Cell-to-Cell Contact with Hepatitis C Virus-Infected Cells Reduces Functional Capacity of Natural Killer Cells[▽][†]

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The distinct feature of hepatitis C virus (HCV) infection is a high incidence of chronicity. The reason for chronic HCV infection has been actively investigated, and impairment of innate and adaptive immune responses against HCV is proposed as a plausible cause. Whereas functional impairment of HCV-specific T cells is well characterized, the role and functional status of natural killer (NK) cells in each phase of HCV infection are still elusive. We therefore investigated whether direct interaction between NK cells and HCV-infected cells modulates NK cell function. HCV-permissive human hepatoma cell lines were infected with cell culture-generated HCV virions and cocultured with primary human NK cells. Cell-to-cell contact between NK cells and HCV-infected cells reduced NK cells' capacity to degranulate and lyse target cells, especially in the CD56$^{\text{dim}}$ NK cell subset, which is characterized by low-density surface expression of CD56. The decrease in degranulation capacity was correlated with downregulated expression of NK cell-activating receptors, such as NKG2D and NKp30, on NK cells. The ability of NK cells to produce and secrete gamma interferon (IFN-γ) also diminished after exposure to HCV-infected cells. The decline of IFN-γ production was consistent with the reduction of NK cell degranulation. In conclusion, cell-to-cell contact with HCV-infected cells negatively modulated functional capacity of NK cells, and the inhibition of NK cell function was associated with downregulation of NK-activating receptors on NK cell surfaces. These observations suggest that direct cell-to-cell interaction between NK cells and HCV-infected hepatocytes may impair NK cell function *in vivo* and thereby contribute to the establishment of chronic infection.

Hepatitis C virus (HCV) is an important human pathogen related to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (13). Of infected patients, 60% to 80% develop chronic hepatitis, which is thought to be a result of impairment of innate and adaptive immune responses (28, 29). Although many aspects of functional impairment in the adaptive immune response to HCV infection are revealed, the role and function of natural killer (NK) cells in the innate immune response to HCV infection is unclear so far (16, 28).

NK cells direct the innate immune response in virus infection by killing infected cells and secreting cytokines, such as gamma interferon (IFN-γ), which inhibits viral replication (22). The antiviral activity of NK cells is controlled by the integration of activating and inhibitory signals. Target cells that are distressed by virus infection display activating ligands on their surfaces, and binding of the ligands to activating receptors on NK cells leads to activation of NK cells and lysis of infected target cells (20, 37). To overcome antiviral NK cell responses, viruses have developed strategies to impair interaction between NK cell-activating receptors and their ligands. For example, human cytomegalovirus (HCMV) evades NK cell responses through inhibiting expression of major histocompat-

ibility complex (MHC) class I-related chain B (MICB), UL-16 binding protein 1 (ULBP-1), and ULBP-2, which are ligands for activating receptor NKG2D, on the surface of infected cells (19). On the other hand, inhibition of activating receptor expression on NK cell surfaces may also suppress NK cell responses (39). Because they are readily involved in the activation of resting NK cells (5), a reduced display of constitutively expressed activating receptors, such as NKG2D, NKp30, NKp46, DNAM-1, and 2B4, may dampen the functional capacity of NK cells to fight against viral infection. Cytokines secreted in response to HCMV infection indeed inhibit NKG2D expression on the surface of NK cells and limit the ability of NK cells to exert cytotoxicity against target cells (25).

The prominent role of NK cells in the innate immune response to virus infection has prompted studies of the NK cell phenotype and functional properties in HCV infection. These include *in vitro* studies using HCV replicon systems, expression vectors encoding HCV proteins, and recombinant HCV proteins (9, 12, 24, 35, 36). For various reasons, these studies yielded contradictory results (12, 24, 35). It is difficult to interpret interaction between NK cells and HCV-infected cells in noninfectious models, which do not completely simulate the natural life cycle of HCV (19). *Ex vivo* studies using peripheral blood mononuclear cells (PBMCs) from patients with hepatitis C also showed discrepancies in the functional status of NK cells in HCV infection (1, 11, 15, 26, 27, 30).

Though small-animal models for immunologic research are not readily available yet, recently developed cell culture systems that generate infectious HCV virions *in vitro* provide more physiological settings in which to study interaction be-

tween HCV and the innate immune system precisely (21, 38, 41). Prior to introduction of the HCV cell culture systems, researchers used recombinant envelope proteins which were immobilized on the surface of a plate to investigate the effect of HCV envelope proteins on the NK cell function. Those studies showed that envelope protein E2 inhibits NK cells *in vitro* (9, 36). The recombinant E2 cannot, however, completely represent natural E2 proteins that are immobilized on the lipid bilayer envelope of HCV virions. In fact, naturally expressed E2 proteins on the surface of cell culture-generated infectious HCV particles do not inhibit NK cell function (8, 40), unless the cell-free virions themselves are immobilized and concentrated on the surface of a plate (8). This shows the utility of HCV cell culture systems for a close examination of interaction between HCV and immune cells. The importance of employing HCV virions is also proved by a recent report that describes inhibition of NKG2D-dependent NK cell responses by HCV nonstructural protein 5A (NS5A). It suggested that NS5A may be released from hepatoma cell line Huh7.5.1 which is infected with cell culture-generated HCV, and the released NS5A inhibits NK cell function through an imbalance of inflammatory cytokines (30).

Even if cell-free HCV particles may affect NK cell function in a specific micromilieu that immobilizes and concentrates the virions and extracellular NS5A may suppress NK cells after release from HCV-infected hepatocytes, the cell-to-cell contact of NK cells with HCV-infected hepatocytes may also inhibit NK cell function in a more general and direct way. The discovery of modulatory effects of HCV proteins on innate immune responses of infected hepatocytes (10) suggests that the virus may control NK cell function through regulatory pathways of infected hepatocytes. We therefore investigated whether HCV modulates NK cell function through direct interaction of NK cells with HCV-infected hepatocytes. To investigate this precisely, we evaluated changes in NK cell function after interplay with HCV-infected cells *in vitro*. Cell-to-cell contact with HCV-infected Huh7 or Huh7.5 cells significantly reduced NK cell degranulation, target cell lysis, and IFN-γ secretion. The reduction of degranulation correlated with the reduced expression of NK cell-activating receptors, such as NKG2D and NKp30, on the surface of NK cells.

MATERIALS AND METHODS

Generation of HCV. HCV (genotype 2a, JFH1 strain) was produced as described previously (40). The JFH1 expression construct (provided by T. Wakita, National Institute of Infectious Diseases, Tokyo, Japan) was linearized, and full-length JFH1 RNA was transcribed using a MEGAscript T7 *in vitro* transcription kit (Ambion, Austin, TX). Huh7.5 cells (provided by C. Rice, The Rockefeller University, New York, NY) were transfected with the transcribed RNA using DMRIE-C reagent (Invitrogen, Carlsbad, CA). The supernatant collected from HCV RNA-transfected Huh7.5 cells was used to infect naïve Huh7.5 cells to make HCV stocks. The supernatant of HCV-infected Huh7.5 cells was filtered through 0.45-μm-pore-size syringe filters (Millipore, Billerica, CA) and concentrated using Centricon Plus-70 (Millipore). HCV titer was determined as previously described (17).

Isolation of primary human NK cells. PBMCs were isolated from buffy coats of healthy, HCV-negative individuals by applying a Ficoll-Paque (GE Healthcare Life Sciences, Piscataway, NJ) density gradient. Separated PBMCs were frozen and stored in a liquid nitrogen tank. All donors were informed for research use of their blood and gave written consent under protocols approved by the institutional review boards of Yonsei University Health System.

Frozen PBMCs were thawed later and suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100

μg/ml streptomycin, and 2.05 mM L-glutamine (HyClone, South Logan, UT) in the presence of 50 U/ml Benzonase (Novagen, Madison, MI), which was added to remove DNA released from dead cells and to prevent clumping of PBMCs (6). Cells were centrifuged and supernatant was removed. After washing with complete RPMI medium without Benzonase, PBMCs were resuspended in complete RPMI medium at approximately 4×10^6 cells/ml. Cells were rested without any stimulation at 37°C in a CO_2 incubator overnight.

Primary human NK cells were isolated from the rested PBMCs by negative selection using a human NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of isolated NK cells was measured by flow cytometry after staining the cells with anti-CD3-fluorescein isothiocyanate (FITC) and anti-CD56-phycoerythrin (PE) antibodies (BD Biosciences, San Jose, CA). When the frequency of $CD3^-CD56^+$ cells was above 90% and that of $CD3^+$ cells was less than 1%, isolated cells were used for subsequent experiments.

