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Hepatitis C virions subvert natural killer cell activation to generate a cytokine environment permissive for infection

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Background & Aims: Hepatitis C virus (HCV) is remarkably successful in establishing persistent infections due to its ability to evade host immune responses through a combination of mechanisms including modulation of interferon (IFN) signalling in infected cells, interference with effector cell function of the immune system and continual viral genetic variation. We have previously demonstrated that natural killer (NK) cells can be inhibited *in vitro* by recombinant HCV glycoprotein E2 via cross-linking of CD81, a cellular co-receptor for the virus.

Methods: Taking advantage of the recently established tissue-culture system for HCV, we have studied the effects of CD81 engagement by the HCV envelope glycoprotein E2 when the protein is part of complete, infectious viral particles. Specifically, we asked whether exposure to HCV viral particles (HCVcc) affects activation of NK cells and whether altered NK cell activation, in turn, impacts on HCV infectivity.

Results: We found that immobilized HCVcc, unlike soluble HCVcc, inhibited IFN-γ production by interleukin (IL)-12 activated NK cells, and that this effect was mediated by engagement of cellular CD81 by HCV-virion displayed E2. In contrast, NK-production of IL-8 was increased in the presence of HCV. The cytokines produced by IL-12 activated NK cells strongly reduced the establishment of productive HCV infection. Importantly, NK-cell derived cytokines secreted in the presence of HCVcc showed a diminished antiviral effect that correlated with IFN-γ reduction, while IL-8 concentrations had no impact on HCV infectivity.

Conclusions: Exposure to HCVcc modulates the pattern of cytokines produced by NK cells, leading to reduced antiviral activity.
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Keywords: Chronic infection; HCV-E2 glycoprotein; IFN-γ; IL-8; Tetraspanin; Natural killer cells.

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Abbreviations: HCV, hepatitis C virus; IFN, interferon; PKR, protein kinase R; IL, interleukin; ISG, IFN-sensitive-gene; NK, natural killer; IL-12, interleukin 12; mAb, monoclonal antibody; HCVcc, cell culture-derived HCV particles.

Introduction

The hepatitis C virus (HCV) is one of the most common causes of liver disease. HCV typically causes persistent infections by undermining virus-specific immunity while leaving intact the host defences to other infectious agents. To achieve this, HCV has evolved complex escape strategies to evade and antagonize the host responses to infection [1]. The antiviral response of the infected organism can be roughly dissected into three levels, first the intracellular antiviral mechanisms in infected cells, second the innate immune response activated by local injury and triggering off the third level, the adaptive immune response. During acute and chronic infection, HCV induces a potent type I interferon response in the liver [2–4], even though the virus appears to be resistant to the effects of this antiviral pathway. Several HCV proteins have been shown to disrupt the interferon-signalling cascade. The NS3/4A protease cleaves the toll-like receptor 3 adaptor protein TRIF as well as IPS-1/MAVS/VISA/Cardif, directly disturbing the phosphorylation and effector action of the transcriptional factor IRF-3 on IFN gene transcription [5,6]. Two other viral proteins, E2 and NS5A, counteract the translational-suppressive activity of IFN that is mediated by the dsRNA-dependent protein kinase PKR by directly binding to PKR and inhibiting its catalytic activity [7,8].

Moreover, NS5A acts as an IFN antagonist, conferring a general attenuation of IFN-sensitive-gene (ISG) expression and function [9]. This effect is partially explained by NS5A ability to transactivate the IL-8 promoter [10], thus inducing IL-8 production and secretion in HCV infected cells. IL-8 has been shown to antagonize *in vitro* the antiviral action of IFN-α by attenuating ISG expression [10,11] and to exert a positive effect on HCV replication in stable replicon cell lines [12]. Moreover, IL-8 levels are elevated in sera of patients with chronic hepatitis C [13].

While escape strategies to the first and third levels are widely studied, less is known about the effects of HCV infection on innate immune responses. Since the quality and efficiency of adaptive immunity, and hence resolution of and protection from infection, directly depend on the input and direction coming from the innate immune response, detailed analysis of early events in the immune response to HCV is crucial.

NK cells represent a major component of early innate immune responses to viruses due to their ability to lyse infected cells and to rapidly produce cytokines that directly inhibit viral replication

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and participate in the recruitment and activation of cells of the adaptive immune system. Under normal conditions, activation of NK cells is controlled by dominant inhibitory signals mediated by recognition of self-MHC class I molecules. This tolerant state can be abrogated during infection or stress, when loss of MHC class I or up-regulation of ligands for activating receptors on target cells can tip the balance towards activation. Moreover, optimal NK-cell responses to pathogens require the presence of accessory cells [14] to provide both contact-dependent and soluble signals, such as IL-12 and type I IFNs, key inducers of IFN-γ secretion and cytotoxicity, respectively. The importance of highly functional NK cells in protection against HCV infection was highlighted in a study showing that composite KIR/HLA haplotypes that predict strong NK cell activation associate with better HCV clearance [15]. Two previous reports demonstrated that natural killer cells of healthy individuals are inhibited *in vitro* by the HCV glycoprotein E2 via cross-linking of the tetraspanin CD81, a cellular co-receptor for the virus [16,17]. The original observations were obtained using high concentrations of truncated, plate-bound HCV-E2, for the unavailability at the time of a robust *in vitro* HCV culture system. Recently, a tissue-culture system for HCV has been developed [18–20], allowing for the study of the effects of CD81 engagement by intact viral particles. Using such virions in solution, it was recently shown that NK cell functions are unaffected and it was suggested that the E2 configuration on the virion may not allow for effective interaction with CD81 on NK cells [21]. Here, we asked whether exposure to HCV viral particles (HCVcc), either soluble or immobilized, affected NK cell activation and whether modulation of NK cell activity could be of physiological relevance for the establishment of HCV infection. We focused on two NK cell produced cytokines: IFN-γ and IL-8. IFN-γ has a direct antiviral role and a non-redundant immunomodulatory function in the establishment of adaptive immune responses. In addition, IFN-γ has been shown to inhibit the replication of an HCV replicon in Huh-7 cells [22]. IL-8 has potent pro-inflammatory activity but has also been described as a pro-viral cytokine [11].

