Hepatitis C Virus–Infected Cells Downregulate NKp30 and Inhibit Ex Vivo NK Cell Functions

Kayla A. Holder, Staci N. Stapleton, Maureen E. Gallant, Rodney S. Russell and Michael D. Grant

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Hepatitis C virus (HCV) successfully evades the immune system and establishes chronic infection in ~80% of cases. Immune evasion may involve modulating NK cell functions. Therefore, we developed a short-term assay to assess immediate effects of HCV-infected cells on ex vivo NK cytotoxicity and cytokine production. Natural cytotoxicity, Ab-dependent cell-mediated cytotoxicity, IFN-γ production, and TNF-α production were all significantly inhibited by short-term direct exposure to HCV-infected hepatoma-derived Huh-7.5 cells. Inhibition required cell-to-cell contact and increased together with multiplicity of infection and HCV protein levels. Blocking potential interaction between HCV E2 and NK CD81 did not abrogate NK cell inhibition mediated by HCV-infected cells. We observed no change in expression levels of NKGD2, NKG2A, NKP46, or CD16 on NK cells exposed to HCV-infected Huh-7.5 cells for 5 h or of human histocompatibility-linked leukocyte Ag E on HCV-infected compared with uninfected Huh-7.5 cells. Inhibition of ex vivo NK functions did correspond with reduced surface expression of the natural cytotoxicity receptor Nkp30, and downregulation of NKP30 was functionally reflected in reduced anti-NKp30 redirected lysis of P815 cells. Infection of Huh-7.5 cells with HCV JFH1T increased surface binding of an NKP30-IgG1 Fc fusion protein, suggesting upregulation of an antagonistic NKP30 ligand on HCV-infected cells. Our assay demonstrates rapid inhibition of critical NK cell functions by HCV-infected cells. Similar localized effects in vivo may contribute to establishment of chronic HCV infection and associated phenotypic and functional changes in the NK population. The Journal of Immunology, 2013, 191: 000–000.

Hepatitis C virus (HCV) is an enveloped, positive-sense RNA virus of the Flaviviridae family (4). Its genome is translated into a polyprotein that is cleaved co- and posttranslationally to form structural proteins comprising the viral particle (core, envelope glycoproteins E1 and E2), p7, and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) that aid in either virion assembly or replication (4, 5). Entry of HCV into permissive hepatocytes requires multiple host receptors, including CD81 (6–10), scavenger receptor class B member 1 (11–13), occludin (14–16), and claudin-1 (17, 18). Entry is mediated primarily by HCV E2, which interacts with conserved residues within both scavenger receptor class B member 1 (13) and CD81 (19, 20). Viral persistence is dependent on successful evasion of host immune defenses. Although the precise mechanism by which HCV accomplishes this is not understood, documented evasion strategies involve diminished type I IFN responses (21–26), impaired cytotoxic CD8+ T cell activity, and suppression of CD4+ Th1 responses (27).

NK cells are enriched in the liver (28) and provide inherent defense against transformed cells and many pathogens, including viruses. Although classically defined as cytolytic lymphocytes (29, 30), NK cells fall into two main functionally distinct subsets. The highly cytotoxic CD56dimCD16bright NK cells are considered more mature, whereas CD56brightCD16dim immature NK cells predominantly secrete proinflammatory cytokines such as IFN-γ and TNF-α. In some instances, the CD56brightCD16dim NK cell population also secretes the immunoregulatory cytokine IL-10 (31, 32). One study showed that CD56dim NK cells can also produce large amounts of IFN-γ promptly (2–4 h) following activation (33). NK cells recognize and target infected or transformed cells through an assortment of germline-encoded receptors. The outcome of engagement of these receptors is determined through the balance of signals from, and interactions between, inhibitory and activating pathways. Inhibitory receptors, such as the killer cell Ig-like receptors (KIRs) or NKG2A/CD94 predominately recognize unaltered “self” cells through...
their expression of MHC class I molecules to prevent cytolyis of healthy autologous cells (34). Stress and infection often reduce surface MHC class I expression, thereby lessening inhibitory signaling and allowing NK cell lysis of altered target cells through stimulation of activating receptors, such as NKG2D and the natural cytotoxicity receptors (NCRs) NKp46, NKp44, and NKp30 (35–40). The best characterized activating NK receptor is the low-affinity receptor for IgG, CD16 (FcγRIII), which recognizes Ab-coated cells and is responsible for Ab-dependent cell-mediated cytotoxicity (ADCC) (34). Although some ligands, such as stress-inducible MICA and MICB, which interact with and stimulate NK cells through NKG2D (34), and human CMV tegument protein pp65, which interacts with and inhibits NK cells through Nkp30 (41), have been identified, many activating and inhibitory ligands remain unknown.