Analysis of NK cell degranulation. Huh7 and Huh7.5 cells were suspended in Dulbecco's modified Eagle medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (HyClone) and seeded in 96-well flat-bottom plates (Nunc, Roskilde, Denmark) at 1×10^4 cells per well. The next day, Huh7 and Huh7.5 cells were infected with HCV at a multiplicity of infection (MOI) of 10 and an MOI of 1, respectively. Three days after infection, when NK cell responses peak in general virus infections *in vivo* (23), supernatant was removed from the culture wells, and NK cells in complete RPMI medium were added to the infected cells at approximately a 1:1 ratio. Recombinant human interleukin 12 (IL-12) (PeproTech, Rocky Hill, NJ) was added to the culture wells for some experiments. To assess direct cytotoxicity of NK cells against HCV-infected cells, NK cells were cocultured with uninfected or HCV-infected Huh7 or Huh7.5 cells for 4 h in the presence of 20 μl/ml anti-CD107a-FITC or anti-CD107a-PE antibodies (BD Biosciences). After the coculture, cells were stained with anti-CD56-PE-Cy7 antibody (BD Biosciences). To evaluate changes in the degranulation capacity of NK cells, NK cells were first cocultured with uninfected or HCV-infected cells for 18 h, and the NK cells were harvested and further cocultured with the same number of K562 cells in the presence of anti-CD107a in 96-well round-bottom plates (Corning Inc., Corning, NY) for 4 h. After the coculture with K562 target cells, NK cells were stained with anti-CD56-PE-Cy7 antibody. Cells were fixed with 4% paraformaldehyde and analyzed with an LSR II flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR). NK cell subsets were selectively analyzed by exclusively gating on $CD56^{dim}$ or $CD56^{bright}$ NK cells among total NK cells by using the analysis software.

Analysis of target cell lysis by NK cells. A specific lysis assay was performed as previously reported (33). To assess direct cytolytic activity of NK cells against HCV-infected cells, uninfected or HCV-infected Huh7.5 cells were harvested after 72 h postinfection and labeled with a PKH-26 red fluorescent cell linker kit according to the manufacturer's instructions (Sigma-Aldrich, Saint Louis, MO). PKH-26-stained Huh7.5 cells were then cocultured with NK cells for 4 h in 96-well round-bottom plates at effector-to-target (E:T) ratios of 10:1, 5:1, 2.5:1, and 1.25:1. To evaluate changes in the cytolytic capacity of NK cells, NK cells were cocultured with uninfected or HCV-infected Huh7.5 cells for 18 h. NK cells were then harvested and cocultured with PKH-26-stained K562 cells for 4 h in 96-well round-bottom plates at E:T ratios of 5:1, 2.5:1, and 1.25:1. After the coculture, cells were stained with 0.2 μM TO-PRO-3–iodide (Invitrogen) to identify permeabilized target cells. Stained cells were analyzed by flow cytometry. Percent specific lysis was calculated as follows: (% TO-PRO-3$^+$PKH-26$^+$ K562 cells in coculture − % TO-PRO-3$^+$PKH-26$^+$ K562 cells in medium)/(% TO-PRO-3$^+$PKH-26$^+$ K562 cells in detergent − % TO-PRO-3$^+$PKH-26$^+$ K562 cells in medium) × 100.

Analysis of IFN-γ production. NK cells were cocultured with uninfected or HCV-infected Huh7 or Huh7.5 cells for 18 h, and then the NK cells were harvested and added to 96-well round-bottom plates. To detect IFN-γ secretion, NK cells were stimulated with the same number of K562 cells and 1 ng/ml IL-12 for 6 h. To measure intracellular IFN-γ, NK cells were treated with the same number of K562 cells, 10 ng/ml IL-12, and 100 ng/ml IL-15 (ATGen, Seongnam, South Korea) in the presence of brefeldin A (BD Biosciences) for 6 h. After the stimulation, supernatant was collected from the cultures and immediately stored at −20°C for later cytokine analysis or cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences). Secreted IFN-γ was measured with a human IFN-γ enzyme-linked immunosorbent assay kit (PBL Interferon Source, Piscataway, NJ) according to the manufacturer's instructions. Intracellular IFN-γ was detected by staining with Alexa Fluor 488-conjugated anti-IFN-γ antibody (BD Biosciences) and flow cytometry.

Contact-free coculture. Huh7 cells were seeded in 12-well culture plates (BD Biosciences) at 1.2×10^5 cells per well and infected with HCV. Seventy-two

hours postinfection, filter well inserts (Transwell; Corning) containing NK cells, the number of which was approximately the same as that for Huh7 cells, were inserted into the 12-well plates. After an 18-h contact-free coculture, NK cell degranulation was assessed.

Analysis of NK ligand and NK receptor expression. To determine the effect of HCV infection on NK ligand expression, Huh7 and Huh7.5 cells were infected with the virus. Seventy-two hours postinfection, cells were stained with combinations of anti-HLA-ABC-PE, anti-HLA-E-PE, anti-PVR-PE (eBioscience, San Diego, CA), anti-MICA/B-Alexa Fluor 647, anti-TRAIL-PE (BioLegend, San Diego, CA), anti-ULBP-1–PE, anti-ULBP-2–APC, and anti-ULBP-3 (R&D Systems, Minneapolis, MN) antibodies, followed by DyLight 649-conjugated F(ab′)$_2$ goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). To determine changes in NK receptor expression induced by HCV-infected cells, NK cells were cocultured with uninfected or HCV-infected Huh7 or Huh7.5 cells and stained with anti-CD158a-FITC, anti-CD158b-PE, anti-NKG2D-APC, anti-DNAM-1–PE, anti-CD56-PE-Cy7 (BD Biosciences), anti-NKp46-PE, anti-NKp30-APC, anti-2B4-APC (BioLegend), anti-NKG2A-PerCP (R&D Systems), and anti-KLRG1 (Abnova, Taipei, Taiwan) antibodies followed by DyLight 649-conjugated F(ab′)$_2$ goat anti-mouse IgG. Cells were fixed with 4% paraformaldehyde and analyzed by flow cytometry.

Statistical analysis. Paired Student's t tests and repeated-measures analysis of variance (ANOVA) with *ad hoc* Newman-Keuls multiple comparison tests were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Two-sided P values less than 0.05 were considered statistically significant.

RESULTS

NK cells are unable to exert cytotoxicity against HCV-infected hepatoma cell lines. Because functional inhibition of NK cells would limit their ability to degranulate and release cytotoxic molecules from cytotoxic granules upon encounter with virus-infected target cells, NK cell degranulation during contact with HCV-infected cells was assessed by measuring the surface expression of CD107a, which is exposed on the NK cell surface when cytotoxic granules fuse with the plasma membrane (2). After a 4-h coculture with HCV-infected Huh7 or Huh7.5 cells, NK cells showed no or low expression of CD107a (Fig. 1A) and CD25, which is an activation marker for NK cells (Fig. 1B). It appeared that HCV-infected cells were protected from NK cell-mediated killing through suppressing NK cell activation and degranulation. To confirm whether this observation is associated with target cell lysis, we performed a specific lysis assay. Consistent with direct degranulation activity against HCV-infected cells (Fig. 1A), NK cells did not exert cytolytic activity against HCV-infected Huh7.5 cells (Fig. 1C). Huh7 and Huh7.5 cells, which are human hepatoma cell lines, however, might inhibit NK cells by themselves (14) and impair NK cell function regardless of HCV infection. If this was the case, activation of NK cells by HCV-infected cells would be masked even though it might in fact exist.

Cytotoxic capacity of NK cells is reduced after interaction with HCV-infected cells. To exclude possible inhibitory effects of the tumor cell lines, we employed an experimental strategy used by *ex vivo* studies which evaluate functional status and capacity of NK cells collected from HCV-infected patients (1, 3, 27). NK cells which interacted with uninfected or HCV-infected Huh7 or Huh7.5 cells were harvested and cocultured with K562 cells to evaluate their functional status and capacity after contact with HCV-infected cells. The interaction of NK cells with uninfected Huh7 or Huh7.5 hepatoma cell lines indeed reduced their degranulation capacity. NK cells which interacted with HCV-infected Huh7 or Huh7.5 cells showed, however, far less degranulation than NK cells that interacted with uninfected cells (Fig. 2A and B). In accordance with the

changes in degranulation capacity after exposure to HCV-infected cells, interaction with HCV-infected Huh7.5 cells reduced the cytolytic capacity of NK cells (Fig. 2C). It is unlikely, however, that the NK inhibitory signal is activated or the number of inhibitory receptors on the NK cells is increased, as the inhibitory NK cell immunological synapse (17) may not form between NK cells and K562 cells, which do not express NK inhibitory ligands, such as HLA-ABC and HLA-E (14). Instead, the observation suggested that NK cells' ability to respond to activating stimuli decreased.

