

deficient mice in which NK cells were not activated after the injection. Mounting lines of evidence suggest the therapeutic usefulness of  $\alpha$ -GalCer, IL-2, and IL-12 administration for inhibiting intrahepatic metastasis of tumor cells, which depends, at least in part, on their ability to activate NKT cells and/or NK cells. However, overdosing of these agents is well known to be potentially toxic to the liver,<sup>32-35</sup> similar to ConA injection. Our present study suggests that a low dose of ConA is capable of preferentially activating innate immune cells in the liver to provoke an antitumor effect without causing liver injury.

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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<sup>1</sup>

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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 TGF $\beta$  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 TGF $\beta$ . The blockade also endowed HCV-NK with an ability to drive DCs to generate Th1-polarized CD4<sup>+</sup> 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)<sup>3</sup> 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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<sup>3</sup> 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<sup>+</sup> 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<sup>+</sup>CD3<sup>-</sup>; >90% purified) as well as naive CD4<sup>+</sup> T cells (CD45RA<sup>+</sup>RO<sup>-</sup>; >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 5.0 × 10<sup>5</sup>/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 antimycotics (Invitrogen Life Technologies) in a humidified atmosphere of 5% CO<sub>2</sub> 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 antimycotics. 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.

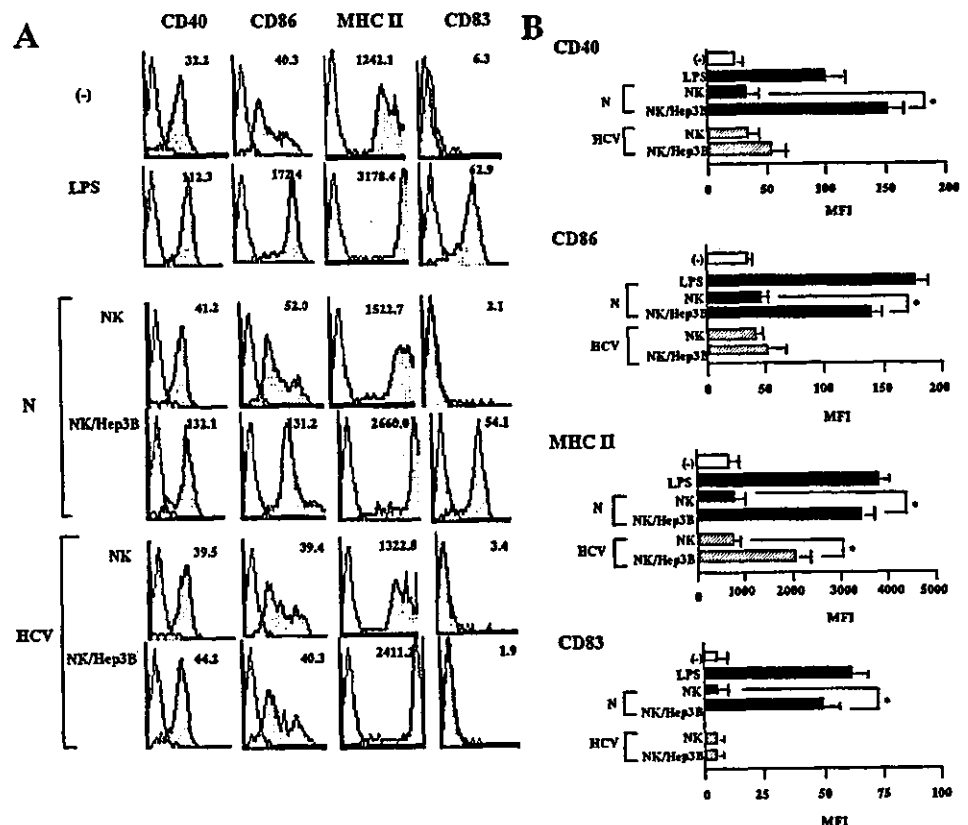
*NK cells, hepatic cells, and DC cocultures*

Resting NK cells and DCs were seeded in the same wells of 24-well plates and cultured with human hepatic cells for 48 h (1 × 10<sup>5</sup> 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 polycarbon 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 (1 × 10<sup>5</sup> cells/well) were seeded in 24-well plates and stimulated with Hep3B cells or K562 cells (1 × 10<sup>5</sup> 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 (1 × 10<sup>5</sup> 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 (1 × 10<sup>5</sup>/well) derived from healthy volunteers (N) or chronic HCV-infected patients (HCV) were cocultured with allogeneic DCs from healthy donors at a ratio of 1:1 in the absence (NK) or presence (NK/Hep3B) of Hep3B cells (1 × 10<sup>5</sup>/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, CDE86, MHC II, and CD83 on DCs after positively gating CD11c<sup>+</sup>CD56<sup>-</sup> 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.



obtained supernatants of NK/hepatic cells or K562 for 24 h and were subjected to further analysis. In some experiments, anti-NKG2A mAb Z199 or isotype-matched control Ab was added during the cocultures of NK cells and Hep3B. Z199 mAb was previously confirmed to block NKG2A-mediated signal (24). The supernatant of NK/Hep3B cocultures was also treated with anti-IL-10 and TGF $\beta$ -neutralizing Ab (R&D Systems, Minneapolis, MN) and used for DC stimulation for 24 h.

#### Flow cytometric analysis

DCs ( $5 \times 10^5$ /ml) were washed and resuspended in PBS containing 1% BSA and 0.05% NaN<sub>3</sub> 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 (KIR2DL1/2DS1), GL186 (KIR2DL2-3/2DS2), p50.3 (KIR2DS4), DX9 (KIR3DL1), HP-3B1 (CD94), Z199 (NKG2A), and 1D11(NKG2D). EB6, GL186, p50.3, HP3B1 and Z199 were purchased from Coulter Immunotech. DX9 was purchased from BD Pharmingen. 1D11 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 cytometric

analysis using a FACScan system (BD Pharmingen), and data analysis was performed using CellQuest software.

The expression of HLA class I as well as HLA-E was examined on hepatic cells and K562 by using w6/32 or 3D12 (IgG1), respectively. 3D12 was kindly provided by Dr. E. Geraghty (Fred Hutchinson Cancer Research Institute) and was used as reported previously (26).

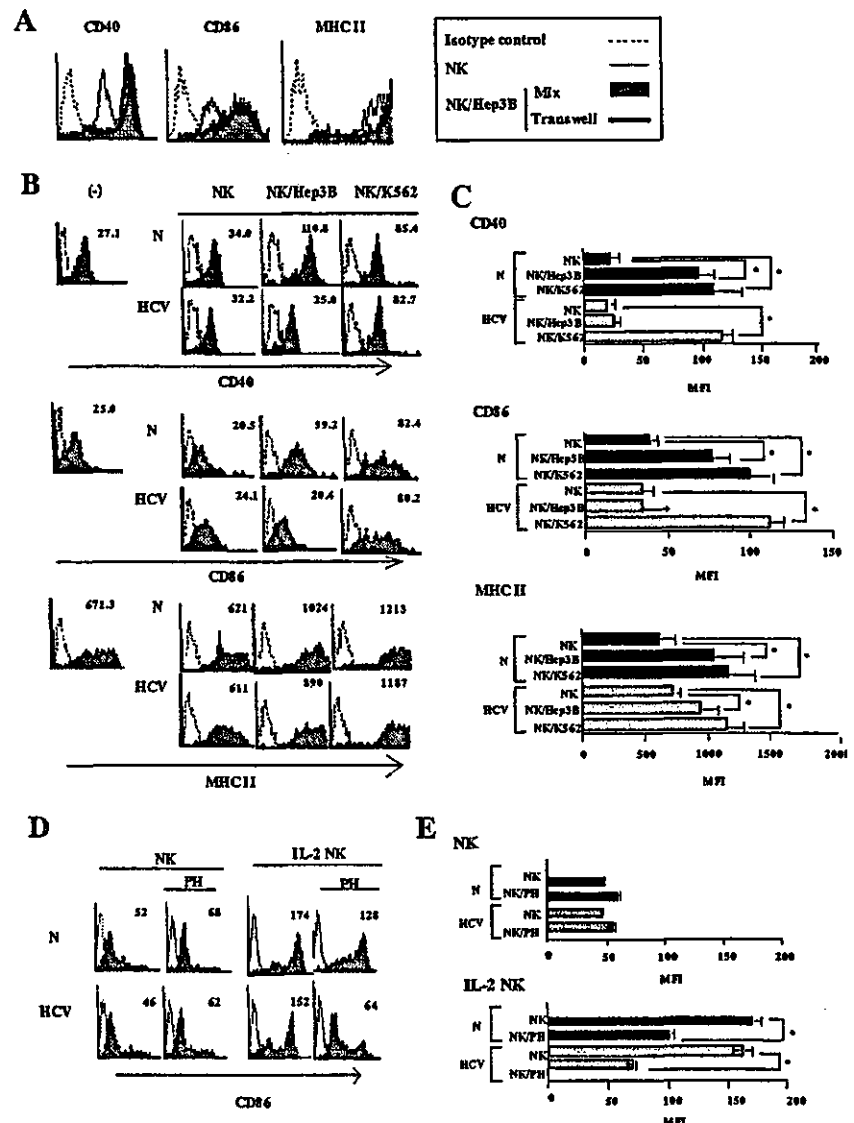
#### Measurements of cytokine production

The culture supernatants of IFN- $\gamma$ , TNF- $\alpha$ , IL-10, and TGF $\beta$  were examined using ELISA kits according to the manufacturer's instructions (IFN- $\gamma$ , TNF- $\alpha$ , and IL-10; Endogen, Woburn, MA; TGF $\beta$ , R&D Systems).