Epidemiological studies suggest that NK cells play a role in determining the outcome of HCV infection (42, 43). Some biomedical studies indicated that although NK cell numbers are reduced in chronically infected individuals, NK-activating receptor and cytolytic functions actually increase (44, 45). Conversely, other studies reported decreases in NK cell–activating receptor expression, IFN-γ production, and cytotoxic activity (28, 46, 47). Mechanistic studies are also inconclusive; some showed that cross-linking NK CD81 receptors with recombinant HCV E2 protein or immobilized HCV virions directly inhibits NK cytotoxicity and cytokine production (48, 49), whereas others reported that NK cell functions remained intact when exposed to infectious cell culture–derived HCV (HCVcc) particles (50). In the absence of an immunocompetent small animal model of HCV infection, in vitro methods of investigating how NK cells behave in the context of HCV infection are required. As most previous systems used peripheral blood NK cells stimulated in vitro with rIL-2, rIL-12, or IFN-α or extended NK cell coculture with HCV-infected cells, the in vivo relevance of the reported effects is uncertain (47–49, 51, 52).

The advent of a robust HCV cell culture system capable of assembly and release of infectious HCV particles (5, 53, 54) enables in vitro study of the immediate effects of HCV-infected human hepatoma cells on freshly isolated NK cells. We explored how HCV-infected cells affect unstimulated NK cells ex vivo in short-term assays and found that direct contact with HCV-infected cells in vitro inhibited NK cytotoxicity and cytokine production coincident with a decline in Nkp30 cell surface expression.

Materials and Methods

**Tissue culture and cell line maintenance**

Human hepatoma Huh-7.5 cells (a gift from Charles Rice, Rockefeller University) were maintained in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin (complete DMEM; all from Invitrogen). Human erythromyeloblastoid leukemia K562 cell line (ATCC CCL 243), murine mastocytoma P815 cell line (ATCC TIB-64), and an EBV-transformed human B lymphoblastoid cell line transfected with HLA-B27, C1R-B27 mastocytoma P815 cell line (ATCC TIB-64), and an EBV-transformed human B lymphoblastoid cell line transfected with HLA-B27, C1R-B27 (a gift from Kelly McDonald, University of Toronto), were propagated in lymphocyte medium consisting of RPMI 1640 supplemented with 10% FCS, 200 IU/ml penicillin/streptomycin, 1% M HEPES, 1% l-glutamine (all from Invitrogen), and 5.5 × 10−3 M 2-ME (Sigma-Aldrich). All cells were cultured at 37°C with 5% CO2.

**Virus stock generation**

To generate infectious virus stock, Huh-7.5 cells were transfected with genomic RNA from a cell culture–adapted JFH1 genotype 2a HCV strain, JFH1G (amino acid mutations N417S, N765D, Q1012R, where amino acid numbering begins with the first residue in the complete polyprotein), constructed as previously described (55), or cell culture–adapted JFH1G1 (amino acid mutations N765D, Q1012R, A.M. Atoom and R.S. Russell, unpublished data). Huh-7.5 cells (1.25 × 105) were seeded in 10-cm tissue culture–treated dishes and then 24 h later transfected with JFH1G or JFH1G1 RNA using DNMRI-C transfection reagent (Invitrogen) (56). Briefly, JFH1G or JFH1G1 DNA plasmid was linearized with restriction enzyme XbaI (Invitrogen) for 2 h at 37°C and then RNA transcribed using the T7 Ribonuclease large-scale RNA production system (Promega). Four microilters of the transcribed RNA was combined with 500 µl serum-free DMEM and 50 µl DmriE-C and then added to cells plated with 2 ml serum-free DMEM and incubated for 4 h. Medium was replaced with fresh complete DMEM. Seventy-two hours after infection, supernatants were harvested, centrifuged, and filtered through a MillexHV 45-µm filter (Millipore). To determine the infectious virus titer of HCVcc in supernatant, 5 × 105 Huh-7.5 cells were seeded in each well of eight-well culture slides (Lab-Tek). Twenty-four hours later, infectious supernatant was serially diluted 10-fold in complete DMEM and 100 µl each dilution in triplicate was added to Huh-7.5 cells in eight-well culture slides. Infectious supernatant was removed after 4 h incubation and replaced with complete DMEM. Seventy-two hours after infection, Huh-7.5 cells were fixed in acetone and stained with anti-HCV core protein (see below) to determine focus forming units (FFU) per milliliter supernatant. Huh-7.5 cells (1.25 × 105) seeded in 10-cm culture–treated dishes were subsequently infected for 4 h with titrated HCVcc supernatant at multiplicity of infection (MOI) of 0.5, after which the infectious supernatant was removed and replaced with 6 ml complete DMEM. Seventy-two hours after infection, supernatants were removed, filtered, and titered as above. Transfection supernatants were passaged twice to eliminate input RNA. The final infectious virus titer, in FFU/ml HCVcc supernatant, was used for all subsequent assays.

