols were approved by the local institutional review board and the Swedish National Ethics Committee. Peripheral blood samples were taken from patients before treatment was initiated. PBMC were isolated from heparinized blood samples by Lymphoprep gradient centrifugation (Axis-Shield). Plasma HCV RNA load was measured using the COBAS TaqMan HCV assay of PBMC from HIV-1-negative individuals. In this study, CD56<sup>+</sup> NK cells have previously been observed in low numbers in healthy control subjects and in high numbers during untreated viremic HIV-1 infection (7–9, 11). This NK cell subset has hitherto not been studied with regard to other viral infections in HIV-1-negative individuals. In this study, CD56<sup>+</sup> CD16<sup>+</sup> NK cells were identified in peripheral blood of HCV-monoinfected and HCV/HIV-1-coinfected subjects using multicolor flow cytometry (Fig. 1A). The gating strategy excluded CD19<sup>+</sup>B cells, CD14<sup>+</sup> monocytes, and CD3<sup>+</sup> T cells. In addition, CD56<sup>+</sup> CD16<sup>+</sup> NK cells were identified as CD4<sup>+</sup>, because CD4<sup>+</sup>CD16<sup>+</sup> cells contain a distinct subset of inflammatory dendritic cells (30). Phenotypic analysis revealed that CD56<sup>+</sup> NK cells had a similar expression of NKP46, CD161, CD81, and NKG2D in HCV-infected patients, the percentages of CD56<sup>+</sup> NK cells were elevated compared with levels in healthy blood donors (Fig. 2A). Mean percentages of CD56<sup>+</sup> NK cells were elevated on average 148% in HCV-monoinfected patients and 478% in HCV/HIV-1-coinfected subjects, with substantial intragroup variability. The levels of CD56<sup>+</sup> NK cells as a percentage of total NK cells were similarly elevated (Fig. 2B). Some of the healthy control subjects had levels of CD56<sup>+</sup> NK cells that permitted phenotypic analysis. CD56<sup>+</sup> NK cells in these healthy subjects did not differ significantly from those in HCV-infected subjects with regard to expression of CD57, CD161, CD94, NKG2C, CD81, NKG2D, NKP30, and NKP46 (data not shown). Because the viral genotype is clinically important, it was of interest to investigate possible differences in the CD56<sup>+</sup> NK cell subset between patients infected with the difficult-to-treat genotype 1 virus and those infected with genotype 2 or 3.

Table I. Characteristics of patient groups

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Healthy Controls</th>
<th>HCV Monoinfected</th>
<th>HCV/HIV-1 Coinfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>16 (47%)</td>
<td>15 (47%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>Male</td>
<td>18 (53%)</td>
<td>17 (53%)</td>
<td>7 (70%)</td>
</tr>
<tr>
<td>Age, median (range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>NA</td>
<td>7 (22%)</td>
<td>6 (60%)</td>
</tr>
<tr>
<td>2</td>
<td>NA</td>
<td>9 (28%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>3</td>
<td>NA</td>
<td>16 (50%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Serum HCV RNA median IU/ml (range)</td>
<td>NA</td>
<td>(0.018–3.6×10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>(0.33–9.4×10&lt;sup&gt;5&lt;/sup&gt;)</td>
</tr>
<tr>
<td>ALAT median IU/L (range)</td>
<td>NA</td>
<td>1.14 (0.35–8.09)</td>
<td>0.75 (0.31–3.22)</td>
</tr>
<tr>
<td>ASAT median IU/L (range)</td>
<td>NA</td>
<td>1.14 (0.41–9.49)</td>
<td>1.11 (0.39–2.5)</td>
</tr>
</tbody>
</table>

Statistical analysis

Data from patient groups were analyzed by one-way ANOVA, one-way ANOVA on ranks, or Mann-Whitney rank sum tests as appropriate. Categorical data were analyzed using Fisher’s exact test. All statistical analyses were performed using GraphPad Prism software.