We demonstrate that CD81 cross-linking by immobilized anti-CD81 mAb or HCVcc inhibits IL-12-induced IFN-γ production, while augmenting IL-8 production. This indicates that the configuration of E2 on the virion surface can engage cellular CD81 with functional consequences. We subsequently tested whether this altered pattern of cytokines would interfere with HCV infectivity. Our data demonstrate that conditioned supernatants from IL-12 activated NK cells strongly suppress HCV infectivity and that this effect is attributable to the presence of IFN-γ. Engagement of NK cells by HCVcc significantly reduced the amount of IFN-γ produced and thereby reduced the antiviral activity of NK cells, while changes in IL-8 production have no direct impact on HCV infectivity. In conclusion, HCV virions are able to subvert NK cell function and counteract the antiviral functions of these cells.

Materials and methods

Antibodies and reagents

Anti-CD81 mAb: JS-81 (BD) and MG-81 [23]. The neutralizing anti-human IL-8/CXCL8 mAb (clone 6217, R&D Systems); the anti-IFN-γ mAbs B133.1 and B133.5 for ELISA, B133.3 for blocking experiments (generously provided by Dr. G. Trinchieri). Recombinant human IL-12, recombinant human IFN-γ, recombinant human IL-8, recombinant human IFN-α and the DouSet ELISA kit for human IL-8 were from R&D Systems; recombinant human IL-2 and the anti-core mAb 3G1-1 (Chiron/Novartis), recombinant purified HCV-E2 [24]; fluorochrome-conjugated secondary antibodies (Alexa Fluor®, Invitrogen).

Cell preparation and cultures

PBMCs were prepared from peripheral blood by Ficoll-Paque density gradient centrifugation.

NK cell bulk cultures were prepared as described [25]. On day 8 of the culture, cells were collected, and NK cells purified by depletion of the magnetically labeled $CD3^+/CD19^+/CD14^+$ cells using MACS Separation Columns (Miltenyi Biotech, Gladbach, Germany) (>98% $CD56^+/CD3^-/CD19^-/CD14^-$). Freshly isolated, untouched NK cells were isolated from PBMCs by the NK Cell Isolation Kit (Miltenyi Biotech). The purity of isolated $CD56^+/CD3^-/CD19^-/CD14^-$ NK cells was >90%, as assessed by flow cytometry.

Antibody coating and cell stimulation

96-Well plates (Greiner) were coated with Abs as previously described [26]. For HCVcc coating, 10^4–10^5 $TCID_{50}$/well, either infectious or β-propiolactone-inactivated, were bound overnight at 4 °C. After washing with PBS, either PBMCs or bulk or freshly isolated NK cells (10^6/ml) were added in complete medium plus IL-2 (10 U/ml). Human IL-12 (10 ng/ml) was added when indicated. For blocking experiments, NK cells were pre-treated with recombinant E2 (5 μg/ml), or the anti-CD81 mAbs JS-81 or MG-81 (5 μg/ml) for 30 min at 37 °C before plating.

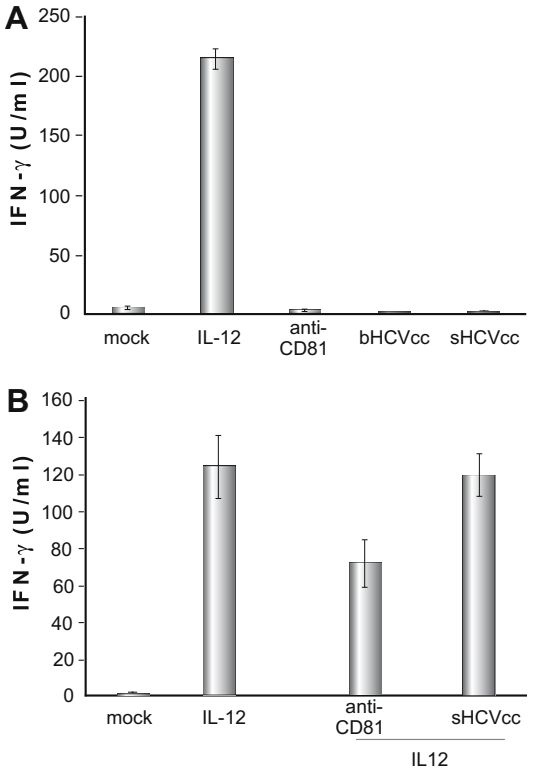

Fig. 1. Soluble infectious HCV particles neither induce IFN-γ production nor block IL-12 induced IFN-γ secretion by NK cells. (A) NK cells from bulk cultures were exposed to plate-bound anti-CD81 (10 mg/ml), to soluble (s) or plate-bound (B) HCVcc, or to IL-12 (10 ng/ml). After 24 h, supernatants were collected and analyzed by ELISA for IFN-γ production. (B) Histograms show the effects of IL-12 (10 ng/ml) alone, IL-12 plus plate-bound anti-CD81 and IL-12 plus soluble HCVcc on the production of IFN-γ by NK cells. These data represent the means ± standard deviation of four replicates. IFN, interferon; HCVcc, cell culture-derived HCV particles; IL-12, interleukin 12; NK, natural killer.