**Indirect immunofluorescence**

Huh-7.5 cells seeded and infected in eight-well chamber slides (as described above) were washed with PBS and fixed for 2 min with 100% acetone. Slides were incubated with the monoclonal anti-HCV core Ab (B2; Anogen) diluted 1:200 in PBS with 1% FCS for 20 min at room temperature, washed with PBS, then incubated with secondary Alexa Fluor 488–conjugated goat anti-mouse IgG Ab (Invitrogen) diluted 1:1000 in PBS for 20 min at room temperature. Washed cells were fixed and mounted using Vectashield with DAPI and mounting medium (Vector Laboratories). Images were visualized at ×10 magnification on a Zeiss Axio Imager.M2 immunofluorescence microscope.

**PBMC isolation and NK cell purification**

PBMCs were freshly isolated from whole blood of consenting healthy donors by Ficoll-Paque (VWR International) density gradient centrifugation on the day of the assay and suspended in lymphocyte medium. PBMCs were used as effector cells unless otherwise stated. For purified NK assays, NK EasySep cells were enriched from isolated PBMCs by negative selection with human NK cell enrichment mixture (Stemcell Technologies) per the manufacturer’s specifications and resuspended in lymphocyte medium. This study received ethical approval from the Health Research Ethics Authority of Newfoundland and Labrador, Canada.

**Cytotoxicity assays**

Five-hour 51Cr-release assays were used to assess the effect of HCV-infected Huh-7.5 cells on NK cytotoxicity. Huh-7.5 cells (5 × 103) were seeded in each well of 96-well round-bottom BD Falcon tissue culture–treated plates, and 24 h later cells were infected at various MOIs with titrated infectious JFH1 HCVcc stocks (as described above). Seventy-two hours later, K562 or C1R-B27 cells were labeled for 90 min with 100 µCi Na51 CrO4 (MP Biomedical) and then washed four times with PBS containing 1% FCS. C1R-B27 cells were subsequently incubated 30 min with 2 ml W6/32 supernatant (ATCC HB-95) to sensitize cells to ADCC (57). Huh-7.5 cells were incubated 5 h with freshly isolated PBMCs and 5 × 103 51Cr-labeled K562 or C1R-B27 target cells at various E:T ratios. Supernatant (125 µl) was then removed from each well, transferred to Kimble tubes (Fisher Scientific), and 51Cr release was measured on a Wallac 1480 Wizard gamma counter. Percentage-specific lysis was calculated by [(experimental 51Cr release – spontaneous 51Cr release)/(maximum 51Cr release – spontaneous 51Cr release)] × 100. Replicates were performed and all values in each condition were compared by Student’s t-test. Spontaneous 51Cr release was <3%. Supernatant (125 µl) was then removed from each well, transfected to kimble tubes (Fisher Scientific), and 51Cr release was measured on a Wallac 1480 Wizard gamma counter. Percentage-specific lysis was calculated by [(experimental 51Cr release – spontaneous 51Cr release)/(maximum 51Cr release – spontaneous 51Cr release)] × 100. Replicates were performed and all values in each condition were compared by Student’s t-test. Spontaneous 51Cr release was <3%.
Transwell coculture assay

Huh-7.5 cells (7.5 × 10³) were seeded and infected as above in a HTS Transwell-96 well receiver microplate (Corning). Seventy-two hours after infection, 51Cr-labeled K562 target cells and freshly isolated PBMCs were incubated 5 h in 3.0-μm membrane inserts placed in receiver plates with uninfected and HCV-infected Huh-7.5 cells and then 51Cr release was measured as above.

CELSA

51Cr-release assay plates contained fixed HCV-infected and uninfected Huh-7.5 cells were washed three times with PBS containing 1% Tween 20 (wash buffer; Sigma-Aldrich). Human plasma from an HCV-infected subject (153) or uninfected control subject (C1) was diluted 1:200 in wash buffer containing 1% FCS (diluent) and 100 μl diluted serum added to each well of the 96-well plate for 90 min at room temperature. Following four washes, 100 μl alkaline phosphatase–conjugated goat anti-human IgG (Jackson ImmunoResearch) diluted 1:5000 was added to each well for 60 min at room temperature. After four more washes, 100 μl substrate (1-p-nitrophenol tablet [Sigma-Aldrich]) dissoloved in 12 ml alkaline phosphatase substrate buffer, which included 10% diethanolamine [BDH Chemicals], 0.02% sodium azide, and 0.01% magnesium chloride (both from Sigma-Aldrich) at pH 9.8 was incubated in each well for 30 min at room temperature in the dark. Absorbance was measured at 405 nm on a Synergy HT BioTek microplate reader. Absorbance at MOI of 0 was considered background and subtracted from all values.

Huh-7.5 cell viability assay

To assess the impact of HCV infection on Huh-7.5 proliferation and viability, 5 × 10² Huh-7.5 cells were seeded per well in 96-well round-bottom tissue culture–treated plates and infected at various MOIs with titered infectious JFH1T HCVcc stock 24 h later (as described above). Seventy-two hours after infection, supernatant was removed and cells were incubated with MTT (Sigma-Aldrich) diluted 1:10 in complete DMEM for 4 h. The MTT medium was then removed and the remaining material solubilized in DMSO. Absorbance was immediately measured at 540 nm on a Synergy HT BioTek microplate reader. Absorbance in wells without Huh-7.5 cells was considered background and subtracted from all values.