#### Chemotactic assay

DC migration capacity was evaluated using the chemotaxis microchamber technique as described previously (8). In brief, human recombinant MIP-3 $\beta$ /CCL19 (R&D Systems) was diluted to 100 ng/ml RPMI 1640 medium and added to the bottom wells and then DCs ( $1 \times 10^5$ /well) were applied to the upper wells, which were separated from the bottom wells with 8- $\mu$ m 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- $\mu$ m 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 ( $1 \times 10^5$ ) 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,  $1 \times 10^5$ ) or those stimulated with IL-2 (50 ng/ml) for 24 h (IL-2 NK,  $1 \times 10^5$ ) 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<sup>+</sup> T cells were pretreated with 1  $\mu$ M GolgiPlug (BD Pharmingen) for 4 h at 37°C. At the end of the incubation period, NK cells and naive CD4<sup>+</sup> T cells were stained with PE5-labeled CD56 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- $\gamma$  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  $\mu$ g/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 ( $10-0.33 \times 10^5$ /well). The DCs were cultured at graded numbers with the responder naive CD4<sup>+</sup> cells ( $1.0 \times 10^5$ /well) for 72 h. The cocultured cells were pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine for 16 h of incubation and collected onto a glass fiber filter. [<sup>3</sup>H]Thymidine incorporation was quantified using a beta-plate liquid scintillation counter. The results were expressed as the mean cpm in triplicate cultures.

### CD4<sup>+</sup> 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  $\mu$ g/ml (27) and were incubated overnight at 37°C. Autologous CD4<sup>+</sup> 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 <sup>51</sup>Cr 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)  $\times$  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

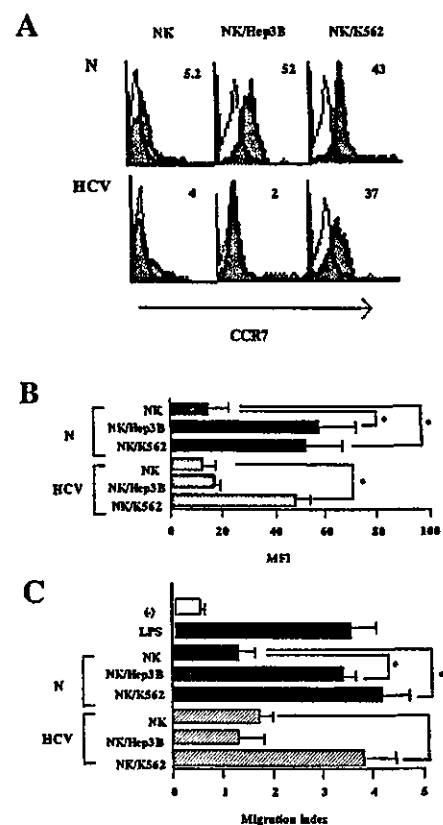
### DCs gained matured phenotypes in the presence of N-NK and Hep3B hepatoma cells

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



**FIGURE 3.** NK/Hep3B coculture supernatants can regulate chemotactic activity of DCs. NK cells from healthy (N) and HCV-infected (HCV) individuals were cultured with Hep3B or K562 at a ratio of 1:1 for 24 h. DCs from healthy donors were stimulated with the obtained supernatant for 24 h and were evaluated for their expression of CCR7 (filled histograms). Open histograms represent the staining with control Ab. All experiments were performed five times and representative results (A) as well as the statistical analysis (B) are shown as MFI of the staining cells. \*, *p* < 0.05. C, CCL19 (100 ng/ml) were loaded in the lower compartment and DCs stimulated as described above were seeded in the upper compartment of Boyden microchambers. After 2 h, the cells that migrated to the lower chambers were collected and counted. These examinations were performed with triplicate cultures, and the results are presented as a migration index (cell number in CCD19-contained medium/cell numbers in medium alone). Similar results were obtained in three independent experiments. \*, *p* < 0.05.

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/K562 (Fig. 3).

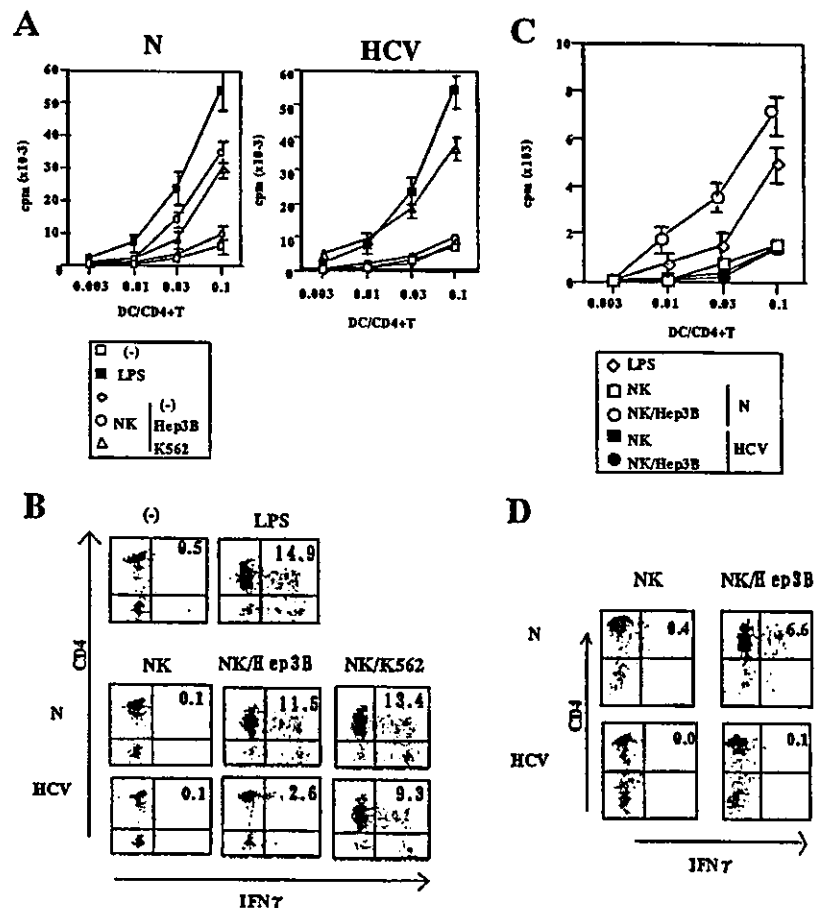
We next evaluated whether the DCs exposed to the NK/Hep3B supernatant could stimulate naive CD4<sup>+</sup> T cells from allogeneic donors. When pretreated with the supernatant of either N-NK/Hep3B or N-NK/K562 mixtures, DCs were capable of stimulating proliferation and IFN- $\gamma$  production of allogeneic CD4<sup>+</sup> T cells (Fig. 4, A and B). In contrast, the allostimulatory capacity of DCs was severely impaired when pretreated with the supernatant of