Results

CD56<sup>+</sup> NK cells are expanded in chronic untreated HCV infection

CD56<sup>+</sup>CD16<sup>+</sup> NK cells have previously been observed in low numbers in healthy control subjects and in high numbers during untreated viremic HIV-1 infection (7–9, 11). This NK cell subset has hitherto not been studied with regard to other viral infections in HIV-1-negative individuals. In this study, CD56<sup>+</sup> CD16<sup>+</sup> NK cells were elevated in peripheral blood of HCV-monoinfected and HCV/HIV-1-coinfected subjects using multicolor flow cytometry (Fig. 1A). The gating strategy excluded CD19<sup>+</sup>B cells, CD14<sup>+</sup> monocytes, and CD3<sup>+</sup> T cells. In addition, CD56<sup>+</sup> CD16<sup>+</sup> NK cells were identified as CD4<sup>+</sup>, because CD4<sup>+</sup>CD16<sup>+</sup> cells contain a distinct subset of inflammatory dendritic cells (30). Phenotypic analysis revealed that CD56<sup>+</sup> NK cells had a similar expression of NKP46, CD161, CD81, and NKG2D in HCV-infected patients, the percentages of CD56<sup>+</sup> NK cells were elevated compared with levels in healthy blood donors (Fig. 2A). Mean percentages of CD56<sup>+</sup> NK cells were elevated on average 148% in HCV-monoinfected patients and 478% in HCV/HIV-1-coinfected subjects, with substantial intragroup variability. The levels of CD56<sup>+</sup> NK cells as a percentage of total NK cells were similarly elevated (Fig. 2B). Some of the healthy control subjects had levels of CD56<sup>+</sup> NK cells that permitted phenotypic analysis. CD56<sup>+</sup> NK cells in these healthy subjects did not differ significantly from those in HCV-infected subjects with regard to expression of CD57, CD161, CD94, NKG2C, CD81, NKG2D, NKP30, and NKP46 (data not shown). Because the viral genotype is clinically important, it was of interest to investigate possible differences in the CD56<sup>+</sup> NK cell subset between patients infected with the difficult-to-treat genotype 1 virus and those infected with genotype 2 or 3.
virus. Interestingly, there was no difference in CD56⁺ NK cell percentages between patients infected with HCV genotype 1 and genotype 2/3 (Fig. 2C). These data indicate that the NK cell compartment is affected by chronic HCV infection in a way leading to accumulation of CD56⁺CD16⁺ NK cells in peripheral blood.

The CD56⁺ NK cells that expand in chronic HCV infection are functionally impaired

We next evaluated the functional capacity of NK cells in blood samples from chronically HCV-infected patients. PBMC were cultured in medium supplemented with 1000 U/ml IL-2 for 10 h to support and maintain NK cells and next stimulated with K562 cells during 6 h. Responses in CD56⁺, CD56dim, and CD56bright subsets were assessed by intracellular cytokine flow cytometry (Fig. 3A). The overall levels of the inflammatory chemokine MIP-1α was comparable between the three subsets (Fig. 3B). However, expression of IFN-γ and TNF-α upon K562 target cell recognition was impaired in the CD56⁺ NK cells. Similarly, there was a tendency toward a lower ability to degranulate, as assessed by CD107a expression, in the CD56⁺ NK cells compared with the CD56dim and

FIGURE 1. Characteristics of CD56⁺ NK cells in peripheral blood of HCV-infected patients. A, PBMC from healthy adult blood donors and adult HCV-infected patients were stained with mAbs against CD14, CD19, CD3, CD4, CD56, and CD16. Data in plots are gated on lymphocytes, showing the gating strategy for CD56⁺ NK cells. Numbers in the figure indicate percentages of cells within the gates. B, Expression of CD57, CD161, CD94, NKG2C, CD81, NKG2D, NKp30, and NKp46 in NK cell subsets as determined by flow cytometry (n = 14). Box plots show median, 25th and 75th percentiles, and 10th and 90th percentiles. Statistical analysis: one-way ANOVA on ranks followed by Dunn’s multiple comparison test, where * indicates p < 0.05, ** indicates p < 0.01, and *** indicates p < 0.005.

FIGURE 2. Elevated levels of CD56⁺ NK cells in peripheral blood of HCV-infected patients. A, The scatter dot plot represents data from healthy blood donors (n = 34), HCV-monoinfected subjects (n = 32), and HCV/HIV-1-coinfected subjects (n = 10), showing the mean and SE for percentage of CD56⁺ NK cells out of lymphocytes. B, The same as in A but data are expressed as percentage out of total NK cells. C, Patients subdivided with regard to HCV genotype: genotype 1 (n = 13) and genotypes 2 and 3 (n = 29). Statistical analysis: one-way ANOVA on ranks followed by Dunn’s multiple comparison test, where ** indicates p < 0.01 and *** indicates p < 0.005. * indicates p < 0.05 using the Mann-Whitney rank sum test. n.s., Not significant.
CD56\textsuperscript{bright} NK cells. The NK cell response to combined IL-12 and IL-15 stimulation was low and focused on cytokine and chemokine production, with no significant differences between the subsets (data not shown). Perforin expression was significantly lower in the CD56\textsuperscript{−} NK cells compared with the CD56\textsuperscript{dim} NK cells, and this was true in both healthy and HCV-infected subjects (Fig. 3D). Function responses in CD56\textsuperscript{−} NK cells from healthy donors (n = 12) and HCV-infected subjects (n = 12) after K562 stimulation or IL-12 + IL-15 stimulation. Statistical analysis in B and C: one-way ANOVA on ranks followed by Dunn’s multiple comparison test, where * indicates p < 0.05, ** indicates p < 0.01 and *** indicates p < 0.001. In D, * indicates p < 0.05 and ** indicates p < 0.01 as determined using the Mann-Whitney rank sum test. Box plots show median, 25th and 75th percentiles, and 10th and 90th percentiles.

