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Negative Regulation of NK Cell Activities by Inhibitory Receptor CD94/NKG2A Leads to Altered NK Cell-Induced Modulation of Dendritic Cell Functions in Chronic Hepatitis C Virus Infection

Masahisa Jinushi, Tetsuo Takehara, Tomohide Tatsumi, Tatsuya Kanto, Takuya Miyagi, Takahiro Suzuki, Yoshiyuki Kanazawa, Naoki Hiramatsu, and Norio Hayashi

NK cells are potent activators of dendritic cells (DCs), but it remains obscure how third-party cells affect the ability of NK cells to modulate DC functions. We show here that NK cells derived from healthy donors (N-NK), when cocultured with human liver epithelial cells, induced maturation as well as activation of DCs, such as increased migratory capacity as well as T cell stimulatory activity. In contrast, NK cells from chronic hepatitis C virus-infected donors (HCV-NK) were not capable of activating DCs under the same conditions. In comparison to N-NK, HCV-NK showed higher expression of CD94/NKG2A and produced IL-10 and TGFβ when cultured with hepatic cells, most of which express HLA-E, a ligand for CD94/NKG2A. Blockade of NKG2A restored the ability of HCV-NK to activate DCs, which appeared to result from the reduced NK cell production of IL-10 and TGFβ. The blockade also endowed HCV-NK with an ability to drive DCs to generate Th1-polarized CD4+ T cells. These findings show that NK cell modulation of DCs is regulated by third-party cells through NK receptor and its ligand interaction. Aberrant expression of NK receptors may have an impact on the magnitude and direction of DC activation of T cells under pathological conditions, such as chronic viral infection. The Journal of Immunology, 2004, 173: 6072–6081.

Innate immunity serves as a first line of defense against pathogens and/or cancer cells during the initial phase of responses before adaptive immunity is sufficiently induced (1, 2). Dendritic cells (DCs) are known to be the most potent cellular lineages to serve as sentinels between innate and adaptive responses (3). The ability of DCs to activate adaptive immune responses is dependent on the state of their maturation, which is promoted by various kinds of innate cytokines as well as pathogen-associated molecular patterns (4–7). In addition, recent studies unveiled NK cell activation of DCs through their cognate interaction or soluble factors (4). NK cells and DCs could be attracted to inflammatory sites by common sets of chemokines, leading to the promotion of NK/DC cross-talk (8, 9). These findings imply that NK cell-mediated regulation of DCs plays an important role in the initiation and extension of adaptive immune responses.

In the inflammatory sites, NK cell functions could be modified in the presence of a wide variety of cytokines as well as by direct contact with infected or tumor cells. NK activities are known to be negatively controlled by signals through intimate interactions of their inhibitory receptors and MHC class I ligands on target cells. NK cells express two major families of molecules: Ig-like superfamily (killer cell Ig-like receptors (KIRs)) and C-type lectin-like molecules (CD94 and NKG2A and -E) (10). Recent studies have suggested that these inhibitory NK cell receptor-mediated down-modulation of NK or CTL effector functions are responsible for the inefficient immune responses against certain pathogens as well as cancer cells (11–13). In addition, the expression levels of these molecules were well correlated with the disease progression in chronic HIV-1 or human T cell leukemia virus 1 infection (14–17). In this context, it is of great interest to assess whether regulation of NK cell activities through their negative signals can affect the magnitude as well as direction of DC functions in certain viral infections.

Hepatitis C virus (HCV), which may cause cirrhosis and hepatic cancer, is the major cause of chronic liver diseases worldwide (18). It has been recognized that the failure of HCV-specific cellular immunity is one of the important factors by which HCV establishes its persistence and causes subsequent liver damage. Several lines of evidence have revealed that HCV can inhibit the NK cell functions by their engagement of molecules, such as CD81 (19–21). However, it remains unclear whether local interaction of NK cells with liver epithelial cells, where HCV can selectively replicate, affects the ability of NK cells to modulate DC functions in HCV infection.

We found that NK cells derived from normal donors (N-NK) have an ability to augment DC functions in the presence of human hepatic cells, and their capacity is severely compromised in NK cells derived from chronic HCV-infected donors (HCV-NK). We also demonstrated that HCV-NK, in comparison to N-NK, express

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3 Abbreviations used in this paper: DC, dendritic cell; KIR, killer Ig-like receptor; HCV, hepatitis C virus; N-NK, NK cells derived from healthy donors; HCV-NK, NK cells derived from chronic HCV-infected donors; PH, primary cultured normal hepatocytes; MHC II, MHC class II; MFI, mean fluorescence intensity.
much higher levels of the inhibitory receptor CD94/NKG2A, which is responsible for the impaired HCV-NK activation of DCs in the presence of third-party cells which appeared to express the NKG2A ligand HLA-E. Furthermore, NKG2A-mediated production of IL-10 and TGFβ from NK cells modified DCs to generate IL-10-producing CD4 T cells. These findings lead to new insight that NK receptor-mediated modulation of innate pathways may determine the magnitude and direction of DCs to induce adaptive immune responses.

