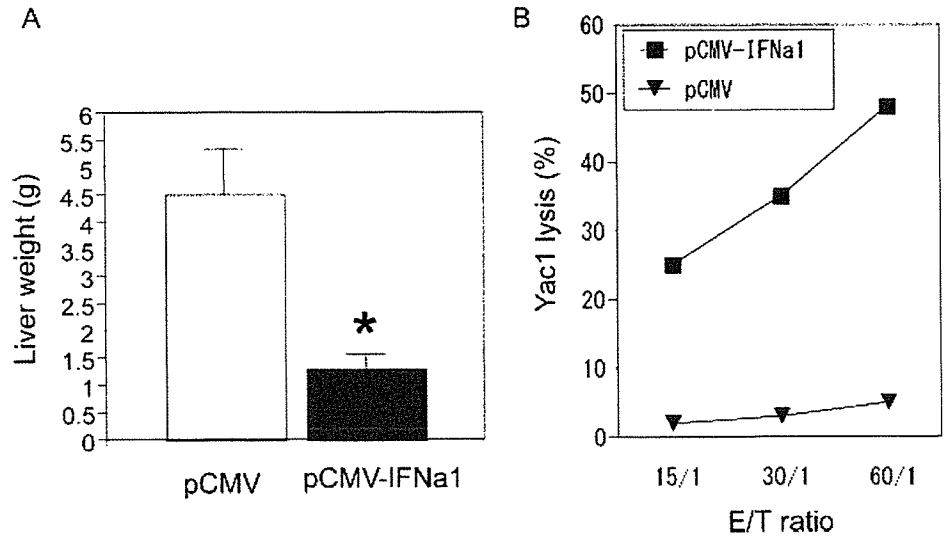

FIGURE 6 – NK cell activation and anti-metastatic effects in GKO mice. (*a*) Anti-metastatic effects. GKO mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) 2 days later. After 14 days, the mice were sacrificed to examine tumor development in the liver. The liver weight was compared between the groups ($n = 8$/*group*). Experiments were performed 3 times and representative data are shown. *, $p < 0.05$ *vs.* pCMV injection group. (*b*) Yac1 lytic ability. GKO mice were hydrodynamically injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles). Four days later, splenocytes isolated from the mice were examined for the lytic ability for Yac1 cells. All experiments were performed at least 3 times and representative data are shown.

effects in SCID mice. Research using a variety of murine models has revealed the direct effects of tumor cells[11] and the CD8 T cell response[12–14] involved in the antitumor effects of IFNα. A recent study[29] using STAT1-deficient animals and STAT1-deficient tumor cells revealed that IFNα activation of host cells, but not tumor cells, is required for antitumor effects in a peritoneal model of melanoma. They also showed the involvement of NK cells in their model. Our data demonstrated that NK cells are critically required and sufficient for IFNα-mediated protection from liver metastasis. However, NK cells are not effective for controlling tumor growth at extrahepatic sites, because IFNα activated splenic (systemic) NK activity but did not elicit antitumor effects against subcutaneously injected CT-26 cells. Subcutaneous tumor growth appeared to be controlled by adaptive immunity rather than innate pathway.

The reason that IFNα-mediated activation of NK cells leads to such a strong antitumor effect in the liver but not under the skin is not known. In the present study, we applied hydrodynamic injection of the IFNα gene to obtain efficient and stable expression of IFNα. Since the hydrodynamic procedure leads to predominant expression of foreign genes in the liver, the concentration of IFNα may be greater in the liver than in circulation. This may be related to the observed strong antitumor effects in the liver. Another possibility is that NK cells are more numerically abundant and functionally potent in the liver than in other organs.[30] In any case, the hydrodynamic injection of the IFNα gene led to higher activation of the NK lytic ability of hepatic mononuclear cells than that of systemic mononuclear cells. This may be related to the stronger antitumor activity in the liver.

An earlier study on STAT1 knockout mice revealed that STAT1 is a critical signaling molecule for IFNα in macrophage and T cells.[9] STAT1-deficient mice showed impairment of NK activity.[31] STAT1-deficient splenocytes did not show increase in NK lytic activity upon IFNα stimulation.[29] Therefore, STAT1 should also play an important role in IFNα-mediated NK cell activation. However, the significance of STAT1 in NK cells on IFNα action had not been fully proven, because splenocytes consist of a variety of lymphocyte subsets. In the present study, we found that NK cells express lower levels of STAT1 than T cells, which is associated with lower levels of STAT1 expression in SCID splenocytes than those in wild-type splenocytes. Importantly, IFNα phosphorylated STAT1 in SCID splenocytes with similar kinetics to that in wild-type splenocytes even if the signal intensities in the former were lower than those in the latter. In agreement with this, IFNα was capable of activating a variety of genes in SCID mononuclear cells. Thus, IFNα does not require other lymphocyte subsets to activate NK cells and to induce NK cell expression of IFN-regulated genes.

IFNγ was shown to be produced in lymphocytes upon IFNα administration, which is dependent on STAT4 signaling.[32] In the present study, IFNγ was produced in serum after pCMV-IFNa1 injection. Furthermore, the IFNγ gene was activated in SCID NK cells upon IFNα stimulation. However, IFNγ is not necessary for NK cell activation in terms of killing ability as well as an IFNα-mediated antimetastatic effect. NK cells, upon IFNα stimulation, expressed well-established IFN-regulated genes[33] as well as killer cell-specific molecules granzyme B or TRAIL. Although our data showed that hepatic mononuclear cells from mice receiving IFNα can kill CT-26 cells *in vitro*, it remains unclear whether NK cells serve as direct effector cells for ablating CT-26 cells *in vivo*. Further study is needed to find whether killer cell-specific molecules are actually involved in the antimetastatic effects of IFNα.

IFNα has achieved a long record of clinical use in the treatment of hematological malignancy and solid tumors such as melanoma, renal cell carcinoma and Kaposi's sarcoma.[34,35] In therapy for colon carcinoma, special attention has been paid to the use of IFNα in the combination with 5-FU, since IFNα has been shown to modulate 5-FU metabolism and to enhance its cytotoxic activity.[36] Although several clinical trials have evaluated the 5-FU plus IFNα combination for adjuvant therapy of colon carcinomas with encouraging results,[37,38] recent randomized trials revealed that addition of IFNα to 5-FU + levamisole marginally increased the recurrence-free survival time compared to 5-FU + levamisole alone, but did not alter the over-all survival.[39] Therefore, use of IFNα as a modulator of 5-FU activity may have some limitations in future clinical use. In the present study, we demonstrated that

IFNα activates both innate and adaptive immunity and ablates microdisseminated colon carcinoma cells in the liver. There may be a variety of reasons which can explain the difference between the present study and the clinical use in the therapy of metastasizing colon cancer. We found CT-26 far less sensitive to NK cells than Yac1 cells but human colon carcinoma cells might be more resistant to NK cells activated by IFNα in a clinical setting. Systemic administration of recombinant IFNα may be less effective than enforced expression of IFNα gene in the liver. In any way, we used CT-26 cells just as a murine model of hepatic metastasis and observed similar therapeutic effect of the IFNα gene when using another cell line such as BL6 melanoma cells in a C57/BL6 background (our unpublished data). Our study raised the possibility that IFNα therapy may be a promising approach for developing future adjuvant therapy for metastatic liver tumors arising from various organs. Immunological aspect of IFNα is important when considering antimetastatic effect of this cytokine.

In conclusion, IFNα-mediated protection of CT-26 hepatic metastasis critically requires NK cells. NK cells, upon IFNα stimulation, do not require other immune cells such as T cells, B cells and NKT cells for their activation and protection against hepatic metastasis. NK cell production of IFNγ is not involved in the increase in NK activity and antitumor effect. Our study has shown NK cells to be important mediators in ablating microdisseminating tumors in the liver in IFNα therapy. Eradication of microdisseminated tumor cells by IFNα led to long-lasting adaptive immune responses which may be important for suppressing tumor growth in extrahepatic sites and overall antitumor effects.