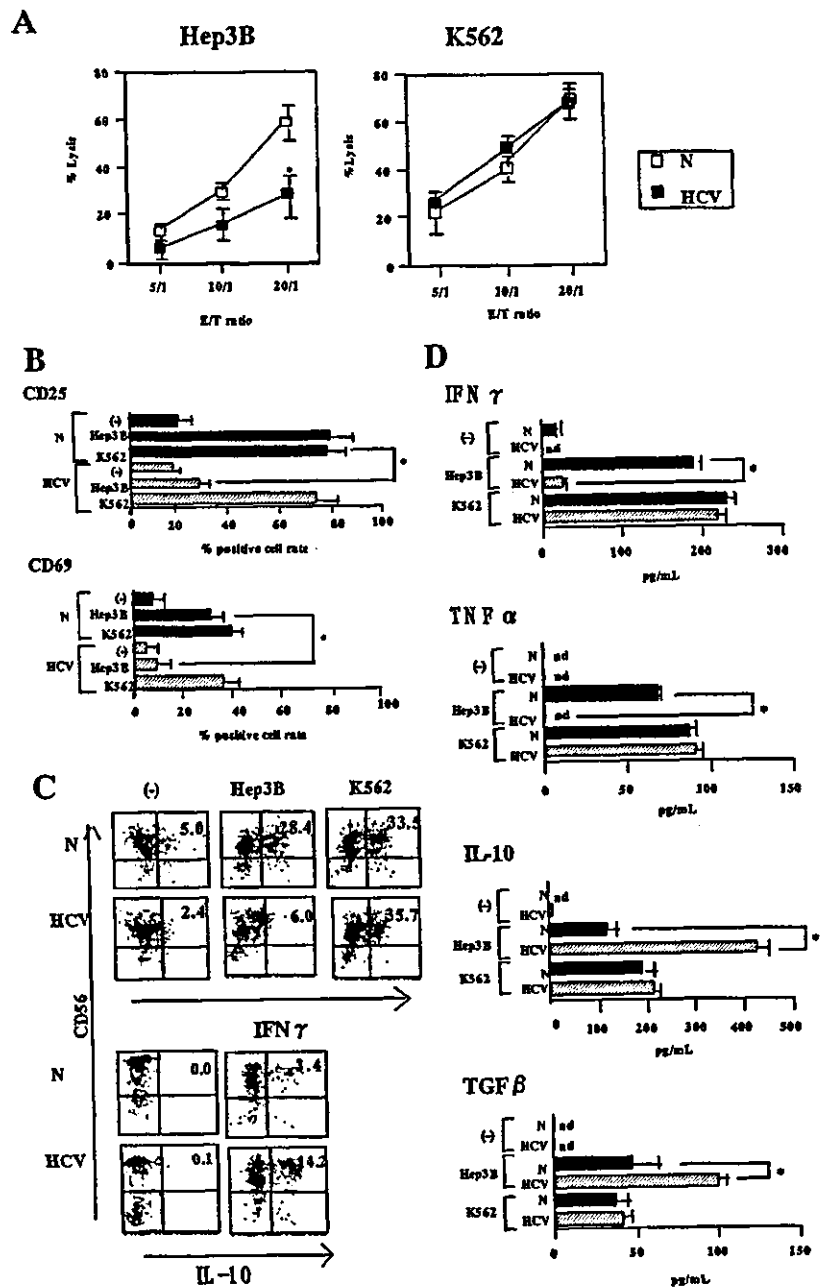
HCV-NK/Hep3B coculture, but not when pretreated with HCV-NK/K562 supernatant. We further examined for differences between N-NK and HCV-NK cultured with Hep3B cells with respect to DC-induced activation of Ag-specific CD4<sup>+</sup> T cells. DCs endowed autologous CD4<sup>+</sup> T cells with the ability to proliferate and 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<sup>+</sup> 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

**FIGURE 4.** N-NK and HCV-NK differ in their ability to trigger DC-mediated induction of CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells for 48 h. In these settings, DCs did not induce autologous CD4<sup>+</sup> T cell responses without recall Ag. CD4<sup>+</sup> T cells ( $1 \times 10^5$ /well) were cultured with graded numbers of DCs and proliferation was examined by [<sup>3</sup>H]thymidine incorporation (*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.





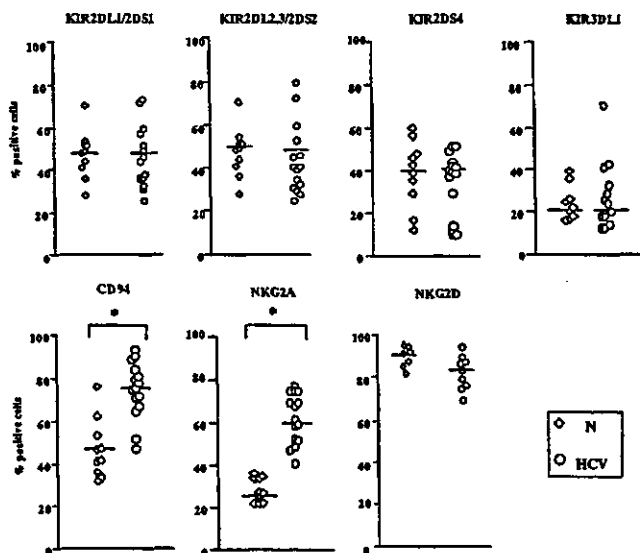
**FIGURE 5.** Human hepatic cells can activate N-NK, but not HCV-NK. **A**, Cytolytic ability of NK cells against Hep3B or K562 cells. NK cells isolated from healthy volunteers (N) or HCV-infected patients (HCV) were cultured with <sup>51</sup>Cr-labeled Hep3B or K562 at the indicated ratios for 4 h and then subjected to the standard <sup>51</sup>Cr-releasing assay. Similar results were obtained for three independent donors, and the data are presented as a composite examined for each group. \*, *p* < 0.05. **B**, The change of CD25 and CD69 expression on NK cells. Resting NK cells from healthy volunteers (N) or HCV-infected patients (HCV) were cultured with or without Hep3B or K562 for 24 h and analyzed for the activation markers CD25 and CD69 by flow cytometry. \*, *p* < 0.05. **C**, Intracellular IFN-γ and IL-10 expression of NK cells. NK cells from healthy volunteers (N) or HCV-infected patients (HCV) were cultured with or without Hep3B or K562 for 24 h, and IFN-γ and IL-10 expression were determined by flow cytometry. The number in the right upper quadrant represents the percentage of CD56-positive cells expressing IFN-γ or IL-10. **D**, NK cells generated from healthy (N) and HCV-infected (HCV) individuals were cultured with or without Hep3B or K562 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.

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 high levels in HCV-NK. It has been reported that human NK cells were composed of CD56<sup>bright</sup> and CD56<sup>dim</sup> subsets and that CD56<sup>bright</sup> NK cells express CD94/NKG2A more than CD56<sup>dim</sup> subsets (28). The percentages of the CD56<sup>bright</sup> 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 CD56<sup>bright</sup> subpopulation.

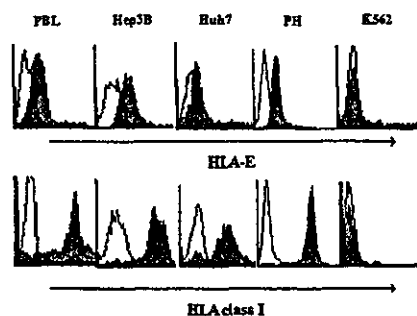


**FIGURE 6.** Profiles of NK receptor expression on NK cells. NK cells isolated from normal volunteers ( $n = 10$ ; N) or from HCV-infected patients ( $n = 15$ ; HCV) were stained with Abs for various KIRs, CD94, NKG2A, and NKG2D as described in *Materials and Methods*. The receptor expression of CD56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

#### *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 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.

NKG2A heterodimer-HLA-E interaction (24), resulted in a marked increase in Hep3B cytotoxicity 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 cytotoxicity 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- $\gamma$  and TNF- $\alpha$ . Again, the blockade of the NKG2A-mediated signal resulted in a substantial decrease in IL-10 and TGF $\beta$  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<sup>+</sup> T cells, whereas the NKG2A blockade slightly enhanced proliferation of CD4<sup>+</sup> 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<sup>+</sup> T cells. Intracellular cytokine staining showed that CD4<sup>+</sup> T cells can produce both IFN- $\gamma$  and IL-10 when stimulated by DCs that had been exposed to N-NK/Hep3B coculture supernatant. DCs pretreated with the HCV-NK/Hep3B supernatant generated CD4<sup>+</sup> T cells, preferentially producing IL-10. Blockade of NKG2A during the coculture of NK cells and Hep3B inhibited IL-10 production and promoted IFN- $\gamma$  production from CD4<sup>+</sup> 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 TGF $\beta$ , act as downstream mediators of NKG2A-induced inhibition of NK cell activation of DCs*