Flow cytometry

Huh-7.5 cells (6.5 × 10⁴) were seeded in each well of 12-well plates and infected with JFH1T HCVcc at an MOI of 3.2 or 4.8. Seventy-two hours later, Huh-7.5 cells were stained for HLA-E expression (3D12; BioLegend) as below or were incubated with freshly isolated PBMCs with or without K562 cells at an E:T ratio of 5:1 for 1 h at 37°C, after which 10 μg/ml brefeldin A (Sigma-Aldrich) was added for an additional 4 h. After 5 h incubation, PBMCs were removed, washed with flow cytometry buffer (0.2% sodium azide, 0.5% FCS, 5 mM EDTA in PBS) and incubated 20 min with 0.4 μg PerCP-conjugated mouse anti-CD3 (BioLegend), 1 μg FITC-conjugated mouse anti-CD56 (eBioscience), 1 μg PE-conjugated mouse anti-CD107a (BioLegend), or anti–NKp46–PE, anti–NKGD2–PE (all from BioLegend), or anti–NKGD2–PE (R&D Systems) for cell surface markers. Cells were then fixed and permeabilized with IntraStain (Dako) and incubated 20 min with 0.1 μg allopocacyocyanin-conjugated mouse anti–IFN-γ (eBioscience) or 1 μg allopocacyocyanin-conjugated anti–TNF-α (MAb11; BioLegend) and then resuspended in 1% paraformaldehyde (Sigma-Aldrich). For fusion protein experiments, uninfected or HCV-infected Huh-7.5 cells were incubated 30 min at 4°C with 1 μg/10⁶ cells Nkp30-IgG1 Fc chimera or control KIR3DS1-IgG1 Fc chimera (both from R&D Systems) and stained with allopocacyocyanin-conjugated AflinnPure goat anti-human IgG1 Fc (Jackson ImmunoResearch). Cells were analyzed on a Becton Dickinson FACSCalibur flow cytometer.

Ab-mediated receptor/ligand masking

HK cells were isolated from PBMCs by negative selection (as described above) and pretreated 30 min at 4°C with either 1 μg/10⁶ cells isotype control (IC) (Ag8; a gift from Chris Ford, Memorial University of Newfoundland) or anti-human CD81 (JS-81; BD Biosciences) mAbs and then incubated at an E:T ratio of 10:1 with 51Cr-labeled K562 cells as described. Alternatively, 96 h after infection with JFH1T HCVcc at MOI of 1.3, Huh-7.5 cells were preincubated for 30 min with either 1 μg/well anti-human E2 (AP33; Genentech, San Francisco, CA) or Ag8 (IC) diluted in

FIGURE 1. Establishment of assay platform. (A) Schematic diagrams of HCV JFH1 cell culture–adapted strains used in this study. JFH1T contains three amino acid substitutions as indicated by * (N417S [E2], N765D [p7], Q1012R [NS2]), which enhance infectious virus production (>10⁵ FFUs/ml) compared with wild-type JFH1. The JFH1D1 construct has two of the above mutations (N765D [p7], Q1012R [NS2]) and yields <10⁵ FFUs/ml. (B) The black bar graph depicts relative fold increase of HCV protein levels at high MOIs compared with an MOI of 0.2 as measured by CELISA on acetone-fixed, HCV-infected Huh-7.5 cells incubated with a 1:200 dilution of plasma containing high levels of anti-HCV Abs. The light bar graph represents results with control plasma. Error bars indicate SEM of 12 replicates. (C) Huh-7.5 cell viability at indicated MOIs with JFH1T HCV was measured on the day of NK assays by MTT assay. Error bars indicate SEM of 12 replicates. (D) Huh-7.5 cells were infected with HCV JFH1T at an MOI of 4.8 and then 72 h later fixed in acetone and stained for HCV core (green) immunofluorescence with DAPI (blue) nuclear staining. Scale bars, 100 μm.
complete DMEM and then incubated with target $^{51}$Cr-labeled K562 and effector PBMC at an E:T ratio of 60:1 as above.

**NKp30-directed lysis**

Huh-7.5 cells ($3 \times 10^4$) were seeded in each well of 96-well round-bottom tissue culture–treated plates and 48 h later infected with JFH1 T HCVcc at an MOI of 3.2. Forty-eight hours after infection, $5 \times 10^4$ PBMCs were incubated with uninfected or infected Huh-7.5 cells for 5 h, after which 40 IU TECIN rIL-2/ml (National Cancer Institute Biological Resource Branch), 1 μg IC, or anti-human NKp30 (clone 210847; R&D Systems) and $^{51}$Cr-labeled P815 cells at an E:T ratio of 100:1 were added to each well. At 8 and 12 h after the addition of $^{51}$Cr-labeled P815 target cells, 50 μl supernatant was removed and transferred to Kimble tubes. $^{51}$Cr release was then measured and percentage-specific lysis calculated as described.