**Materials and Methods**

**Subjects**

Ten healthy volunteers and 15 patients with chronic hepatitis C were enrolled in this study after informed consent had been obtained. The patients were positive for HCV RNA and were histologically diagnosed as having mild or moderate chronic hepatitis. They did not display any evidence of other types of liver diseases.

**Isolation of PBL populations**

PBL, obtained from healthy volunteers or patients with chronic hepatitis C infection, were isolated by Ficoll-Hypaque density centrifugation. Resting NK cells (CD56+CD3-; >90% purified) as well as naive CD4 T cells (CD45RA+RO; >95% purified) were further isolated using StemSep-negative selection systems (Stem Cell Technologies, Vancouver, Canada).

**Generation of monocyte-derived DCs**

Monocyte-derived DCs were generated from the peripheral venous blood of healthy volunteers as described previously (22, 23). In brief, PBMCs were centrifuged on a Percoll (Sigma-Aldrich, St. Louis, MO) gradient. The light density fraction floating on the middle layer, which contained highly purified monocytes, was seeded in 24-well culture plates at a density of 5.0x10⁵/well. After 45 min of incubation at 37°C, nonadherent cells were removed and the adherent cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies, Gaithersburg, MD) containing 10% FBS, 10 U/ml penicillin/streptomycin, and 2 mM l-glutamine and supplemented with GM-CSF and IL-4 (10 ng/ml: PeproTech, Rocky Hill, NJ) for 6 days to generate DCs of an immature phenotype.

**Cell lines**

The Hep3B human hepatoma cell line was purchased from the American Type Culture Collection (Manassas, VA). The HuH7 human hepatoma cell line was a generous gift from Dr. M. Nanba (Institute for Molecular and Cellular Biology, Okayama University, Okayama, Japan). These hepatic cells were propagated in DMEM supplemented with 10% heat-inactivated FBS, antibiotics, and antimitotics (Invitrogen Life Technologies) in a humidified atmosphere of 5% CO₂ at 37°C. We also used the human chronic myeloid leukemia cell line K562 (American Type Culture Collection), propagated in RPMI 1640 supplemented with 10% heat-inactivated FBS, antibiotics, and antimitotics. Human nontransformed hepatocytes (PH) were purchased from Applied Cell Biology Research Institute (Kirkland, WA) and cultured in CS-C complete medium according to the manufacturer’s instructions.

**NK cells, hepatic cells, and DC cocultures**

Resting NK cells and DCs were seeded in the same wells of 24-well plates and cultured with human hepatic cells for 48 h (1x10⁶ of each type of cell/well). As a control, DCs were plated in 24-well culture plates. A Transwell insert was also used to prevent direct contact of the mixtures of NK cells, human hepatic cells, and DCs. In these Transwell systems, the soluble factors could freely pass through a microporous polycarbonate membrane (0.4 μm). DCs were plated in an upper chamber, and NK cells and Hep3B cells were seeded in the bottom chamber at a ratio of 1:1 for 48 h. As a control, DCs (upper chamber) and NK cells alone (bottom chamber) were plated in 24-well culture plates.

**Stimulation of DCs by coculture supernatants of NK/third-party cells**

NK cells (1x10⁶ cells/well) were seeded in 24-well plates and stimulated with Hep3B cells or K562 cells (1x10⁶ cells/well) at a ratio of 1:1 for 24 h. In the case of nontransformed normal hepatocytes (PH), NK cells were stimulated with or without 50 ng/ml IL-2 for 24 h and then cocultured with PH (1x10⁵ cells/well) for 24 h. DCs were cultured in presence of the

**FIGURE 1.** Phenotypic change of DCs cocultured with N-NK and HCV-NK in the presence of third-party cells. NK cells (1x10⁵/well) derived from healthy donors or chronic HCV-infected patients were cocultured with allogenic DCs from healthy donors at a ratio of 1:1 in the absence (NK) or presence (NK/Hep3B) of Hep3B cells (1x10⁵/well) for 48 h. As negative and positive controls, DCs were unstimulated (−) or were stimulated with 10 ng/ml LPS for 48 h, respectively. FACS analysis was performed to evaluate the expression of CD40, CD86, MHC II, and CD83 on DCs after positively gating CD11c+ CD56- cell population (filled histograms). Open histograms represent the staining of control Ab. All experiments were performed at least three times and representative results (A) as well as the statistical analysis (B) are shown as the MFI of the staining cells. *, p < 0.05.
Flow cytometric analysis

DCs (5 × 10^5/ml) were washed and resuspended in PBS containing 1% BSA and 0.05% NaN₃ and incubated with a series of mAbs at 4°C for 30 min. The following mAbs were used for immunofluorescent staining on DCs: anti-CD11c, anti-CD40, anti-CD86, anti-HLA-DR (BD Pharmingen, San Diego, CA), anti-CD83, and anti-CCR7 (Coulter Immunotech, Marseille, France). DCs were positively gated from triple cell cultures by CD11c expression.