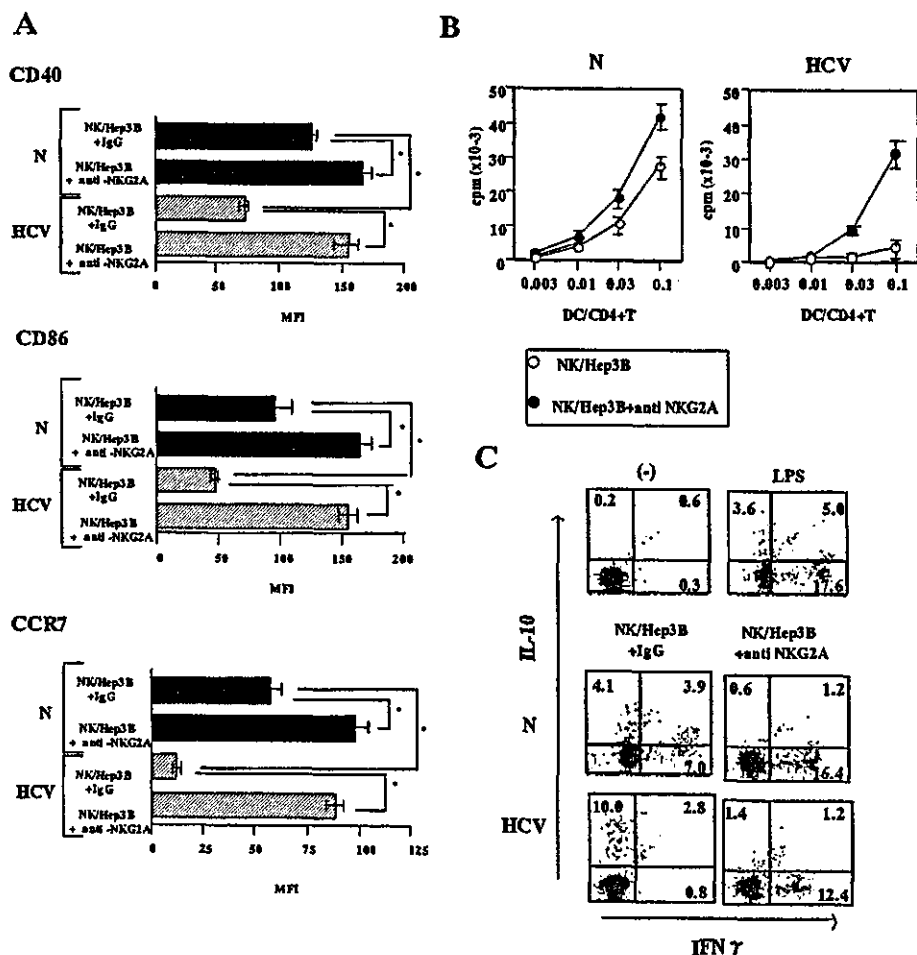
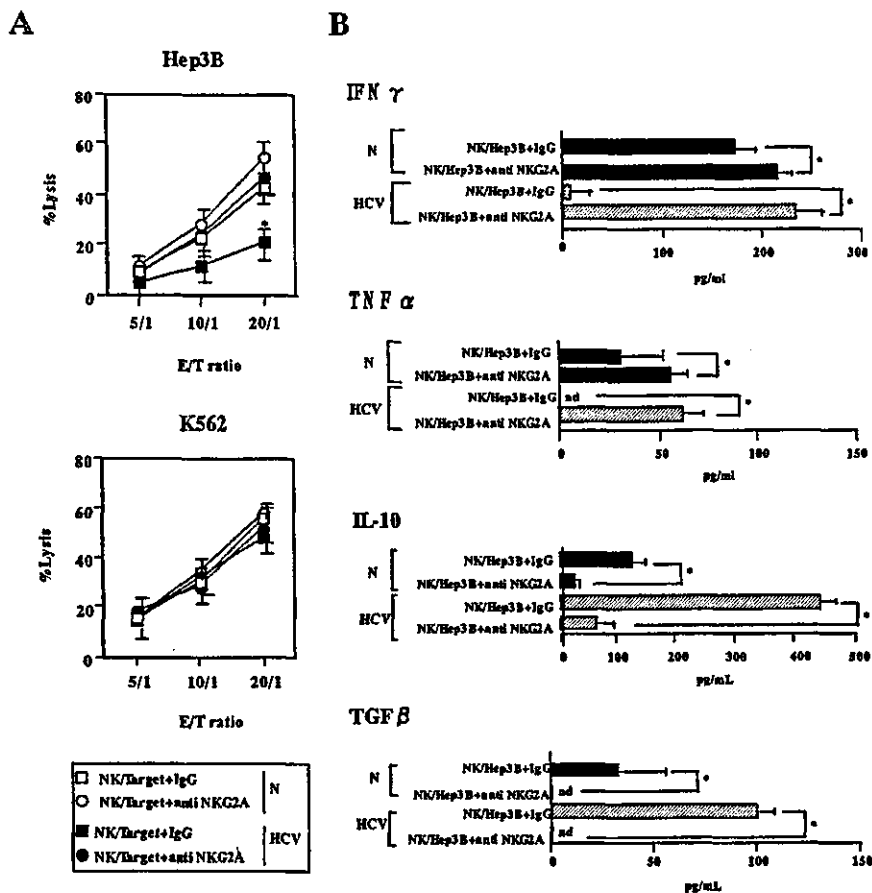
IL-10 and TGF $\beta$  are known to act as suppressive factors of DC activation to induce the anergic states (33, 34). We examined whether IL-10 and TGF $\beta$ , produced in large amounts by HCV-NK, are responsible for the impaired DC activation. Toward this goal, the culture supernatant of HCV-NK/Hep3B cells was treated with neutralizing Abs of IL-10 and TGF $\beta$  and was used for the stimulation of DCs. The blockade of IL-10/TGF $\beta$  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/TGF $\beta$  blockade had little additional effects on DC maturation (Fig. 10). These results indicated that the IL-10/TGF $\beta$  production, triggered by the interaction of NKG2A and its ligand, could directly interfere with DC activation.

## **Discussion**

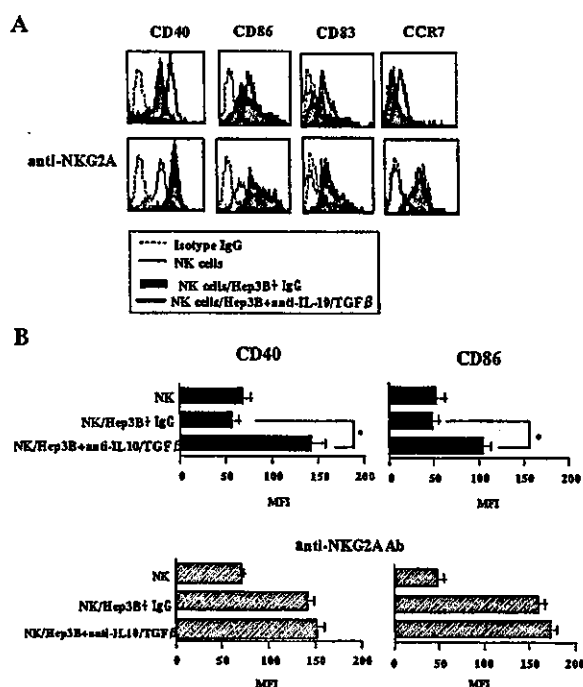
Several lines of evidence have revealed that NK cell-mediated regulation of DCs plays an important role in the recognition of pathogens and malignancies at the early phase of immune responses



**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 <sup>51</sup>Cr-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 <sup>51</sup>Cr-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 ( $1 \times 10^5$ ) 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 ( $1 \times 10^5$ ) 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<sup>+</sup> T cells ( $1 \times 10^5$ /well) were cultured for 72 h by graded numbers of DCs pretreated as described above and then the proliferation of CD4<sup>+</sup> T cells was examined by [<sup>3</sup>H]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 ( $1 \times 10^5$ /well) were cocultured with allogeneic naive CD4<sup>+</sup> T cells ( $5 \times 10^5$ /well) for 48 h. After positive gating as CD4<sup>+</sup> cells to exclude the DC population, intracellular expression of IFN-γ and IL-10 in CD4<sup>+</sup> 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.



**FIGURE 10.** IL-10 and TGF $\beta$  are responsible for NKG2A-mediated inhibition of HCV-NK activation of DCs. NK cells ( $1 \times 10^5$ ) 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  $\mu$ g/ml; anti-NKG2A). DCs ( $1 \times 10^5$ ) were stimulated with the culture supernatant in the presence or absence of both anti-IL-10 (20  $\mu$ g/ml) and anti-TGF $\beta$ -neutralizing Ab (30  $\mu$ g/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. \*,  $p < 0.05$ .

(4–6, 35). In these studies, NK cell activation, triggered by various kinds of cytokines, such as IL-2 and IFN- $\gamma$ , 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 prohistiocytic 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 serum TGF $\beta$  production were observed in chronic HCV-infected patients (36) and that TGF $\beta$  can up-regulate the expression of NKG2A on NK cells (37), TGF $\beta$  may contribute to the high expression of NKG2A on NK cells in chronic HCV infection. 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 TGF $\beta$  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/TGF $\beta$ -stimulated 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/TGF $\beta$ -stimulated DCs. Indeed, NKG2A-mediated suppression of the ability of HCV-NK to activate DCs was largely dependent on the production of IL-10 and TGF $\beta$ . It has been reported that IL-10/TGF $\beta$ -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 TGF $\beta$  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 explored 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-specific adaptive 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). Since aberrant expression of CD94/NKG2A on NK cells could affect not only their activation status but also acquired immune responses, it will be of great interest to evaluate NK cell expression of CD94/NKG2A in other viral infection in future study.

In summary, we have demonstrated that NKG2A-mediated negative regulation of NK cell activities represents a novel innate pathway for determining the magnitude and direction of DC functions. These findings imply the attractive concept that modulation of the interplay between inhibitory and activating signals mediated by NK receptors can dramatically change the properties of DC-primed Ag-specific T cell immunity against various kinds of infectious diseases as well as cancers.

