deficient mice in which NK cells were not activated after the injection. Mounting lines of evidence suggest the therapeutic usefulness of α-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,[32–35] 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.

References

1. Tiegs G, Hentschel J, Wendel A. A T cell-dependent experimental liver injury in mice inducible by concanavalin A. J Clin Invest 1992;90:196–203.
2. Mizuhara H, O'Neill E, Seki N, Ogawa T, Kusunoki C, Otsuka K, et al. T cell activation-associated hepatic injury: mediation by tumor necrosis factors and protection by interleukin 6. J Exp Med 1994;179:1529–1537.
3. Schumann J, Wolf D, Pahl A, Brune K, Papadopoulos T, van Rooijen N, et al. Importance of Kupffer cells for T-cell-dependent liver injury in mice. Am J Pathol 2000;157:1671–1683.
4. Kaneko Y, Harada M, Kawano T, Yamashita M, Shibata Y, Gejyo F, et al. Augmentation of Valpha14 NKT cell-mediated cytotoxicity by interleukin 4 in an autocrine mechanism resulting in the development of concanavalin A-induced hepatitis. J Exp Med 2000;191:105–114.
5. Takeda K, Hayakawa Y, Van Kaer L, Matsuda H, Yagita H, Okumura K. Critical contribution of liver natural killer T cells to a murine model of hepatitis. Proc Natl Acad Sci U S A 2000;97:5498–5503.
6. Sass G, Heinlein S, Agli A, Bang R, Schumann J, Tiegs G. Cytokine expression in three mouse models of experimental hepatitis. Cytokine 2002;19:115–120.
7. Fiorucci S, Santucci L, Antonelli E, Distrutti E, Del Sero G, Morelli O, et al. NO-aspirin protects from T cell-mediated liver injury by inhibiting caspase-dependent processing of Th1-like cytokines. Gastroenterology 2000;118:404–421.
8. Faggioni R, Jones-Carson J, Reed DA, Dinarello CA, Feingold KR, Grunfeld C, et al. Leptin-deficient (ob/ob) mice are protected from T cell-mediated hepatotoxicity: role of tumor necrosis factor alpha and IL-18. Proc Natl Acad Sci U S A 2000;97:2367–2372.
9. Mizuhara H, Uno M, Seki N, Yamashita M, Yamaoka M, Ogawa T, et al. Critical involvement of interferon gamma in the pathogenesis of T-cell activation-associated hepatitis and regulatory mechanisms of interleukin-6 for the manifestations of hepatitis. HEPATOLOGY 1996;23:1608–1615.
10. Tagawa Y, Sekikawa K, Iwakura Y. Suppression of concanavalin A-induced hepatitis in IFN-gamma(-/-) mice, but not in TNF-alpha(-/-) mice: role for IFN-gamma in activating apoptosis of hepatocytes. J Immunol 1997; 159:1418–1428.
11. Kusters S, Gantner F, Kunstle G, Tiegs G. Interferon gamma plays a critical role in T cell-dependent liver injury in mice initiated by concanavalin A. Gastroenterology 1996;111:462–471.
12. Wiltrout RH. Regulation and antimetastatic functions of liver-associated natural killer cells. Immunol Rev 2000;174:63–76.
13. Smyth MJ, Hayakawa Y, Takeda K, Yagita H. New aspects of natural-killer-cell surveillance and therapy of cancer. Nat Rev Cancer 2002;2:850–861.
14. Waldmann TA, Dubois S, Tagaya Y. Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. Immunity 2001;14:105–110.
15. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol 1999;17:189–220.
16. Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. Annu Rev Immunol 2003;21:483–513.
17. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science 1997;278:1626–1629.
18. Yoshimoto T, Min B, Sugimoto T, Hayashi N, Ishikawa Y, Sasaki Y, et al. Nonredundant roles for CD1d-restricted natural killer T cells and conventional CD4+ T cells in the induction of immunoglobulin E antibodies in response to interleukin 18 treatment of mice. J Exp Med 2003;197:997–1005.
19. Ohteki T, Ho S, Suzuki H, Mak TW, Ohashi PS. Role for IL-15/IL-15 receptor beta-chain in natural killer 1.1+ T cell receptor-alpha beta+ cell development. J Immunol 1997;159:5931–5935.
20. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, et al. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science 1997;278:1623–1626.
21. Miyagi T, Takehara T, Tatsumi T, Kanto T, Suzuki T, Jinushi M, et al. CD1d-mediated stimulation of natural killer T cells selectively activates hepatic natural killer cells to eliminate experimentally disseminated hepatoma cells in murine liver. Int J Cancer 2003;106:81–89.
22. Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, Takeda K, et al. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. Blood 2002;99:1259–1266.
23. Hashimoto W, Takeda K, Anzai R, Ogasawara K, Sakihara H, Sugiura K, et al. Cytotoxic NK1.1 Ag+ alpha beta T cells with intermediate TCR induced in the liver of mice by IL-12. J Immunol 1995;154:4333–4340.
24. Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. J Exp Med 2000;191:661–668.
25. Lafreniere R, Rosenberg SA. Successful immunotherapy of murine experimental hepatic metastases with lymphokine-activated killer cells and recombinant interleukin 2. Cancer Res 1985;45:3735–3741.
26. Tsuruo T, Yamori T, Naganuma K, Tsukagoshi S, Sakurai Y. Characterization of metastatic clones derived from a metastatic variant of mouse colon adenocarcinoma 26. Cancer Res 1983;43:5437–5442.
27. Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, Wang CR, et al. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. J Exp Med 2000;192:741–754.
28. Risso A, Smilovich D, Capra MC, Baldissarro I, Yan G, Bargellesi A, et al. CD69 in resting and activated T lymphocytes. Its association with a GTP binding protein and biochemical requirements for its expression. J Immunol 1991;146:4105–4114.
29. Eberl G, MacDonald HR. Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. Eur J Immunol 2000;30:985–992.
30. Nishimura T, Kitamura H, Iwakabe K, Yahata T, Ohta A, Sato M, et al. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. Int Immunol 2000;12:987–994.
31. Toyabe S, Seki S, Iiai T, Takeda K, Shirai K, Watanabe H, et al. Requirement of IL-4 and liver NK1+ T cells for concanavalin A-induced hepatic injury in mice. J Immunol 1997;159:1537–1542.
32. Nakagawa K, Miller FN, Sims DE, Lentsch AB, Miyazaki M, Edwards MJ. Mechanisms of interleukin-2-induced hepatic toxicity. Cancer Res 1996; 56:507–510.
33. Osman Y, Kawamura T, Naito T, Takeda K, Van Kaer L, Okumura K, et al. Activation of hepatic NKT cells and subsequent liver injury following administration of alpha-galactosylceramide. Eur J Immunol 2000;30: 1919–1928.
34. Atkins MB, Robertson MJ, Gordon M, Lotze MT, DeCoste M, DuBois JS, et al. Phase I evaluation of intravenous recombinant human interleukin 12 in patients with advanced malignancies. Clin Cancer Res 1997;3:409–417.
35. Matsushita T, Ando K, Kimura K, Ohnishi H, Imawari M, Muto Y, et al. IL-12 induces specific cytotoxicity against regenerating hepatocytes in vivo. Int Immunol 1999;11:657–665.

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[1]

Masahisa Jinushi, Tetsuo Takehara, Tomohide Tatsumi, Tatsuya Kanto, Takuya Miyagi, Takahiro Suzuki, Yoshiyuki Kanazawa, Naoki Hiramatsu, and Norio Hayashi[2]

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)[3] 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

Department of Molecular Therapeutics, Osaka University Graduate School of Medicine, Osaka, Japan

Received for publication April 15, 2004. Accepted for publication September 10, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and for Research on Hepatitis and Bovine Spongiform Encephalomyelitis from the Ministry of Health Labor and Welfare of Japan. This work was also partially supported by the 21st Century Center of Excellent Program from the Ministry of Education, Science, and Technology of Japan.

[2] Address correspondence and reprint requests to Dr. Norio Hayashi, Department of Molecular Therapeutics, Osaka University Graduate School of Medicine, Yamadaoka 2-2, Suita, Osaka 565-0871, Japan. E-mail address: hayashin@moltx.med.osaka-u.ac.jp

[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.