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IMMUNOLOGY ORIGINAL ARTICLE

Natural killer cell and hepatic cell interaction via NKG2A leads to dendritic cell-mediated induction of CD4$^+$ CD25$^+$ T cells with PD-1-dependent regulatory activities

Masahisa Jinushi, Tetsuo Takehara, Tomohide Tatsumi, Shinjiro Yamaguchi, Ryotaro Sakamori, Naoki Hiramatsu, Tatsuya Kanto, Kazuyoshi Ohkawa and Norio Hayashi

Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka, Japan

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Correspondence: Norio Hayashi MD, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2–2 Yamada-oka, Suita, Osaka 565-0871, Japan.
Email: hayashin@gh.med.osaka-u.ac.jp
Senior author: Tetsuo Takehara, email: takehara@gh.med.osaka-u.ac.jp

Summary

Natural killer (NK) cells have the ability to control dendritic cell (DC)-mediated T cell responses. However, the precise mechanisms by which NK receptor-mediated regulation of NK cells determines the magnitude and direction of DC-mediated T cell responses remain unclear. In the present study, we applied an *in vitro* co-culture system to examine the impact of NK cells cultured with hepatic cells on DC induction of regulatory T cells. We found that interaction of NK cells and non-transformed hepatocytes (which express HLA-E) via the NKG2A inhibitory receptor resulted in priming of DCs to induce CD4$^+$ CD25$^+$ T cells with regulatory properties. NKG2A triggering led to characteristic changes of the cytokine milieu of co-cultured cells; an increase in the transforming growth factor (TGF)-β involved in the generation of this specific type of DC, and a decrease in the tumour necrosis factor-α capable of antagonizing the effect of TGF-β. The regulatory cells induced by NK cell-primed DCs exert their suppressive actions through a negative costimulator programmed death-1 (PD-1) mediated pathway, which differs from freshly isolated CD4$^+$ CD25$^+$ T cells. These findings provide new insight into the role of NK receptor signals in the DC-mediated induction of regulatory T cells.

Keywords: NK receptor; regulatory T cell; HLA-E; liver; HCV

Introduction

CD4$^+$ CD25$^+$ regulatory T (Treg) cells have been identified as the main suppressors of immune responses.[1–5] Although the mechanisms by which CD4$^+$ CD25$^+$ Treg cells exert their suppressive actions have not been fully elucidated, negative costimulatory signals via cytotoxic T lymphocyte antigen-4 (CTLA-4) or inducible costimulator (ICOS)-mediated signals, have been suggested to play a key role in the activation of CD4$^+$ CD25$^+$ Treg cells.[6,7] Programmed death-1 (PD-1), another molecule identified as a negative costimulatory receptor, has also serves as a negative regulator for effector immune responses.[8] Recent reports have demonstrated that PD-1 is expressed in CD4$^+$ CD25$^+$ Treg cells, suggesting its potential roles in the regulation of T cell tolerance.[9] However, the precise roles of PD-1 in CD4$^+$ CD25$^+$ Treg cell functions remain elusive.

The mechanisms by which CD4$^+$ CD25$^+$ Treg cells are generated have been extensively investigated. Dendritic cells (DCs), the sentinels between innate and adaptive immunity, have recently emerged as candidate cells involved in the differentiation and/or activation of CD4$^+$ CD25$^+$ Treg cells.[10] Various kinds of factors have been identified as involved in DC induction of CD4$^+$ CD25$^+$ Treg cells. Mouse immature DC promotes the differentiation of CD4$^+$ CD25$^+$ Treg cells through the DEC 205-mediated targeting of self-antigen in the steady state.[10,11] The immune regulatory cytokines interleukin (IL)-10/transforming growth factor (TGF)-β have also been reported to play important roles in DC generation and activation of CD4$^+$ CD25$^+$ Treg cells.[12–14]

Abbreviations: CTLA-4, cytotoxic T lymphocyte antigen-4; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; GITR, glucocorticoid-induced TNF receptor; HCV, hepatitis C virus; HLA, human leucocyte antigen; NH, human non-transformed hepatocyte; NK, natural killer; PD-1, programmed death-1; PDL-1, programmed death ligand 1; PBMC, peripheral blood mononuclear cell; Treg, regulatory T.

Several lines of evidence have revealed that natural killer (NK) cell-mediated innate immunity regulates DC functions to determine the direction and magnitude of adaptive T cell immunity.[15–18] It has also been established that NK cell function is regulated by positive and negative signals through their receptor and ligand interactions.[19] We previously reported that, upon exposure to non-transformed hepatocytes (NHs), IL-2-primed NK cells negatively regulated DC functions, which appeared to be dependent on NKG2A inhibitory signals during co-culture of NK cells and NHs. Immunosuppressive cytokines such as IL-10 and TGF-β, but not direct NK–DC contact, were responsible for this action.[20] However, it remains unclear whether these NK/hepatocyte co-cultures can also influence the induction as well as activation of $CD4^+$ $CD25^+$ Treg cells.

In the present study, we investigated whether DCs stimulated with the co-culture supernatant of IL-2-prestimulated NK cells and NHs can modulate Treg cell functions. We found that TGF-β produced from NK cell/hepatocyte co-culture via NKG2A activation is responsible for modulating DCs to induce and maintain regulatory phenotypes and functions of $CD4^+$ $CD25^+$ Treg cells. Furthermore, the generated $CD4^+$ $CD25^+$ Treg cells suppressed T cell activation via interaction between PD-1 and programmed death ligand 1 (PDL-1). These findings represent new evidence that NK receptor-mediated modulation of NK cells may dictate DC-induced adaptive immunity toward an immunogenic or tolerogenic status via induction of Treg cells.

Materials and methods

Antibodies

Anti-NKG2A monoclonal antibody (mAb) (Z199), PC5-labelled CD25 mAb or isotype-matched control IgG1 and IgG2a mAb were purchased from Beckmann-Coulter (Fullerton, CA). Anti-IL-10, anti-TGF-β, anti-CTLA-4, anti-GITR (glucocorticoid-induced TNF receptor) and anti-PD-1 polyclonal Abs were purchased from R & D Systems (Minneapolis, MN) and phycoerythrin (PE)-labelled mAb CTLA-4 from BD Biosciences (San Jose, CA). Anti-HLA-E mAb 3D12 was kindly provided by Dr E. Geraghty (Fred Hutchinson Cancer Research Institute, Seattle, WA) and used as reported previously.[21] Anti-MIC mAb 6D4, anti-ULBP1 mAb 3F1 and anti-ULBP2 mAb DH1 were kindly provided by Drs T. Spies and V. Groh (Fred Hutchinson Cancer Research Institute) and used as reported previously.[22]

Human hepatic cells

Human non-transformed hepatocytes (NHs) derived from mixed heterogeneous donors were purchased from the Applied Cell Biology Research Institute (Kirkland, WA) and cultured in CS-C complete medium according to the manufacturer's instructions.

Isolation of peripheral blood lymphocyte populations

Resting NK cells ($CD56^+$ $CD3^+$), naive $CD4^+$ T cells ($CD45RA^+$ RO^+) or $CD8^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) with a positive cell isolation kit according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ $CD25^+$ T cells were further separated from naive $CD4^+$ T cells using anti-CD25 microbeads (Miltenyi Biotech). Their purity was >90% by flow cytometry. Informed consent was obtained from all blood donors.

Generation of monocyte-derived DC

Monocytes were isolated by plastic adherence from PBMCs and cultured in RPMI-1640 supplemented with granulocyte–macrophage colony stimulating factor (GM-CSF) (PeproTech, London, UK) and IL-4 (PeproTech). At day 6, they were stimulated with or without the co-culture supernatant of NK cells and hepatic cells. At day 7, non-adherent cells were harvested and used as described below.

Stimulation of DCs by co-culture supernatants of NK cells and hepatic cells

Freshly isolated NK cells were cultured with or without IL-2 for 24 hr. IL-2-prestimulated or non-stimulated NK cells were seeded in 24-well plates and then co-cultured for 24 hr with NHs (1×10^5 cells/well), respectively. Monocyte-derived DCs were cultured for 24 hr with 1 ml of the co-culture supernatant of IL-2-prestimulated NK cells and NHs (NH/IL-2 NK-primed DC). In some experiments, anti-NKG2A mAb (Z199) or isotype-matched control Ab was added during the co-cultures of NK cells and hepatic cells. Z199 mAb was previously confirmed to block the NKG2A-mediated signal.[23] In some experiments, the supernatant of NK/hepatic cell co-cultures was also treated with anti-IL-10 or anti-TGF-β neutralizing Ab and used for DC stimulation for 24 hr. In some experiments, tumour necrosis factor (TNF)-α, TGF-β or both were used for DC stimulation for 24 hr.