Importantly, the reduced functional capacity was not related to homeostatic downregulation of NK cell activity or exhaustion of NK cells following initial activation and degranulation, because coculture of NK cells with HCV-infected Huh7 or Huh7.5 cells for 4 h did not result in an initial increase of degranulation and CD25 expression (Fig. 1A and B). Moreover, coculture for 18 h did not increase expression of KLRG1, which is an exhaustion marker (4), on NK cells (Fig. 2D). Taken together, the observations indicated that the interaction with HCV-infected cells reduced NK cells' capacity to degranulate against target cells. Degranulation capacity was, however, slightly recovered, and the difference between NK cells interacting with uninfected cells and those interacting with HCV-infected cells was no longer statistically significant when a high concentration of IL-12, which enhances NK cell cytotoxicity (37), was added (Fig. 2E).

In addition to degranulation, NK cells may exert cytotoxicity to HCV-infected target cells by inducing TRAIL expression on their surfaces (31). TRAIL expression, however, did not increase on NK cells after exposure to HCV-infected cells under our experimental setting (Fig. 2F).

CD56^{dim} NK cells are responsible for the reduced degranulation. Human NK cells comprise two major subsets. CD56^{dim} NK cells, which express low-level CD56, mediate cytotoxicity upon proper recognition of target cells and secrete a small amount of cytokines. In contrast, CD56^{bright} NK cells, which express high-level CD56, secrete a large amount of cytokines upon stimulation and acquire cytotoxicity after prolonged activation (7, 32). We therefore analyzed the two subsets separately to identify their respective responses to HCV-infected cells (Fig. 3). CD56^{dim} NK cells showed a reduced degranulation capacity after interaction with HCV-infected cells, as did the total NK cell preparation, and the differences among groups were significant (Fig. 3A and B, left). On the contrary, CD56^{bright} NK cells showed insignificant changes after contact with HCV-infected cells (Fig. 3A and B, right). Reduced degranulation capacity was, however, no longer statistically significant in CD56^{dim} NK cells in the presence of a high concentration of IL-12 (Fig. 3C, left).

NK cells' ability to produce IFN-γ is reduced in accordance with decreased cytotoxic capacity. Since NK cells produce IFN-γ to control viral replication (32), we evaluated changes in IFN-γ synthesis and secretion by NK cells after exposure to HCV-infected cells. To exclude the inhibitory effect of hepatoma cells on NK cell function, NK cells which interacted with uninfected or HCV-infected Huh7 or Huh7.5 cells in the absence of cytokines were used for coculture with K562 cells in the presence of 1 ng/ml IL-12 or 10 ng/ml IL-12 and 100 ng/ml IL-15 for 6 h. As expected from previous reports (7), the simultaneous engagement of activating NK receptors by acti-
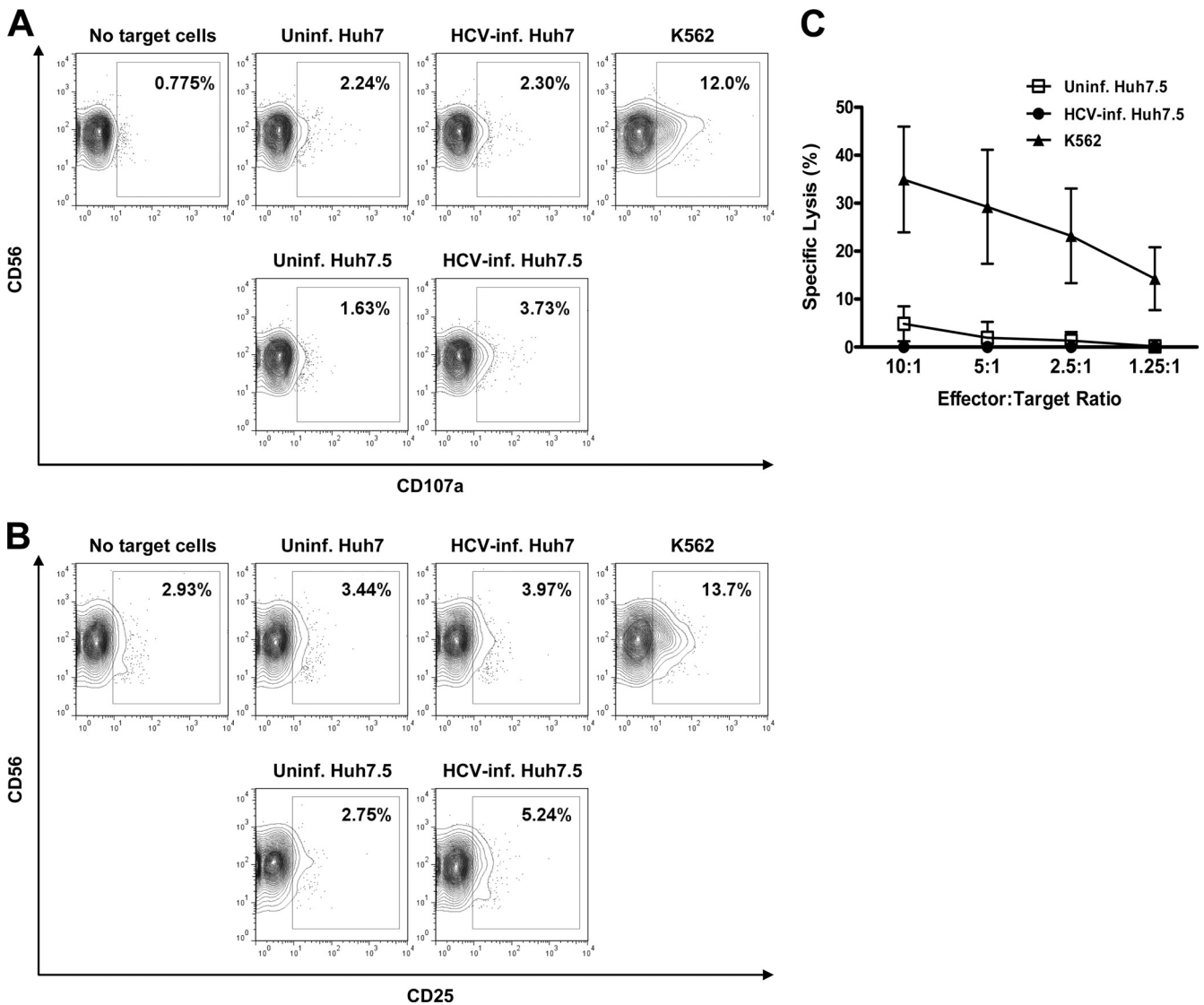
FIG. 1. NK cell cytotoxicity and activation upon direct exposure to HCV-infected cells. Primary human NK cells were cocultured with uninfected (uninf.) or HCV-infected (inf.) Huh7 or Huh7.5 cells for 4 h. (A) NK cell degranulation upon exposure to HCV-infected cells. Degranulation was evaluated by measuring the surface expression of CD107a. NK cells directly cocultured with K562 cells were included as a positive control. Representative contour plots of results for three independent subjects are shown. (B) NK cell activation upon exposure to HCV-infected cells was evaluated by measuring the surface expression of activation marker CD25. As a negative control, NK cells were cultured without target cells. Activation upon direct exposure to K562 cells was measured as a positive control. Representative contour plots of results for three independent subjects are shown. (C) Specific lysis of HCV-infected cells by NK cells. Uninfected or HCV-infected Huh7.5 cells were used as target cells. Direct lysis of K562 cells was included as a positive control. Percent specific lysis was calculated as follows: (% TO-PRO-3$^+$PKH-26$^+$ target cells in coculture − % TO-PRO-3$^+$PKH-26$^+$ target cells in medium)/(% TO-PRO-3$^+$PKH-26$^+$ target cells in detergent − % TO-PRO-3$^+$PKH-26$^+$ target cells in medium) × 100. Each point represents the mean ± standard deviation ($n = 3$ experiments).

vating ligands on K562 cells and cytokine receptors by proinflammatory cytokines induced IFN-γ production (Fig. 4A). The exposure of NK cells to the hepatoma cell lines, however, downregulated IFN-γ production (Fig. 4A). Moreover, the interaction of NK cells with HCV-infected cells further reduced IFN-γ synthesis and secretion by NK cells (Fig. 4A and B). These findings were consistent with the changes in the cytotoxic capacity of NK cells upon exposure to uninfected or HCV-infected Huh7 or Huh7.5 cells (Fig. 2A and B).