0022-1767/04/$02.00

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 5.0×10^5/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_2 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 (1×10^5 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 (1×10^5 cells/well) were seeded in 24-well plates and stimulated with Hep3B cells or K562 cells (1×10^5 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 (1×10^5 cells/well) for 24 h. DCs were cultured in presence of the

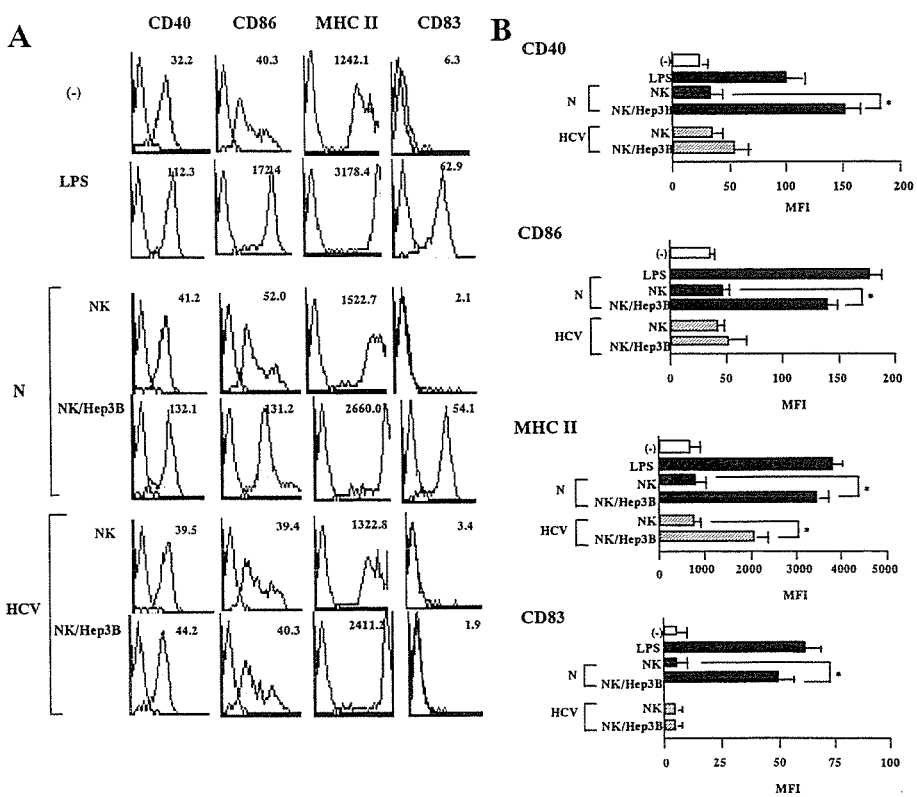

FIGURE 1. Phenotypic change of DCs cocultured with N-NK and HCV-NK in the presence of third-party cells. NK cells (1×10^5/well) derived from healthy volunteers (N) or chronic HCV-infected patients (HCV) were cocultured with allogeneic DCs from healthy donors at a ratio of 1:1 in the absence (NK) or presence (NK/Hep3B) of Hep3B cells (1×10^5/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 $CD11c^+$ $CD56^-$ 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. *, $p < 0.05$.

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 TGFβ-neutralizing Ab (R&D Systems, Minneapolis, MN) and used for DC stimulation for 24 h.

Flow cytometric analysis

DCs (5×10^5/ml) were washed and resuspended in PBS containing 1% BSA and 0.05% NaN_3 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-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.

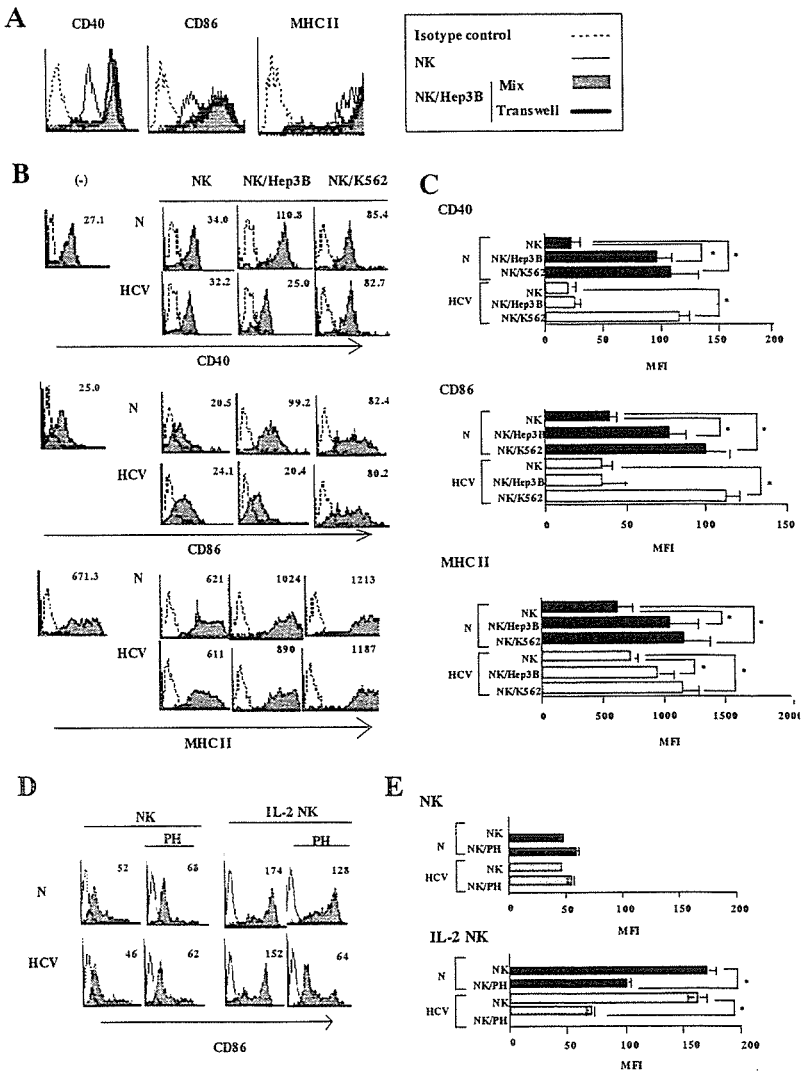

FIGURE 2. Soluble factors produced from NK cells/Hep3B mixtures are responsible for promoting DC maturation. *A*, NK cells, in the presence or absence of Hep3B (NK/Hep3B or NK, respectively), were added to DC cultures either directly (NK/Hep3B-Mix) or to the bottom compartment separately through 0.4-μm 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 μl/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 PC5-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-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 ($10-0.33 \times 10^3$/well). The DCs were cultured at graded numbers with the responder naive $CD4^+$ cells (1.0×10^5/well) for 72 h. The cocultured cells were pulsed with 1 μCi/well [^{3}H]thymidine for 16 h of incubation and collected onto a glass fiber filter. [^{3}H]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 ^{51}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) × 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. 1*A*). Neither NK cells nor Hep3B cells alone had any impact on DC phenotypes under our experimental conditions (Fig. 1*A* 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. 2*A*). 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. 2*D*). At the next step, NK cells were stimulated with IL-2 for 24 h and then cocultured with PH. The obtained supernatant

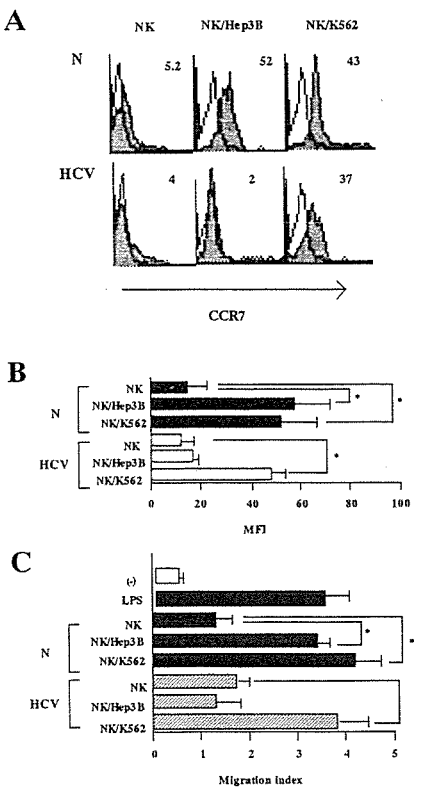

FIGURE 3. NK/Hep3B coculture supernatants can regulate chemotactic activity of DCs. NK cells from healthy (N) and HCV-infected (HCV) individuals were cultured with Hep3B or K562 at a ratio of 1:1 for 24 h. DCs from healthy donors were stimulated with the obtained supernatant for 24 h and were evaluated 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. 3*C*). 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 $CD4^+$ 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-γ 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/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 $CD4^+$ T cells. DCs endowed autologous $CD4^+$ T cells with the ability to proliferate and secrete IFN-γ 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. 5*A*). 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. 5*B*). We next examined the cytokine production profiles of NK cells cultured with tumor cells. Intracellular cytokine staining revealed that N-NK produced IFN-γ when stimulated with Hep3B or K562. In contrast, HCV-NK did not efficiently produce IFN-γ 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. 5*C*). ELISA data confirmed that N-NK produced significantly higher levels of IFN-γ as well as TNF-α than HCV-NK when cultured with Hep3B cells. The levels