Isolation of $CD4^+$ $CD25^+$ T cells

DCs (1×10^5) were stimulated for 24 hr with the supernatant obtained from the co-cultured medium. After washing three times, DCs were cultured with allogeneic $CD4^+$ T cells for 48 hr; $CD4^+$ $CD25^+$ fractions were isolated from DC and $CD4^+$ co-culture and subjected to further analysis. $CD4^+$ $CD25^+$ fractions were also isolated

from PBMCs and cultured with 1 μg/ml plate-bound anti-CD3 mAb (UCHT1; Beckmann-Coulter) for 24 hr to efficiently induce their suppressive properties as described previously.[3] These cells are referred to as natural $CD4^+$ $CD25^+$ T cells.

Flow cytometry

The expression of NK inhibitory ligands (human leucocyte antigen, HLA, class I, HLA-E) was examined on NHs by using w6/32 or 3D12, respectively. MIC, ULBP1 or ULBP2 expression on hepatocytes was also evaluated by mAb 6D4, 3F1 or DH1, respectively. For $CD4^+$ $CD25^+$ T cell staining, the cells were costained with PC5-labelled CD25 mAb with PE-labelled mAb of CTLA-4, GITR or PD-1 polyclonal Ab. The cells were analysed by flow cytometry using a fluorescence-activated cell sorter (FACScan) system, and data analysis was performed using CELLQUEST software.

Measurements of cytokine production in culture supernatant

The culture supernatants of interferon (IFN)-γ, TNF-α, IL-10 and TGF-β were examined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions (IFN-γ, TNF-α and IL-10, Endogen, Tokyo, Japan; TGF-β, R & D Systems).

Analysis of Foxp3 mRNA expression

Polymerase chain reaction (PCR) analysis was performed to determine Foxp3 mRNA expression of $CD4^+$ T cells using a commercial PCR panel according to the manufacturer's instructions (Gibco BRL, Rockville, MD). The following primers were used: 5′-CCCACTTACAGGCACT CCTC-3′ (forward) and 5′-CTTCTCCTTCTCCAGCAC CA-3′ (reverse).[24] Amplification was carried out for 35 cycles of 20 seconds at 95°, 20 seconds at 58 ° and 30 seconds at 72°. As a control for the integrity of mRNA, primers specific for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were used as follows: 5′-GCCACCCAGAAGACTGTGGATGGC-3′ (forward) and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (reverse). The PCR products were analysed by ethidium bromide-stained 1·5% agarose gel electrophoresis.

Analysis of $CD4^+$ $CD25^+$ T cell suppressor functions

DCs (5×10^4/well) were cultured with allogeneic $CD4^+$ T cells (5×10^5/well) for 48 hr, after which $CD4^+$ $CD25^+$ T cells were isolated from the co-cultured cells. $CD4^+$ $CD25^-$ T cells were freshly isolated from the same donors and activated with 1 μg/ml plate-bound anti-CD3 mAb in the presence or absence of autologous $CD4^+$ $CD25^+$ T cells for 48 hr. The ability of $CD4^+$ $CD25^+$ T cells to suppress proliferation and IFN-γ production of activated $CD4^+$ $CD25^-$ T cells was determined by [^{3}H]thymidine incorporation and ELISA assay, respectively. To further examine the mechanisms of $CD4^+$ $CD25^+$ T cell suppressive actions, neutralizing Ab of IL-10 or TGF-β, anti-CTLA-4, anti-GITR or anti-PD-1 was added at the beginning of $CD4^+$ $CD25^+$ T cell and $CD4^+$ $CD25^-$ T cell co-cultures.

Statistical analysis

Comparisons between groups were analysed by *t*-test with Welch's correction or ANOVA for experiments with more than two subgroups. Differences were considered significant when the *P*-value was $< 0{\cdot}05$.

Results

IL-2-primed NK cells upon exposure to NH-modulated DCs on the induction of regulatory $CD4^+$ $CD25^+$ T cells

Natural $CD4^+$ $CD25^+$ T cells from human peripheral blood lymphocytes (PBLs) expressed CTLA-4 and GITR, both of which have been identified as regulatory markers,[6,25] but did not express PD-1 (Fig. 1a). To examine whether DCs can modulate the expression of these regulatory markers on $CD4^+$ $CD25^+$ T cells, we stimulated monocyte-derived DCs for 24 hr, either by the culture supernatant of IL-2-stimulated NK cells (IL-2 NK) or by the co-culture supernatant of NH/IL-2 NK. After washing, the resulting DCs were cultured for 48 hr with $CD4^+$ T cells isolated from allogeneic donors. $CD4^+$ $CD25^+$ T cells were isolated from the DC and $CD4^+$ T cell co-culture and subjected to analysis for regulatory markers. The expression levels of CTLA-4 and GITR decreased on $CD4^+$ $CD25^+$ T cells after stimulation of IL-2 NK-primed DCs (Fig. 1b). By contrast, $CD4^+$ $CD25^+$ T cells stimulated with NH/IL-2 NK-primed DCs remained positive for CTLA-4 and GITR on their surface. Of note is the finding that PD-1 was induced on these cells, showing their phenotypic properties to differ from natural $CD4^+$ $CD25^+$ T cells (Fig. 1b, c). The induction of PD-1 on $CD4^+$ $CD25^+$ T cells was further confirmed when IL-2NK/NH-primed DCs from different donors were used as stimulators (Fig. 1d). The supernatant of NH without NK cells had little effect on phenotypic changes of $CD4^+$ $CD25^+$ T cells by DCs (data not shown).

The forkhead transcription factor Foxp3 has been recently identified as a master gene for defining Treg cells.[26] We therefore performed reverse transcription-PCR (RT-PCR) analysis of $CD4^+$ T cells to evaluate the mRNA expression of Foxp3. Foxp3 expression was detected in natural $CD4^+$ $CD25^+$ T cells. When $CD4^+$ T cells were