To identify which NK cell subset is responsible for the reduced IFN-γ production, we analyzed the two NK cell subsets

separately (Fig. 4C). The hepatoma cell lines Huh7 and Huh7.5 downregulated IFN-γ production by both NK cell subsets. Reduction of IFN-γ production after contact with HCV-infected cells was, however, due mostly to decreased IFN-γ production by CD56^{dim} NK cells. This agreed with the results from degranulation assays, since the decrease in cytotoxicity of total NK cells was due mainly to the reduced degranulation of CD56^{dim} NK cells (Fig. 3A and B).

Continuity of NK cell modulation requires persistent cell-to-cell contact between NK cells and HCV-infected cells. To determine whether the changes induced in NK cells are revers-
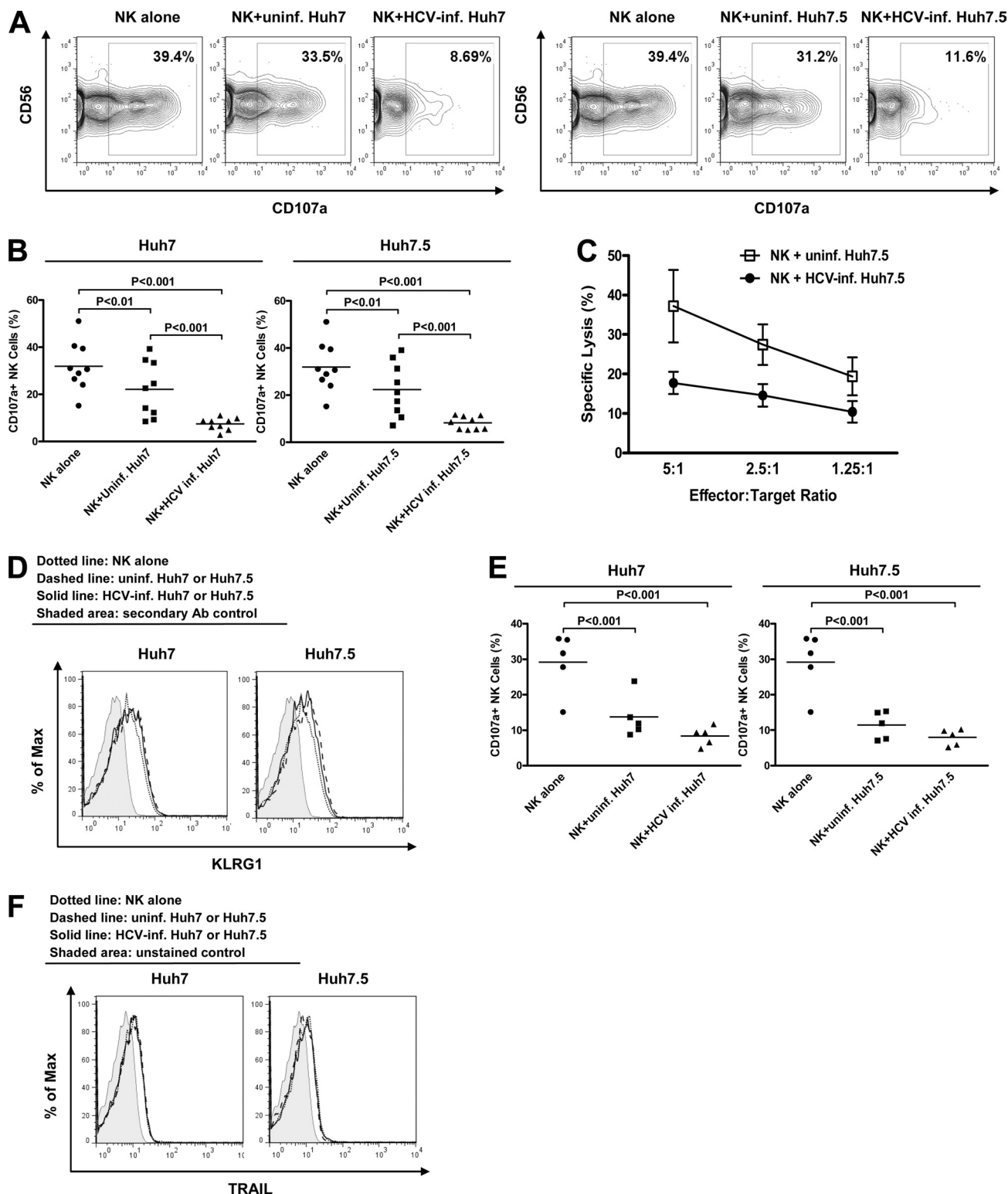
FIG. 2. Reduced cytotoxic capacity of NK cells after interaction with HCV-infected cells. Primary human NK cells were preincubated with uninfected or HCV-infected Huh7 or Huh7.5 cells for 18 h and cocultured with K562 cells at a 1:1 ratio for 4 h in the absence of Huh7 or Huh7.5 cells. (A, B) Reduced degranulation capacity of NK cells after interaction with HCV-infected cells. Representative contour plots of results for nine independent subjects (A) and percentage of CD107a^{+} NK cells for all subjects (B) are shown. NK-alone plots for Huh7 and Huh7.5 experiments are duplicates of the same contour plot (A), and NK-alone columns for Huh7 and Huh7.5 graphs are copies of the same data (B). (C) Decreased cytolytic capacity of NK cells after exposure to HCV-infected cells. Each point represents the mean ± standard deviation ($n = 3$ experiments). (D) Expression of exhaustion marker KLRG1 on NK cells after an 18-h coculture with uninfected or HCV-infected Huh7 or Huh7.5 cells. Representative histograms for two independent subjects are shown. (E) Changes in NK cell degranulation capacity after interaction with HCV-infected cells in the presence of 50 ng/ml IL-12 for 18 h. NK alone columns for Huh7 and Huh7.5 graphs are copies of the same data. (F) Expression of TRAIL on NK cells after an 18-h coculture with uninfected or HCV-infected Huh7 or Huh7.5 cells. Representative histograms for two independent subjects are shown.