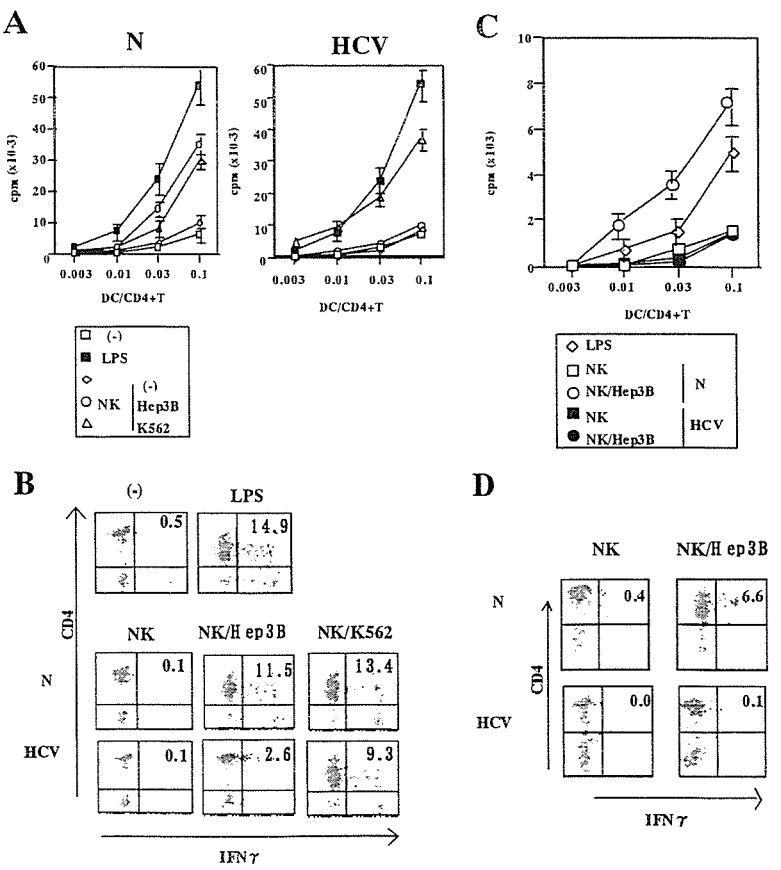

FIGURE 4. N-NK and HCV-NK differ in their ability to trigger DC-mediated induction of $CD4^+$ T cell responses. *A* and *B*, DCs were cultured with the supernatant of N-NK (N) or HCV-NK (HCV)/Hep3B coculture medium for 24 h and then used for the stimulation of allogeneic $CD4^+$ T cells for 48 h. *C* and *D*, DCs cultured with N-NK (N) or HCV-NK (HCV)/Hep3B coculture supernatant were pulsed with or without influenza A Ag (10 μg/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 (1×10^5/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-γ was determined by FACS analysis (*B* and *D*). The number in the *right upper quadrant* represents the percentages of CD4-positive cells expressing IFN-γ (*B* and *D*). Similar results were obtained in two independent experiments and representative data are shown.

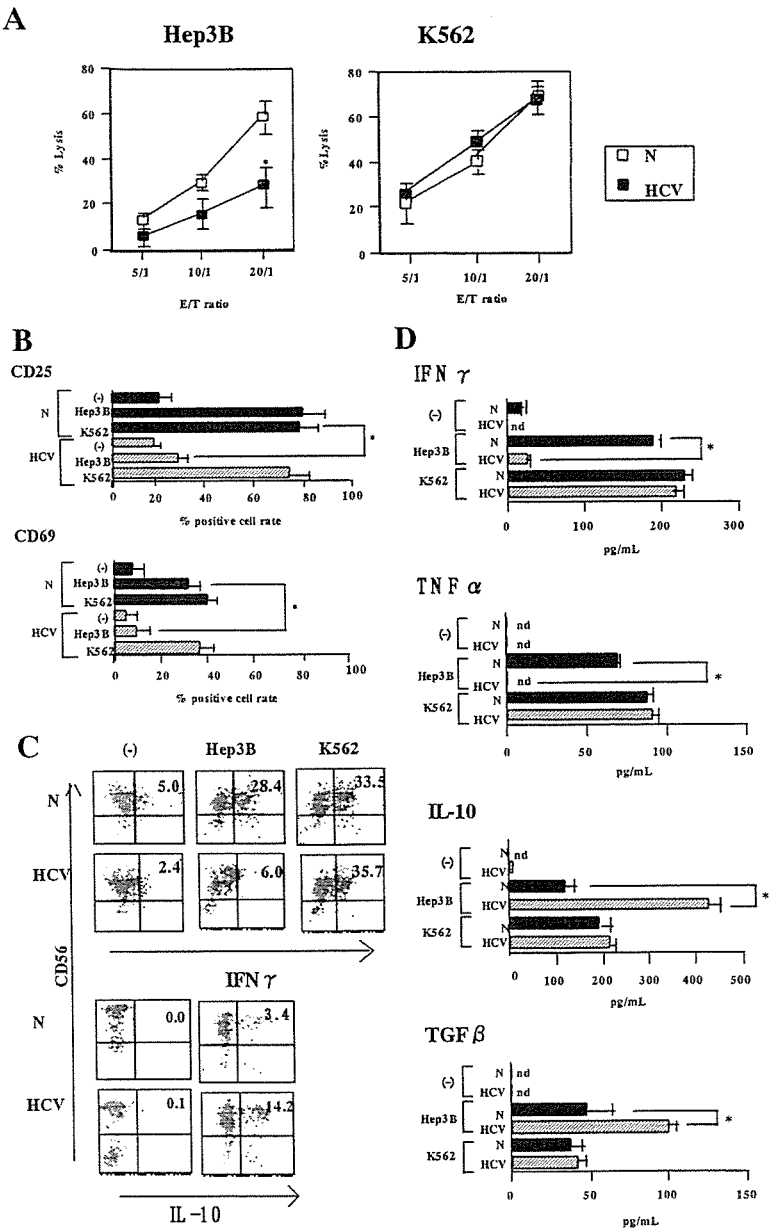

FIGURE 5. Human hepatic cells can activate N-NK, but not HCV-NK. *A*, Cytolytic ability of NK cells against Hep3B or K562 cells. NK cells isolated from healthy volunteers (N) or HCV-infected patients (HCV) were cultured with ^{51}Cr-labeled Hep3B or K562 at the indicated ratios for 4 h and then subjected to the standard ^{51}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. 5*D*). 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^{bright} and CD56^{dim} subsets and that CD56^{bright} NK cells express CD94/NKG2A more than CD56^{dim} subsets (28). The percentages of the CD56^{bright} 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^{bright} subpopulation.

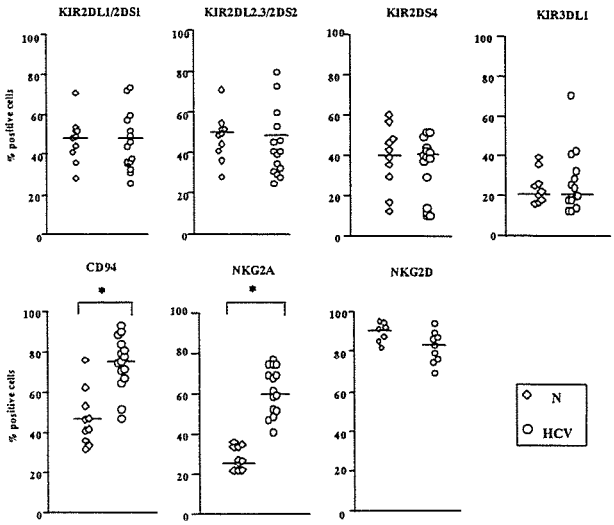

FIGURE 6. Profiles of NK receptor expression on NK cells. NK cells isolated from normal volunteers (n = 10; N) or from HCV-infected patients (n = 15; HCV) were stained with Abs for various KIRs, CD94, NKG2A, and NKG2D as described in *Materials and Methods.* The receptor expression of $CD56^+$ cells 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/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. 8*A*). 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. 8*B*).

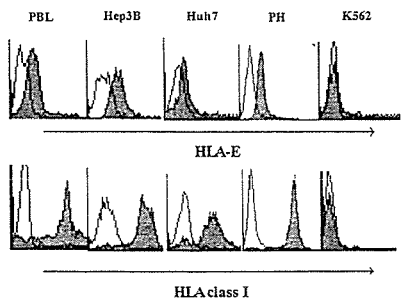

FIGURE 7. Surface expression of HLA-E and HLA class I in Hep3B, Huh7, primary hepatocyte, and K562 was assessed by flow cytometry (filled histograms). Peripheral blood leukocytes (PBL) were used as positive control for HLA-E and HLA class I expression. Open histograms show isotype control staining.