For NK cell staining, the cells were washed and incubated at 4°C for 30 min in PC5-labeled CD56 mAb (Coulter Immunotech) and PE-labeled mAbs as follows: EB6 (KIR2DL2/2DS1), GL186 (KIR2DL2-3/2DS2), p50.3 (KIR2DS4), DX9 (KIR3DL1), HP-3B1 (CD94), Z199 (NKG2A), and ID11(NKG2D). EB6, GL186, p50.3, HP3B1 and Z199 were purchased from Coulter Immunotech. DX9 was purchased from BD Pharmingen. ID11 was kindly provided by Dr. V. Groh and Dr. T. Spies (Fred Hutchinson Cancer Research Institute, Seattle, WA) and was used as previously reported (25). The cells were then washed twice and fixed with 2% paraformaldehyde solution. The cells were analyzed by flow cytometry.

Measurements of cytokine production

The culture supernatants of IFN-γ, TNF-α, IL-10, and TGFβ were examined using ELISA kits according to the manufacturer’s instructions (IFN-γ, TNF-α, and IL-10; Endogen, Woburn, MA; TGFβ, R&D Systems).

Chemotactic assay

DC migration capacity was evaluated using the chemotaxis microchamber technique as described previously (8). In brief, human recombinant MIP-3β/CCL19 (R&D Systems) was diluted to 100 ng/ml RPMI 1640 medium and added to the bottom wells and then DCs (1 × 10^5/well) were applied to the upper wells, which were separated from the bottom wells with 8-μm pore polycarbonate filters (Costar, Corning, NY). After 2 h of incubation at 37°C, the cells that migrated to the bottom chamber were collected and counted manually. As a control, the lower compartment of the control chambers contained medium alone.

**FIGURE 2.** Soluble factors produced from NK cells/Hep3B mixtures are responsible for promoting DC maturation. A, NK cells, in the presence or absence of Hep3B (NK/Hep3B or NK, respectively), were added to DC cultures either directly (NK/Hep3B-Mix) or to the bottom compartment separately through 0.4-μm pore size membrane (NK/Hep3B-Transwell) for 48 h. The expression of CD40, CD86, and MHC II on DCs was examined by flow cytometry. B and C, NK cells (1 × 10^5) from healthy donors (N) and HCV-infected individuals (HCV) were cocultured in the absence (NK) or presence of Hep3B (NK/Hep3B) or K562 (NK/K562) at a ratio of 1:1 for 24 h. DCs from healthy donors were stimulated with or without ((−)) the supernatant obtained from the cocultured medium. The expression of CD40, CD86, and MHC II (filled histogram) were evaluated by FACS analysis. Open histograms represent the staining with control Ab. All experiments were performed at least five times and representative results (B) as well as the statistical analysis (C) are shown as MFI of the staining cells. *, p < 0.05. D and E, NK cells (NK, 1 × 10^5) or those stimulated with IL-2 (50 ng/ml) for 24 h (IL-2 NK, 1 × 10^5) were cocultured in the absence (NK) or presence of nontransformed normal hepatocytes (NK/PH) at a ratio of 1:1 for 24 h. DCs were then stimulated with the supernatant obtained from the cocultured medium. The expression of CD86 was evaluated by FACS analysis (filled histograms). Open histograms represent the staining with control Ab. All experiments were performed at least five times and representative results (D) as well as the statistical analysis (E) are shown as MFI of the staining cells. *, p < 0.05.
Intracellular cytokine analysis

Intracellular staining was performed as described previously (22, 23). In brief, NK cells or CD4⁺ T cells were pretreated with 1 μg/ml GolgiPlug (BD Pharmingen) for 4 h at 37°C. At the end of the incubation period, NK cells and naive CD4⁺ T cells were stained with PCs5-labeled CD86 mAb and CD4 mAb (Beckman Coulter) for 30 min, respectively. The cells were then fixed and permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen) for 15 min at room temperature. Permeabilized cells were stained with FITC-labeled anti-IFN-γ mAb and PE-labeled anti-IL-10 mAb (mouse IgG1; BD Pharmingen) or isotype-matched control IgG. The stained cells were analyzed by flow cytometry.

Proliferation assay

DCs were cultured with 10 μg/ml LPS or the supernatant of NK cells cultured with tumor cells (Hep3B and K562) for 48 h, and then were seeded in round-bottom 96-well plates for use as stimulator cells (10–3.33 × 10⁵/well). The DCs were cultured at graded numbers with the responder naïve CD4⁺ cells (1.0 × 10⁵/well) for 72 h. The cocultured cells were pulsed with 1 μCi/well [³²P]thymidine for 16 h of incubation and collected onto a glass fiber filter. [³²P]Thymidine incorporation was quantified using a beta-plate liquid scintillation counter. The results were expressed as the mean cpm in triplicate cultures.

CD4⁺ T cell responses against recall Ag

DCs stimulated with the supernatant of the NK cell/Hep3B coculture medium for 24 h were given influenza Ag of the Texas 1/77 strain (Chemicon International, Temecula, CA) at 10 μg/ml (27) and were incubated overnight at 37°C. Autologous CD4⁺ T cells were stimulated by Ag-pulsed or unpulsed DCs for 48 h and subjected to proliferation or intracellular cytokine assay as described above.