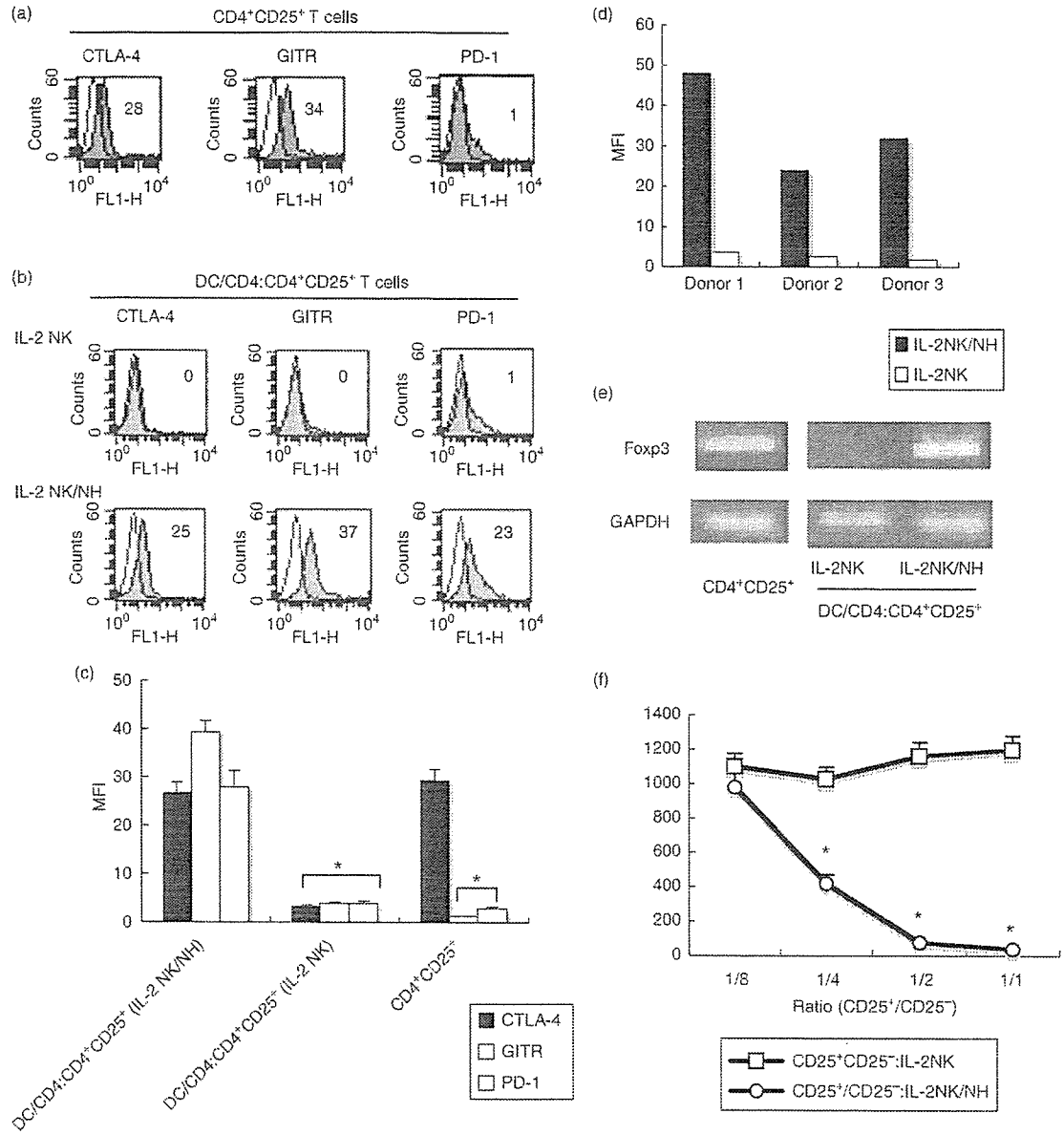

Figure 1. Human non-transformed hepatocyte (NH) modulation of activated natural killer (NK) cells endows dendritic cells (DCs) with the ability to induce $CD4^+$ $CD25^+$ regulatory T cells. (a) Freshly isolated $CD4^+$ $CD25^+$ T cells were cultured in the presence of plate-bound anti-CD3 antibody (Ab) for 24 hr, and then subjected to flow cytometry to examine their expression of cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR) and programmed death-1 (PD-1) (closed histograms). Open histograms represent the staining of control Ab. Numbers on the upper right indicate the mean fluorescence intensity (MFI) of each type of stained cells. (b) NK cells were preactivated with 50 ng/ml interleukin (IL)-2, and co-cultured in the absence (IL-2 NK) or presence (IL-2 NK/NH) of NHs at a ratio of 1 : 1 for 24 hr. DCs (1×10^5) were stimulated for 24 hr with the supernatant obtained from the co-cultured medium. After washing three times, DCs were cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ fractions were isolated from the DC/$CD4^+$ T co-culture and subjected to flow cytometry for expression of CTLA-4, GITR or PD-1 (closed histograms). Open histograms show isotype control staining. Numbers on the upper right indicate the MFI of each type of stained cell. (c) All experiments in (a) and (b) were performed three times and the composite results with statistical analysis are shown as the MFI of the staining cells. $^*P < 0{\cdot}05$ vs. responses of IL-2 NK/NH group. The experiment was performed with a different set of donors and similar results were obtained. (d) PD-1 expression on $CD4^+$ $CD25^+$ T cells stimulated with allogeneic DCs from three different donors, shown as the MFI. (e) $CD4^+$ $CD25^+$ T cells were prepared as described above. The mRNA expression of Foxp3 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was examined by reverse transcription-polymerase chain reaction (RT-PCR). (f) $CD4^+$ $CD25^+$ fractions were isolated from DC/$CD4^+$ T cell co-cultures. Different numbers of these $CD4^+$ $CD25^+$ T cells were co-cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells (1×10^5/well) in the presence of plate-bound anti-CD3 Ab ($CD4^+$ $CD25^+$/$CD4^+$ $CD25^-$). The anti-CD3 Ab-activated $CD4^+$ $CD25^-$ T cells alone were used as a positive control ($CD4^+$ $CD25^-$). IFN-γ was measured for each supernatant obtained after 48 hr of co-culture by enzyme-linked immunosorbent assay. $^*P < 0{\cdot}05$.

stimulated with IL-2 NK-primed DCs for 24 hr, Foxp3 was not expressed on $CD4^+$ $CD25^+$ T cells. By contrast, they dominantly transcribed Foxp3 at levels comparable with those of natural $CD4^+$ $CD25^+$ T cells when stimulated with NH/IL-2 NK-primed DCs (Fig. 1e). Taken together, $CD4^+$ $CD25^+$ T cells, when stimulated by NH/IL-2 NK-primed DCs, maintained regulatory phenotypes such as CTLA-4, GITR and Foxp3, and properties distinct from those of natural $CD4^+$ $CD25^+$ Treg cells in terms of PD-1 expression.

$CD4^+$ $CD25^+$ T cells on stimulation of NH/IL-2 NK-primed DC suppressed effector cell functions

We next analysed the functions of $CD4^+$ $CD25^+$ T cells stimulated by NH/IL-2 NK-primed DC. $CD4^+$ $CD25^+$ T cells were co-cultured for 72 hr with $CD4^+$ $CD25^-$ T cells freshly isolated from the same donors. During the co-cultures, $CD4^+$ $CD25^-$ T cells were stimulated with plate-bound anti-CD3 Ab. The $CD4^+$ $CD25^+$ T cells induced by NH/IL-2 NK-primed DCs dose-dependently suppressed the proliferation of co-cultured cells, whereas those induced by IL-2 NK-primed DC did not (data not shown). $CD4^+$ $CD25^+$ T cells induced by NH/IL-2 NK-primed DCs also dose-dependently inhibited IFN-γ production of the co-cultured cells, by contrast with those induced by IL-2 NK-primed DCs (Fig. 1f). The suppressive activities of these $CD4^+$ $CD25^+$ Treg cells were similar to those of natural $CD4^+$ $CD25^+$ Treg cells (data not shown). These results demonstrate that $CD4^+$ $CD25^+$ T cells induced by NH/IL-2 NK-primed DCs exert suppressive actions to effector cell functions, consistent with their expression of regulatory markers. Taken together, these results indicated that NK cell modulation of DCs leads to the $CD4^+$ $CD25^+$ Treg cell-mediated suppression of effector cell responses when NK cells encounter hepatocytes.

NKG2A signal of NK cells is responsible for the modulation of DCs to activate $CD4^+$ $CD25^+$ Treg cells

We examined the expression of various ligands for NK cell receptors on NHs. NHs expressed HLA-E, the ligand of NKG2A, but did not express NKG2D receptor ligands, MIC and ULBP1-2 (Fig. 2a). Given our previous findings that NHs negatively regulated IL-2 NK-mediated modulation of DC functions through the interaction of the NKG2A inhibitory receptor and its ligand HLA-E,[20] we evaluated the role of these receptor signals in the induction of $CD4^+$ $CD25^+$ Treg cells by DCs. When anti-NKG2A Ab was added during the co-culture of NH and IL-2 NK and DCs were stimulated with the resultant supernatant, the expression of CTLA-4, GITR and PD-1 was diminished on $CD4^+$ $CD25^+$ T cells (Fig. 2b, c). NKG2A blockade also suppressed PD-1 expression on $CD4^+$ $CD25^+$ T cells stimulated with IL-2NK/NH-primed DCs from three different donors (Fig. 2d). The anti-NKG2A neutralizing Ab treatment also abrogated Foxp3 expression in $CD4^+$ $CD25^+$ Treg cells (Fig. 2e). Moreover, the blockade of NKG2A signals during NH and IL-2 NK co-cultures resulted in inhibition of the DC ability to induce $CD4^+$ $CD25^+$ T cells with regulatory functions; these $CD4^+$ $CD25^+$ T cells did not suppress proliferation or IFN-γ production (Fig. 2f and data not shown) of $CD4^+$ $CD25^-$ T cells. Altogether, the activation of NKG2A inhibitory signals during NK cell and hepatocyte interaction was required for the DC induction of $CD4^+$ $CD25^+$ T cells with regulatory phenotypes and functions.