**FIGURE 3.** CD56\textsuperscript{−} NK cells display impaired function. A. PBMC from patients with chronic HCV infection were stimulated for 10 h with the classical NK cell target K562 cell line in the presence of brefeldin A and monensin, and production of MIP-1\textbeta, IFN-\gamma, TNF-\alpha, and CD107a was assessed by intracellular cytokine flow cytometry. B. Comparison of responses to K562 in CD56\textsuperscript{−}, CD56\textsuperscript{dim}, and CD56\textsuperscript{bright} NK subsets in HCV-infected subjects (n = 12). C. Perforin expression as assessed by intracellular staining in unstimulated CD56\textsuperscript{−} and CD56\textsuperscript{dim} NK cells from healthy donors (n = 7) and HCV-infected patients (n = 7). D. Functional responses in CD56\textsuperscript{−} NK cells from healthy donors (n = 12) and HCV-infected subjects (n = 12) after K562 stimulation or IL-12 + IL-15 stimulation. Statistical analysis in B and C: one-way ANOVA on ranks followed by Dunn’s multiple comparison test, where * indicates p < 0.05, ** indicates p < 0.01 and *** indicates p < 0.001. In D, * indicates p < 0.05 and ** indicates p < 0.01 as determined using the Mann-Whitney rank sum test. Box plots show median, 25th and 75th percentiles, and 10th and 90th percentiles.

**CD56\textsuperscript{−} NK cells display sharply reduced polyfunctionality**

In studies of human T cell immunity, it has been realized that T cells display significant functional heterogeneity (31) and that this can be important in the context of viral infection (32). This has been much less studied in NK cells. In this study, the composition of the NK cell response in HCV-infected patients was investigated by analysis of the coexpression pattern of MIP-1\textbeta, IFN-\gamma, TNF-\alpha, and CD107a as a measure of degranulation in response to K562 stimulation. The CD56\textsuperscript{−} NK cell subset displayed limited polyfunctionality compared with both CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cell subsets (Fig. 4A). This was most pronounced in the very limited ability to express all four functions (Fig. 4B). However, several of the triple and double combinations of functions were also reduced in the CD56\textsuperscript{−} NK cells. MIP-1\textbeta monofunctionality was the only functional profile which was significantly more common in the CD56\textsuperscript{−} NK cells. Thus, the CD56\textsuperscript{−} NK cell subset displays a restricted functional profile focused on MIP-1\textbeta expression.

**High pretreatment levels of CD56\textsuperscript{−} NK cells correlate with IFN-\alpha and ribavirin treatment failure**

We next investigated whether the marked intragroup variability in CD56\textsuperscript{−} NK cell levels among infected patients before initiation of peg-IFN-\alpha and ribavirin treatment was associated with the variable treatment outcome observed in HCV infection. Patients were subdivided according to treatment outcome into one group that obtained a SVR and a second group that were nonresponders or responders with relapse, here collectively referred to as having a non-SVR. Both groups displayed higher percentages of CD56\textsuperscript{−} NK cells than the uninfected controls (Fig. 5A). However, levels in the non-SVR group exceeded those in the SVR group, suggesting an association between a high pretreatment percentage of CD56\textsuperscript{−} NK cells and the probability of treatment failure. This pattern was also consistent in the 13 patients with HCV genotype 1 infection, where the percentages of CD56\textsuperscript{−} NK cells were 0.82 ± 0.29% and 2.27 ± 0.74% (mean ± SE) in the SVR and non-SVR groups, respectively (data not shown).

The cohort with HCV-infected subjects comprised both monoinfected and HCV/HIV-1-coinfected patients. We therefore subdivided the monoinfected and coinfected groups into subgroups with...
FIGURE 5. Elevated levels of CD56\textsuperscript{−} NK cells in peripheral blood of HCV-infected patients correlate with HCV treatment failure. A. Before treatment with peg-IFN-\alpha and ribavirin, levels of CD56\textsuperscript{−} NK cells were significantly increased in subjects not reaching a SVR (n = 14) and to a lesser extent also in subjects with a SVR (n = 28), in comparison to healthy controls (n = 34). Mean and SEs are shown, where * indicates p < 0.05 and ** indicates p < 0.001 as determined by one-way ANOVA on ranks. * p < 0.05 by means of a Mann-Whitney U test in a direct comparison between SVR and non-SVR groups. B. High percentages of CD56\textsuperscript{−} NK cells are associated with low SVR rates. The threshold for high levels of CD56\textsuperscript{−} NK cells (1.8%) was defined for the HCV-monoinfected group as the level where 95% of healthy donors fell below this threshold. This level coincided with three times the mean percentage of CD56\textsuperscript{−} NK cells. Elevated CD56\textsuperscript{−} NK cells show no strong association with known risk factors for poor treatment outcome.