Cytokine production assays

NK cells were cultured in 96-well plates for 24 h and supernatants analyzed by ELISA for IFN-γ and IL-8. Samples from freshly purified NK cells were tested using multiplex-plates from Meso Scale Discovery (Gaithersburg, MD), according to the manufacturer's protocol.

Production of HCVcc

HCVcc production was obtained in S6.1 cells [27], after electroporation with *in vitro* transcribed HCV RNA as previously described [28]. Supernatants were harvested, cleared from cellular debris by filtration (0.45 μm) and concentrated by ultracentrifugation. $TCID_{50}$ (median tissue culture infective dose) was determined on Huh-7 cells by 10-fold serial dilutions, done in eight replicas, according to the Spearman and Kaerber fit.

HCVcc infection

Huh-7 cells grown in 96-well plates (10,000/well) were pre-treated with diluted (1:4) supernatants from NK cells for 1 h and then infected with HCVcc, 100 $TCID_{50}$/well. At 72 h post-infection, Huh-7 cells were fixed, permeabilized and processed for immunofluorescent detection of the HCV-core protein, using the anti-core mAb 3G1-1 and fluorochrome-conjugated secondary antibodies. Infection was determined by enumerating HCV-core positive foci under a fluorescence microscope.

Statistical analysis

Statistical analyses were performed with a two-tailed paired Student *t* test. Two-sided *p* value of less than 0.05 was considered significant.