Cytolytic assay

Target cells labeled with ⁵¹Cr were incubated in the NK cells for 4 h at various E:T ratios. The supernatants were obtained after the incubation and subjected to gamma counting. The maximum or spontaneous release was defined as counts from samples incubated with 5% Triton X-100 or medium alone, respectively. Cytolytic activity was calculated with the following formula: percent lysis = (experimental release – spontaneous release) × 100/(maximum release – spontaneous release). The spontaneous release in all assays was ~20% of the maximum release.

Statistical analysis

Comparisons between groups were analyzed using the compared t test with Welch’s correction or Mann-Whitney U test. Differences were considered to be significant when the p < 0.05.

Results

DCs gained matured phenotypes in the presence of N-NK and Hep3B hepatoma cells

To investigate whether NK cells could modulate DCs in the presence of human hepatic cells, monocyte-derived DCs were cultured with both NK cells and Hep3B human hepatoma cells for 48 h and analyzed for their phenotypes by flow cytometry. Unstimulated DCs displayed immature phenotypes, such as with moderate expression of costimulatory molecules (CD40 and CD86) as well as MHC class II (MHC II) and no expression of CD83 (Fig. 1A). Neither NK cells nor Hep3B cells alone had any impact on DC phenotypes under our experimental conditions (Fig. 1A and data not shown). DCs cultured with both N-NK and Hep3B cells exhibited the matured phenotype, exhibiting the elevated expression of CD40, CD86, MHC II, and CD83, at comparable levels to LPS-stimulated DCs (Fig. 1). These results indicated that the coexistence of NK cells and Hep3B, but not NK cells alone, can induce maturation of DCs. In striking contrast, DCs cultured with both HCV-NK and Hep3B cells displayed little phenotypic changes of CD40, CD86, and CD83, whereas they showed up-regulation of MHC II expression.

These observed effects of DC maturation do not require direct cell-cell contact of DCs and NK/Hep3B cells, because the insertion of a Transwell membrane between DCs and cocultured NK/Hep3B cells induced DC maturation at levels similar to mixed cultures of DCs, NK cells, and Hep3B (Fig. 2A). Next, we examined whether the supernatant of NK cells/Hep3B culture could affect the DC phenotypes. As shown in Fig. 2, B and C, up-regulation of CD40, CD86, and MHC II was observed on DCs treated with the supernatant of N-NK/Hep3B cocultures. These results further confirmed that direct cell-to-cell contact is not necessary for NK cells to regulate DC maturation upon human hepatic cells. In contrast, the supernatant of HCV-NK/Hep3B up-regulated MHC II expression on DCs, but had little effect on CD40 or CD86 expression. The differences of DC maturation status between N-NK and HCV-NK were similarly observed when NK cells were cocultured with another human hepatoma cell line, Huh7 (data not shown). In contrast, the maturation of DCs was similarly observed in the coculture of either N-NK or HCV-NK with K562 cells (Fig. 2, B and C).

We examined whether primary-cultured, nontransformed human hepatocytes (PH) could modify NK cell activation of DCs. NK cells did not induce DC maturation either in the presence or absence of PH (Fig. 2D). At the next step, NK cells were stimulated with IL-2 for 24 h and then cocultured with PH. The obtained supernatant
of IL-2-stimulated HCV-NK alone up-regulated CD86 expression of DCs at levels similar to those of IL-2-stimulated N-NK alone. However, the maturation of DCs was clearly inhibited, especially in chronic HCV-infected patients when IL-2-stimulated NK cells were cocultured with PH (Fig. 2E). Taken together, these results showed that HCV-NK reduced their ability to induce DC maturation when cultured with human hepatic cells.

DC functions were up-regulated by N-NK/Hep3B coculture supernatant, but not HCV-NK/Hep3B culture supernatant

One of the important aspects of DC activation is their acquisition of the migratory ability to regional lymph nodes as well as the stimulatory capacity of T cells (3). Therefore, we examined whether NK cells can modulate the DC chemotactic activity in the presence of human hepatic cells. Flow cytometric analysis revealed that DCs stimulated with the N-NK/Hep3B coculture supernatant showed significantly higher expression of CCR7 than those stimulated with the supernatant of NK cells alone (Fig. 3, A and B). Furthermore, DCs stimulated with N-NK/Hep3B or N-NK/K562 supernatant gained the ability to migrate in response to CCL19, the specific ligand of CCR7 (Fig. 3C). In contrast, CCR7 expression and the migratory activity of DCs were not induced by the treatment of HCV-NK/Hep3B culture supernatant, but were increased by the coculture supernatant of HCV-NK/H9253 (Fig. 3).