**Results**

**Development of a novel assay platform to detect HCV effects on NK cells**

To study the effects of HCV-infected Huh-7.5 cells on NK cell functions, we developed a microtiter assay by infecting Huh-7.5 cells in 96-well microtiter plates with JFH1 T or JFH1D1 HCVcc (Fig. 1A). Seventy-two hours after infection, we performed a CELISA to determine the extent of infection. As MOI increased, we observed a 6-fold increase in relative HCV protein levels (Fig. 1B). To determine the impact of HCV infection on Huh-7.5 cell proliferation and viability, we assessed cellular metabolic activity with an MTT assay. At an MOI of 4.8, there was an ∼30% reduction in MTT metabolism at 72 h, reflecting reduced cell viability or proliferation (Fig. 1C). Despite the decline in Huh-7.5 cell metabolism or proliferation, there was little overt cell death indicated by indirect immunofluorescence (Fig. 1D). Consequently, a high MOI of either 3.2 or 4.8 was chosen for subsequent experiments to optimize virus infection levels in this system.

**HCV-infected Huh-7.5 cells reduce NK cytotoxicity**

Using the microtiter system described above, we measured NK cell cytotoxicity in the presence of uninfected and HCV-infected Huh-7.5 cells by adding $^{51}$Cr-labeled target cells and freshly isolated PBMCs from 12 consenting healthy donors in 5-h $^{51}$Cr-release assays. The presence of HCV-infected Huh-7.5 cells reduced NK cell cytotoxicity against K562 cells by a mean of 23 ± 2.2% (SEM; $p < 0.0001$, Student paired $t$ test; $n = 12$) (Fig. 2A). To determine whether other mechanisms of NK cell cytotoxicity were affected by HCV-infected Huh-7.5 cells, we used a $^{51}$Cr-labeled anti–HLA class I-coated B lymphoblastoid cell line (CIR-B27) as targets and measured ADCC. The presence of HCV-infected Huh-7.5 cells reduced ADCC by a mean of 18 ± 6% (SEM; $p = 0.001$, Student paired $t$ test; $n = 6$) (Fig. 2B). Although donors had varying levels of NK cytotoxicity, the percentage decline in cytotoxicity was consistently between 20 and 27% and all healthy donors displayed a reduction of NK cell cytotoxicity in the presence of HCV-infected cells. Furthermore, we repeated assays with purified NK cells to test whether the inhibition was mediated directly against the NK cells or whether another PBMC subset was involved. Specific lysis of K562 cells by purified NK cells (85–95% purity, data not shown) declined by a mean of 15 ± 2.4% (SEM; $p = 0.001$, Student paired $t$ test; $n = 6$) in the presence of HCV-infected compared with uninfected Huh-7.5 cells (Fig. 2C).

**HCV-infected Huh-7.5 cells reduce NK cell IFN-γ and TNF-α production**

We next investigated whether HCV-infected cells perturbed NK cell cytokine production during 5 h incubation. Flow cytometry of CD56$^+$CD3$^-$ PBMCs revealed reduced NK degranulation (CD107a expression) in the presence of HCV-infected Huh-7.5 cells by adding $^{51}$Cr-labeled target cells and freshly isolated PBMCs from 12 consenting healthy donors in 5-h $^{51}$Cr-release assays. The presence of HCV-infected Huh-7.5 cells reduced NK cell IFN-γ and TNF-α production.
cells compared with uninfected Huh-7.5 cells (Fig. 3A), corroborating the effect seen on NK cytotoxicity by 51Cr-release assays. Although the decline in NK cell cytotoxicity mediated by HCV-infected Huh-7.5 cells, as measured by CD107a production, was less consistent than as measured in 51Cr-release assays, NK degranulation was inhibited by a mean of 33% ($p = 0.0313$, one-

FIGURE 3. Effect of HCV-infected cells on NK degranulation and cytokine production. PBMCs were incubated as described with uninfected or HCV-infected Huh-7.5 cells and K562 at an E:T ratio of 5:1 and then removed for analysis by flow cytometry. Representative plots are shown ($n = 5–6$) with gating on the CD16$^+$CD3$^-$ or CD56$^+$CD3$^-$ lymphocyte population. (A) Degranulation was assessed by measuring CD107a expression, and (A, B) cells treated with brefeldin A for 4 h were analyzed for IFN-$\gamma$ and TNF-$\alpha$ production. (C) Summary plots showing effects of HCV-infected cells on CD107a expression (left panel) or IFN-$\gamma$ and TNF-$\alpha$ production (right panel).
tailed Wilcoxon signed rank test; \( n = 5 \) (Fig. 3C, left panel). Fig. 3A and 3B show representative plots of CD56\(^+\)CD3\(^+\) PBMC NK IFN-\( \gamma \) and TNF-\( \alpha \) production. Analysis of permeabilized CD56\(^+\)CD3\(^+\) PBMCs also indicated that production of NK IFN-\( \gamma \) and TNF-\( \alpha \) was reduced by HCV-infected Huh-7.5 cells a mean of 26 and 18\%, respectively (\( p = 0.0156 \), one-tailed Wilcoxon signed rank test; \( n = 6 \)) (Fig. 3C, right panel). We observed no difference in NK IL-4 or IL-10 production in the presence of uninfected versus HCV-infected Huh-7.5 cells (data not shown). These data demonstrate a generalized effect of HCV-infected cells on NK activation affecting both cytotoxicity and cytokine release.