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# Reduced Numbers and Impaired Ability of Myeloid and Plasmacytoid Dendritic Cells to Polarize T Helper Cells in Chronic Hepatitis C Virus Infection

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Hepatitis C virus (HCV) infection induces a wide range of chronic liver injuries. The mechanism by which HCV evades the immune surveillance system remains obscure. Blood dendritic cells (DCs) consist of myeloid and plasmacytoid subsets that play distinct roles in the regulation of antiviral immune responses; however, their roles in the pathogenesis of HCV infection are yet to be determined. We compared the numbers and functions of myeloid and plasmacytoid DCs between 43 patients with chronic hepatitis and 26 age-matched healthy volunteers. Absolute numbers of myeloid DCs, plasmacytoid DCs, and DC progenitors in the periphery were significantly lower in patients with chronic hepatitis than in healthy volunteers. Myeloid and plasmacytoid DCs from the patients had impaired abilities to stimulate allogeneic CD4 T cells and to produce interleukin (IL)-12 p70 and interferon- $\alpha$ , compared with those from healthy volunteers. After exposure to naive CD4 T cells, myeloid DCs from the patients were less able to drive the T helper type 1 response, whereas myeloid and plasmacytoid DCs from the patients primed more IL-10-producing cells than did those from healthy volunteers. In conclusion, in chronic HCV infection, both types of blood DCs are reduced and have an impaired ability to polarize T helper cells.

Hepatitis C virus (HCV) is a double-stranded RNA virus that causes a wide range of chronic liver diseases in humans, from mild or active chronic hepatitis to liver cirrhosis and hepatocellular carcinoma [1]. Epidemiological studies have revealed that the incidence of HCV-related hepatocellular carcinoma is increasing

in general, not only in areas where HCV is endemic, showing that HCV infection is a worldwide health problem [2]. To prevent the progression of liver injury and the subsequent occurrence of hepatocellular carcinoma, HCV should be eradicated. At present, a combination of pegylated interferon (IFN)- $\alpha$  and ribavirin is used as the standard treatment for chronic HCV infection [3]. This protocol has significantly improved the rate of HCV eradication, compared with that attained by IFN- $\alpha$  monotherapy; however, more than half of the patients are refractory to the combination therapy [3]. To improve the prognosis of chronically infected patients, the reason for HCV persistently infecting humans and resisting clearance, even with repeated administration of antiviral agents, needs to be elucidated. It has been suggested that HCV has strategies for escaping from the immune surveillance system—for example, by disabling antigen-presenting cell (APC) function [4] or inhibiting CD4 and CD8 T cell responses [5, 6].

Dendritic cells (DCs) are professional APCs that stim-

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ulate innate and adaptive immune reactions by priming other types of blood cells [7]. The existence of ontogenetically distinct DC subsets has been reported in humans and mice, and the subsets are grouped into at least 2 types—myeloid DCs and lymphoid (i.e., plasmacytoid) DCs [8]. In general, in humans, myeloid DCs produce interleukin (IL)-12 or tumor necrosis factor- $\alpha$  when stimulated and induce Th1 polarization, whereas plasmacytoid DCs produce a considerable amount of type-I IFN after viral infection and mainly induce Th2 polarization [8, 9]. However, the properties of each DC subset are flexible in vivo and vary according to their activation state or the nature of the maturation stimuli given to DCs [8, 9]. Several reports have focused on DC dysfunction and its involvement in the pathogenesis of a variety of disorders [10–12]. We and other groups have shown that, in chronic HCV infection, DCs have impaired allostimulatory capacities [4, 13], suggesting that DCs themselves are a reciprocal target of HCV-induced immune dysfunction.

Recently, the roles of blood DC subsets in HIV infection have been studied extensively [11, 14, 15]. A clear correlation was reported between plasmacytoid DC counts and HIV quantity in HIV-infected patients [14]. More importantly, plasmacytoid DC counts decrease in parallel with the progression of HIV-related disorders [15], suggesting that plasmacytoid DCs protect against the development of disease. To clarify the roles of DC subsets in HCV infection, we compared the numbers and functions of blood DCs between patients with chronic hepatitis and healthy volunteers. We were able to demonstrate that, in chronic HCV infection, both types of DCs are numerically and functionally impaired. Of note is the finding that myeloid DCs from patients with chronic hepatitis show impaired ability to induce Th1 polarization and that myeloid and plasmacytoid DCs from patients with chronic hepatitis can prime more IL-10-producing cells than can those from healthy volunteers.

## SUBJECTS, MATERIALS, AND METHODS

**Subjects.** Among the patients with chronic hepatitis monitored at Osaka University Hospital (Osaka, Japan), 43 (27 men and 16 women; mean  $\pm$  SD age, 48  $\pm$  8 years) were enrolled in the present study after their written, informed consent had been obtained. Patients were confirmed to be positive for both serum anti-HCV antibody (Ab) and HCV RNA but were negative for other viral infections, including hepatitis B virus (HBV) and HIV. None of them had been treated with antiviral agents, including IFN- $\alpha$  or ribavirin. All patients had shown persistent or fluctuating alanine aminotransferase (ALT) abnormalities at enrollment (mean  $\pm$  SD ALT levels, 91  $\pm$  72 IU/L). The presence of other causes of liver disease, such as autoimmune deficiency, alcohol abuse, and metabolic disorders, was excluded by use of laboratory and imaging analyses. In all

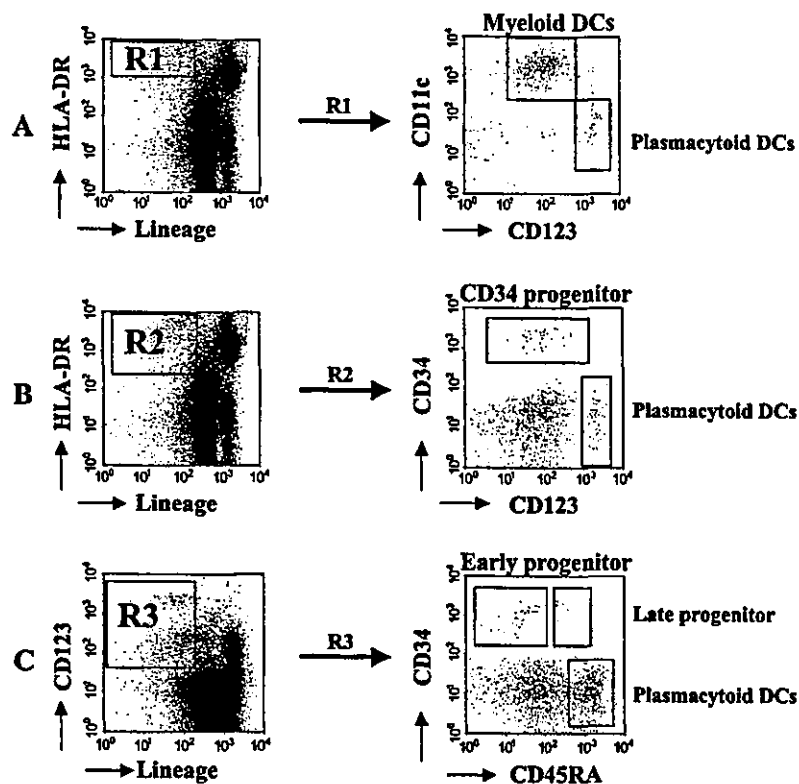
patients, a combination of biochemical testing and ultrasonography or computer tomography scan analysis ruled out the presence of cirrhosis and tumors in the liver. As control subjects, 26 age-matched, healthy volunteers who were all negative for HCV, HBV, and HIV were examined (20 men and 6 women; mean  $\pm$  SD age, 45  $\pm$  10 years). HCV RNA quantity was assayed by use of a branched DNA probe assay (Chiron HCV-RNA). HCV serotyping was performed as described elsewhere [16]. The median HCV RNA titer in patients was 5.7 million genome equivalents/mL (Meq/mL) (range, 0–37 Meq/mL). Thirty-three patients had HCV serotype 1, and 4 patients had HCV serotype 2; HCV serotype was not determined for the remaining 6 patients.

**Reagents.** Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 were provided by Kirin Brewery or were purchased from PeproTech. Recombinant human IL-3 was purchased from R&D Systems. Recombinant human IL-2 was purchased from Genzyme-Techne. IFN- $\gamma$  was purchased from Strathman-Biotech. Human lymphoblastoid IFN- $\alpha$  was provided by Sumitomo Pharmaceuticals.

**Analysis of myeloid DCs, plasmacytoid DCs, and DC progenitors in the periphery.** Twenty milliliters of heparinized venous blood was drawn from patients and volunteers. Peripheral blood mononuclear cells (PBMCs) were collected by use of density-gradient centrifugation on a ficoll-hypaque cushion. After the live PBMCs had been counted, the cells were subsequently stained with Abs. Blood DCs were defined as lineage marker (Lin; CD3, CD14, CD16, CD20, and CD56)-negative and HLA-DR<sup>+</sup> cells. After setting the gate on these cells, myeloid and plasmacytoid DCs were further defined by the pattern of CD11c and CD123 expression. Myeloid DCs are Lin<sup>-</sup>, HLA-DR<sup>+</sup>, CD11c<sup>+</sup>, and CD123<sup>low</sup> cells, and plasmacytoid DCs are Lin<sup>-</sup>, HLA-DR<sup>+</sup>, CD11c<sup>-</sup>, and CD123<sup>high</sup> cells. From the percentages of these cells determined by use of fluorescence-activated cell sorter (FACS; Becton Dickinson Immunocytometry Systems) analyses, the absolute numbers of the DC subset in the periphery were calculated by multiplying the PBMC counts.