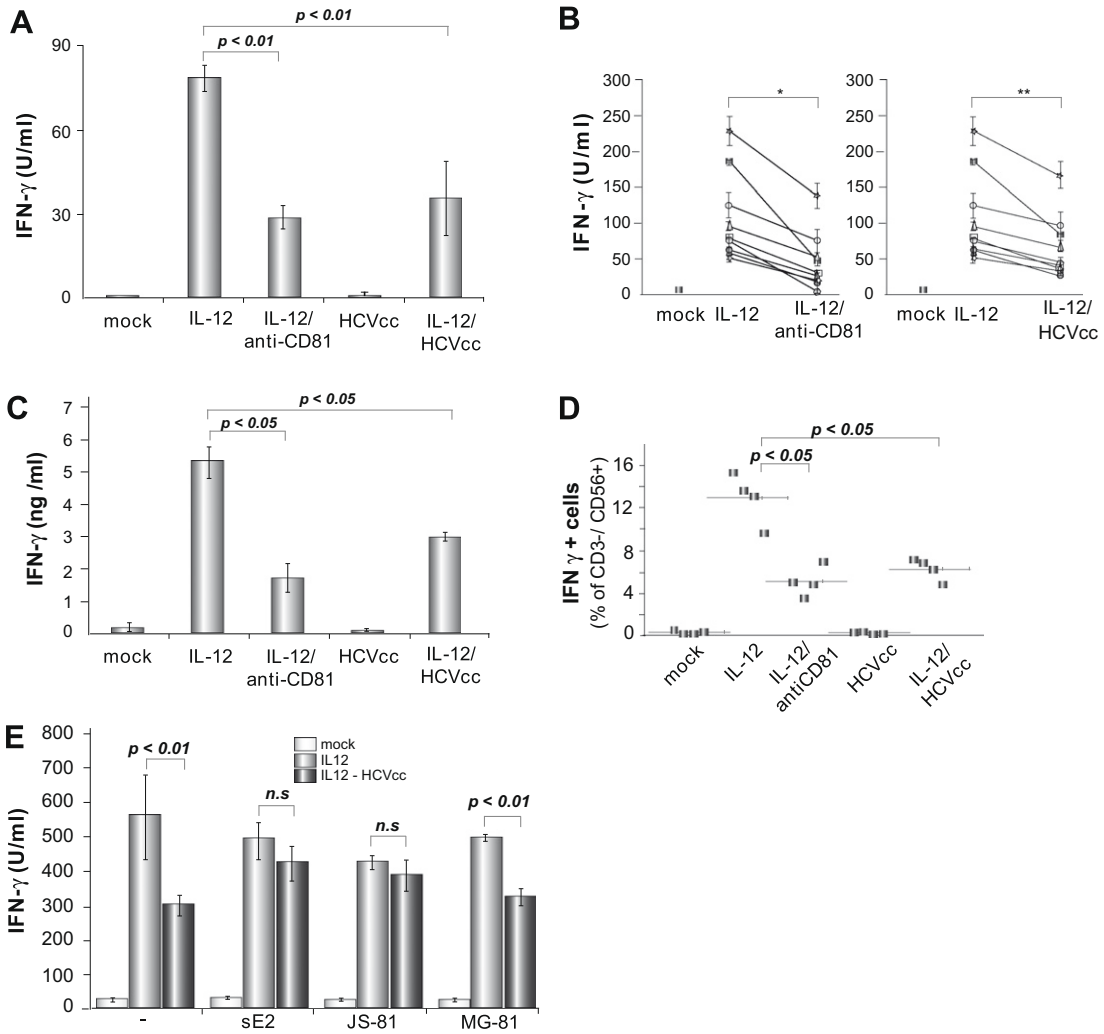

Fig. 2. CD81 cross-linking by virion-displayed HCV-E2 is responsible for HCVcc-mediated inhibition of IFN-γ production by NK cells. (A) Bulk NK cells were stimulated by IL-12 (10 ng/ml) alone or in the presence of plate-bound anti-CD81 mAb or HCVcc. After 24 h, supernatants were collected and analyzed by ELISA. Data represent the means ± standard deviation of four replicates. (B) The effect of plate-bound anti-CD81 or HCVcc on IL-12 dependent IFN-γ production by NK cells from nine different donors was determined as in (A). Each symbol indicates a different donor ($^{*}p$ value < 0.0007, $^{**}p$ value < 0.002, as determined by two-tailed, paired Student *t* test). (C) $CD56^{+}/CD3^{-}/CD19^{-}/CD14^{-}$ NK cells freshly isolated from PBMCs were stimulated as indicated for 24 h, and supernatants analyzed by multispot-plates (Meso Scale). (D) PBMCs were stimulated as indicated in the presence of Brefeldin A for 4 h and then stained for CD3, CD56 and intracellular IFN-γ. (E) Purified bulk NK cells were mock treated or treated with soluble, recombinant E2 (10 μg/ml), a neutralizing anti-CD81 mAb (JS-81, 10 μg/ml), or a non-neutralizing anti-CD81 mAb (MG-81, 10 μg/ml) and then stimulated by IL-12 alone or in the presence of plate-bound HCVcc. After 24 h supernatants were collected and analyzed by ELISA. Data represent the means ± standard deviation of four replicates and were reconfirmed in two additional experiments. IFN, interferon; HCVcc, cell culture-derived HCV particles; IL-12, interleukin 12; NK, natural killer.

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Results

Exposure to HCV modulates NK cell functions

We showed previously [16], that CD81 engagement by anti-CD81 mAb or recombinant HCV-E2 did not have by itself any effect on NK cell functions. Similarly, both freshly isolated peripheral blood NK cells (not shown) or NK cells purified from bulk cultures, were not induced to secrete IFN-γ after exposure to either soluble or plate-bound HCVcc (Fig. 1A). In agreement with previous reports [29], we did not find any evidence of infection and sustained HCVcc replication in NK cells, as assessed by flow cytometry (not shown). Since IL-12 has been described as the pivotal mediator of NK-cell responses to many pathogens, including MCMV and influenza [30–32], we tested whether infectious HCVcc particles in solution could modulate the functions of IL-12 activated NK cells. As shown in Fig. 1B, while plate-bound anti-CD81 inhibited IL-12 mediated IFN-γ secretion, soluble HCVcc did not cause any significant reduction in the amount of secreted cytokine, in agreement with a recent study [21]. However, when HCVcc particles were immobilized on culture plates at a ratio of 0.05–0.5 $TCID_{50}$ unit/cell (10^4–10^5 $TCID_{50}$ units/2×10^5 cells), they induced a significant reduction in the amount of IFN-γ produced by IL-12 stimulated NK cells, comparable to the effect of plate-bound anti-CD81 mAb (Fig. 2A), and consistently in all donors tested (Fig. 2B). The β-propiolactone-inactivated viral particles had the same effect on IFN-γ production while isotype-matched control antibody or supernatants from cells transfected with a subgenomic replicon had no effect on NK cell functions (not shown). A similar reduction in IFN-γ production was obtained when freshly purified $CD56^+/CD3^-/CD19^-/CD14^-$ cells (Fig. 2C) or total PBMCs (Fig. 2D) were stimulated by IL-12 in the presence of plate-bound HCVcc.

To verify whether HCVcc-mediated inhibition of IFN-γ production was due to CD81 cross-linking by virion-displayed HCV-E2, we blocked these interactions by pre-treating NK cells with soluble E2 or with anti-CD81 mAbs. We used two different anti-CD81 mAbs, JS-81 that can neutralize HCV-E2 binding to CD81 and the non-neutralizing MG-81 mAb [23]. Fig. 2E shows that NK cell inhibition was prevented only by treatment of NK cells with soluble E2 or with the neutralizing anti-CD81 mAb. As the inhibitory effect of plate-bound HCVcc was titrated away by soluble E2, these results indicate that the natural E2 protein displayed on the surface of HCV virions and its soluble form have similar configurations, and that inhibition of IFN-γ production requires engagement of CD81 on the cellular surface of NK cells by virion-associated E2 molecules.

Modulation of NK cell function influences HCV infectivity

To test whether the observed reduction in IFN-γ production could be of physiological relevance for the establishment of HCV infection, supernatants from bulk NK cells stimulated by IL-12 in the presence or absence of plate-bound anti-CD81 Ab or HCVcc were analyzed for their ability to support or counteract HCV infection of the permissive cell line Huh-7. For this purpose, naïve Huh-7 were pre-treated for 1 h with diluted NK cell supernatants and then infected with 100 $TCID_{50}$ units of HCVcc. The efficiency of HCV infection was determined 72 h post-infection by counting the number of foci that stained positive for the HCV-core protein. Supernatants from IL-12-stimulated NK cells consistently and significantly inhibited the ability of HCVcc to establish productive infection in Huh-7 cells, compared to supernatants derived from unstimulated NK cells. This inhibitory activity, however, was partially abrogated in supernatants derived from NK cells that have been stimulated by IL-12 in the presence of anti-CD81 or HCVcc particles (Fig. 3A and C). In contrast, soluble HCVcc particles that do not interfere with IFN-γ production by IL-12 stimulated NK cells (Fig. 1B), did not have any effect on HCV infectivity (Fig. 3B).