Change of cytokine milieu, triggered by NKG2A signals, plays a critical role in DC-mediated induction of $CD4^+$ $CD25^+$ Treg cells

TNF-α has been well known as a critical factor for NK cell-mediated maturation of DCs.[27] By contrast, IL-10 and TGF-β are known to act as suppressive factors of effector immune responses, and their roles in modulating DCs for Treg cell induction has recently been validated.[12–14] These findings led us to evaluate the change in cytokine production patterns in NH and IL-2 NK co-cultures in the presence or absence of anti-NKG2A Ab. ELISA data showed that the production of IFN-γ and TNF-α from NH and IL-2 NK co-cultures were substantially increased in the presence of anti-NKG2A Ab. By contrast, the addition of NKG2A masking Ab during the co-culture resulted in the marked reduction of IL-10 and TGF-β from co-cultured cells (Fig. 3a).

We next examined whether these changes of cytokine profiles were responsible for the DC induction of the $CD4^+$ $CD25^+$ Treg cells. For this purpose, the NH and IL-2 NK co-culture supernatant was treated with neutralizing Ab of IL-10 or TGF-β before DC stimulation, and suppressive activity was evaluated by analysing $CD4^+$ $CD25^+$ T cells obtained from $CD4^+$ and DC mixtures. The neutralization of IL-10 did not reverse the suppressive actions of $CD4^+$ $CD25^+$ Treg cells, but the blockade of TGF-β led to reversal of $CD4^+$ $CD25^+$ Treg cell activities (Fig. 3b).

We directly examined the effect of TGF-β on the modulation of DC ability to induce $CD4^+$ $CD25^+$ Treg cells. TGF-β endowed DCs with the ability to induce $CD4^+$ $CD25^+$ Treg cells. TNF-α inhibited TGF-β-mediated DC induction of $CD4^+$ $CD25^+$ Treg cells (Fig. 3c). By contrast, IFN-γ had little effect on the modulation of DC by TGF-β (data not shown). Taken together, these results strongly suggest that increased TGF-β and decreased TNF-α production, the change of cytokine profiles mediated by the NKG2A signals, are involved in DC-mediated $CD4^+$ $CD25^+$ Treg cell induction.

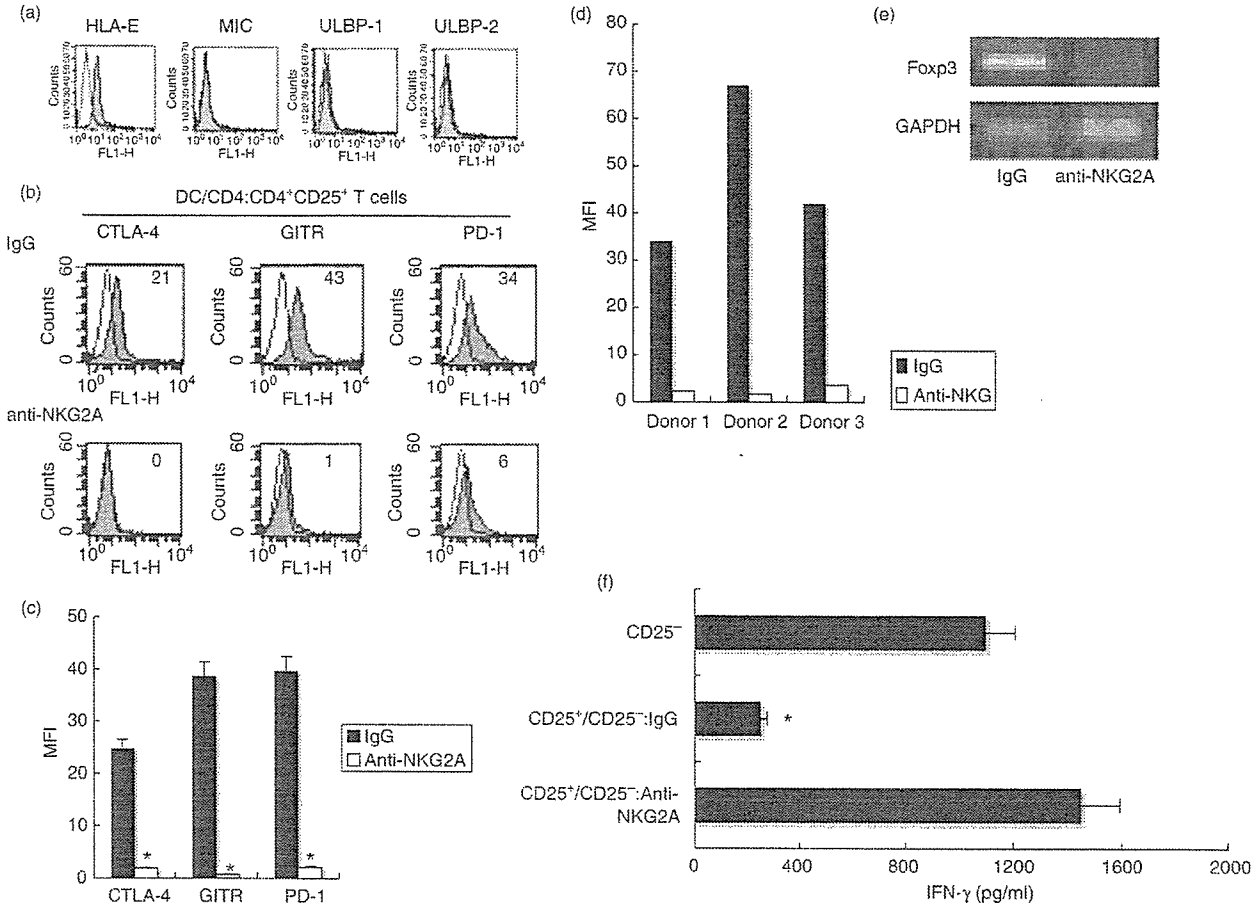

Figure 2. NKG2A signals of natural killer (NK) cells are required for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ T cells with the regulatory phenotype. (a) Surface expression of the ligands of NKG2A (HLA-E) as well as NKG2D (MIC, ULBP1 and ULBP2) in human non-transformed hepatocytes (NHs) were assessed by flow cytometry (closed histograms). Open histograms show isotype control staining. (b, c) Interleukin (IL)-2-preactivated NK cells were co-cultured with NHs in the presence of 30 μg/ml of anti-NKG2A neutralizing antibody (Ab) (anti-NKG2A) or control IgG. DCs (1×10^5) were then stimulated with the supernatant obtained from the co-cultured medium for 24 hr. After washing three times, DCs were cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ cells isolated from the co-culture were subjected to FCM for their surface expression of cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR) and programmed death-1 (PD-1) (closed histograms). Open histograms show isotype control staining. Numbers on the upper right indicate the mean fluorescence intensity (MFI) of each type of stained cell. All experiments were performed three times. Representative data (b) and composite results with statistical analysis (c) are shown as the MFI of the staining cells. $^*P < 0.05$ vs. responses of IgG group. The experiment was performed in different set of donors and similar results were obtained. (d) The inhibitory effect of anti-NKG2A Ab on PD-1 expression of $CD4^+$ $CD25^+$ T cells stimulated with allogeneic DCs from three different donors. Data are shown as MFI. (e) $CD4^+$ $CD25^+$ T cells were stimulated and purified as described above. The mRNA expression of Foxp3 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was examined by reverse transcription-polymerase chain reaction (RT-PCR. (f) $CD4^+$ $CD25^+$ T cells (1×10^5/well) isolated from DC and $CD4^+$ T cell co-cultures were cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells at a ratio of 1 : 1 in the presence of plate-bound anti-CD3 Ab ($CD25^+/CD25^-$). The anti-CD3 Ab-activated $CD4^+$ $CD25^-$ T cells alone were used as a positive control ($CD25^-$). Interferon (IFN)-γ was measured for each supernatant obtained after 48 hr of co-culture by enzyme-linked immunosorbent assay (ELISA). $^*P < 0.05$. All experiments were performed three times; representative results are shown.