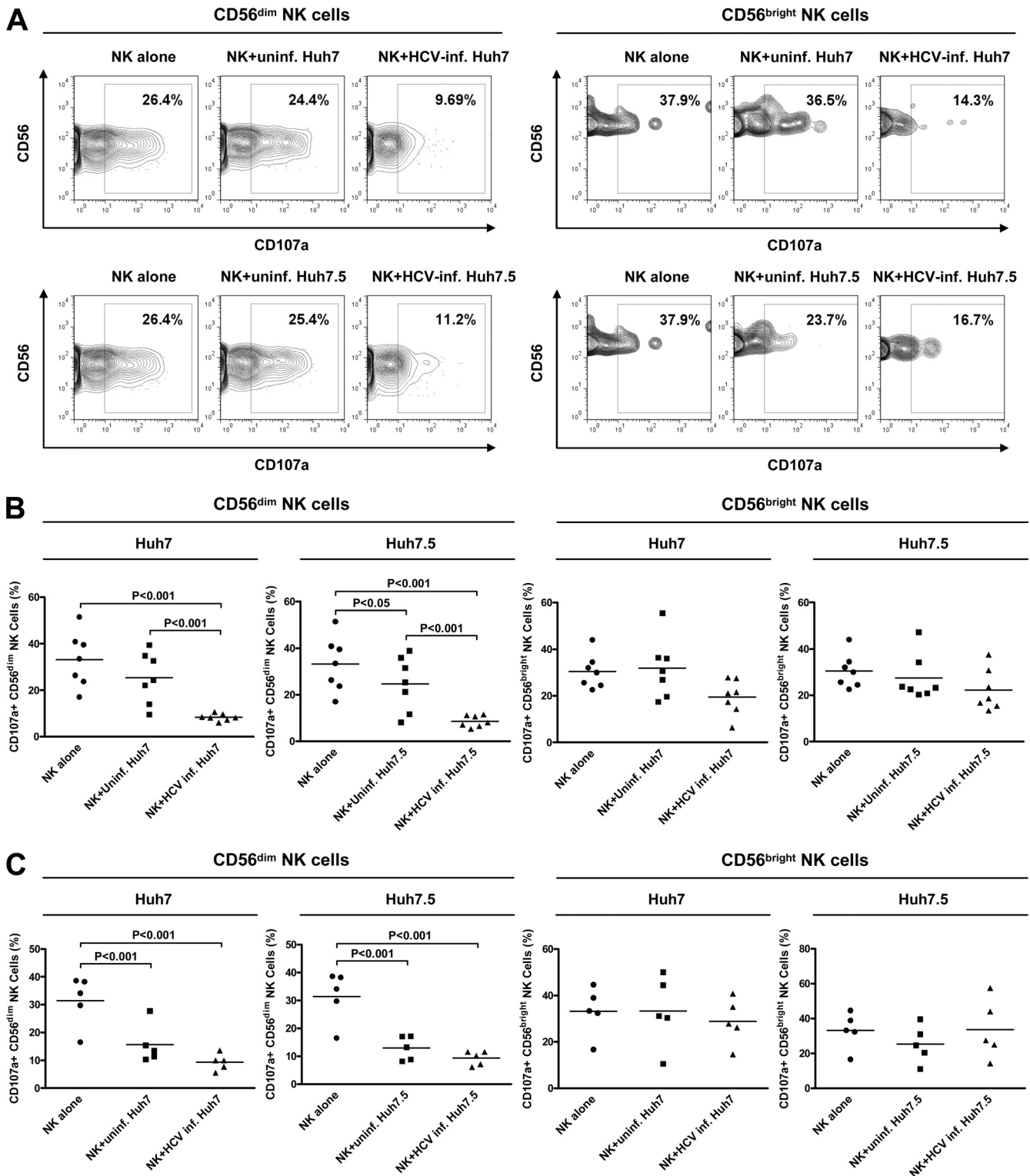
FIG. 3. Decreased degranulation of CD56^{dim} NK cells after exposure to HCV-infected cells. (A, B) Degranulation capacities of CD56^{dim} and CD56^{bright} NK cells were analyzed separately. Representative contour plots of results for seven independent subjects (A) and percentage of CD107a^{+} NK cell subsets for all subjects (B) are shown. (C) Changes in degranulation capacity of NK cell subsets after interaction with HCV-infected cells in the presence of 50 ng/ml IL-12 for 18 h. NK-alone plots for Huh7 and Huh7.5 experiments are duplicates of the same contour plot (A), and NK-alone columns for Huh7 and Huh7.5 graphs are copies of the same data (B, C).
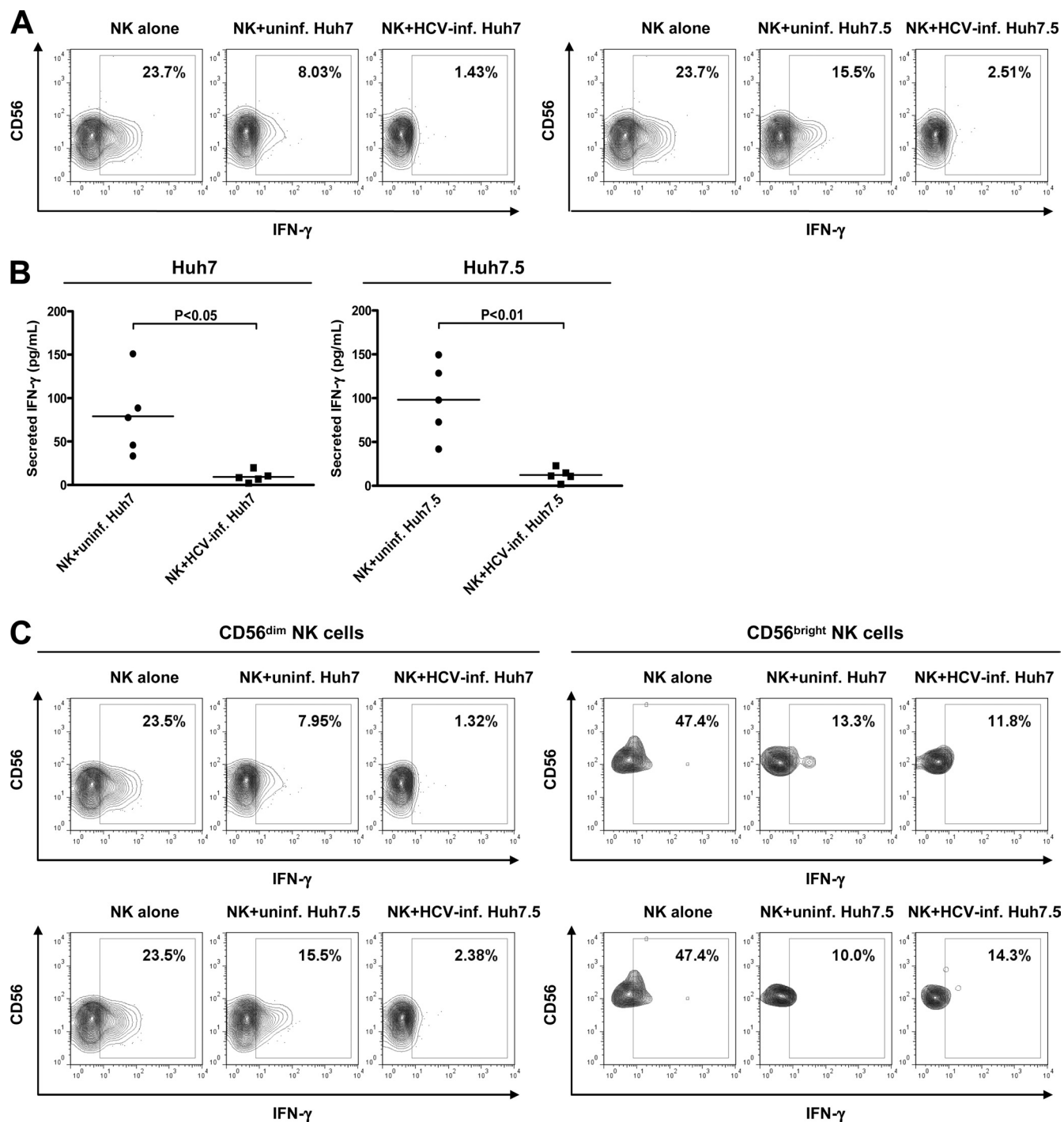
FIG. 4. Reduced IFN-γ production by NK cells after interaction with HCV-infected cells. (A) Primary human NK cells were preincubated with uninfected or HCV-infected Huh7 or Huh7.5 cells in the absence of cytokines for 18 h and cocultured with K562 cells at a 1:1 ratio in the presence of 10 ng/ml recombinant human IL-12 and 100 ng/ml recombinant human IL-15 for 6 h. IFN-γ production was measured by intracellular staining of IFN-γ and flow cytometric analysis. Representative contour plots of the results for three independent subjects are shown. NK-alone plots for Huh7 and Huh7.5 experiments are duplicates of the same contour plot. (B) Primary human NK cells were preincubated with uninfected or HCV-infected Huh7 or Huh7.5 cells for 18 h and cocultured with K562 cells at a 1:1 ratio in the presence of 1 ng/ml recombinant human IL-12 for 6 h. IFN-γ secreted into the culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). (C) IFN-γ productions by CD56dim and CD56bright NK cells were analyzed separately. Representative contour plots of the results for three independent subjects are shown. NK-alone plots for Huh7 and Huh7.5 experiments are duplicates of the same contour plot.
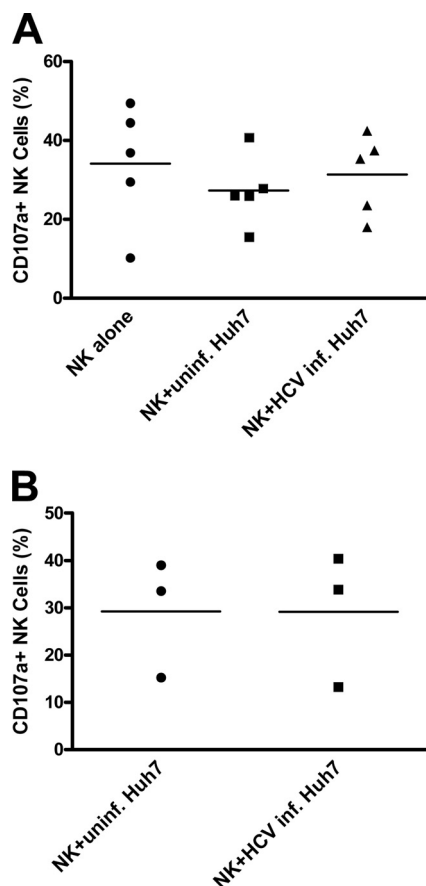
A