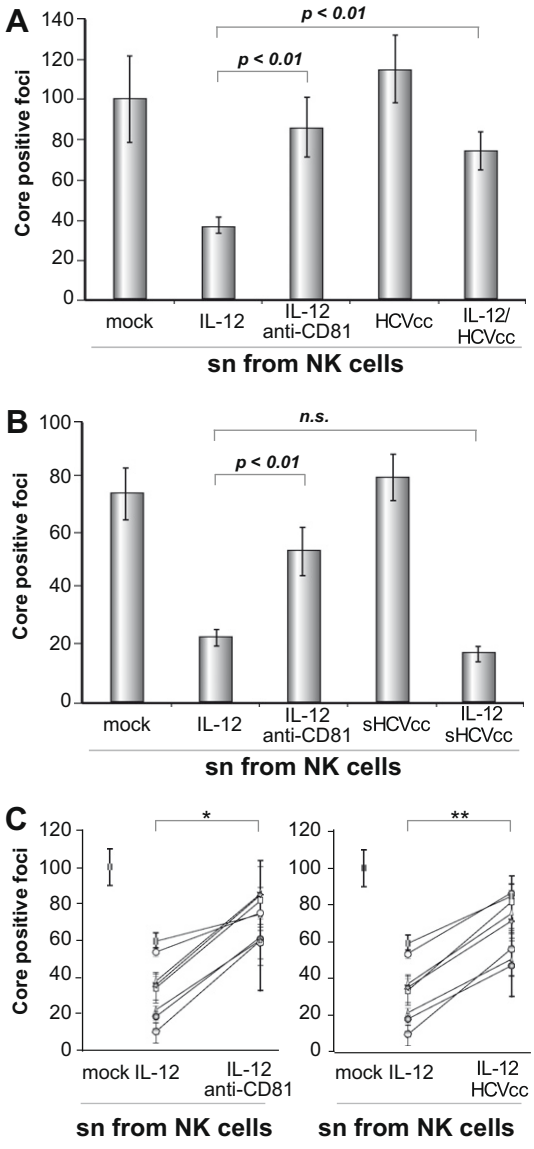

Fig. 3. Supernatants from NK cells influence the ability of HCVcc to infect Huh-7 cells. (A and B) Diluted supernatants from bulk NK cells stimulated as indicated were used to treat Huh-7 cells 1 h before and during HCVcc infection (100 $TCID_{50}$ units/sample). The number of HCV-core positive foci was determined 72 h post-infection by immunofluorescence. Data represent the means ± standard deviation of four replicates. (C) Bulk NK cells from nine different donors were stimulated as indicated. Supernatants were then tested for their ability to inhibit HCVcc infectivity as in (A) ($^*p$ value $< 9 \times 10^{-6}$, $^{**}p$ value $< 3 \times 10^{-7}$, as determined by two-tailed, paired Student *t* test). NK, natural killer; HCVcc, cell culture-derived HCV particles; IL-12, interleukin 12.

These results establish an inverse correlation between the number of HCV-core positive foci and the amount of IFN-γ produced by NK cells, and thus indicate that NK cell exposure to HCV particles negatively affects the cell ability to produce antiviral mediators.

IFN-γ but not IL-8 modulates HCVcc infectivity

We have previously described that CD81 cross-linking inhibits a wide range of NK cell activities, including proliferation, cytotoxicity and cytokine production. Remarkably, IL-8 production did not follow this pattern and was augmented upon CD81 cross-linking by anti-CD81 mAb or HCVcc, independently of the presence or absence of external IL-12 (Fig. 4A and B). Previous reports have described IL-8 as a pro-viral cytokine that partially inhibits the antiviral actions of type I IFN *in vitro* and exerts a positive effect on HCV replication [11,12]. We therefore tested whether NK-produced IL-8 influences the ability of HCVcc to infect Huh-7 cells. To dissect the relative contribution of IFN-γ and IL-8, both present in NK-conditioned media (Fig. 5, inset), supernatants from NK cells were pre-treated with blocking antibodies to the two cytokines prior to use. As shown in Fig. 5 and Table 1, the modulation of HCVcc infectivity determined by NK-conditioned media can be totally attributed to the amount of IFN-γ present, since a neutralizing anti-IFN-γ mAb completely abolished the inhibitory effects of NK-conditioned media on virus infectivity. In contrast, an anti-IL-8 blocking mAb did not influence the efficiency of HCV infection in the presence of NK culture supernatants.

IL-8 does not rescue HCV infectivity upon IFN treatment

While IL-8 protein levels have been found to correlate positively with the levels of HCV RNA replication in a series of replicon lines, we have shown here that removal of IL-8 by blocking antibody did not result in inhibition of HCV infectivity. However, as the levels of IL-8 present in NK-conditioned media were not excessively high, we tested whether higher amounts of the cytokine could influence HCV capability to establish productive infection and whether IL-8 could counteract the antiviral activity of both type I and type II interferons. For this purpose, target Huh-7 cells were treated with recombinant IL-8 in the presence or absence of increasing concentrations of either IFN-γ or IFN-α and subsequently exposed to the virus. Seventy-two hours later the number of foci that stained positive for the HCV-core protein was determined. Our results indicate that both in the presence or absence of interferons, IL-8 did not exert any significant effect, either positive or negative, on the ability of HCV to establish a productive infection (Fig. 6A–C).

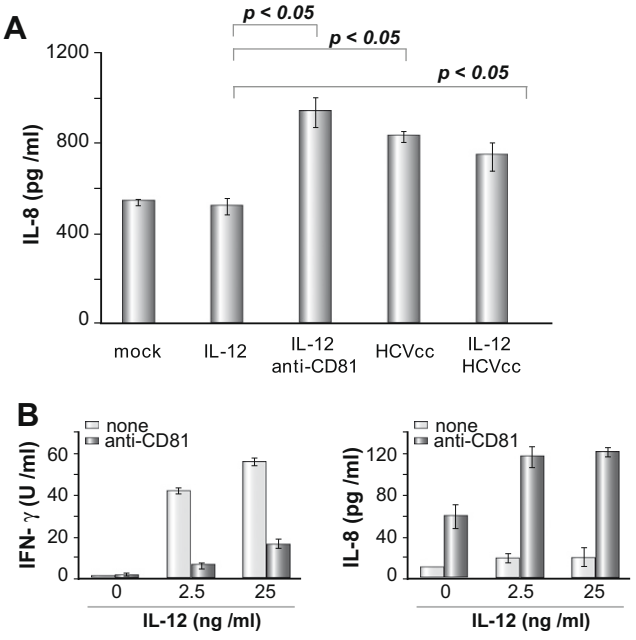

Fig. 4. CD81 cross-linking by either plate-bound anti-CD81 antibody or HCVcc particles increases IL-8 production by NK cells. (A) Bulk NK cells were stimulated by IL-12 (10 ng/ml) alone or in the presence of plate-bound anti-CD81 mAb or HCVcc. After 24 h supernatants were analyzed by ELISA. The results represent the means ± standard deviations from four replicates and are representative of three different experiments. (B) Amount of secreted IFN-γ and IL-8 upon NK cell stimulation by different concentrations of IL-12, in the presence or absence of plate-bound anti-CD81. IL-12, interleukin 12; HCVcc, cell culture-derived HCV particles; IFN, interferon; IL-8, interleukin 8; NK, natural killer.