We next evaluated whether the DCs exposed to the NK/Hep3B supernatant could stimulate naive CD4\(^+\) T cells from allogeneic donors. When pretreated with the supernatant of either N-NK/Hep3B or N-NK/K562 mixtures, DCs were capable of stimulating proliferation and IFN-\(\gamma\) production of allogeneic CD4\(^+\) T cells (Fig. 4, A and B). In contrast, the allostimulatory capacity of DCs was severely impaired when pretreated with the supernatant of HCV-NK/Hep3B coculture, but not when pretreated with HCV-NK/H9253 (Fig. 4, C and D). HCV-NK/Hep3B supernatant did not induce DCs to secrete IFN-\(\gamma\) in the presence of the influenza A Ag when cultured in the supernatant of N-NK/Hep3B mixtures. However, the addition of HCV-NK/Hep3B supernatant did not induce DCs to stimulate the influenza A Ag-dependent autologous CD4\(^+\) T cell responses (Fig. 4, C and D).

**Hep3B cells activated N-NK cells, but not HCV-NK cells**

We evaluated whether the differences between N-NK and HCV-NK in their ability to modulate DC functions are associated with the NK cell effector functions. N-NK and HCV-NK efficiently killed K562 target cells at similar levels, but the cytolytic activity of HCV-NK against Hep3B cells was much lower than that of N-NK (Fig. 5A). In addition, the cell surface expressions of activation markers CD25 and CD69 increased when N-NK were cultured with K562 or Hep3B cells. In contrast, these enhancements were observed when HCV-NK were cultured with K562 cells, but not when they were cultured with Hep3B cells (Fig. 5B). We next examined the cytokine production profiles of NK cells cultured with tumor cells. Intracellular cytokine staining revealed that N-NK produced IFN-\(\gamma\) when stimulated with Hep3B or K562. In contrast, HCV-NK did not efficiently produce IFN-\(\gamma\) upon stimulation with Hep3B, although they responded to K562 cells. IL-10 production was much higher in HCV-NK than in N-NK when cultured with Hep3B cells (Fig. 5C). ELISA data confirmed that N-NK produced significantly higher levels of IFN-\(\gamma\) as well as TNF-\(\alpha\) than HCV-NK when cultured with Hep3B cells. The levels of IL-2-stimulated HCV-NK alone up-regulated CD86 expression of DCs at levels similar to those of IL-2-stimulated N-NK alone. However, the maturation of DCs was clearly inhibited, especially in chronic HCV-infected patients when IL-2-stimulated NK cells were cocultured with PH (Fig. 2E). Taken together, these results showed that HCV-NK reduced their ability to induce DC maturation when cultured with human hepatic cells.

**FIGURE 4.** N-NK and HCV-NK differ in their ability to trigger DC-mediated induction of CD4\(^+\) T cell responses. A and B, DCs were cultured with the supernatant of N-NK (N) or HCV-NK (HCV)/Hep3B coculture medium for 24 h and then used for the stimulation of allogeneic CD4\(^+\) T cells for 48 h. C and D, DCs cultured with N-NK (N) or HCV-NK (HCV)/Hep3B coculture supernatant were pulsed with or without influenza A Ag (10 \(\mu\)g/ml) and used for the stimulation of autologous CD4\(^+\) T cells for 48 h. In these settings, DCs did not induce autologous CD4\(^+\) T cell responses without recall Ag. CD4\(^+\) T cells (1 \times 10^5/well) were cultured with graded numbers of DCs and proliferation was examined by [\(^{3}H\)thymidine corporation (A and C), and intracellular expression of IFN-\(\gamma\) was determined by FACS analysis (B and D). The number in the right upper quadrant represents the percentages of CD4-positive cells expressing IFN-\(\gamma\) (B and D). Similar results were obtained in two independent experiments and representative data are shown.
of IL-10 production from NK cells were higher in HCV-infected patients than those in healthy donors. Similarly, HCV-NK produced much higher amounts of TGFβ than N-NK (Fig. 5D). The different patterns of these cytokine productions between N-NK and HCV-NK were not observed when NK cells were cocultured with K562 cells (Fig. 5, A–C). These results indicate that HCV-NK, upon stimulation of Hep3B cells, show a decrease in NK effector functions, such as cytolytic activity and production of proinflammatory cytokines, correlating well with their decreased ability to activate DCs. Furthermore, HCV-NK, in response to Hep3B cells, produced higher levels of immunoregulatory cytokines IL-10 and TGFβ than N-NK.

Increased frequency of NK cells expressing the inhibitory receptor CD94/NKG2A in HCV-infected individuals

NK cell activities have been reported to be regulated by the interaction of NK cell inhibitory and activating receptors and their ligands (10). Thus, we next examined the expression of NK cell receptors on N-NK as well as HCV-NK. As shown in Fig. 6, flow cytometric analysis revealed that all of the KIRs tested and activating receptor NKG2D were expressed on HCV-NK at levels similar to those on N-NK. The expression of C-type lectin receptor CD94 and NKG2A were significantly elevated in HCV-NK compared with N-NK. These results demonstrated that CD94 and NKG2A are molecules expressed at much higher levels in HCV-NK. It has been reported that human NK cells were composed of CD56bright and CD56dim subsets and that CD56bright NK cells express CD94/NKG2A more than CD56dim subsets (28). The percentages of the CD56bright subset of HCV-NK were similar to those of N-NK (data not shown), indicating that the higher expression levels of CD94/NKG2A of HCV-NK were not due to expansion of the CD56bright subpopulation.
isotype control staining. tive control for HLA-E and HLA class I expression. Open histograms show (filled histograms). Peripheral blood leukocytes (PBL) were used as posi-

Huh7, primary hepatocyte, and K562 was assessed by flow cytometry

FIGURE 7. Surface expression of HLA-E and HLA class I in Hep3B, Huh7, primary hepatocyte, and K562 was assessed by flow cytometry (filled histograms). Peripheral blood leukocytes (PBL) were used as positive control for HLA-E and HLA class I expression. Open histograms show isotype control staining.