B

FIG. 5. NK cell modulation requires persistent cell-to-cell contact of NK cells with HCV-infected cells. (A) Recovery of degranulation. Primary human NK cells were preincubated with uninfected or HCV-infected Huh7 cells for 18 h and rested for 24 h in the absence of Huh7 cells. The NK cells were cocultured with K562 cells for 4 h after the rest. Degranulation was determined by detecting CD107a expression on NK cells. (B) Degranulation after contact-free coculture. Primary human NK cells were preincubated with uninfected or HCV-infected Huh7 cells for 18 h in filter wells to prevent cell-to-cell contact and cocultured with K562 cells for 4 h. Degranulation was determined by detecting CD107a expression on NK cells.

ible, NK cells were cocultured with uninfected or HCV-infected Huh7 cells for 18 h and rested for 24 h in the absence of Huh7 cells. After the rest, degranulation of the NK cells showed recovery, in that the difference between NK cells exposed to uninfected cells and those exposed to HCV-infected cells was no longer statistically significant (Fig. 5A).

To investigate whether soluble factors, such as cytokines secreted by the infected cells, soluble NK ligands released from the infected cells, and cell-free HCV virions, play a role in the reduction of NK cell degranulation (9, 14, 25, 36), we cocultured NK cells and HCV-infected cells in adjacent filter wells. In this system, we found that the degranulation capacity of NK cells did not change and therefore that the HCV-infected Huh7 and Huh7.5 cells require cell-to-cell contact with NK cells to mediate their effects (Fig. 5B). We concluded that changes in NK cell function require continued cell-to-cell contact between NK cells and HCV-infected cells.

NK ligand expression is unassociated with the functional modulation of NK cells. Altered expression levels of inhibitory NK ligands, activating NK ligands, or both on the surface of HCV-infected target cells may modulate signals for NK cell activation. Thus, we assessed the expression levels of these ligands on HCV-infected Huh7 and Huh7.5 cells. To ensure infection of all cells, Huh7 cells were infected at an MOI of 10. Huh7.5 cells were infected at an MOI of 1, however, because higher MOI killed Huh7.5 cells within 3 days of infection. Nevertheless, about 70% of Huh7.5 cells were positive for the HCV core at 72 h postinfection, while about 30% of Huh7 cells were positive for the HCV core (see Fig. S1 in the supplemental material). The high core positivity observed in Huh7.5 cells might be due to the retinoic acid-inducible gene I mutation found in Huh7.5 cells (34).

Three days postinfection, the surface expression levels of inhibitory ligands, HLA-ABC and HLA-E, and activating ligands, MICA/B and ULBPs, were determined. An increase of inhibitory ligands would inactivate NK cells, and a decrease of activating ligands would lower the chance of NK cell activation. The expression of activating ligands, however, did not change after HCV infection, and ULBP-1 expression on Huh7.5 cells rather slightly increased after HCV infection (Fig. 6A). The expression of inhibitory ligands did not change either, regardless of cell type (Fig. 6B).

Surface expression of activating receptors on NK cells is downregulated after interaction with HCV-infected cells. Because an altered display of NK receptors may affect NK cell function (15, 25), we evaluated the expression of inhibitory receptors, such as KIR2DL1, KIR2DL2/3, and NKG2A (Fig. 7A). Coculture with HCV-infected cells did not change the expression of these NK cell surface proteins. It was indeed consistent with the results from the cytotoxicity assays, which suggested that upregulation of inhibitory receptors would not occur because K562 cells do not express HLA-ABC (ligand for KIR) and HLA-E (ligand for CD94/NKG2A) (15).

In contrast, the surface expression of activating receptors, such as NKG2D and NKp30, which are constitutively expressed on resting NK cells (5), decreased after exposure to HCV-infected cells (Fig. 7B and C). The expression of DNAM-1, NKp46, and 2B4, which are other constitutively expressed activating receptors, did not decrease, except after interplay with HCV-infected Huh7.5 cells in the case of DNAM-1 and NKp46 (Fig. 7D to F). The result suggested that the downregulated surface expression of several constitutively expressed activating receptors might be associated with the reduction in functional capacity of NK cells after contact with HCV-infected cells.

Correlation between degranulation and activating receptor expression reveals the effect of receptor downregulation on NK cell functional modulation. To determine direct correlation between the downregulation of activating receptors and the reduction of degranulation capacity, we specifically examined changes in activating receptor expression on degranulation competent NK cells, which could express CD107a upon exposure to K562 cells. Since it was likely that the degranulation changes shown in Fig. 1 were caused when previously degranulation competent NK cells lost their degranulation capacity, alterations in the frequencies of degranulation competent NK cells rather than those of total NK cells, which include NK cells without degranulation capacity, were needed to be examined.

Huh7

Dashed line: uninf. Huh7
Solid line: HCV-inf. Huh7
Shaded area: unstained control

Huh7.5

Dashed line: uninf. Huh7.5
Solid line: HCV-inf. Huh7.5
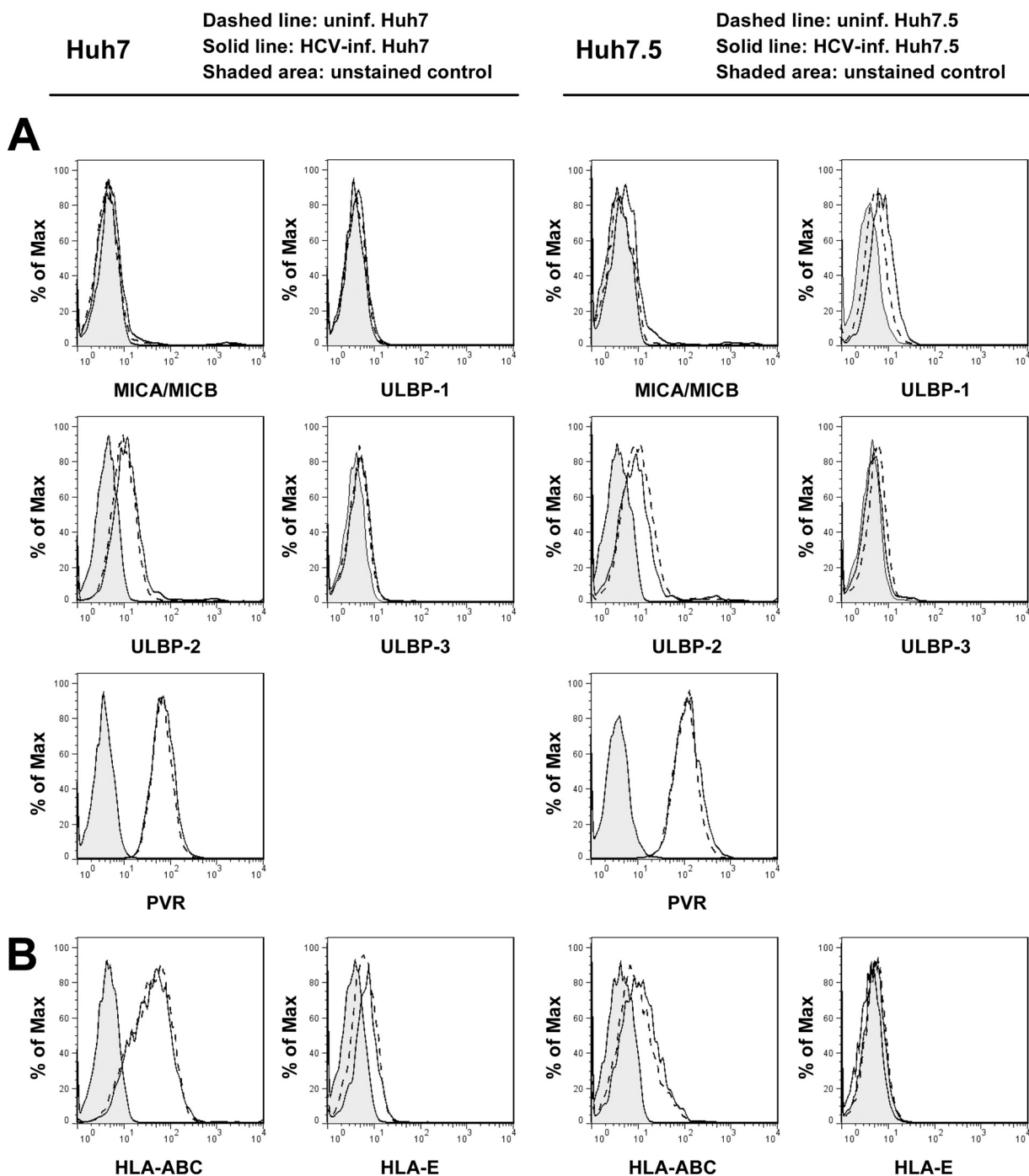Shaded area: unstained control

FIG. 6. NK ligand expression on Huh7 and Huh7.5 cells after HCV infection. Huh7 and Huh7.5 cells were infected with HCV at an MOI of 10 and an MOI of 1, respectively. (A) Expression of NK-activating ligands MICA/B, ULBP-1 to -3, and PVR on uninfected or HCV-infected Huh7 and Huh7.5 cells was measured at 3 days postinfection. Representative histograms from three independent experiments are shown. (B) Expression of NK inhibitory ligands HLA-ABC and HLA-E on uninfected or HCV-infected Huh7 and Huh7.5 cells was measured at 3 days postinfection. Representative histograms from three independent experiments are shown.