Discussion

With this study, we show for the first time that infectious HCV particles, through binding of the glycoprotein E2 to CD81 on NK cells, are able to reduce IFN-γ production by NK cells and shift the cytokine pattern of NK cells towards a virus-permissive profile. Our data are strongly suggestive of an HCV immune evasion strategy based on modulation of NK cell antiviral activities that may ultimately contribute to HCV chronicity.

In the course of pathogen-induced inflammation in peripheral tissues, various chemokines and cytokines are released by resident cells, favouring extravasation of NK cells and DCs. Following endocytic uptake of both free virus and viral components from lysed cells, different subsets of dendritic cells are stimulated to secrete IL-12, the most potent soluble inducer of IFN-γ production by NK cells. Here we demonstrate that upon IL-12 stimulation, NK-derived IFN-γ reduces HCV infectivity and that this effect is subverted when CD81 on NK cells is engaged by HCV particles. We also checked for effects on NK cytotoxicity and found a trend towards reduced target cell killing in the presence of immobilized HCV. However, effects of CD81 engagement on cytotoxicity were more subtle than those on cytokines, in agreement with previous reports [17,21]. It remains to be determined whether this divergence truly reflects differential modification of the diverse NK functions.

Our results extend what we previously described using high concentrations of soluble, recombinant HCV-E2 and show that even as part of intact, infectious virions, E2 is able to engage CD81 on the NK cell surface with functional consequences. Indeed, we could detect inhibition of NK cell function when low amounts of HCV virions were immobilized in the cell culture dish and not when they were directly seeded onto NK cells, results in line with a recent publication [21] which reported no effect of soluble HCV virions on NK cell activation.

The different effects of soluble versus plate-bound HCVcc suggest that the inhibition of NK cells requires an extensive cross-linking of CD81 on the cellular surface. It is worth noticing that,

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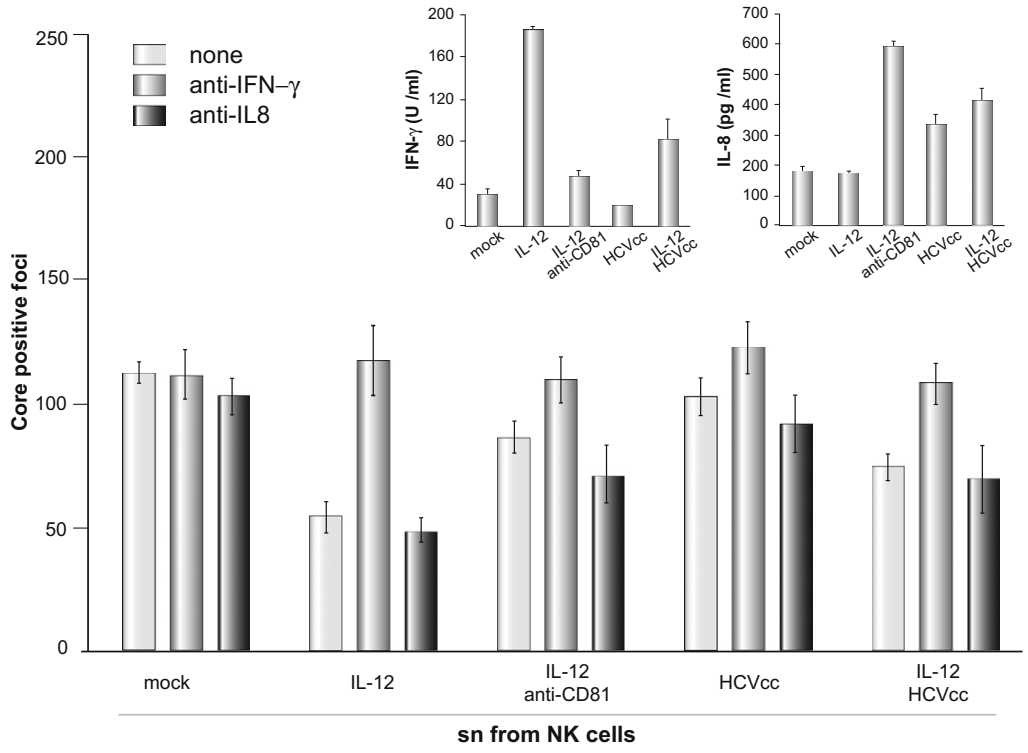

Fig. 5. IFN-γ but not IL-8 modulates HCVcc infectivity. Bulk NK cells were treated as indicated. IFN-γ and IL-8 in cell culture supernatants were measured by ELISA (Insets). Supernatants from NK cells were used to treat Huh-7 cells for 1 h before and during HCVcc infection (100 $TCID_{50}$ units/sample) alone or in the presence of 5 μg/ml monoclonal anti-IFN-γ or anti-IL-8 Abs. Seventy-two hours post-infection, the number of HCV-core positive foci was determined by immunofluorescence. Data represent the means ± standard deviation of four replicates and are representative of three similar experiments. IFN, interferon; IL-8, interleukin 8; NK, natural killer; HCVcc, cell culture-derived HCV particles.