To address whether high levels of CD56\textsuperscript{−} NK cells were associated with previously described factors influencing treatment outcome, we analyzed possible relationships between known risk factors and the levels of CD56\textsuperscript{−} NK cells in peripheral blood. We observed higher HCV loads in the HCV/HIV-1-coinfected subjects compared with monoinfected subjects, but there was no significant difference between the subgroups with high or low percentages of CD56\textsuperscript{−} NK cells (Fig. 6A). Neither did baseline levels of CD56\textsuperscript{−} NK cells directly correlate with viral load or alanine aminotransferase levels (data not shown). Although the patient cohort did not have an equal distribution of HCV genotypes, the data indicated that CD56\textsuperscript{−} NK cell levels above the threshold were not directly related to a specific viral genotype (Fig. 6B). Elderly and male individuals tended to have higher levels of CD56\textsuperscript{−} NK cells, but this difference did not reach statistical significance (Fig. 6C and D). Thus, none of the known risk factors addressed here seemed to be strongly linked to higher levels of CD56\textsuperscript{−} NK cells.

Discussion

HCV preferentially infects hepatocytes and NK cells are abundant in the liver. Considerable evidence also indicates that NK cells take part in the immune response against HCV, although many...
aspects of their precise role in immune control of the virus and in the progressive immunopathology of the liver remain to be clarified (3,33). In this study, we document that an aberrant expansion of a subset of functionally skewed NK cells lacking the classical NK cell marker CD56 occurs in some, but not all, patients with chronic HCV infection. Notably, this expansion was greater in patients who failed to clear HCV in response to peg-IFN-α and ribavirin treatment. Furthermore, elevated levels of CD56+ NK cells before treatment were associated with a significantly lower likelihood of HCV clearance in response to treatment. These data suggest that elevated levels of this aberrant NK cell subset is an indication of a disturbance in the innate cellular immune system which is associated with an inability to suppress or clear the HCV infection and to respond effectively to peg-IFN-α.

HCV and HIV-1 are both chronic RNA virus infections and both are associated with expansion of CD56+ NK cells. Nevertheless, there seems to be some differences between CD56+ NK cells from HIV-1-infected subjects and the similar cells from HCV-infected patients. We observed that CD56+ NK cells express NKP46 and NKP30 at similar percentages as those seen in the CD56dim subset, whereas CD56+ NK cells in HCV+ subjects was previously found to display a profound decrease in these activating receptors (9). Our data on poor perforin expression and IFN-γ production is consistent with the functional phenotype observed in HIV-1 infection. However, our data also clearly show that CD56+ NK cells in HCV-infected patients retain the capacity to respond with MIP-1β production upon recognition of K562 target cells. The cells thus display a functional skewing rather than complete dysfunction. We also provide an additional dimension to the analysis of this subset by the simultaneous assessment of four functions. The defect in polyfunctionality we observed in HCV-infected patients remains to be investigated in HIV-1 infection.

Independently of the immunological interpretations, the data indicate that pretreatment measurement of CD56+ NK cells could potentially be used as a clinical predictor of peg-IFN-α and ribavirin treatment outcome provided that the results can be verified in a larger prospective study. In the present study, CD56+ NK cell expansion appeared to have no strong link with viral genotype and load, host age, or sex, suggesting that there might be no direct biological link between CD56+ NK cells and these four known risk factors. A degree of association among such predictive factors would be expected in a larger prospective trial. Nevertheless, the present data suggest that the percentage of CD56+ NK cells is a predictor of treatment failure. It is thus possible that quantification of this cell subset could be of significant clinical value and may allow identification of patients who are less likely to respond to
antiviral therapy and in need of higher doses and longer treatment and those perhaps most likely to benefit from novel combination regimens including HCV protease inhibitors. Taken together, our findings show that functionally skewed CD56^+ NK cells expand in patients with chronic HCV infection. When compared with similar cells previously observed in HIV-1-infected patients, these cells show some similarities in their impaired functional capacity. Nevertheless, they are distinct in their expression of NCRs and ability to respond with MIP-1β production, which are retained in HCV-infected patients. The finding that the levels of CD56^+ NK cells correlate with peg-IFN-α and ribavirin treatment failure identifies a possible cellular immunological biomarker of HCV treatment outcome that merits further investigation.

**Acknowledgments**

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**Disclosures**

The authors have no financial conflict of interest.

**References**

5. ...