The interaction with HCV-infected Huh7 or Huh7.5 cells downregulated NKG2D and NKp30 on the surface of degranulation competent NK cells and made them lose degranulation capacity. The interaction with HCV-infected cells increased the proportion of double-negative cells at the same time (Fig. 8A and B). Analysis of NK cell subsets showed that CD56^{dim}

NK cells were responsible for the reduction of NKG2D and NKp30 on the surface of CD107a$^+$ NK cells (see Fig. S2 in the supplemental material). Moreover, unlike total NK cells, the expression of NKG2D and NKp30 was reduced on the CD107a$^+$ subset of NK cells after interaction with uninfected Huh7 or Huh7.5 cells (Fig. 8A and B). The para-
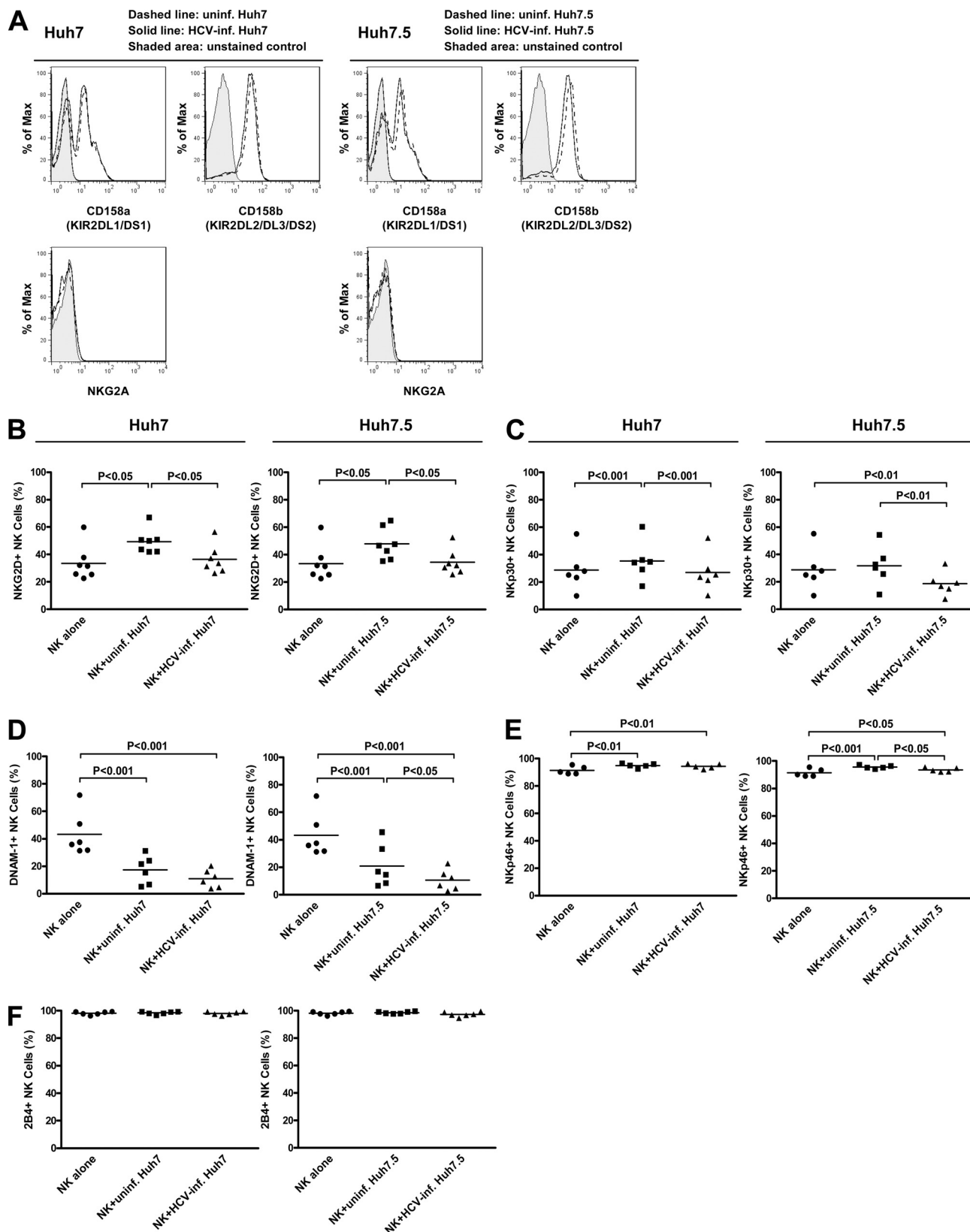
FIG. 7. NK receptor expression on NK cells after interaction with HCV-infected cells. (A) Expression of NK inhibitory receptors on NK cells after interaction with HCV-infected cells. Primary human NK cells were cocultured with uninfected or HCV-infected Huh7 or Huh7.5 cells for 18 h, and expression of inhibitory receptors KIR2DL1, KIR2DL2/DL3, and NKG2A on NK cells was determined. Representative histograms for five independent subjects are shown. (B to F) Downregulation of NK-activating receptors NKG2D, NKp30, and DNAM-1 on the surface of NK cells after interaction with HCV-infected cells. Primary human NK cells were cocultured with uninfected or HCV-infected Huh7 or Huh7.5 cells for 18 h, and expression of activating receptors was determined. Frequencies of NKG2D^{+} (B), NKp30^{+} (C), DNAM-1^{+} (D), NKp46^{+} (E), and 2B4[+] (F) NK cells are shown. NK-alone columns for Huh7 and Huh7.5 graphs are copies of the same data (B to F).
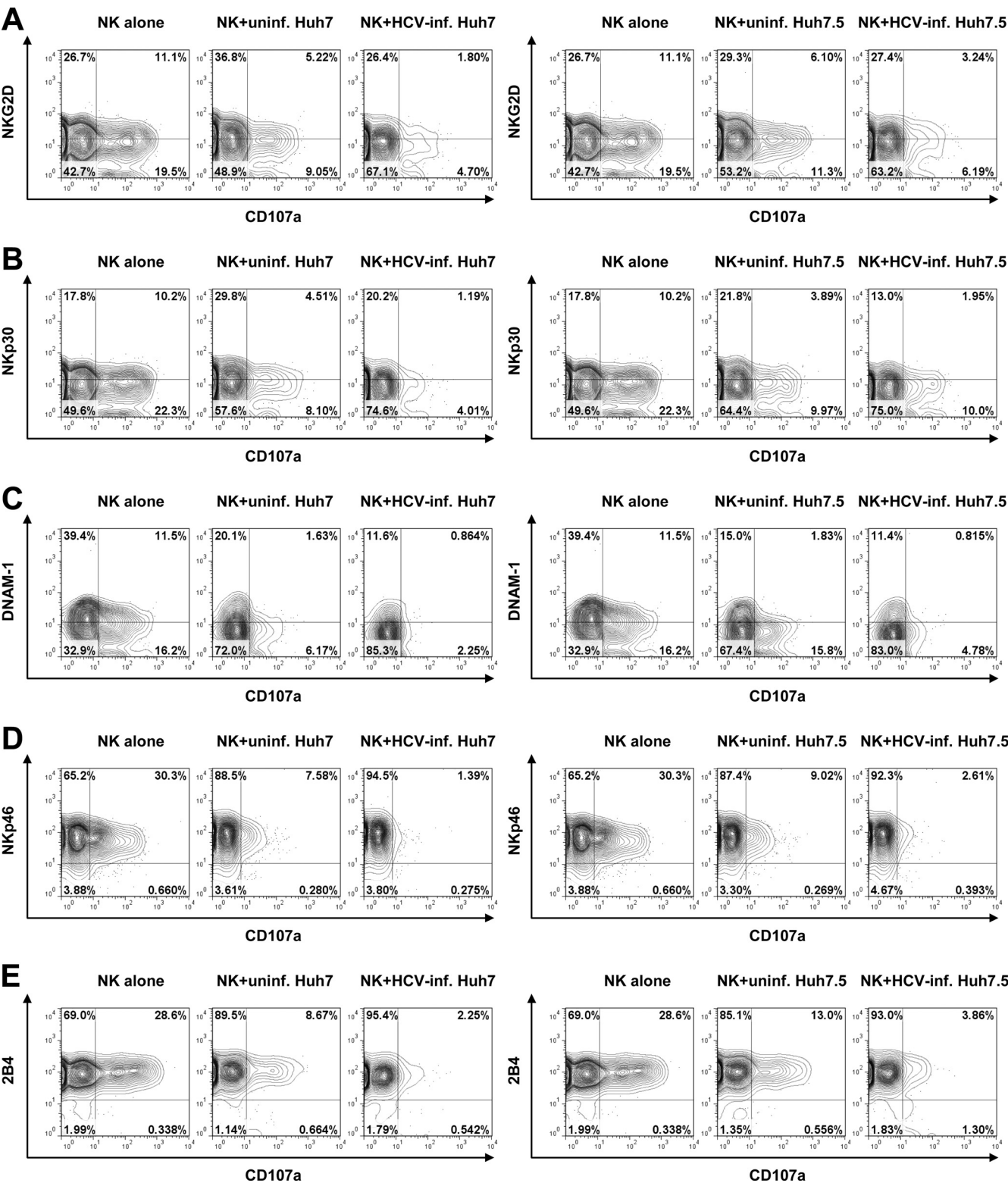
FIG. 8. Correlation of activating receptor downregulation with reduced degranulation. Primary human NK cells were preincubated with uninfected or HCV-infected Huh7 or Huh7.5 cells for 18 h and cocultured with K562 cells at a 1:1 ratio for 4 h. Interrelation between degranulation and activating receptor expression was analyzed for NKG2D (A), NKp30 (B), DNAM-1 (C), NKp46 (D), and 2B4 (E). Representative contour plots of results for five (A to C) or three (D, E) independent subjects are shown. NK-alone plots for Huh7 and Huh7.5 experiments are duplicates of the same contour plot (A to E).