**HCV-infected cells inhibit NK cell functions by direct contact**

Because infectious virus particles are released from HCV-infected Huh-7.5 cells, we next determined whether HCV particles, soluble factors released from HCV-infected cells, or the HCV-infected Huh-7.5 cells themselves mediated NK cell inhibition. PBMCs were incubated in a 5 h \( ^{51}\text{Cr} \)-release assay with titered infectious cell-free HCVcc or complete DMEM as control. Cell-free virus had no effect on NK cell cytotoxicity assessed by K562 lysis (Fig. 4A). To determine whether cytokines or other soluble factors were downregulating NK cell functions, we performed a 5 h transwell assay in which both PBMCs and target K562 cells were sequestered from direct contact with uninfected or HCV-infected Huh-7.5 cells. Whereas NK cytotoxicity declined a mean of 13 \( \pm \) 3.0\% (SEM; \( p = 0.0034 \), Student paired \( t \) test; \( n = 6 \)) when in direct contact with HCV-infected Huh-7.5 cells, an insignificant decline of 3.5 \( \pm \) 1.6\% (SEM; \( n = 6 \)) in NK cell cytotoxicity occurred when NK cells were sequestered from HCV-infected Huh-7.5 cells (Fig. 4B). These results indicate that neither cell-free virus nor...

**FIGURE 4.** Effect of cell-free virus and infected cell supernatant on NK cytotoxicity. (A) PBMCs were incubated with \( ^{51}\text{Cr} \)-labeled K562 cells at an E:T ratio of 60:1 for 5 h with DMEM control (dark shading) or infectious HCV supernatant (\( \sim 50,000 \text{ FFUs/well} \), light shading). Percentage inhibition is represented by a black line graph and calculated as in Fig. 2. (B) NK cell cytotoxicity was compared with direct exposure to HCV-infected Huh-7.5 cells (left graphs) or when PBMCs were physically separated from HCV-infected Huh-7.5 cells by a semipermeable membrane (right graphs). Error bars indicate SEM of four to six replicates.

**FIGURE 5.** Relationship between inhibition of NK cytotoxicity and HCV infection levels. Percentage NK inhibition (gray line graph) was assessed in a cytotoxicity assay as described at various HCV MOIs. Following the assay, HCV-infected Huh-7.5 cells were fixed, permeabilized, and relative HCV protein levels compared by CELISA (black bar graph). Error bars indicate SEM of 12 replicates.

**FIGURE 6.** The effect of blocking NK CD81 or HCV E2 on NK cell cytotoxicity. (A) Huh-7.5 cells infected at an MOI of 1.3 with JFH1\(_{\text{D1}}\) HCV were pretreated with 1 mg/well anti-E2 mAb or IC prior to \( ^{51}\text{Cr} \)-release assays. The dark and light shading of the bars shows cytotoxicity in the presence of uninfected and HCV-infected Huh-7.5 cells, respectively. The effect of pretreating HCV-infected Huh-7.5 cells with anti-E2 (right graphs) is compared with pretreatment with an IC (left graphs). (B) Purified NK cells were pretreated with 1 mg/10\(^6\) cells of either anti-CD81 (right graphs) or IC (left graphs) prior to cytotoxicity assays. Error bars indicate SEM of four to six replicates.
soluble factors released from HCV-infected Huh-7.5 cells inhibit NK cytotoxicity. Thus, direct cell-to-cell contact between NK cells and HCV-infected Huh-7.5 cells is necessary to inhibit NK cell functions. We also noted that NK cell inhibition was directly related to the level of HCV infection of Huh-7.5 cells. At a high MOI with the greatest relative levels of HCV proteins, we observed maximum inhibition of NK cell cytotoxicity (Fig. 5). Therefore, the inhibition of NK cytotoxicity mediated by HCV-infected Huh-7.5 cells is dependent on direct cell-to-cell contact and related to the extent of HCV infection.