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. 9*A* 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. 9*B*). We also examined whether any differences existed between anti-NKG2A Ab and control IgG treatment in DC-stimulated cytokine production from $CD4^+$ T cells. 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 pretreated 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. 8*C*). 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 regulation of DCs plays an important role in the recognition of pathogens and malignancies at the early phase of immune responses

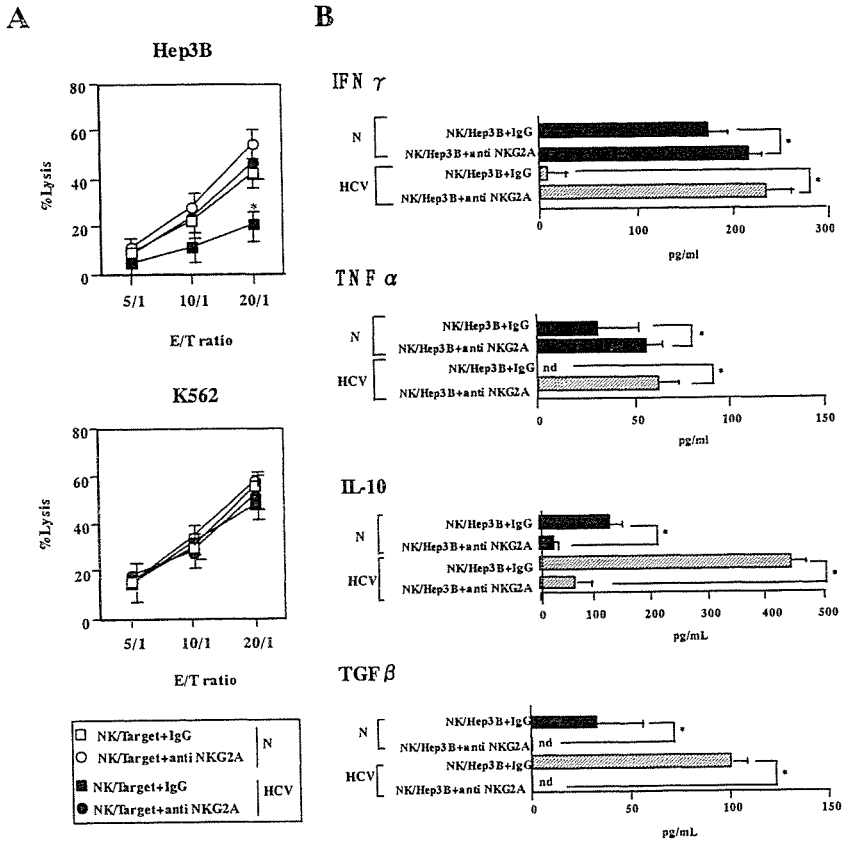

FIGURE 8. Blockade of NKG2A enhances NK cell activities. *A,* NK cells isolated from healthy volunteers (N) or HCV-infected patients (HCV) were cultured with ^{51}Cr-labeled Hep3B or K562 in the presence of the masking Ab of NKG2A or isotype control IgG (20 μg/ml) at the indicated ratios in 96-well culture plates for 4 h, and then were subjected to standard ^{51}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. *, $p < 0.05$.

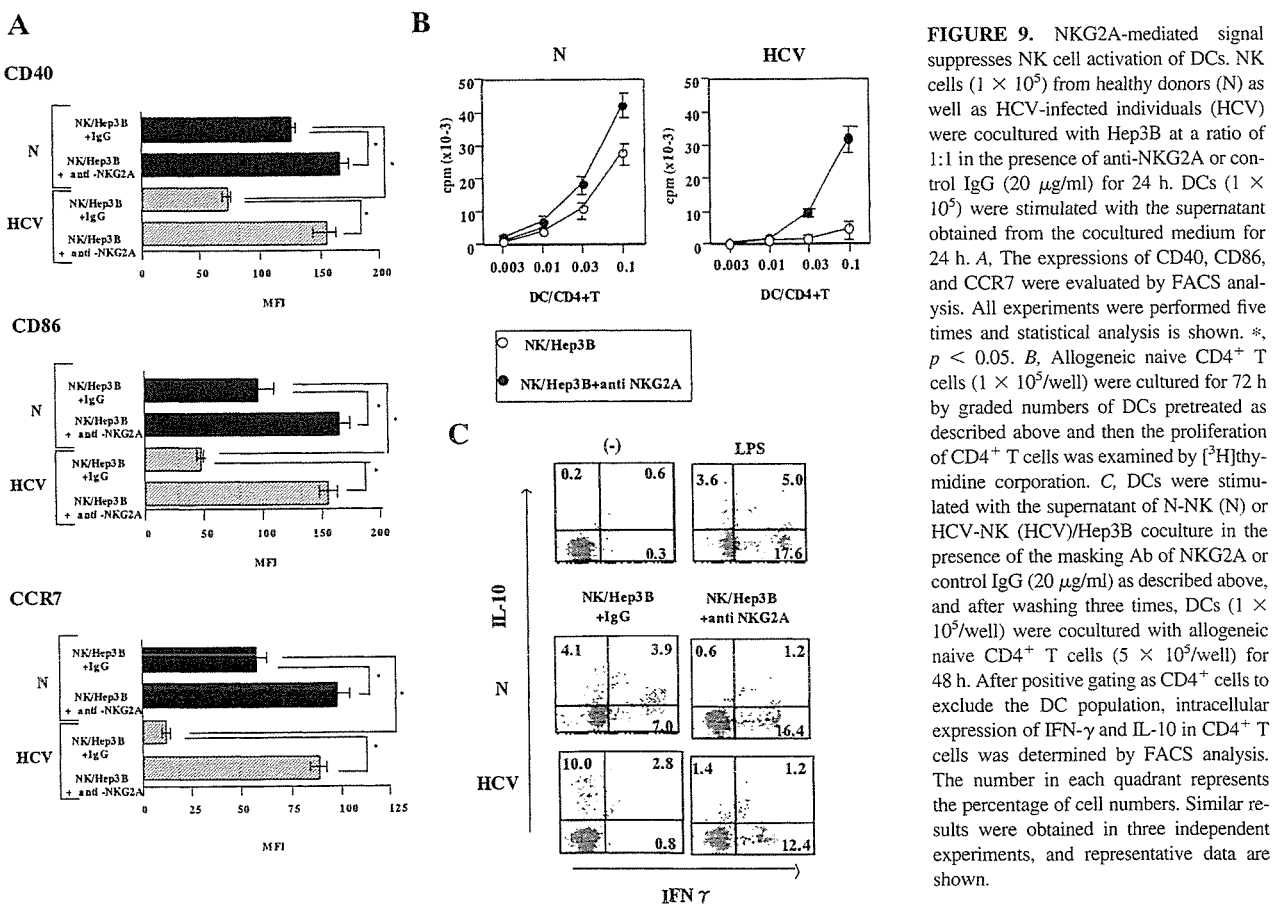

FIGURE 9. NKG2A-mediated signal suppresses NK cell activation of DCs. NK cells (1×10^5) from healthy donors (N) as well as HCV-infected individuals (HCV) were cocultured with Hep3B at a ratio of 1:1 in the presence of anti-NKG2A or control IgG (20 μg/ml) for 24 h. DCs (1×10^5) were stimulated with the 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$^+$ T cells (1×10^5/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 [^{3}H]thymidine corporation. *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 (1×10^5/well) were cocultured with allogeneic naive CD4$^+$ T cells (5×10^5/well) for 48 h. After positive gating as CD4$^+$ 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.

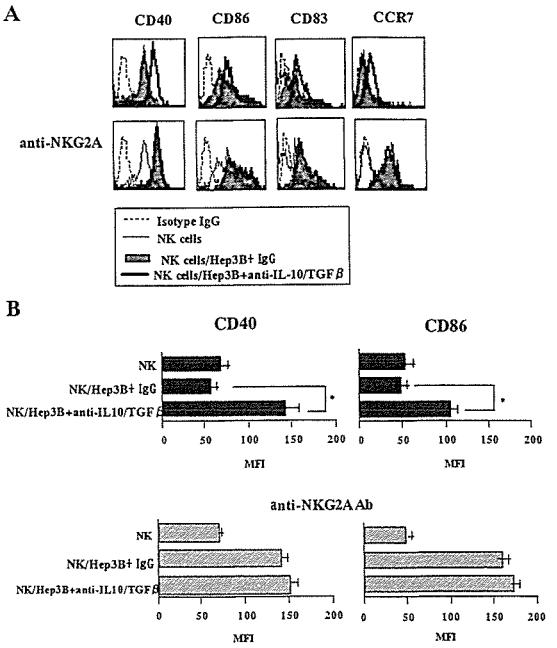

FIGURE 10. IL-10 and TGFβ are responsible for NKG2A-mediated inhibition of HCV-NK activation of DCs. NK cells (1×10^5) from HCV-infected individuals (HCV-NK) were cocultured with Hep3B at a ratio of 1:1 for 24 h. In some experiments, NK cells were cocultured with Hep3B in the presence of anti-NKG2A Ab (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. *, $p < 0.05$.