Suppressive actions of $CD4^+$ $CD25^+$ Treg cells, induced by NH/IL-2 NK-primed DCs, depends on PD-1-mediated negative costimulatory signals

The suppressive activities of $CD4^+$ $CD25^+$ Treg cells reportedly depend on various kinds of mediators, such as CTLA-4, IL-10 and/or TGF-β, but the exact mechanisms of the actions have not been fully elucidated.[1,6,12–14] PD-1, recently identified as a negative costimulatory receptor of the B-7 family, is expressed in $CD4^+$ $CD25^+$ Treg cells, indicating that PD-1-mediated negative signals may be involved in the regulatory functions of $CD4^+$ $CD25^+$ Treg cells.[9] Thus, we evaluated the involvement of these molecules in the suppressive activities of $CD4^+$ $CD25^+$ Treg cells. For this purpose, the blocking Ab of CTLA-4, GITR, PD-1, TGF-β or IL-10 was added

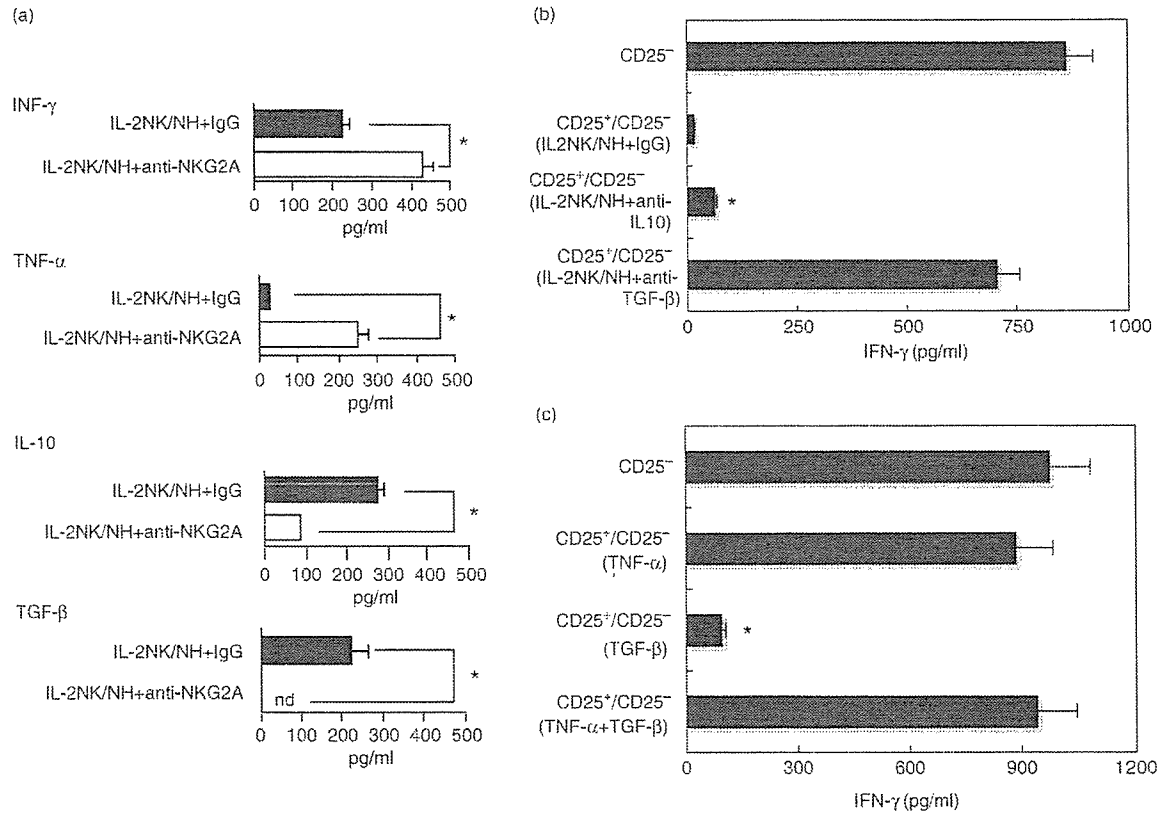

Figure 3. Change of cytokine production pattern of natural killer (NK) cells through NKG2A signals is responsible for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ Treg cells. (a) NK cells prestimulated with interleukin (IL)-2 were cultured with human non-transformed hepatocytes (NHs) in the presence of masking antibodies (Abs) of NKG2A (IL-2 NK/NH + anti-NKG2A) or isotype control IgG (IL-2 NK/NH + IgG) for 24 hr. $^*P < 0{\cdot}05$. (b) IL-2 activated NK cells were co-cultured with NHs (IL-2 NK/NH). DCs (1×10^5) were stimulated with the culture supernatant in the presence of anti-IL-10, anti-transforming growth factor (TGF)-β neutralizing Ab or control IgG for 24 hr. DCs were washed thoroughly and co-cultured with allogeneic $CD4^+$ T cells for 48 hr. Next, the isolated $CD4^+$ $CD25^+$ T cells (1×10^5/well) were co-cultured with autologous $CD4^+$ $CD25^-$ T cells in the presence of plate-bound anti-CD3 Ab at a ratio of 1 : 1. Interferon (IFN)-γ production from the culture supernatant was examined by enzyme-linked immunosorbent assay. $^*P < 0{\cdot}05$ vs. responses of anti-CD3 Ab-stimulated $CD4^+$ $CD25^-$ T cells. (c) DCs (1×10^5) were stimulated with 50 ng/ml TNF-α, 100 ng/ml TGF-β or both for 24 hr. After thorough washing, they were co-cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ T cells (1×10^5/well) were isolated from the DC and $CD4^+$ co-cultures and cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells at a ratio of 1 : 1 in the presence of plate-bound anti-CD3 Ab. IFN-γ production was examined as described above. $^*P < 0{\cdot}05$ vs. responses of anti-CD3 Ab-stimulated $CD4^+$ $CD25^-$ T cells.

during co-cultures of $CD4^+$ $CD25^+$/$CD4^+$ $CD25^-$ T cells in the presence of anti-CD3 Ab. In case of natural $CD4^+$ $CD25^+$ T cells, their suppressive action was partially reversed on addition of anti-CTLA-4 Ab. By contrast, they preserved their suppressive capacity even in the presence of the blocking Ab of GITR, PD-1, TGF-β or IL-10 (Fig. 4a). When $CD4^+$ $CD25^+$ Treg cells induced by NH/IL-2 NK-primed DCs were used instead of natural $CD4^+$ $CD25^+$ T cells, their suppressive activity was markedly reduced on addition of the blocking Ab of PD-1 but not CTLA-4, IL-10, TGF-β or GITR (Fig. 4a). The regulatory functions of these Treg cells were required for direct cell-to-cell contact because separation of $CD4^+$ $CD25^+$ Treg cells and $CD4^+$ $CD25^-$ T cells in transwell chambers virtually abolished their suppressive effects (data not shown). We also confirmed the presence of PDL-1 expression on $CD4^+$ $CD25^-$ T cells when they were activated with anti-CD3 Ab (Fig. 4b), suggesting that effector cells themselves induce suppressive activities of $CD4^+$ $CD25^+$ Treg cells. Taken together, these results further reinforced the hypothesis that $CD4^+$ $CD25^+$ Treg cells induced by NH/IL-2 NK-primed DCs were different from natural $CD4^+$ $CD25^+$ Treg cells in their PD-1-dependent suppressive functions.