Recent reports have shown that myeloid and plasmacytoid DCs develop from CD34<sup>+</sup> cells with the aid of various hematopoietic factors [17, 18]. Throughout the process, intermediate plasmacytoid DC progenitors are present in the periphery, and their phenotypes were determined [18]. We defined CD34 progenitors as Lin<sup>-</sup>, HLA-DR<sup>+</sup>, CD123<sup>+</sup>, and CD34<sup>+</sup> cells. The early and late progenitors of DCs were defined as Lin<sup>-</sup>, CD34<sup>+</sup>, CD123<sup>+</sup>, and CD45RA<sup>-</sup> cells and Lin<sup>-</sup>, CD34<sup>+</sup>, CD123<sup>+</sup> and CD45RA<sup>+</sup> cells, respectively. The numbers of these cells in the periphery were calculated from their percentages in PBMCs, as described above.

**Separation of DC subsets from the blood.** For the functional analyses, myeloid and plasmacytoid DCs were separated from PBMCs or buffy coat (provided by the Osaka Red Cross Blood



**Figure 1.** Identification of blood dendritic cell (DC) subsets, CD34 progenitors, and early and late progenitors of plasmacytoid DCs in peripheral blood mononuclear cells. *A*, Myeloid DCs are lineage marker-negative ( $\text{Lin}^-$ ), HLA-DR $^+$ , CD11c $^+$ , and CD123 $^{\text{low}}$ ; plasmacytoid DCs are  $\text{Lin}^-$ , HLA-DR $^+$ , CD11c $^-$ , and CD123 $^{\text{high}}$ . *B*, CD34 progenitors are  $\text{Lin}^-$ , HLA-DR $^+$ , CD34 $^+$ , and CD123 $^-$ . *C*, Early progenitors of plasmacytoid DCs are  $\text{Lin}^-$ , CD123 $^+$ , CD34 $^+$ , and CD45RA $^-$ ; late progenitors are  $\text{Lin}^-$ , CD123 $^+$ , CD34 $^+$ , and CD45RA $^+$ .

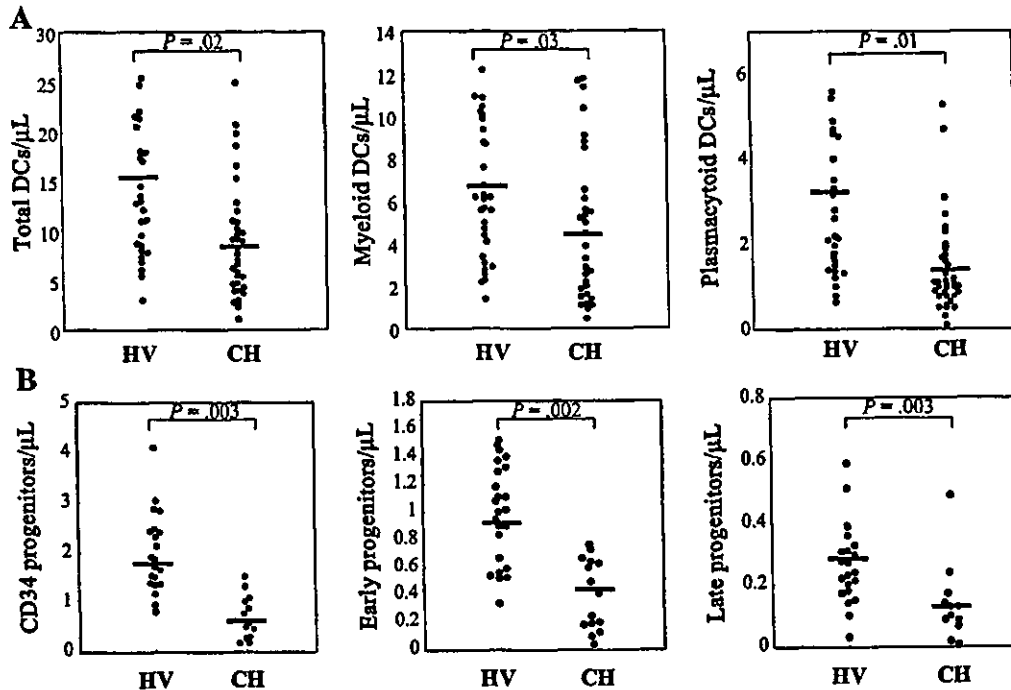
Center, Osaka, Japan) by use of blood DC antigen (BDCA)-1 and BDCA-4 separation kits (Miltenyi Biotec) with some modifications [19]. Alternatively, after  $\text{Lin}^+$  cells were depleted magnetically with the cocktail Abs containing CD3, CD14, CD16, CD20, CD56, and glycoaphorins (StemCell Technologies), myeloid and plasmacytoid DCs were sorted separately by use of a FACS Vantage SE (Becton Dickinson Immunocytometry Systems). The purity of myeloid and plasmacytoid DCs was >95%, as assessed by FACS analysis.

**DC culture.** Sorted myeloid DCs were cultured in DC media (DCM) (Isocove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 100  $\mu\text{g}/\text{mL}$  streptomycin, 100 U/mL penicillin, 2 mmol/L L-glutamine, 5 mmol/L HEPES, and 5 mmol/L nonessential amino acid) containing 50 ng/mL GM-CSF and 10 ng/mL IL-4 for 3–5 days at 37°C in 5%  $\text{CO}_2$ . To analyze cytokine production, day-3 myeloid DCs (i.e., DCs that had been incubated for 3 days) were stimulated with the same numbers of human CD40 ligand (CD40L)-transfected L cells. The culture supernatants were collected 24 h after stimulation and were used for the cytokine ELISA. Plasmacytoid DCs were cultured in DCM in the presence of 50 ng/mL IL-3 for 2–3 days. To stimulate plasmacytoid DCs to produce IFN- $\alpha$ , we added 5  $\mu\text{mol}/\text{L}$  stimulatory cytosine-phosphodiester-guanine oligodeoxynucleotide

2216 [20] to the freshly collected plasmacytoid DCs, and the supernatants were collected after 24 h.

**Flow cytometric analysis.** The expression of surface molecules on DCs was analyzed by use of a FACSCaliber (Becton Dickinson Immunocytometry Systems). At each step of the staining,  $5 \times 10^5$  cells were stained with specific Abs for 30 min at 4°C in PBS containing 2% bovine serum albumin and 0.1% sodium azide. We used fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin chlorophyll protein (Per-CP)-, cytochrome (Cy-chrome)-, or allophycocyanin-labeled mouse monoclonal Abs for the staining of HLA-DR (L243), lineage cocktail (CD3, CD14, CD16, CD19, and CD56), CD3 (HIT3a), CD4 (13B8.2), CD11c (KB90), CD14 (mC5-3), CD34 (581), CD40 (5C3), CD45RA (HI100), CD80 (L307.4), CD83 (HB15a), CD86 (IT2.2), and CDw123 (IL-3 receptor  $\alpha$ -chain and 7G3). FITC- or PE-labeled mouse IgG was substituted for specific Abs, to obtain negative controls. All Abs except CD11c (DAKO) and CD83 (Coulter Immunotech) were purchased from BD Pharmingen.

**Allogeneic mixed lymphocyte reaction (MLR).** Responder naive CD4 T cells (CD4 $^+$ , CD45RO $^-$ , and CD45RA $^+$  cells) were separated from PBMCs of healthy volunteers by use of the StemSep system (StemCell Technologies). The purity of naive CD4 T cells was >95%. The graded numbers of day-3 myeloid



**Figure 2.** Reduction of blood dendritic cells (DCs) and their progenitors in patients with chronic hepatitis (CH). Absolute nos. of blood DCs (A) and progenitors (B) were determined from their frequencies in peripheral blood mononuclear cells. Horizontal bars, median. Statistical significance was analyzed by use of the Mann-Whitney *U* test. HV, healthy volunteers.

or plasmacytoid DCs were cultured for 5 days with  $2 \times 10^4$  allogeneic naive CD4 T cells/well. In the final 16–20 h of culture, 1  $\mu$ Ci/well of [ $^3$ H]-thymidine (ICN Biomedicals) was pulsed. The uptake of [ $^3$ H]-thymidine to T cells was measured by use of a  $\beta$ -counter (Wallac).

**Analysis of T helper cell polarization by DC subsets.** After myeloid or plasmacytoid DCs were cultured in the presence of cytokines for 3 days,  $1 \times 10^4$  DCs/well were cultured for an additional 7 days with  $1 \times 10^5$  allogeneic naive CD4 T cells/well. On day 4 of the coculture, 5 ng/mL IL-2 was added. On day 7, the cells were stimulated with 50 ng/mL PMA and 1  $\mu$ g/mL ionomycin and incubated for 24 h. The supernatants of the stimulated cells were collected and used for cytokine ELISA.