Table 1. Modulation of HCVcc infectivity by NK cell conditioned supernatants in the presence or absence of anti-IFN-γ or anti-IL-8 neutralizing antibody.

Treatment	None	Anti-IFN-γ	Anti-IL-8
Mock	109 ± 7	107 ± 11	102 ± 5
IL-12	49 ± 5	116 ± 9**	50 ± 2
IL-12/anti-CD81	74 ± 9	104 ± 6**	68 ± 8
HCVcc	101 ± 8	112 ± 6	91 ± 9
IL-12/HCVcc	69 ± 5	137 ± 22*	71 ± 4

Supernatants from bulk NK cells treated as indicated were used on Huh-7 cells during HCVcc infection, alone or in the presence of 5 μg/ml monoclonal anti-IFN-γ or anti-IL-8 Abs. The number of HCV-core positive foci was determined by immunofluorescence. Data represent the means ± standard deviation of four different experiments. The asterisks indicate p values < 0.05 versus control (same treatment without cytokine neutralizing antibody), as determined by two-sided, paired Student t test: $^{*}p < 0.05$, $^{**}p < 0.005$.

for technical reasons associated to virus yields, the virus-to-cell ratio in our experiments was never higher than 1, rendering the presence of solid support necessary to achieve enforced cross-linking. It remains to be determined whether during disease, local concentrations of HCV in the infected liver are high enough to induce the effects described here. Alternatively, our experimental setting could be representative of particular physiological niches where HCV virions are enriched and immobilized. The hepatic extracellular matrix or liver resident cells such as liver sinusoidal lining cells, that express the HCV binding proteins dendritic cell-specific (DC-SIGN) or liver/lymph node-specific (L-SIGN) intercellular adhesion molecule-3-grabbing non-integrin [33], could represent these potential virion accumulation sites.

Previous works [17,34] demonstrated that CD81 engagement provides a global inhibition of NK cell activities triggered by signals as diverse as CD16, NKG2A, β1-integrin and IL-12. The activities blocked include proliferation, up-regulation of CD25 and CD69, production of TNF-α and IFN-γ and cytotoxicity. Here, we show one exception to this general phenomenon, IL-8, whose production was increased in response to CD81 engagement.

As inhibition of IL-8 production has been shown to inhibit chronic HCV replication in stable replicon cell lines [12], we tested whether, upon CD81 engagement on NK cells, the sustained level of IL-8 and the concomitant down-modulation of IFN-γ could be both responsible for the reduced antiviral activity of activated NK cells.

However, we could not detect any significant effect of IL-8 on HCV infectivity. IL-8 blocking mAbs did not influence the effects of NK supernatants, and pre-treatment of target Huh-7 cells with recombinant IL-8 did not render them more refractory to the action of interferons in blocking the establishment of productive infection.

In conclusion, our data show that cytokines produced by activated NK cells block HCV infectivity and that HCV-E2 as part of intact, infective HCV particles is able to down-modulate IFN-γ production by NK cells through engagement with CD81. This down-modulation of IFN-γ strictly correlates with the decreased antiviral activity of the NK cytokine mix. We suggest that in the infected liver, either high local HCV concentrations or virus

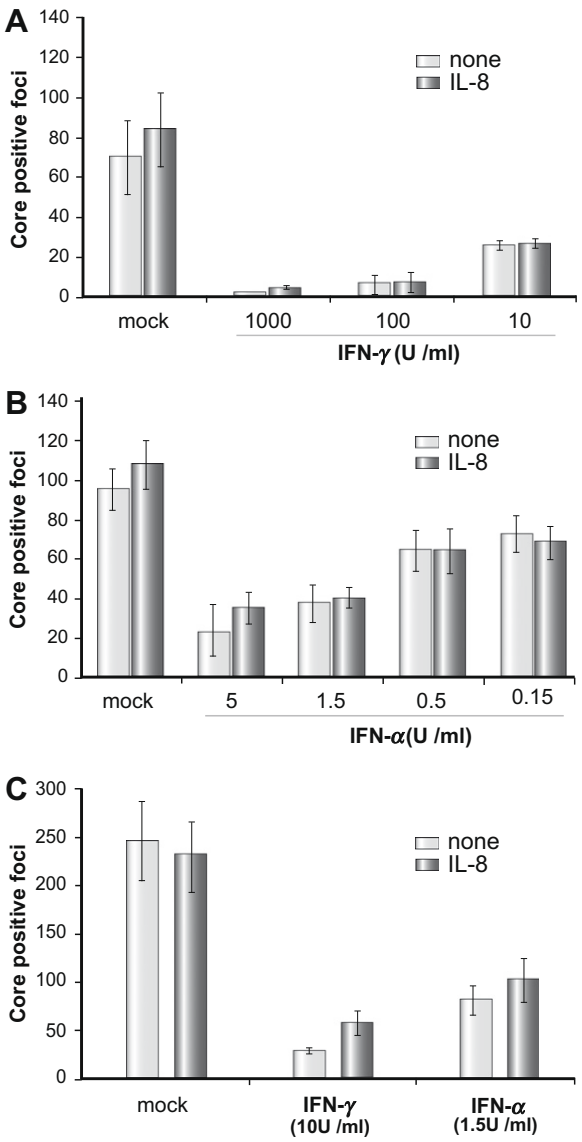

Fig. 6. IL-8 does not interfere with the antiviral action of IFN-γ and IFN-α on HCVcc infectivity. Huh-7 cells mock treated or treated with different concentrations of recombinant IFN-γ (A and C) or IFN-α (B and C) were infected with 100 $TCID_{50}$ units, in the presence or absence of IL-8 (A and B: 40 ng/ml; C: 400 ng/ml). Seventy-two hours post-infection the number of HCV-core positive foci was determined by immunofluorescence. Data represent the means ± standard deviation of four replicates. IFN, interferon; IL-8, interleukin 8.

particles immobilized on cells or on the extracellular matrix have the potential to inhibit NK cells and thereby contribute to the establishment of chronic hepatitis C.