Discussion

Recent studies have revealed that activated NK cells positively regulate DC activation and maturation either through direct contact via NK cell receptors (NKp30, NKG2D, etc.) or in co-ordination with various kinds of cytokines (IFN-γ, TNF-α, etc.).[15–18] However, the issue of

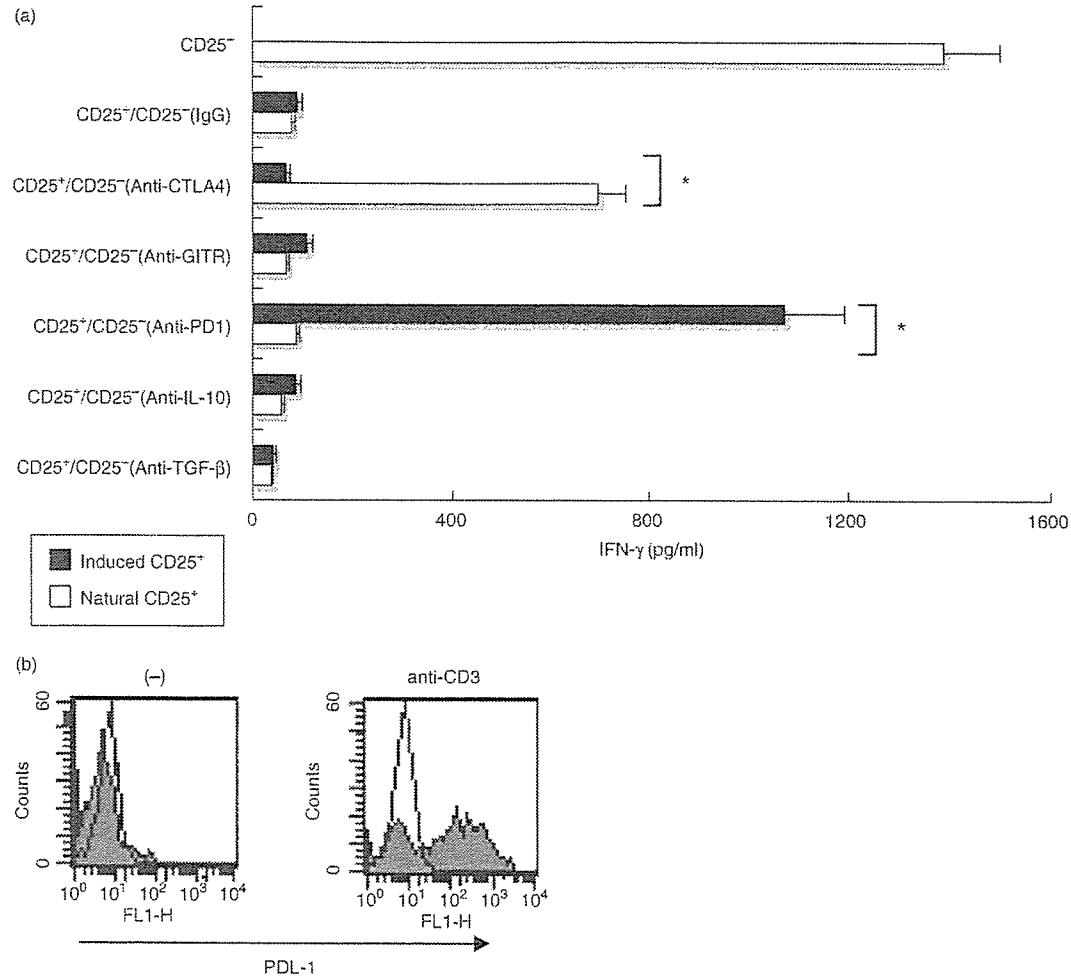

Figure 4. $CD4^+$ $CD25^+$ Treg cells induced by interleukin (IL)-2 natural killer (NK)/human non-transformed hepatocytes (NH)-treated dendritic cell (DC) suppressed T cell activation through programmed death-1 (PD-1)/programmed death ligand-1 (PDL-1) interactions. (a) DCs (1×10^5) were stimulated with the IL-2 NK/NH supernatant for 24 hr, and then cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ fractions were isolated from the DC/$CD4^+$ T cell mixtures. Freshly isolated $CD4^+$ $CD25^+$ T cells (natural $CD25^+$) or $CD4^+$ $CD25^+$ T cells induced by NK/NH-primed DCs (induced $CD25^+$) were co-cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells at a ratio of 1 : 1 upon stimulation of plate-bound anti-CD3 antibody (Ab). Anti-CTLA-4 (cytotoxic T lymphocyte antigen-4) Ab, anti-GITR (glucocorticoid-induced TNF receptor) Ab, anti-PD-1 Ab, anti-IL-10 Ab, anti-TGF-β Ab or isotype control IgG (20 μg/ml for each) were incubated during $CD4^+$ $CD25^+$/$CD4^+$ $CD25^-$ T cell co-cultures. Interferon (IFN)-γ was measured for each supernatant obtained after 72 hr of co-culture by enzyme-linked immunosorbent assay. $^*P < 0{\cdot}05$ vs. responses of anti-CD3 Ab-stimulated $CD4^+$ $CD25^-$ T cells. (b) Freshly isolated $CD4^+$ $CD25^-$ T cells were incubated with (anti-CD3) or without (–) plate-bound anti-CD3 Ab for 24 hr. PDL-1 expression was assessed by flow cytometry (closed histograms). Open histograms show isotype control staining.

whether NK cells are involved in DC-mediated Treg cell induction has not been resolved. In the present study, we report that the expression of regulatory markers and functions was markedly decreased on $CD4^+$ $CD25^+$ T cells upon exposure to IL-2 NK-primed DCs. By contrast, the interaction of activated NK cells and NH through the NKG2A inhibitory receptor led to DC induction of $CD4^+$ $CD25^+$ T cells with regulatory properties. Furthermore, NKG2A-mediated increase in TGF-β as well as decrease in TNF-α in an NH and NK cell mixture contributed to DC induction of $CD4^+$ $CD25^+$ Treg cells. This is consistent with previous reports showing that TGF-β plays a role in generating the specific DC that activates $CD4^+$ $CD25^+$ Treg cells.[10,11] The findings that TNF-α suppressed TGF-β-mediated priming of DCs to induce Treg cells also extended the previously identified role of TNF-α as a positive regulator of DC activation. In line with our findings, previous reports showed that impairment of $CD4^+$ $CD25^+$ Treg cell activities restored their suppressive functions after blocking TNF-α signals in non-obese diabetic (NOD) mice or in patients with Crohn's disease.[28,29] To our knowledge, the present study is the first description of modulation of NK cells and human hepatocytes through NKG2A-mediated inhibitory

signals that profoundly affect DC functions towards $CD4^+$ $CD25^+$ Treg cells. Because NK cell functions are regulated by the balance between inhibitory and activating signals, any future clarification of the role of other NK inhibitory and activating receptors in DC modulation and Treg cell activation will be of great interest.

The cross-presentation of self-antigens by major histocompatibility complex (MHC) class II pathways constitutes an important step towards generating and/or expanding peripheral Treg cells.[30] However, we initially settled our experimental design by using DCs and Treg cells from different donors, and DCs encountered $CD4^+$ T cells in an 'antigen-free' condition. Therefore, Treg cells induced by NK/NH-primed DCs are generated independently of MHC class II-mediated self-antigen recognition. These results give rise to the possibility that the cross-talk of NK cells, DCs and hepatocytes represents an alternative pathway in the generation and expansion of peripheral Treg cells. However, it should be noted that these results may not apply to all donors because of the complexity of the allogeneic system and the relatively few donors tested.

PD-1-mediated suppressive activities were characteristic for $CD4^+$ $CD25^+$ Treg cells generated by NH/IL-2 NK-primed DCs. By contrast, natural $CD4^+$ $CD25^+$ Treg cells exerted their suppressive function, at least in part, in a CTLA-4-dependent fashion. Recent reports have clarified the existence of two subtypes of Treg cells: natural and inducible $CD4^+$ $CD25^+$ Treg cells. Inducible Treg cells exert suppressive activities by using molecular mechanisms distinct from those of natural regulatory cells.[31] Our findings further identify the novel pathways by which inducible $CD4^+$ $CD25^+$ Treg cell activities triggered by NKG2A inhibitory signals are dependent on PD-1-mediated negative costimulation. A recent report identified the interaction of B7 on effector T cells with costimulatory molecules CD28/CTLA-4 on $CD4^+$ $CD25^+$ Treg cells as molecular mechanisms of their suppressor activity.[32] Thus, it is possible that reverse signalling of PDL-1 on effector cells may also be crucial for the negative costimulator-mediated suppressive action of $CD4^+$ $CD25^+$ Treg cells. In the present study, we did not address the mechanisms by which NH/IL-2 NK-primed DCs induce $CD4^+$ $CD25^+$ Treg cells with PD-1-dependent suppressive functions. Further study will be needed to clarify this issue.