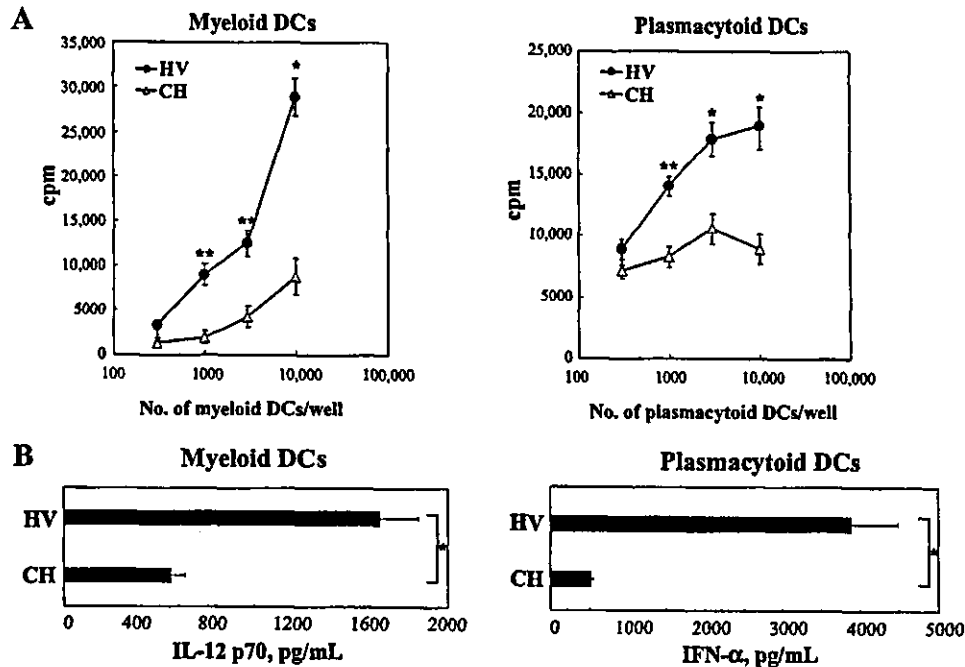
**ELISA.** The cytokine concentration was measured by use of ELISA with matched Ab pairs for human IFN- $\gamma$ , IL-4, IL-10, and IL-12 p70 (Endogen), according to the manufacturer's instructions. IFN- $\alpha$  was assayed by use of the ELISA kit (BioSource). The range of the limits of detection of the assay was 15–1000 pg/mL.

**Statistical analysis.** The differences of various parameters between the healthy volunteers and the patients were analyzed by use of the Mann-Whitney *U* test. The nonparametric Spearman's test was used to explore correlations. All analyses were performed with StatView software (version 5; SAS Institute).  $P < .05$  was considered to be statistically significant.

## RESULTS

**Reduction of blood DCs and their progenitors in patients with chronic hepatitis.** After setting the gate on Lin $^-$  and HLA-DR $^+$  DCs, myeloid and plasmacytoid DCs in the periphery were clearly identified by the expression pattern of CD11c and CD123 (figure 1A). Total DC, myeloid DC, and plasmacytoid DC counts in the patients with chronic hepatitis were lower than those in healthy volunteers (figure 2A). To exclude the possibility that the reduction of DC subsets was due to a decrease in the total PBMC count in patients with chronic hepatitis, we compared the numbers of PBMCs, CD4, and monocytes among the groups and found no differences (data not shown). Therefore, the low blood DC counts are not simply due to low PBMC counts; DCs are selectively reduced in patients with chronic hepatitis. Furthermore, in the patients with chronic hepatitis, no correlation was found between DC counts and the serum ALT levels or HCV RNA titers (data not shown).

Next, we analyzed the progenitor and precursor populations of DC subsets. In accordance with the phenotypes reported by Blom et al. [18], we identified CD34 progenitors (Lin $^-$ , HLA-DR $^+$ , CD123 $^+$ , and CD34 $^+$ ), early progenitors (Lin $^-$ , CD123 $^+$ , CD34 $^+$ , and CD45RA $^-$ ), and late progenitors (Lin $^-$ , CD123 $^+$ , CD34 $^+$ , and CD45RA $^+$ ) in the PBMCs (figure 1B and 1C). The numbers of CD34 progenitors and early and late progenitors



**Figure 3.** Functional impairment of both types of blood dendritic cells (DCs) from hepatitis C virus-infected patients. *A*, Allogeneic mixed lymphocyte reaction. Vertical bars, mean  $\pm$  SD. \* $P < .01$  and \*\* $P < .05$ , vs. patients with chronic hepatitis (CH). *B*, Day-3 myeloid DCs were stimulated with equal nos. of CD40L-expressing L cells for 24 h. Day-1 plasmacytoid DCs were cultured for 24 h in the presence of 5  $\mu$ mol/L cytosine-phosphodiester-guanine oligodeoxynucleotide 2216. The supernatants were collected from both cultures and examined for interleukin (IL)-12 p70 and interferon (IFN)- $\alpha$  by use of ELISA. Bars, mean  $\pm$  SD. \* $P < .05$  (Mann-Whitney *U* test). HV, healthy volunteers.

in patients with chronic hepatitis were significantly lower than those in healthy volunteers (figure 2B). In addition, a positive correlation was observed between Lin<sup>-</sup> and HLA-DR<sup>+</sup> DC counts and those of CD34<sup>+</sup> progenitors or early progenitors (DCs vs. CD34<sup>+</sup> progenitors,  $R^2 = 0.535$ , and  $P < .05$ ; DCs vs. early progenitors,  $R^2 = 0.533$ , and  $P < .05$ ). These results suggest that the low number of blood DCs in patients with chronic hepatitis is due, in part, to the reduction in DC progenitors.

**Functional impairment of blood DC subsets in patients with chronic hepatitis.** After myeloid and plasmacytoid DCs had been sorted magnetically and had been cultured in the presence of relevant cytokines, the phenotypes of these cells were examined. On both myeloid and plasmacytoid DCs, the expressions of CD40, CD80, CD83, CD86, and HLA-DR were not different between patients with chronic hepatitis and healthy volunteers (data not shown).

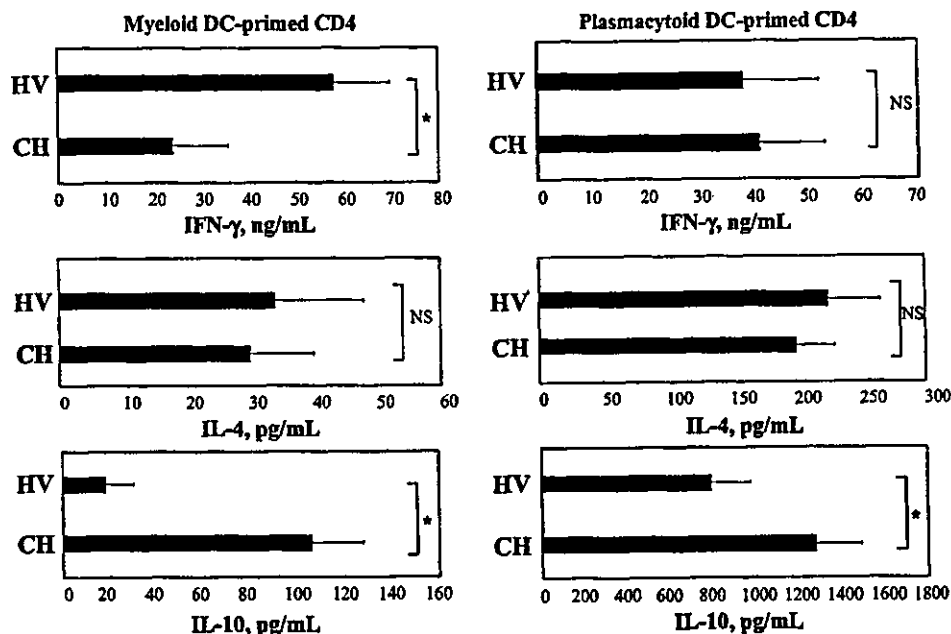
To compare the allostimulatory capacity of day-3 DCs from each group, we performed MLR. The proliferative responses of CD4 T cells with myeloid or plasmacytoid DCs from patients with chronic hepatitis were lower than those from healthy volunteers (figure 3A).

We analyzed the production of IL-12 p70 by myeloid DCs and that of IFN- $\alpha$  by plasmacytoid DCs. After stimulation, myeloid and plasmacytoid DCs from patients with chronic

hepatitis released lower amounts of these cytokines than did DCs from healthy volunteers (figure 3B). Therefore, blood DC subsets from patients with chronic hepatitis were functionally impaired in the stimulation of allogeneic T cells and cytokine production.