Inhibition of NKG2A during culture with HLA-E-bearing Hep3B cells restored the activation status of HCV-NK

We next analyzed the expression of HLA class I and HLA-E, which function as ligands of KIRs and CD94/NKG2A, respectively (10, 29), on human hepatic cells or K562. As shown in Fig. 7, all human hepatic cells expressed HLA-E as well as HLA class I molecules on their cell surface, and the levels of HLA-E expression varied among them. Neither HLA-E nor HLA class I was detected on K562, consistent with previous reports (30, 31). The above findings raise the possibility that the interaction of CD94/NKG2A and HLA-E may attenuate NK cell effector functions against HLA-E-bearing human hepatic cells in patients with HCV infection.

Although the formation of a heterodimer complex is necessary to express NKG2A and CD94 at cell surface levels to exert their biological effects, CD94 does not trigger any functions, and NKG2 families are critical for exerting their biological effects (32). We next investigated the cytolytic activity of NK cells with masking Ab of NKG2A during coculture of NK cells and Hep3B cells. Addition of anti-NKG2A Ab, which can specifically block CD94/

FIGURE 6. Profiles of NK receptor expression on NK cells. NK cells isolated from normal volunteers (n = 10; N) or from HCV-infected patients (n = 15; HCV) were stained with Abs for various KIRs, CD94, NKG2A, and NKG2D as described in Materials and Methods. The receptor expression of CD56+ cells was examined by flow cytometry and was expressed as a percentage of the positive cell rate. *, p < 0.05.

NKG2A heterodimer–HLA-E interaction (24), resulted in a marked increase in Hep3B cytolysis of HCV-NK, but to a lesser extent for N-NK. In contrast, the NKG2A blockade had no effect on N-NK or HCV-NK cytolysis of HLA-E-negative K562 cells (Fig. 8A). We also examined the effect of anti-NKG2A Ab on cytokine production from NK cells. The addition of anti-NKG2A Ab during NK cells/Hep3B cocultures increased production of proinflammatory cytokines, such as IFN-γ and TNF-α. Again, the blockade of the NKG2A-mediated signal resulted in a substantial decrease in IL-10 and TGFβ production from HCV-NK, but to a lesser extent for N-NK (Fig. 8B).

Blockade of NKG2A signals enhanced the ability of NK cells to activate DCs

To investigate the involvement of the NKG2A-mediated signal in NK cell activation of DCs, NK cells and Hep3B cells were cultured in the presence of anti-NKG2A Ab for 24 h. Next, DCs were stimulated with the obtained NK/Hep3B supernatant for 24 h. FACS analysis showed that the expression levels of CD86, CD40, CD83, and CCR7 were elevated on DCs when anti-NKG2A Ab was added during the cocultures of NK cells and Hep3B; the extent was greater in HCV-NK than in N-NK. The NKG2A blockade during IL-2-stimulated NK cells and nontransformed hepatocytes also restored DC maturation, indicating that the ability of normal hepatocytes to inhibit NK cell activation of DCs relied mainly upon the NKG2A-mediated inhibitory signal (Fig. 9A and data not shown). Moreover, the blockade of NKG2A during HCV-NK/Hep3B coculture restored the ability of the supernatant-treated DCs to stimulate allogeneic CD4+ T cells, whereas the NKG2A blockade slightly enhanced proliferation of CD4+ T cells by N-NK/Hep3B supernatant (Fig. 9B). We also examined whether any differences existed between anti-NKG2A Ab and control IgG treatment in DC-stimulated cytokine production from CD4+ T cell lines. Intracellular cytokine staining showed that CD4+ T cells can produce both IFN-γ and IL-10 when stimulated by DCs that had been exposed to N-NK/Hep3B coculture supernatant. DCs pre-treated with the HCV-NK/Hep3B supernatant generated CD4+ T cells, preferentially producing IL-10. Blockade of NKG2A during the coculture of NK cells and Hep3B inhibited IL-10 production and promoted IFN-γ production from CD4+ T cells (Fig. 8C).

Taken together, these findings identified high expression levels of NK inhibitory receptor NKG2A as the critical factor for suppressing the ability of NK cells to activate DCs.