(4–6, 35). 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 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 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 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 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-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/TGFβ-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 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 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.

Acknowledgments

We thank Dr. E. Geraghty and N. Lee for providing human HLA-E mAb 3D12 and Dr. T. Spies and V. Groh for providing human NKG2D mAb 1D11.

References

1. Janeway, C. A., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol. 20:197.*
2. Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and P. Salazar-Mather. 1999. Natural killer cells in antiviral defence: function and regulation by innate cytokines. *Annu. Rev. Immunol. 17:189.*
3. Bachereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245.*
4. Piccioli, D., S. Sbrana, E. Melandri, and N. M. Valiance. 2002. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J. Exp. Med. 3:335.*
5. Ferlazzo, G., M. L. Tsang, L. Moretta, G. Meliole, R. M. Steinman, and C. Munz. 2002. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J. Exp. Med. 195:343.*
6. Mailliard, R. B., Y. I. Son, R. Redlinger, P. T. Coates, A. Giermasz, P. A. Morel, W. J. Storkus, and P. Kalinski. 2003. Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for induction of NK cell helper function. *J. Immunol. 171:2366.*
7. Fujii, S., K. Shimizu, C. Smith, L. Bonifaz, and R. M. Steinman. 2003. Activation of natural killer T cells by α-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J. Exp. Med. 198:267.*
8. Moretta, A. 2003. Natural killer cells and dendritic cells: rendezvous in abused tissues. *Nat. Rev. Immunol. 2:957.*
9. Dieu, M. C., B. Vanbervliet, A. Vicari, J. M. Bridon, E. Oldham, S. Ait-Yahia, F. Briere, A. Zlotnik, S. Lebecque, and C. Caux. 1998. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med. 188:373.*
10. Cerwenka, A., and L. L. Lanier. 2001. Natural killer cells, viruses and cancer. *Nat. Rev. Immunol. 1:41.*
11. Moser, J. M., J. Gibbs, P. E. Jensen, and A. E. Lukacher. 2002. CD94-NKG2A receptors regulate antiviral $CD8^+$ T cell responses. *Nat. Immunol. 3:189.*
12. Speiser, D. E., M. J. Pittet, D. Valmori, R. Dunbar, D. Rimoldi, D. Lienard, H. R. MacDonald, J. C. Cerottini, V. Cerundolo, and P. Romero. 1999. In vivo expression of natural killer inhibitory receptors by human melanoma-specific cytotoxic T cells. *J. Exp. Med. 190:775.*
13. Malmberg, K. J., V. Levitsky, H. Norell, C. T. de Matos, M. Carlsten, K. Schedvins, H. Rabbani, A. Morreta, K. Soderstrom, J. Levitskaja, and R. Kiessling. 2002. IFNγ protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism. *J. Clin. Invest. 110:1515.*
14. Costello, R. T., S. Sivori, E. Marcenaro, M. Lafage-Pochitaloff, M. Mozziconacci, D. Reviron, J. Gastaut, D. Pende, and A. Moretta. 2002. Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia. *Blood 99:3661.*
15. Andre, P., C. Brunet, S. Guia, H. Gallais, J. Sampol, E. Vivier, and F. Dignat-George. 1999. Differential regulation of killer cell Ig-like receptors and CD94 lectin-like dimers on NK and T lymphocytes from HIV-1 infected individuals. *Eur. J. Immunol. 29:1076.*
16. Mavilio, D., J. Benjamin, M. Daucher, G. Lombardo, S. Kottilil, M. A. Planta, E. Marcenaro, C. Bottino, L. Morretta, A. Morretta, and A. S. Fauci. 2003. Natural killer cells in HIV-1 infection: dichotomous effects of viremia on inhibitory and activating receptors and their functional correlates. *Proc. Natl. Acad. Sci. USA 100:15011.*
17. Saito, M., V. M. Braud, P. Goon, E. Hanon, G. P. Taylor, A. Saito, N. Eiraku, Y. Tanaka, K. Usuku, J. N. Weber, et al. 2003. Low frequency of CD94/$NKG2A^+$ T lymphocytes in patients with HTLV-1-associated myelopathy/tropical spastic pareresis, but not in asymptomatic carriers. *Blood 102:577.*
18. Liang, J. T., B. Rhermann, L. B. Sheeff, and J. H. Hoofnagle. 2000. Pathogenesis, natural history, treatment, and prevention of hepatitis C. *Annu. Int. Med. 132:296.*
19. Bertolettti, A., and C. Ferrari. 2003. Kinetics of the immune response during HBV and HCV infection. *Hepatology 38:4.*
20. Crotta, S., A. Stilla, A. Wack, A. D'Andrea, S. Nuti, U. D'Oro, M. Mosca, F. Filliponi, R. M. Brunetto, F. Bonino, et al. 2002. Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. *J. Exp. Med. 195:35.*
21. Tseng, C. K., and G. R. Klimpel. 2002. Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. *J. Exp. Med. 195:43.*
22. Jinushi, M., T. Takehara, T. Kanto, T. Tatsumi, V. Groh, T. Spies, T. Miyagi, T. Suzuki, Y. Sasaki, and N. Hayashi. 2003. Critical role of MHC class I-related chain A and B expression on IFNα-stimulated dendritic cells in NK cell activation: impairment in chronic hepatitis C infection. *J. Immunol. 170:1249.*
23. Jinushi, M., T. Takehara, T. Tatsumi, T. Kanto, V. Groh, T. Spies, T. Suzuki, T. Miyagi, and N. Hayashi. 2003. Autocrine IL-15 that is required for type I IFN-mediated dendritic cell expression of MHC class I-related chain A and B is impaired in hepatitis C virus infection. *J. Immnunol. 171:5423.*
24. Pende, D., S. Sivori, L. Accame, L. Pareti, M. Falco, D. Geraghty, P. Le Bouteiller, L. Moretta, and A. Moretta. 1997. HLA-G recognition by human natural killer cells: involvement of CD94 both as inhibitory and as activating receptor complex. *Eur. J. Immunol. 27:1875.*
25. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science 285:727.*
26. Lee, N., D. R. Goodlett, A. Ishitani, H. Marquardt, and D. E. Geraghty. 1998. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J. Immunol. 160:4951.*
27. Kanto, T., N. Hayashi, T. Takehara, T. Tatsumi, N. Kuzushita, A. Ito, Y. Sasaki, A. Kasahara, and M. Hori. 1999. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J. Immunol. 162:5584.*
28. Cooper, M. A., T. A. Fehniger, and M. A. Caligiuri. 2001. The biology of human natural killer cell subsets. *Trends Immunol. 22:633.*
29. Braud, V. M., D. S. J. Allan, C. A. O'Callaghan, K Soderstrom, A. D'Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, et al. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature 391:795.*
30. Ulbrecht, M., S. Martinozzi, M. Grzeschik, H. Hengel, J. W. Ellwart, M. Pla, and E. H. Weiss. 2000. The human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. *J. Immunol. 164:5019.*
31. Marin, R., F. Ruiz-Cabello, S. Pedrinaci, R. Mendez, P. Jimenez, D. E. Geraghty, and F. Garrido. 2003. Analysis of HLA-E expression in human tumors. *Immunogenetics 54:767.*
32. Lazetic, S., C. Chang, J. P. Houchins, L. L. Lanier, and J. H. Phillips. 1996. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J. Immunol. 157:4741.*
33. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol. 19:683.*
34. Kobie, J. J., R. S. Wu, Kurt, R. A., S. Lou, M. K. Adelman, L. J. Whitesell, L. V. Ramanathapuram, C. L. Ateaga, and E. T. Akporiaye. 2003. Transforming growth factor β inhibits the antigen-presenting functions and anti-tumor activity of dendritic cell vaccines. *Cancer Res. 63:1860.*
35. Mocikat, R., H. Braumuller, A. Gumy, O. Egeter, H. Ziegler, U. Reusch, A. Bubeck, J. Louis, R. Maihammer, G. Riethmuller, et al. 2003. Natural killer cells activated by MHC^{low} targets prime dendritic cells to induce protective CD8 T cell responses. *Immunity 19:561.*
36. Nelson, D. R., R. P. Gonzalez-Peralta, K. Qian, Y. Xu, C. G. Marousis, G. L. Davis, and J. Y. Lau. 1997. Transforming growth factor-β1 in chronic hepatitis C. *J. Viral Hepat. 4:29.*
37. Bertone, S., F. Schiavetti, R. Bellomo, C. Vitale, M. Ponte, L. Moretta, and M. C. Mingari. 1999. Transforming growth factor-β-induced expression of CD94/NKG2A inhibitory receptors in human T lymphocytes. *Eur. J. Immunol. 29:23.*
38. Sato, K., N. Yamashita, M. Baba, and T. Matsuyama. 2003. Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse. *Immunity 18:367.*
39. Boussiotis, V. A., G. J. Freeman, P. A. Taylor, A. Berezovskaya, I. Grass, B. R. Blazar, and L. M. Nadler. 2000. $p27^{kip1}$ functions as an anergy factor inhibiting interleukin 2 transcription and clonal expansion of allloreactive human and mouse helper T lymphocytes. *Nat. Med. 6:290.*
40. Steinman, R. M., D. Hawiger, and M. C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annu. Rev. Immunol. 21:685.*
41. Shevach, E. M. 2002. $CD4^+CD25^+$ suppressor T cells: more questions than answers. *Annu. Rev. Immunol. 2:389.*
42. Yamagiwa, S., J. Gray, S. Hashimoto, and D. Horwitz. 2001. A role of TGFβ in the generation and expansion of $CD4^+CD25^+$ T regulatory T cells from human peripheral blood. *J. Immunol. 166:7282.*
43. Goudy, K. S., B. R. Burkhardt, C. Wasserfall, S. Song, A. L. Campbell-Thompson, T. Brusko, M. A. Powers, M. J. Clare-Salzler, E. S. Sobel, T. M. Ellis, et al. 2003. Systemic overexpression of IL-10 induces $CD4^+CD25^+$ cell populations in vivo and ameliorates type I diabetes in nonobese diabetic mice in dose-dependent fashion. *J. Immunol. 171:2270.*
44. Kamal, S. M., J. Fehr, B. Roesler, T. Peters, and J. W. Rasenack. 2002. Peginterferon alone or ribavirin enhances HCV-specific $CD4^+$ T helper 1 responses in patients with chronic hepatitis C. *Gastroenterology 123:1070.*
45. Tsai, S. L., Y. F. Liaw, M. H. Chen, C. Y. Huang, and G. C. Kuo. 1997. Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. *Hepatology 25:449.*
46. Wedemeyer, H., X.-S. He, M. Nascimbeni, A. R. Davis, H. B. Greenberg, J. H. Hoofnagle, T. J. Liang, H. Alter, and B. Rehermann. 2002. Impaired effector function of hepatitis C virus-specific $CD8^+$ T cells in chronic hepatitis C infection. *J. Immunol. 169:3447.*