**Decreased ability of myeloid DCs from patients with chronic hepatitis to induce Th1 polarization and higher capacity of myeloid DCs and plasmacytoid DCs to induce IL-10-producing cells.** DCs prime naive CD4 T cells to differentiate into Th1 and Th2 or regulatory T cells. In our system, myeloid DCs predominantly induced IFN- $\gamma$ -producing Th1 cells, whereas plasmacytoid DCs primed Th1- and IL-4-positive Th2 cells and IL-10-producing cells. Myeloid DCs from patients with chronic hepatitis were less able to drive the Th1 response than were myeloid DCs from healthy volunteers (figure 4). Plasmacytoid DCs from patients with chronic hepatitis induce IFN- $\gamma$  or IL-4-producing cells at levels comparable to those induced by plasmacytoid DCs from healthy volunteers (figure 4). Both types of DCs from patients with chronic hepatitis primed more IL-10-producing cells than did those from healthy volunteers, although the levels of IL-10 were much higher in plasmacytoid DC-primed CD4 T cells than in myeloid DC-primed CD4 T cells (figure 4).





**Figure 4.** Inability of myeloid dendritic cells (DCs) from patients with chronic hepatitis (CH) to drive Th1-polarization. Myeloid and plasmacytoid DCs from patients with CH can prime more interleukin (IL)-10 producing cells than do those from healthy volunteers (HV). Day-3 myeloid or plasmacytoid DCs were cultured with allogeneic naive CD4 T cells for an additional 7 days. CD4 T cells were stimulated with PMA and ionomycin for 24 h, and the supernatants were collected for cytokine ELISA. Bars, mean + SD. \* $P < .05$  (Mann-Whitney  $U$  test). NS, not significant.

## DISCUSSION

DCs are well known central regulators in both innate and adaptive immune responses against viruses [7, 8]. Several studies, including our own [4], have demonstrated that dysfunction of monocyte-derived DCs occurs during HCV infection and is implicated in the persistence of HCV [13]. In the present study, we have determined the reduction in and dysfunction of blood DC subsets in patients with chronic hepatitis.

The effect of DC counts on the pathogenesis of virus-related disorders has been well documented in HIV infection. Plasmacytoid DC counts decrease in parallel with an increase in HIV quantity and with the progression of disease severity [14]. On the other hand, long-term suppressors, who have been healthy despite HIV infection, exhibit higher plasmacytoid DC counts than do control subjects [15]. These observations strongly suggest that plasmacytoid DCs are able to control replication of HIV and HIV-related disorders. In contrast, no correlation was observed between DC counts and HCV RNA titers in patients with chronic hepatitis, implying that DC count is not a primary factor influencing HCV replication. In addition, the lack of correlation between DC counts and ALT levels suggests that the decrease in DC counts is not simply due to liver inflammation. In patients who cleared HCV after receiving IFN- $\alpha$ -based therapy, DC and progenitor counts were comparable to those of healthy volunteers (authors' unpublished data), showing that the presence of HCV principally contributes to the reduction in DCs.

There are several possible explanations for the mechanisms that cause DC deficiency during HCV infection. One hypothesis is that HCV aims to infect DCs and enhances their susceptibility to apoptosis. By means of polymerase chain reaction, the HCV genome has been detected in myeloid and plasmacytoid DCs recovered from some chronically infected patients, although the replication level in cells appeared to be low [13, 21]. The next possible explanation for the low numbers of circulating DCs is their enhanced mobilization to other tissues. In patients with systemic lupus erythematoses or tuberculosis, plasmacytoid DCs accumulate in cutaneous or granulomatous lesions, resulting in reduction of the circulating DC pool [22, 23]. Thus, during HCV infection, DCs may disappear from the periphery to move into the inflamed liver or lymphoid tissues. Finally, the reduction of DC progenitors and/or precursors may be involved. It is generally accepted that DCs are derived from hematopoietic stem cells or CD34<sup>+</sup> progenitors [17]. Blom et al. reported that myeloid and plasmacytoid DCs were generated from CD34<sup>+</sup> cells with the aid of hematopoietic factors, such as fms-like tyrosine kinase 3 ligand [17, 18]. The present study has demonstrated that numbers of CD34<sup>+</sup> and early progenitor cells are decreased in patients with chronic hepatitis, a fact that correlates well with the results of numerical DC analyses. Therefore, the development of DCs may be impeded in chronically infected patients with chronic hepatitis, and the mechanism needs to be further investigated.

An imbalance between Th1 and Th2 subsets is crucially in-

involved in the pathogenesis of chronic hepatitis [24, 25]. Several lines of evidence have clearly demonstrated that a Th1 response, either spontaneous or IFN- $\alpha$  induced, is required for eradication of HCV [26–28]. One of the possible mechanisms causing defective Th1 responses by myeloid DCs is the lower amount of IL-12 produced by them, since a critical role of IL-12 in Th1 polarization has been reported elsewhere [29]. Direct HCV infection of DCs may be involved in the impaired production of IL-12, which is supported by the finding that the expression of HCV proteins in monocyte-derived DCs suppresses the release of IL-12 [30]. In addition to dysfunction of myeloid DCs, the impaired capacity of plasmacytoid DCs to produce IFN- $\alpha$  plays a role in inducing the low-grade Th1 response in HCV infection. It has been reported that plasmacytoid DC-derived IFN efficiently promotes Th1 by stimulating monocytes to produce Th1-attracting chemokines [31]. Thus, plasmacytoid DCs may be less able to offer a Th1-inducing environment for the shortage of interaction with other cells via IFN- $\alpha$ .

Another feature of the DC system in HCV infection is the profound ability of both types of DCs to prime IL-10-producing T cells. IL-10 either inhibits the antigen-specific T cell response by suppressing APC function [32] or directly suppresses the Th1-polarizing ability of myeloid DCs. The mechanisms that allow plasmacytoid DCs to prime more IL-10-producing cells are yet to be identified. Kadowaki et al. reported that herpes simplex virus-activated plasmacytoid DCs induce IL-10- and IFN- $\gamma$ -producing cells [33], whereas CD40L-activated plasmacytoid DCs can prime IL-10-producing regulatory CD8 T cells [34]. These observations show that plasmacytoid DCs alter their T cell-priming abilities in response to a variety of maturation stimuli. Thus, it is conceivable that plasmacytoid DCs in patients with chronic hepatitis are already primed by certain HCV-derived signals.

In summary, we have demonstrated that, in patients with chronic hepatitis, blood DC counts are reduced, and DCs are functionally impaired. Most notably, myeloid DCs from such patients are less able to induce Th1, and both myeloid and plasmacytoid DCs from these patients can prime more IL-10-producing cells than can those from healthy volunteers, thus favoring HCV persistence.

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## Interferon treatment improves survival in chronic hepatitis C patients showing biochemical as well as virological responses by preventing liver-related death

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**SUMMARY.** Interferon therapy for chronic hepatitis C reduces the risk of hepatocellular carcinoma, especially among virological and biochemical responders. However, little is known about the effect of interferon therapy on mortality. We studied the long-term effect of interferon therapy on mortality in patients with chronic hepatitis C. For this retrospective cohort study, 2954 patients with chronic hepatitis C were recruited, of whom 2698 received interferon therapy and 256 did not. The effect of interferon therapy on survival was assessed by standardized mortality ratio (SMR) based on published mortality data for the general Japanese population and by risk ratio calculated by proportional hazard regression. Over  $6.0 \pm 2.2$  years follow-up, death from liver-related diseases was observed in 69 (68%) of 101 deaths among interferon-treated patients and in 42 (81%) of 52 deaths among untreated patients. Compared with the general population, overall mortality was high among untreated patients (SMR: 2.7; 95% CI: 2.0–3.6) but not among interferon-treated patients (SMR: 0.9; 95% CI: 0.7–1.1). Liver-related mortality was extremely high among

untreated patients (SMR: 22.2; 95% CI: 16.0–30.0) and less among interferon-treated patients (SMR: 5.5; 95% CI: 4.3–6.9). The risk of death from all causes was lower for interferon-treated than untreated patients (risk ratio: 0.47; 95% CI: 0.261–0.836;  $P = 0.01$ ). The risk of death from liver-related diseases was significantly lower for sustained virological responders (risk ratio: 0.04; 95% CI: 0.005–0.301;  $P = 0.002$ ) compared with untreated patients, but not for nonsustained virological responders. Sustained biochemical responders (risk ratio: 0.03; 95% CI: 0.004–0.230;  $P < 0.001$ ) and transient biochemical responders (risk ratio: 0.18; 95% CI: 0.063–0.532;  $P = 0.002$ ) showed a significantly reduced risk of death from liver-related death, whereas biochemical nonresponders did not. Hence interferon treatment improved survival in chronic hepatitis C patients showing a biochemical as well as a virological response by preventing liver-related deaths.

**Keywords:** chronic hepatitis C, interferon, liver-related mortality, multivariate analysis, standardized mortality ratio.

Abbreviations: HCC, hepatocellular carcinoma; SMR, standardized mortality ratio.

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