Blocking HCV E2 or NK CD81 does not abrogate NK cell inhibition

Previous studies indicated that HCV E2 protein binding to NK CD81 can affect NK function (48, 49). Although we could not detect extracellular HCV E2 expression on HCV-infected Huh-7.5 cells by immunofluorescence microscopy (data not shown), we performed Ab-masking experiments to determine whether an E2/CD81 interaction could be downregulating NK cell functions in our system. JFH1T has a mutation at amino acid 417 in E2 (N→S) that prevents anti-HCV E2 mAb AP33 binding and could not be used for this experiment. JFH1D1 lacks the E2 adaptive mutation but invokes the same effects on NK cell cytotoxicity as JFH1T (shown in Fig. 6A). HCV JFH1D1-infected Huh-7.5 cells were pretreated with an anti-HCV E2 mAb (AP33) that binds E2 at amino acid residues 412–423 and blocks CD81/E2 interactions (58, 59), then incubated with PBMCs and 51Cr-labeled K562 cells in a 5 h 51Cr-release assay. Pretreating HCV-infected cells with anti-HCV E2 to block potential HCV E2/NK CD81 interaction had no effect on NK cell inhibition (Fig. 6A). Because CD81 is expressed on lymphocytes other than NK cells, we tested the effect of anti-human CD81 treatment on purified NK cells in our assay system. Treating purified NK cells with anti-human CD81 Ab also did not prevent the decline in NK cytoxicity mediated by HCV-infected Huh-7.5 cells (Fig. 6B). The intent was not to cross-link NK CD81 receptors, but to have soluble CD81 Ab block potential E2/CD81 interactions that could be involved in NK cell inhibition. Pretreatment of PBMCs with soluble anti-CD81 alone had no effect on NK cell cytotoxicity assessed by 51Cr-labeled K562 cell lysis in the absence of Huh-7.5 cells compared with 51Cr-labeled K562 cell lysis by IC-pretreated PBMCs (data not shown). Treatment of HCV-infected Huh-7.5 cells with anti-E2 and treatment of purified NK cells with anti-CD81 both indicate that E2/CD81 interactions are not involved in the inhibition of NK cells by HCV-infected cells in this system.

NKp30 is downregulated by direct interaction with HCV-infected cells

To address the mechanism by which HCV-infected Huh-7.5 cells inhibit NK cell functions, we examined surface expression of various NK cell receptors. Flow cytometric data showed that NKp46, NKG2A, CD16, and NKG2D expression levels were unaltered over a 5 h incubation of PBMCs with HCV-infected compared with uninfected Huh-7.5 cells (data not shown). Previous studies indicated

![Figure 7](http://www.jimmunol.org/)
Student paired through NKp30 declined a mean of 20.6% (SEM; \( p = 0.003 \), Student paired \( t \) test; \( n = 6 \)) as assessed by mean fluorescence intensity (MFI) following a 5 h incubation with HCV-infected Huh-7.5 cells (representative histogram shown in Fig. 7A, summary in Fig. 7B). We established a redirected lysis assay system to directly test whether the downregulation of NKp30 mediated by HCV-infected cells affected NK cytotoxicity. After incubating PBMCs with uninfected or HCV-infected Huh-7.5 cells for 5 h, we assessed NKp30-redirected lysis of P815 cells infected Huh-7.5 cells for 5 h, we assessed NKp30-redirected lysis of P815 cells through NKp30 declined a mean of 20.6 ± 4.4% (SEM; \( p = 0.0111 \), Student paired \( t \) test; \( n = 6 \)) in the presence of HCV-infected compared with uninfected Huh-7.5 cells (Fig. 7C). There was no significant reduction of background P815 cell lysis in assays with an IC. Thus, downregulation of NKp30 expression following exposure of NK cells to HCV-infected Huh-7.5 cells affects NKp30-mediated NK cytotoxicity.

Because NKp30 surface expression was reduced, and direct cell-cell contact with HCV-infected cells was required to inhibit NK cell functions, we examined the possible upregulation of an NKp30 ligand on Huh-7.5 cells following HCV infection. We observed an increase in surface binding of an NKp30-IgG1 Fc fusion protein to HCV-infected compared with uninfected Huh-7.5 cells (Fig. 8A). To account for potential nonspecific binding of the NKp30-IgG1 Fc fusion protein to the HCV-infected Huh-7.5 cells, we compared binding of a control KIR3DS1-IgG1 Fc fusion protein to uninfected and HCV-infected Huh-7.5 cells. Although there was no increased binding of the control KIR3DS1-IgG1 Fc fusion protein to HCV-infected compared with uninfected Huh-7.5 cells (Fig. 8A), we noted a 2-fold (\( p = 0.0286 \), Mann–Whitney \( U \) test; \( n = 4 \)) increase in NKp30-IgG1 Fc protein binding upon exposure to HCV-infected Huh-7.5 cells (Fig. 8B). This indicates that HCV infection of Huh-7.5 cells results in upregulation of a cell surface molecule that binds NKp30 and potentially antagonizes NK cell function.

**Discussion**

Through interaction with host innate and adaptive immune responses, HCV has evolved mechanisms to circumvent immune pressures and establish chronic infection. Cytokines produced by HCV-infected cells. Furthermore, NK cells play a role in clearance or control of HCV infection (42, 62, 63).