Immunosuppressive cytokines, IL-10 and TGFβ, act as downstream mediators of NKG2A-induced inhibition of NK cell activation of DCs

IL-10 and TGFβ are known to act as suppressive factors of DC activation to induce the anergic states (33, 34). We examined whether IL-10 and TGFβ, produced in large amounts by HCV-NK, are responsible for the impaired DC activation. Toward this goal, the culture supernatant of HCV-NK/Hep3B cells was treated with neutralizing Abs of IL-10 and TGFβ and was used for the stimulation of DCs. The blockade of IL-10/TGFβ clearly enhanced the expression of CD40, CD86, CD83, and CCR7 on DCs. In contrast, when DCs were pretreated with the supernatant of the anti-NKG2A Ab-added culture, the IL-10/TGFβ blockade had little additional effects on DC maturation (Fig. 10). These results indicated that the IL-10/TGFβ production, triggered by the interaction of NKG2A and its ligand, could directly interfere with DC activation.

Discussion

Several lines of evidence have revealed that NK cell-mediated reg-

ulation of DCs plays an important role in the recognition of patho-

gens and malignancies at the early phase of immune responses
FIGURE 8. Blockade of NKG2A enhances NK cell activities. A, NK cells isolated from healthy volunteers (N) or HCV-infected patients (HCV) were cultured with 51Cr-labeled Hep3B or K562 in the presence of the masking Ab of NKG2A or isotype control IgG (20 μg/ml) at the indicated ratios in 96-well culture plates for 4 h, and then were subjected to standard 51Cr-releasing assay. Similar results were obtained for three independent donors, and the data are presented as a composite examined for each groups. *, p < 0.05. B, NK cells isolated from healthy (N) or HCV-infected (HCV) individuals were cultured with Hep3B in the presence of masking Ab of NKG2A or isotype control IgG (20 μg/ml) for 24 h (n = 3 for each group). IFN-γ, TNF-α, IL-10, and TGFβ were measured in each culture supernatant by ELISA. *, p < 0.05.

FIGURE 9. NKG2A-mediated signal suppresses NK cell activation of DCs. NK cells (1 × 10⁵) from healthy donors (N) as well as HCV-infected individuals (HCV) were cocultured with Hep3B at a ratio of 1:1 in the presence of anti-NKG2A or control IgG (20 μg/ml) for 24 h. DCs (1 × 10⁵) were stimulated with the supernatant obtained from the cocultured medium for 24 h. A. The expressions of CD40, CD86, and CCR7 were evaluated by FACS analysis. All experiments were performed five times and statistical analysis is shown. *, p < 0.05. B. Allogeneic naive CD4⁺ T cells (1 × 10⁵/well) were cultured for 72 h by graded numbers of DCs pretreated as described above and then the proliferation of CD4⁺ T cells was examined by [³H]thymidine incorporation. C. DCs were stimulated with the supernatant of N-NK (N) or HCV-NK (HCV)/Hep3B coculture in the presence of the masking Ab of NKG2A or control IgG (20 μg/ml) as described above, and after washing three times, DCs (1 × 10⁵/well) were cocultured with allogeneic naive CD4⁺ T cells (5 × 10⁵/well) for 48 h. After positive gating as CD4⁺ T cells to exclude the DC population, intracellular expression of IFN-γ and IL-10 in CD4⁺ T cells was determined by FACS analysis. The number in each quadrant represents the percentage of cell numbers. Similar results were obtained in three independent experiments, and representative data are shown.
In the presence of anti-NKG2A Ab (20 μg/ml), infected individuals (HCV-NK) were cocultured with Hep3B at a ratio of 1:1 for 24 h. In some experiments, NK cells were cocultured with Hep3B in the presence of anti-NKG2A Ab (20 μg/ml; anti-NKG2A). DCs (1 × 10^5) were stimulated with the culture supernatant in the presence or absence of both anti-IL-10 (20 μg/ml) and anti-TGFβ-neutralizing Ab (30 μg/ml) for 24 h. The expressions of CD40, CD86, CD83, and CCR7 were evaluated by FACS analysis. All experiments were performed at least three times and representative results (A) as well as the statistical analysis of CD40 and CD86 (B) are shown as the MFI of the stained cells. *p < 0.05.

FIGURE 10. IL-10 and TGFβ are responsible for NKG2A-mediated inhibition of HCV-NK activation of DCs. NK cells (1 × 10^5) from HCV-infected individuals (HCV-NK) were cocultured with Hep3B at a ratio of 1:1 for 24 h. In some experiments, NK cells were cocultured with Hep3B in the presence of anti-NKG2A Ab (20 μg/ml; anti-NKG2A). DCs (1 × 10^5) were stimulated with the culture supernatant in the presence or absence of both anti-IL-10 (20 μg/ml) and anti-TGFβ-neutralizing Ab (30 μg/ml) for 24 h. The expressions of CD40, CD86, CD83, and CCR7 were evaluated by FACS analysis. All experiments were performed at least three times and representative results (A) as well as the statistical analysis of CD40 and CD86 (B) are shown as the MFI of the stained cells. *p < 0.05.