MAJOR ARTICLE

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

Tatsuya Kanto,[1,2] Michiyo Inoue,[1,2] Hideki Miyatake,[1] Aki Sato,[1] Mitsuru Sakakibara,[1] Takayuki Yakushijin,[1] Chika Oki,[1] Ichiyo Itose,[1] Naoki Hiramatsu,[1] Tetsuo Takehara,[1] Akinori Kasahara,[3] and Norio Hayashi[1]

Departments of [1]Molecular Therapeutics, [2]Dendritic Cell Biology and Clinical Application, and [3]General Medicine, Osaka University Graduate School of Medicine, Osaka, Japan

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 antivirus 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-α, 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)–α 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-α 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-

Received 11 April 2004; accepted 8 June 2004; electronically published 27 October 2004.

Presented in part: 54th annual meeting of the American Association for the Study of Liver Diseases, Boston, 24–28 October 2003 (abstract 380); 10th International Meeting of Hepatitis C Virus and Related Viruses, Kyoto Japan, 2–6 December 2003 (abstract O-2).

Financial support: Ministry of Health, Labor and Welfare of Japan (grant-in-aid for Research on Emerging and Re-emerging Infectious Diseases); Ministry of Education, Science and Culture of Japan (grant-in-aid); Organization for Pharmaceutical Safety and Research.

Reprints or correspondence: Dr. Tatsuya Kanto, Dept. of Dendritic Cell Biology and Clinical Application, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan (kantot@moltx.med.osaka-u.ac.jp) or Dr. Norio Hayashi, Dept. of Molecular Therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan (hayashin@moltx.med.osaka-u.ac.jp).

The Journal of Infectious Diseases 2004;190:1919–26

0022-1899/2004/19011-0006$15.00

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–α 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 ± SD age, 48 ± 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-α or ribavirin. All patients had shown persistent or fluctuating alanine aminotransferase (ALT) abnormalities at enrollment (mean ± SD ALT levels, 91 ± 72 IU/L). The presence of other causes of liver disease, such as autoimmunodeficiency, 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 ± SD age, 45 ± 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-γ was purchased from Strathman-Biotech. Human lymphoblastoid IFN-α 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^+ 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^-, HLA-DR^+, $CD11c^+$, and $CD123^{low}$ cells, and plasmacytoid DCs are Lin^-, HLA-DR^+, $CD11c^-$, and $CD123^{high}$ 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 $CD34^+$ 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^-, HLA-DR^+, $CD123^+$, and $CD34^+$ cells. The early and late progenitors of DCs were defined as Lin^-, $CD34^+$, $CD123^+$, and $CD45RA^-$ cells and Lin^-, $CD34^+$, $CD123^+$ and $CD45RA^+$ 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

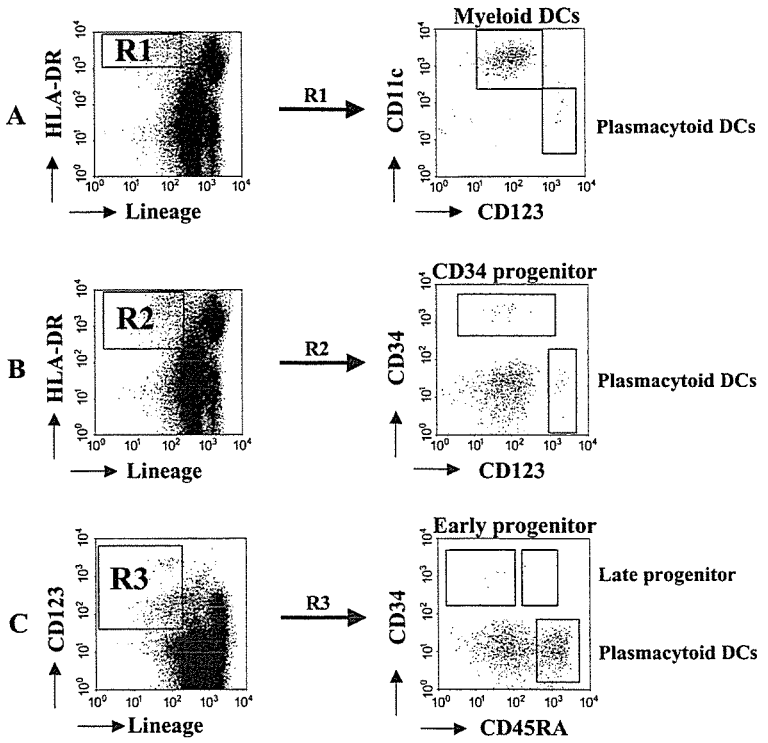

Figure 1. Identification of blood dendritic cell (DC) subsets, CD34 progenitors, and early and late progenitors of plasmacytoid DCs in peripheral blood mononuclear cells. *A*, Myeloid DCs are lineage marker–negative (Lin^-), $HLA\text{-}DR^+$, $CD11c^+$, and $CD123^{low}$; plasmacytoid DCs are Lin^-, $HLA\text{-}DR^+$, $CD11c^-$, and $CD123^{high}$. *B*, CD34 progenitors are Lin^-, $HLA\text{-}DR^+$, $CD34^+$, and $CD123^+$. *C*, Early progenitors of plasmacytoid DCs are Lin^-, $CD123^+$, $CD34^+$, and $CD45RA^-$; late progenitors are 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 Lin^+ cells were depleted magnetically with the cocktail Abs containing CD3, CD14, CD16, CD20, CD56, and glycophorins (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 μg/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% 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-α, we added 5 μmol/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, 5×10^5 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 allophycocianin-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 (rmC5-3), CD34 (581), CD40 (5C3), CD45RA (HI100), CD80 (L307.4), CD83 (HB15a), CD86 (IT2.2), and CDw123 (IL-3 receptor α-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