doxical increase of NKG2D and NKp30 on the surface of total NK cells after exposure to uninfected Huh7 or Huh7.5 cells compared to that of unexposed NK cells (Fig. 7B and C) was due largely to the increase of NKG2D- or NKp30-expressing noncytotoxic (CD107a⁻) NK cells. Although the reason for the increased surface expression of NKG2D and NKp30 on degranulation incompetent NK cells was not clear, such upregulation of NKG2D⁺ or NKp30⁺ degranulation incompetent NK cells did not cause any increase in the cytotoxic capacity of total NK cells (Fig. 8A and B).

Reduced expression of DNAM-1 might be, however, much less responsible for the HCV-infected-cell-mediated reduction in degranulation capacity than that of NKG2D and NKp30, since the frequency of DNAM-1⁺ degranulation competent NK cells was considerably reduced after interaction with the uninfected hepatoma cell lines, Huh7 or Huh7.5 cells, and already quite low even before contact with HCV-infected cells (Fig. 8C). It appeared, therefore, that DNAM-1 downregulation might be rather involved in the tumor cell-mediated decrease of NK cell cytotoxic capacity.

In contrast to NKG2D and NKp30, the expression of NKp46 and 2B4 did not decrease, and the percentage of double-negative cells did not increase after interaction with HCV-infected cells, except a slight increase in the percentage of NKp46⁻CD107a⁻ NK cells after interaction with HCV-infected Huh7.5 cells (Fig. 8D and E). The result suggested that NKp46 and 2B4 might be unassociated with the observed NK cell cytotoxicity changes.

DISCUSSION

We performed this study to investigate whether HCV causes functional modulation of NK cells through a direct interplay with HCV-infected hepatocytes. If the NK cell response to HCV infection is suppressed, viral containment will be impaired, and this may contribute to diminished cellular immune responses and a high incidence of chronic hepatitis C after HCV infection (11). Our study showed that the interaction with HCV-infected cells resulted in a reduced functional capacity of NK cells. The ability to degranulate, lyse target cells, and produce and secrete IFN-γ was impaired, and it was associated with the downregulated expression of activating receptors on NK cells.

The frequency of infected cells did not affect the inhibitory effect of HCV-infected cells, because Huh7 and Huh7.5 cells showed similar results, though frequencies of HCV core-positive cells were different from each other. The inhibitory effect of HCV-infected cells, therefore, may be based on cellular changes that are induced by HCV infection, because cells that clear HCV infection spontaneously by 72 h postinfection may retain HCV-induced changes even after clearance of the virus. This hypothesis should be, however, investigated in further studies.

To exclude the inhibitory effect of tumor cells on NK cells, we separated the NK cells from HCV-infected hepatoma cells before interaction with K562 cells. This implies that the functional inhibition of NK cells is unassociated with a transient inhibition of NK cells by the inhibitory NK cell immunological synapse (iNKIS) (18) formed between NK cells and HCV-infected cells. The absence of any considerable increase in the

expression of inhibitory ligands on HCV-infected cells supports the conclusion. Instead, the functional modulation must correspond to stable changes in regulation, such as activating receptor downregulation on the NK cell surfaces. The NK cell degranulation did not last indefinitely, however, which points to a requirement for sustained cell-to-cell contact between NK cells and HCV-infected cells.

The functional capacity of resting NK cells is dependent largely on the surface expression of constitutively expressed activating receptors. Although there have been discrepancies among studies, several *ex vivo* studies showed that NK-activating receptors are downregulated in HCV infection *in vivo*. For example, Nattermann et al. identified that the proportion of NKp30- and NKp46-expressing NK cells is reduced in patients with chronic hepatitis C (26), and Sene et al. reported that NKG2D expression is reduced on circulating NK cells from patients with chronic hepatitis C (30). In conjunction with our study, these reports indicate that downregulation of NK-activating receptors is a plausible mechanism of functional inhibition of NK cells in HCV infection.

IFN-γ is the major cytokine that NK cells secrete and is a critical factor for inhibition of viral replication in the infected cells (22). Although CD56ᵇʳⁱᵍʰᵗ NK cells readily secrete IFN-γ after stimulation with proinflammatory cytokines, such as IL-12 and IL-15, CD56ᵈⁱᵐ NK cells can also secrete IFN-γ upon recognition of target cells in the presence of proinflammatory cytokines (32). Since CD56ᵈⁱᵐ NK cells are readily recruited from peripheral blood to the inflammation sites, whereas CD56ᵇʳⁱᵍʰᵗ NK cells are enriched in secondary lymphoid organs (32, 37), inhibition of CD56ᵈⁱᵐ NK cells' ability to secrete IFN-γ may be more directly related to uncontrolled replication of viruses. Our results therefore suggest that CD56ᵈⁱᵐ NK cells which are recruited to the infection sites and contact with HCV-infected hepatocytes may lose their capacity to secrete IFN-γ *in vivo*.

The direct correlation of decreased IFN-γ production with downregulated activating receptor expression could not be evaluated, because brefeldin A treatment for intracellular cytokine detection blocked not only IFN-γ secretion but also surface expression of activating receptors (data not shown). The consistency between the results from cytotoxicity assays and the observations from IFN-γ assays implies, however, that the reduction in IFN-γ secretion by CD56ᵈⁱᵐ NK cells may be associated with the decrease in activating receptor expression. It is, in fact, known that concurrent engagement of activating receptors and cytokine receptors on NK cells induces IFN-γ secretion by NK cells (7). A decrease in activating receptor expression would, therefore, likely be correlated with IFN-γ production by CD56ᵈⁱᵐ NK cells. Further investigations are, however, needed to confirm the relationship.

Although we proposed downregulation of activating receptors as the cause of reduced NK cell functional capacity, interaction of immobilized E2 with CD81 might be another possible answer if E2 could be immobilized on the surface of HCV-infected hepatocytes. Moreover, other factors, such as cytokines secreted by immune cells or the infected hepatocytes, may also control NK cell function in addition to direct cell-to-cell contact between NK cells and HCV-infected hepatocytes. Our results from experiments using high-dose IL-12 implied this possibility. Other reports showed that exposure to inhibi-

tory cytokines, such as IL-10 and transforming growth factor β (TGF-β), suppresses NK cell cytotoxicity (30), while chronic exposure to activating cytokine IFN-α contributes to the polarization of NK cells toward cytotoxicity (1, 27). The fate of NK cells in HCV infection may be therefore determined by the integration of signals generated by direct cell-to-cell interaction and cytokine stimulation. Further studies using *in vivo* models of HCV infection are needed to identify the outcome of the integrated signals in various stages of HCV infection.

Thus far, there is no evidence that NK cells are inhibited in the HCV-infected liver, though our *in vitro* study suggests that functional capacity of liver-infiltrating NK cells which interacted with HCV-infected hepatocytes may be reduced *in vivo*. Especially in the incubation phase and the acute phase of HCV infection, it is difficult to investigate whether liver-infiltrating NK cells are functionally impaired, because liver biopsy during these phases is impossible due to ethical reasons. We therefore hope that our study prompts further studies employing chimpanzee models or humanized mouse models for immunologic research to assess functional status of liver NK cells in various phases of HCV infection.

In summary, this study revealed a significant modulation of NK cell function in HCV infection *in vitro*, which may be based on the downregulation of activating receptors, such as NKG2D and NKp30. *In vivo*, reduced functional capacity would in principle make NK cells fail to prevent HCV replication and spread of the virus. Further studies that investigate detailed mechanisms may provide a way to find molecules or signaling processes which could be targeted to restore NK cell function.

ACKNOWLEDGMENTS

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