In this study, we directly assessed the ability of HCV-infected cells to inhibit NK cell functions, we examined the possible upregulation of an NKp30 ligand on Huh-7.5 cells following HCV infection. In contrast, we found that NCR NKp30 expression was downregulated by a mean of 18 ± 4.5% (SEM; \( p = 0.003 \), Student paired \( t \) test; \( n = 6 \)) when compared with uninfected Huh-7.5 cells (Fig. 8B). This indicates that HCV infection of Huh-7.5 cells results in upregulation of a cell surface molecule that binds NKp30 and potentially antagonizes NK cell function.

**Toxicity and cytokine production via the cross-linking of NK CD81.** As a consequence, inhibition of NK cell functions through HCV E2/NK CD81 cross-linking is often proposed as a means to HCV persistence (64–67). Although cross-linking NK CD81 results in a negative signaling cascade, which dampens NK cell cytotoxicity and cytokine production (68), we used soluble anti-CD81 in our system to prevent potential E2/CD81 interactions. Treating purified NK cells with an anti-CD81 Ab did not relieve the inhibition of NK cell cytotoxicity mediated by HCV-infected cells. Furthermore, we found no evidence of surface HCV E2 expression on HCV-infected Huh-7.5 cells, and disruption of potential HCV E2/NK CD81 interactions by blocking HCV E2 also did not relieve the decline in NK cell cytotoxicity mediated by HCV-infected cells. Our results, combined with previous studies indicating that HCV E2 molecules as displayed on HCV JFH1 virions do not inhibit NK cell functions (50), suggest that an HCV E2/NK CD81 interaction is not the primary cause of impaired NK cell functions mediated in vitro or in vivo by HCV-infected cells.
Although HLA-E can be stabilized and upregulated by HCV core peptide (amino acids 35–44) and provides a ligand for inhibitory NK receptor KIR2DL1/CD158a (59), we and others did not observe increased expression of HLA-E on HCV-infected cells (52). Despite inhibition of ADCC, CD16 expression levels were also unaltered on NK cells exposed to HCV-infected cells. NKp30 was the only NK receptor we examined with decreased cell surface expression, which was reflected in a significant decrease of NKp30-redirected lysis of P815 cells. These in vitro data corroborate the ex vivo observation of reduced circulating NKp30-expressing NK cells in individuals with chronic HCV infection (47). Interestingly, CD16 and NKp30 associate with the same ε-chain adapter molecule to facilitate the signaling cascade that ultimately results in NK cytotoxic secretion and cytotoxicity (69). Antagonism of this signaling pathway through interaction with a putative NKp30 ligand upregulated on HCV-infected cells could undermine the rapid NK cell inhibition described in this study. Although this ligand may interact selectively with NKp30, disrupted signaling through this receptor could also affect ε-chain–dependent signaling through CD16 to decrease ADCC. A previous study by Yoon et al. (52) showed that NK cytotoxicity and IFN-γ production was reduced following an 18 h coculture with HCV-infected cells and noted a decline in NKp30 and NKG2D expression on NK cells. We did not observe a reduction in NKG2D expression on NK cells after a 5 h incubation with infected cells.

Our data show impairment of NK cytotoxicity and cytokine production in the presence of HCV-infected Huh-7.5 cells. This corresponds with downregulation of the NCR NKp30 and increased binding of an NKp30-IgG1 Fcγ fusion protein to the surface of HCV-infected cells. An antagonistic NKp30 ligand induced on HCV-infected cells could inhibit NK cell–mediated immune mechanisms and favor chronic HCV infection in a manner similar to that observed for CMV. Arnon et al. (41) demonstrated a direct antagonistic interaction between human CMV tegument protein pp65 and NK cell–activating receptor NKp30 that reduced NK cell cytotoxicity by dissociation of the ε-chain adapter protein from NKp30. Further studies addressing the nature of a putative antagonistic ligand for the NKp30 receptor expressed on HCV-infected cells are required. NKp30 exhibits structural homology to programmed cell death protein 1 (PD-1), CTLA-4, and CD28 receptors, all of which recognize members of the B7 family of ligands (70–72). NKp30 itself recognizes B7-H6 ligands, which trigger NK cell activation. Homologs of the B7-H6 ligand, such as B7-H1 (also called programmed cell death ligand 1 or CD274) and B7-H4 (also called B7S1 or B7x) or other yet unidentified ligands may bind NKp30 and antagonize NK cell activation (73–76).

The negative influence of HCV-infected cells on NK cell cytokine release and cytotoxicity reported in this study represents yet another example of a chronic pathogen subverting NK cell behavior, affirming a potentially important role for NK cells in viral containment. Elucidation of an antagonistic NKp30 ligand of host or viral origin on HCV-infected cells could introduce new strategies to preserve NK cell function in HCV-infected individuals and treat or prevent chronic HCV infection.

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Disclosures

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