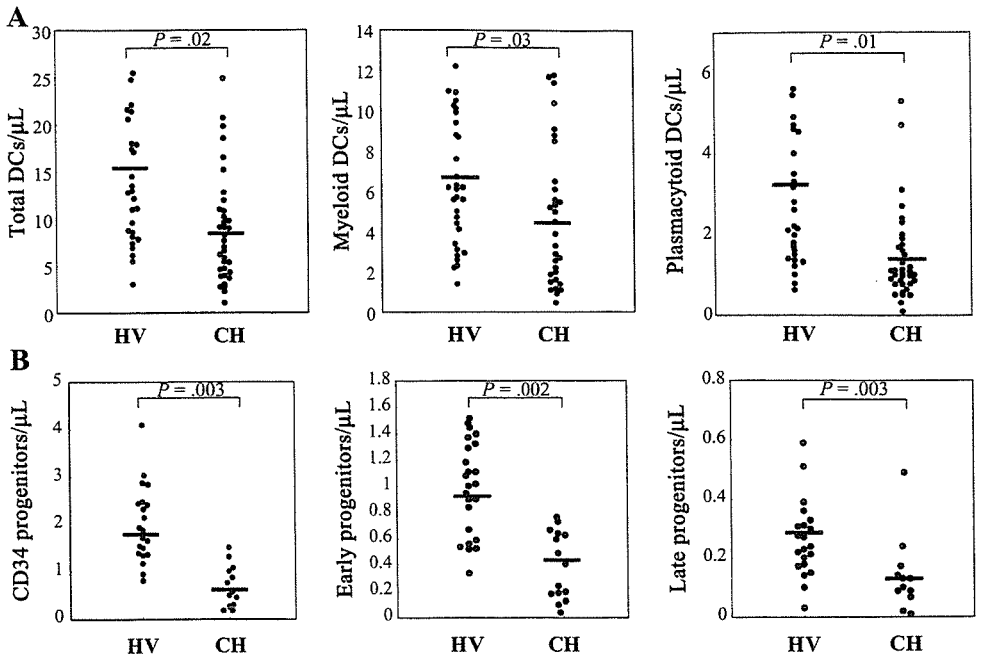

Figure 2. Reduction of blood dendritic cells (DCs) and their progenitors in patients with chronic hepatitis (CH). Absolute nos. of blood DCs *(A)* and progenitors *(B)* were determined from their frequencies in peripheral blood mononuclear cells. *Horizontal bars,* median. Statistical significance was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

or plasmacytoid DCs were cultured for 5 days with 2×10^4 allogeneic naive CD4 T cells/well. In the final 16–20 h of culture, 1 μCi/well of [^{3}H]-thymidine (ICN Biomedicals) was pulsed. The uptake of [^{3}H]-thymidine to T cells was measured by use of a β-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, 1×10^4 DCs/well were cultured for an additional 7 days with 1×10^5 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 μg/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-γ, IL-4, IL-10, and IL-12 p70 (Endogen), according to the manufacturer's instructions. IFN-α 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 1*A*). Total DC, myeloid DC, and plasmacytoid DC counts in the patients with chronic hepatitis were lower than those in healthy volunteers (figure 2*A*). 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 1*B* and 1*C*). The numbers of CD34 progenitors and early and late progenitors

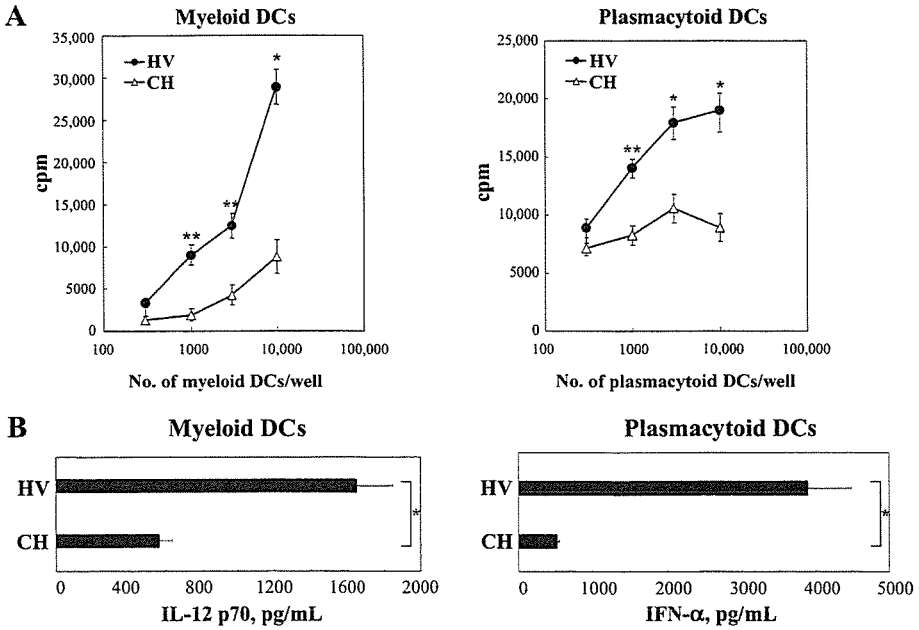

Figure 3. Functional impairment of both types of blood dendritic cells (DCs) from hepatitis C virus–infected patients. *A*, Allogeneic mixed lymphocyte reaction. *Vertical bars,* mean ± SD. $*P < .01$ and $**P < .05$, vs. patients with chronic hepatitis (CH). *B*, Day-3 myeloid DCs were stimulated with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

in patients with chronic hepatitis were significantly lower than those in healthy volunteers (figure 2*B*). In addition, a positive correlation was observed between Lin^- and $HLA\text{-}DR^+$ DC counts and those of $CD34^+$ progenitors or early progenitors (DCs vs. $CD34^+$ 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 3*A*).

We analyzed the production of IL-12 p70 by myeloid DCs and that of IFN-α 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 3*B*). 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-γ–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-γ 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).

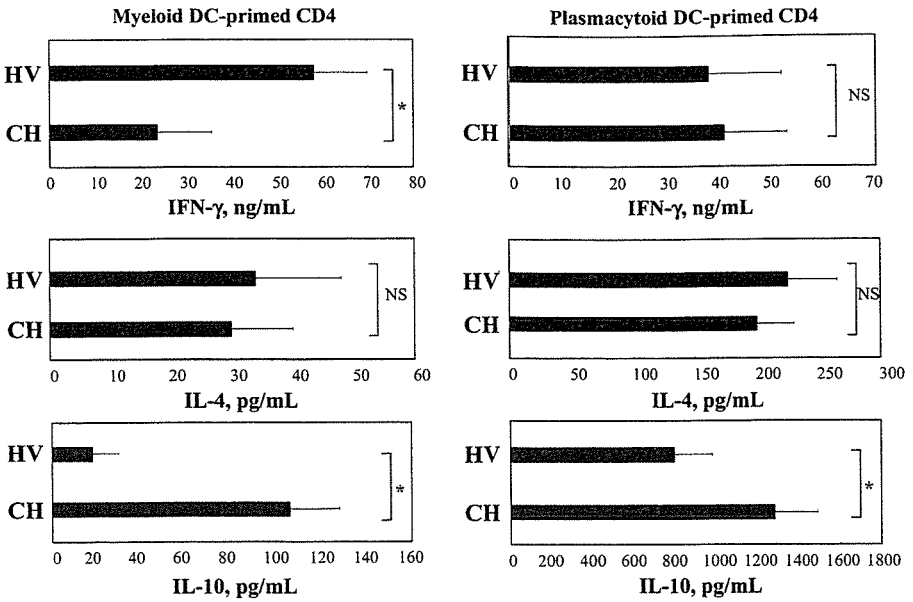

Figure 4. Inability of myeloid dendritic cells (DCs) from patients with chronic hepatitis (CH) to drive Th1-polarization. Myeloid and plasmacytoid DCs from patients with CH can prime more interleukin (IL)–10 producing cells than do those from healthy volunteers (HV). Day-3 myeloid or plasmacytoid DCs 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-α–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 $CD34^+$ progenitors [17]. Blom et al. reported that myeloid and plasmacytoid DCs were generated from $CD34^+$ 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 $CD34^+$ 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 patients with chronic hepatitis, and the mechanism needs to be further investigated.

An imbalance between Th1 and Th2 subsets is crucially in-

volved in the pathogenesis of chronic hepatitis [24, 25]. Several lines of evidence have clearly demonstrated that a Th1 response, either spontaneous or IFN-α 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-α 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-α.

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-γ–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.