Acknowledgements

All the authors are or were are employees in Novartis Vaccine and Diagnostics.

References

[1] Dustin LB, Rice CM. Flying under the radar: the immunobiology of hepatitis C. Annu Rev Immunol 2007;25:71–99.
[2] Bigger CB, Brasky KM, Lanford RE. DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. J Virol 2001;75:7059–7066.
[3] Bigger CB, Guerra B, Brasky KM, Hubbard G, Beard MR, Luxon BA, et al. Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees. J Virol 2004;78:13779–13792.
[4] Su AI, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Thimme R, et al. Genomic analysis of the host response to hepatitis C virus infection. Proc Natl Acad Sci USA 2002;99:15669–15674.
[5] Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, et al. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc Natl Acad Sci USA 2005;102:2992–2997.
[6] Foy E, Li K, Wang C, Sumpter Jr R, Ikeda M, Lemon SM, et al. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science 2003;300:1145–1148.
[7] Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science 1999;285:107–110.
[8] Gale Jr M, Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, et al. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. Mol Cell Biol 1998;18:5208–5218.
[9] Geiss GK, Carter VS, He Y, Kwieciszewski BK, Holzman T, Korth MJ, et al. Gene expression profiling of the cellular transcriptional network regulated by alpha/beta interferon and its partial attenuation by the hepatitis C virus nonstructural 5A protein. J Virol 2003;77:6367–6375.
[10] Polyak SJ, Khabar KS, Paschal DM, Ezelle HJ, Duverlie G, Barber GN, et al. Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. J Virol 2001;75:6095–6106.
[11] Khabar KS, Al-Zoghaibi F, Al-Ahdal MN, Murayama T, Dhalla M, Mukaida N, et al. The alpha chemokine, interleukin 8, inhibits the antiviral action of interferon alpha. J Exp Med 1997;186:1077–1085.
[12] Koo BC, McPoland P, Wagoner JP, Kane OJ, Lohmann V, Polyak SJ. Relationships between hepatitis C virus replication and CXCL-8 production *in vitro*. J Virol 2006;80:7885–7893.
[13] Polyak SJ, Khabar KS, Rezeiq M, Gretch DR. Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy. J Virol 2001;75:6209–6211.
[14] Newman KC, Riley EM. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. Nat Rev Immunol 2007;7:279–291.
[15] Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science 2004;305:872–874.
[16] Crotta S, Stilla A, Wack A, D'Andrea A, Nuti S, D'Oro U, et al. Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. J Exp Med 2002;195:35–41.
[17] Tseng CT, Klimpel GR. Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. J Exp Med 2002;195:43–49.
[18] Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623–626.
[19] Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791–796.
[20] Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection *in vitro*. Proc Natl Acad Sci USA 2005;102:9294–9299.
[21] Yoon JC, Shiina M, Ahlenstiel G, Rehermann B. Natural killer cell function is intact after direct exposure to infectious hepatitis C virions. Hepatology 2009;49:12–21.
[22] Frese M, Schwarzle V, Barth K, Krieger N, Lohmann V, Mihm S, et al. Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. Hepatology 2002;35:694–703.
[23] Rosa D, Saletti G, De Gregorio E, Zorat F, Comar C, D'Oro U, et al. Activation of naïve B lymphocytes via CD81, a pathogenetic mechanism for HCV-associated B-lymphocyte disorders. Proc Natl Acad Sci USA 2005;102:18544–18549.
[24] Heile JM, Fong YL, Rosa D, Berger K, Saletti G, Campagnoli S, et al. Evaluation of hepatitis C virus glycoprotein E2 for vaccine design: an endoplasmic reticulum-retained recombinant protein is superior to secreted recombinant protein and DNA-based vaccine candidates. J Virol 2000;74:6885–6892.
[25] Perussia B, Ramoni C, Anegon I, Cuturi MC, Faust J, Trinchieri G. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines. Nat Immun Cell Growth Regul 1987;6:171–188.

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[26] Wack A, Soldaini E, Tseng C, Nuti S, Klimpel G, Abrignani S. Binding of the hepatitis C virus envelope protein E2 to CD81 provides a co-stimulatory signal for human T cells. Eur J Immunol 2001;31:166–175.
[27] Zhu Q, Oei Y, Mendel DB, Garrett EN, Patawaran MB, Hollenbach PW, et al. Novel robust hepatitis C virus mouse efficacy model. Antimicrob Agents Chemother 2006;50:3260–3268.
[28] Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110–113.
[29] Marukian S, Jones CT, Andrus L, Evans MJ, Ritola KD, Charles ED, et al. Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. Hepatology 2008;48:1843–1850.
[30] Orange JS, Biron CA. An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. J Immunol 1996;156:1138–1142.
[31] Monteiro JM, Harvey C, Trinchieri G. Role of interleukin-12 in primary influenza virus infection. J Virol 1998;72:4825–4831.
[32] Krug A, French AR, Barchet W, Fischer JA, Dzionek A, Pingel JT, et al. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. Immunity 2004;21:107–119.
[33] Ludwig IS, Lekkerkerker AN, Depla E, Bosman F, Musters RJ, Depraetere S, et al. Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. J Virol 2004;78:8322–8332.
[34] Crotta S, Ronconi V, Ulivieri C, Baldari CT, Valiante NM, Abrignani S, et al. Cytoskeleton rearrangement induced by tetraspanin engagement modulates the activation of T and NK cells. Eur J Immunol 2006;36:919–929.