In these studies, NK cell activation, triggered by various kinds of cytokines, such as IL-2 and IFN-γ, were required for efficiently priming DCs. In the present study, we extended these concepts by demonstrating that NK cells are capable of activating DCs in the presence of third-party cells. N-NK cells up-regulated DC functions, such as the promotion of their maturation status, migratory as well as CD4+ T cell stimulatory capacities, when they were stimulated with Hep3B or K562 cells. Furthermore, soluble factors released from cocultures of NK cells and human hepatic cells were responsible for controlling the DC activation status. In contrast, HCV-NK, upon stimulation of Hep3B cells, had little ability to induce DC activation, whereas they could do so at similar levels to N-NK when they were cultured with K562 cells. These findings demonstrated that there is a marked difference between N-NK and HCV-NK in their ability of DC activation when stimulated with human hepatoma cells.

NK cell functions are regulated by inhibitory and activating signals through their receptor-ligand interaction (10). A recent report by Mocikat et al. (35) described that tumor cells expressing low levels of NK inhibitory ligand MHC class I could activate NK cells, which, in turn, could prime DCs to efficiently induce CD8+ T cell activities. We have demonstrated in this study that the high expression levels of CD94/NKG2A on HCV-NK contributed to insufficient NK cell-mediated DC activation after interacting with HLA-E-bearing hepatoma cells. Although nontransformed hepatocytes were different from hepatoma cells in terms of NK cell activation, they were quite similar to hepatoma cells in terms of HLA-E expression and the ability to suppress NK cell activation of DCs through the NKG2A inhibitory signal. It should also be noted that this is not hepatic cell specific but rather depends on HLA-E expression on third-party cells, because HCV-NK cocultured with the histiocytic lymphoma cell line U937, which expresses HLA-E (31), could not activate DCs (M. Jinushi, unpublished data). Under pathological conditions, NK cells may be activated not only by transformed hepatocytes via NKG2D ligands, but also by inflammatory/immune regulatory cytokines. Whatever the mechanisms of NK cell activation, NK cell activity and DC maturation could be negatively regulated by hepatoma cells as well as nontransformed hepatocytes via NK G2A/HLA-E pathways. In this regard, NK cell triggering and subsequent DC activation may be down-regulated in the HLA-E-bearing liver epithelial cells of HCV-infected patients during the course of inflammation and transformation.

The mechanisms by which HCV-NK express CD94/NKG2A at considerable high levels remain elusive. One possibility is that the expression levels of NK receptors may be modified by various types of cytokines released under chronically inflamed conditions. Given previous findings that high levels of NK receptors may be modified by various types of cytokines released under chronically inflamed conditions. Given previous findings that high levels of serum TGFβ production were observed in chronic HCV-infected patients (36) and that TGFβ can up-regulate the expression of NKG2A on NK cells (37), TGFβ may contribute to the high expression of NKG2A. However, we cannot exclude the possibility of other factors, including the virus itself, having roles in the modification of NKG2A.

Other interesting observations are that high amounts of IL-10 and TGFβ were produced from HCV-NK upon the stimulation of hepatic cells, which appeared to be dependent on an HLA-E and NKG2A interaction. Previous reports have described that IL-10/ TGFβ-modified DCs showed up-regulation of MHC II and little induction of costimulatory molecules (38, 39). Our phenotypic analysis showed that DCs up-regulate MHC II, but not CD40 or CD86, upon stimulation of HCV-NK/Hep3B supernatants; these phenotypes are similar to those of IL-10/TGFβ-modified DCs. Indeed, NKG2A-mediated suppression of the ability of HCV-NK to activate DCs was largely dependent on the production of IL-10 and TGFβ. It has been reported that IL-10/TGFβ-modified DCs can induce IL-10-producing T cells as well as CD25+CD4+ regulatory T cells (40–43). Taken together, production of IL-10 and/or TGFβ from NK cells after NKG2A triggering may lead to generation of DCs with regulatory properties in HCV infection.

The underlying mechanisms by which HCV causes persistent infection in a vast majority of patients have been largely unknown. There is much evidence that HCV-specific adaptive immune responses have an important role in the recognition and elimination of virus-infected cells. Although direct proof of NK cell involvement in the control of HCV infection has been lacking, our current study explores the possibility of NK cells indirectly suppressing adaptive T cell responses by modulating DC activities; HCV-NK activity was down-modulated through NKG2A-mediated inhibitory actions, and the suppression of NK cell activity could suppress DC functions. Furthermore, HCV-NK/Hep3B-stimulated DCs preferentially generated IL-10-producing CD4+ T cells. These results are also consistent with the concept that the perturbation of immune functions, such as the dominant shift of cytokine profiles toward Th2 type, may enable HCV to evade antiviral responses (44–46). Therefore, expression levels of NKG2A on NK cells may be one of the important factors interfering with the ability of DCs to generate HCV-specificadaptive immune responses.

A question arises whether increased NKG2A expression and subsequent modulation of DC functions are specific for NK cells from chronic HCV-infected patients. In the case of patients with chronic hepatitis B virus infection, the expression levels of NKG2A on NK cells were not different from those of N-NK, and...
NK cell triggering and DC maturation were quite similar between HBV-infected patients and healthy donors (our unpublished data).

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References