References

1. Houghton M, Weiner A, Han J, Kuo G, Choo QL. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology **1991**; 14:381–8.
2. Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin Liver Dis **2000**; 20:1–16.
3. Di Bisceglie AM, Hoofnagle JH. Optimal therapy of hepatitis C. Hepatology **2002**; 36(Suppl 1):S121–7.
4. Kanto T, Hayashi N, Takehara T, et al. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus–infected individuals. J Immunol **1999**; 162:5584–91.
5. Wedemeyer H, He XS, Nascimbeni M, et al. Impaired effector function of hepatitis C virus–specific $CD8^+$ T cells in chronic hepatitis C virus infection. J Immunol **2002**; 169:3447–58.
6. Chang KM, Thimme R, Melpolder JJ, et al. Differential $CD4^+$ and $CD8^+$ T-cell responsiveness in hepatitis C virus infection. Hepatology **2001**; 33:267–76.
7. Banchereau J, Briere F, Caux C, et al. Immunobiology of dendritic cells. Annu Rev Immunol **2000**; 18:767–811.
8. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell **2001**; 106:259–62.
9. Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. Nat Rev Immunol **2002**; 2:151–61.
10. Fugier-Vivier I, Servet-Delprat C, Rivailler P, Rissoan MC, Liu YJ, Rabourdin-Combe C. Measles virus suppresses cell-mediated immunity by interfering with the survival and functions of dendritic and T cells. J Exp Med **1997**; 186:813–23.
11. Chehimi J, Campbell DE, Azzoni L, et al. Persistent decreases in blood plasmacytoid dendritic cell number and function despite effective highly active antiretroviral therapy and increased blood myeloid dendritic cells in HIV-infected individuals. J Immunol **2002**; 168:4796–801.
12. Ratta M, Fagnoni F, Curti A, et al. Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6. Blood **2002**; 100:230–7.
13. Bain C, Fatmi A, Zoulim F, Zarski JP, Trepo C, Inchauspe G. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. Gastroenterology **2001**; 120:512–24.
14. Donaghy H, Pozniak A, Gazzard B, et al. Loss of blood $CD11c^+$ myeloid and $CD11c^-$ plasmacytoid dendritic cells in patients with HIV-1 infection correlates with HIV-1 RNA virus load. Blood **2001**; 98:2574–6.
15. Soumelis V, Scott I, Gheyas F, et al. Depletion of circulating natural type 1 interferon-producing cells in HIV-infected AIDS patients. Blood **2001**; 98:906–12.
16. Shiratori Y, Kato N, Yokosuka O, et al. Predictors of the efficacy of interferon therapy in chronic hepatitis C virus infection. Tokyo-Chiba Hepatitis Research Group. Gastroenterology **1997**; 113:558–66.
17. Blom B, Ligthart SJ, Schotte R, Spits H. Developmental origin of pre-DC2. Hum Immunol **2002**; 63:1072–80.
18. Blom B, Ho S, Antonenko S, Liu YJ. Generation of interferon α–producing predendritic cell (Pre-DC)2 from human $CD34^+$ hematopoietic stem cells. J Exp Med **2000**; 192:1785–96.
19. Dzionek A, Fuchs A, Schmidt P, et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol **2000**; 165:6037–46.
20. Krug A, Rothenfusser S, Hornung V, et al. Identification of CpG oligonucleotide sequences with high induction of IFN-α/β in plasmacytoid dendritic cells. Eur J Immunol **2001**; 31:2154–63.
21. Navas MC, Fuchs A, Schvoerer E, Bohbot A, Aubertin AM, Stoll-Keller F. Dendritic cell susceptibility to hepatitis C virus genotype 1 infection. J Med Virol **2002**; 67:152–61.
22. Farkas L, Beiske K, Lund-Johansen F, Brandtzaeg P, Jahnsen FL. Plasmacytoid dendritic cells (natural interferon-α/β–producing cells) accumulate in cutaneous lupus erythematosus lesions. Am J Pathol **2001**; 159:237–43.
23. Uehira K, Amakawa R, Ito T, et al. Dendritic cells are decreased in blood and accumulated in granuloma in tuberculosis. Clin Immunol **2002**; 105:296–303.
24. Godkin A, Jeanguet N, Thursz M, Openshaw P, Thomas H. Characterization of novel HLA-DR11-restricted HCV epitopes reveals both qualitative and quantitative differences in HCV-specific $CD4^+$ T cell responses in chronically infected and non-viremic patients. Eur J Immunol **2001**; 31:1438–46.
25. Tsai SL, Liaw YF, Chen MH, Huang CY, Kuo GC. Detection of type 2–like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. Hepatology **1997**; 25:449–58.
26. Cramp ME, Rossol S, Chokshi S, Carucci P, Williams R, Naoumov NV. Hepatitis C virus–specific T-cell reactivity during interferon and ribavirin treatment in chronic hepatitis C. Gastroenterology **2000**; 118: 346–55.

27. Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med **2002**; 347:975–82.
28. Gerlach JT, Diepolder HM, Jung MC, et al. Recurrence of hepatitis C virus after loss of virus-specific CD4$^+$ T-cell response in acute hepatitis C. Gastroenterology **1999**; 117:933–41.
29. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol **2003**; 3:133–46.
30. Sarobe P, Lasarte JJ, Casares N, et al. Abnormal priming of CD4$^+$ T cells by dendritic cells expressing hepatitis C virus core and E1 proteins. J Virol **2002**; 76:5062–70.
31. Blackwell SE, Krieg AM. CpG-A–induced monocyte IFN-γ–inducible protein–10 production is regulated by plasmacytoid dendritic cell–derived IFN-α. J Immunol **2003**; 170:4061–8.
32. Steinbrink K, Graulich E, Kubsch S, Knop J, Enk AH. CD4$^+$ and CD8$^+$ anergic T cells induced by interleukin-10–treated human dendritic cells display antigen-specific suppressor activity. Blood **2002**; 99:2468–76.
33. Kadowaki N, Antonenko S, Lau JY, Liu YJ. Natural interferon α/β–producing cells link innate and adaptive immunity. J Exp Med **2000**; 192:219–26.
34. Gilliet M, Liu YJ. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. J Exp Med **2002**; 195:695–704.

Original Paper

Intervirology

Intervirology 2005;48:77–83
DOI: 10.1159/000081732

Received: November 19, 2003
Accepted after revision: February 13, 2004

Alteration in Gene Expression Profile by Full-Length Hepatitis B Virus Genome

Fumihiko Nakanishi[a] Kazuyoshi Ohkawa[b] Hisashi Ishida[b] Atsushi Hosui[b]
Aki Sato[b] Naoki Hiramatsu[b] Keiji Ueda[c] Tetsuo Takehara[b]
Akinori Kasahara[d] Yutaka Sasaki[e] Masatsugu Hori[a] Norio Hayashi[b]

Departments of [a]Internal Medicine and Therapeutics, [b]Molecular Therapeutics, [c]Microbiology and [d]General Medicine, Osaka University Graduate School of Medicine, Suita, Osaka, and [e]Department of Gastroenterology and Hepatology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan

Key Words
Hepatitis B virus · Hepatitis B virus-expressing cell · DNA array assay · Corroboration assay · Hepatocarcinogenesis

Abstract
Persistent expression of hepatitis B virus (HBV) proteins is thought to be involved in virus-related hepatocarcinogenesis. Here, we compared the gene expression profile of cells persistently expressing the full-length HBV with that of negative control cells to comprehensively investigate virus-mediated changes in the gene expression of the host cells. RNA samples from both virus-expressing and negative control cells were used for the DNA array assay. DNA array assay and subsequent corroboration assays revealed that expression of 14 of 1,176 genes (1.2%) was altered in response to virus expression. The upregulated genes included CD44, high mobility group protein-I, thymosin beta-10 and 27-kD heat shock protein, while the downregulated genes included NM23-H1, all of which are thought to be associated with the development or progression of carcinoma in the liver or other organs. Furthermore, virus expression resulted in the decrease of two apoptosis-inducing molecules, caspase-3 and BAX, which may also contribute to carcinogenesis through prolonged survival of the host cell. Thus, expression of the virus genome caused carcinogenesis-related changes in host cell gene expression. HBV expression may change the host cell to a malignant phenotype through alterations in the expression levels of a set of genes.

Introduction

Hepatitis B virus (HBV) is a major causative agent of acute and chronic liver diseases. Chronic HBV infection eventually results in more serious liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. HBV is a circular, partially double-stranded DNA virus of approximately 3.2 kb in length that encodes four kinds of viral proteins, i.e. preS/S, precore/core, polymerase and X proteins. Among these HBV proteins, the role of the X protein (HBx) in HBV-mediated pathogenesis has been studied most extensively. HBx displays tumorigenic transforming activity in vitro [3] and in vivo [4]. Also, HBx is thought to considerably modify cellular apoptotic processes under various apoptosis-inducing stimuli [5–7]. HBx acts as a transcription activator for many cellular and virus promoters and enhancers [reviewed in ref. 8]. In

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0300–5526/05/0483–0077$22.00/0

Accessible online at:
www.karger.com/int

Norio Hayashi, MD
Department of Molecular Therapeutics
Osaka University Graduate School of Medicine
Suita, Osaka 565-0871 (Japan)
Tel. +81 6 6879 3440, Fax +81 6 6879 3449, E-Mail hayashin@moltx.med.osaka-u.ac.jp