We previously showed that NKG2A is expressed at higher levels from NK cells isolated from peripheral blood in patients with chronic hepatitis C virus (HCV) infection than from those in healthy donors.[20] HCV frequently persists in humans, at least in part, due to inefficient induction of NK activity as well as specific T cell responses.[33–35] The small percentage of patients who spontaneously clear the virus and recover from chronic hepatitis C mount vigorous HCV-specific $CD4^+$ and $CD8^+$ T cell responses.[36,37] Research has described an increased frequency of $CD4^+$ $CD25^+$ T cells in the blood of patients with persistent HCV infection compared with those who have spontaneously cleared HCV.[38,39] Our current findings raise the interesting possibility that increased NKG2A expression on NK cells may lead to DC-mediated induction of Treg cells, leading to the inhibition of adaptive responses to HCV and failure to eliminate this virus. Indeed, $CD4^+$ $CD25^+$ T cells induced by HCV-NK/Hep3B hepatoma cell-primed DCs expressed and suppressed effector T cell functions at greater levels than those induced by N-NK/Hep3B-primed DCs (our unpublished data). Interestingly, a recent study identified PD-1-mediated signals as a critical pathway to induce anergic $CD8^+$ T cells and impair antiviral CTL responses in chronic viral infection.[40] In this regard, the therapeutic modification of the PD-1 pathway may synergistically augment antiviral immunity by suppressing Treg activity and recovering CTL responses. It is important to establish whether the PD-1 pathway in liver lymphocytes may be operable *in vivo* and play a critical role in suppression of virus-specific immunity in HCV infection.

In conclusion, we have demonstrated that interaction of NK cells and hepatic cells via NKG2A leads to DC induction of $CD4^+$ $CD25^+$ T cells with PD-1-dependent regulatory activities. These findings also imply that NK receptor signals of NK cells may dictate DC-mediated adaptive immune responses towards tolerogenic or immunogenic status via induction of Treg cells.

Acknowledgements

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9. 肝癌免疫療法

大阪大学大学院医学系研究科消化器内科学助教授　竹原徹郎
同　消化器内科学教授　林　紀夫

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動　向

肝癌は限局した病変であれば肝切除や内科的ablation治療が可能であり，肝予備能および技術的な問題が充分にクリアされ，安全なマージンをとって切除・焼灼することができれば，根治的な治療効果を得ることができる．しかし，肝癌は時間的あるいは空間的に多発性を示すという生物学的な特徴があり，このことが肝癌の治療をきわめて難しいものにしている．肝両葉に多発する癌に対しては，局所治療は無効である．このような場合，肝動脈塞栓術や抗癌剤の動注などが考慮されるが，治療効果には限界がある．また，肝癌はたとえ局所治療に成功しても，異所再発をきたすことが多く，再発抑止法の確立が急務の課題となっている．したがって，進行肝癌の予後を改善し，局所治療後の無再発生存を向上させるためには，新たな視点での全肝治療法の開発が必要であり，このような背景から肝癌に対する免疫治療に期待が高まっている．

癌の免疫治療の歴史は非特異的なものから特異的なものへ，メカニズムの不明なものから明確なものへと進化してきている．生体の免疫応答を全般的に活性化する試みは，菌体成分による免疫賦活療法からサイトカインの利用へと進み，LAK (lymphokine-activated killer cell) による養子免疫治療は腫瘍特異的なTIL (tumor-infiltrating lymphocyte) を用いた方法へ，さらに，非特異的な免疫賦活は樹状細胞や腫瘍特異的抗原を用いたより特異的な治療へと変化してきている．また，従来から免疫賦活に用いられてきた菌体成分が実はToll様レセプターのリガンドであり，これが樹状細胞の成熟をうながすことにより，先天免疫あるいは獲得免疫を活性化することが明らかになり，このような方法の現代的な意味での見直しも行われている．肝癌に対してもサイトカイン治療，養子免疫治療，樹状細胞治療，腫瘍抗原ペプチド療法の各方面において，前臨床研究の成果に基づいて臨床試験が行われており一定の効果が報告されるようになってきている[1-3]．

A. サイトカイン治療

非特異的に免疫系全般の活性化をはかる方策として，先天免疫や獲得免疫を制御する種々のサイトカインの効果が検討されている．

進行肝癌に対するIFNα単独治療については1980年代から1990年代にかけていくつかの報告があるが，有効であるという報告とそれを否定する報告が混在している．最近，Llovetら[4]は58人の進行肝癌例に対して，IFNα-2b (3MU)

を1年間投与する場合と保存的な治療をする場合の効果についての無作為比較試験を行っている．彼らはIFNαの投与は副作用中止率も高く，1年および2年生存率において，対照群と有意差がなかったと結論している．一方，Kuboら[5]はC型肝炎ウイルスの感染を基礎疾患とする30例の肝癌切除患者を無作為に2群に振り分け，15例に対してIFNα（6MU）の2年間投与を行い，対照15例との間での肝癌の再発率を検討している．それによると，IFNα治療群からの肝癌の発生が5例，対照群からは12例であり，IFNα投与群において有意に発癌率が低かったと報告している．IFNαの投与が画像上認識されない微小な肝癌に対して治療効果があるのか，HCV感染を基盤とした新規の再発を抑制しているのかは明らかではないが，肝癌切除後のアジュバント治療としては，無再発生存に寄与することが示唆されている．

このように進行肝癌に対するIFNαの単独治療については限界があることから，他の抗癌剤との併用治療についての検討がなされている．パイロットスタディにおいてMTX＋5-FU＋CDDP＋IFNα[6]，CDDP＋DXR＋5-FU＋IFNα[7]　の併用治療効果が報告されている．これらの治療法はある程度の奏効率が報告されているものの，副作用も強く忍容性が低いことが問題となっている[8]．このようなIFNα併用化学療法のなかで5-FUがキードラッグであることが認識されるようになり，IFNαと5-FUの併用治療法が，進行肝癌の約半数において腫瘍縮小効果が得られることが報告されている[9-11]．最近，38例の進行肝癌を無作為に2群に割付け，5-FU＋IFNα治療と5-FU単独治療の無作為比較試験が行われた．併用群の奏効率が26%，単独治療が11%であり，両群間に有意差はなかったが，累積生存率では併用治療群が有意に良好であったと報告されている．5-FUとIFNαの併用治療効果は腫瘍細胞に対する直接作用が強調されているが[10]，IFNαにはNK細胞活性化能などの多彩な免疫賦活作用があり[12]，進行肝癌に対しても抗癌剤と併用することによりIFNα免疫治療が有効である可能性があると考えられる．

その他のサイトカインとして，Lygidakisら[13]はIL-2とIFNγを抗癌剤と併用する効果について検討している．Stage III～IVの肝癌のうち70%に腫瘍の縮小とAFP値の低下が得られたと報告している．Reinischら[14]は15例の進行肝癌に対してIFNγとGM-CSFとの併用治療法を行い，1例に腫瘍縮小効果があったとしている．

サイトカイン単独による肝癌治療については，再発予防を目的にしたIFNαによるアジュバント治療効果が示唆される以外は，進行癌に対する有効性は示されていない．進行肝癌に対しては化学療法剤との併用を行うことにより，治療効果を上昇させる可能性があると考えられる．

B. 養子免疫治療

生体内で抗腫瘍効果を示す免疫細胞はT細胞やNK細胞である．このようなエフェクター細胞を体外に取り出して活性化しこれを生体に戻して抗腫瘍効果を期待する治療法を養子免疫療法とよんでいる．1980年代中頃にRosenbergら[15]が進行癌患者（肝癌を含まない）を対象に臨床応用した免疫治療法である．我々は治療不能肝癌患者を対象に自己のLAK細胞を週2回4週間にわたってIL-2とともに肝動脈に注入するパイロットスタディを行い，奏効例があることを報告した[16]．最近，Takayamaら[17]は肝癌切除後の患者に対して，末梢血リンパ球を取り出し，これをIL-2，OKT3で刺激し，これを戻すことにより再発率が低下するかどうかを無作為化比較試験で検討している．それによると，無再発生存率は免疫群が対照群に比べて有意に良好であり，再発リスクが41%低下したと報告している．その後，活性化T

リンパ球を用いた肝癌切除後の肝動注免疫療法の有効性も報告されている[18]．

C. 樹状細胞治療

樹状細胞（DC）は生体内で最も強力な抗原提示細胞であり，獲得免疫の形成に必須の役割を担っている．また，DCはNK細胞やNKT細胞の活性化能もあり，先天免疫の制御においても重要な役割をもっている．DCは流血中にはきわめて微量にしか存在しないが，末梢血単球をGM-CSFやIL-4存在下に約1週間培養することにより，分化誘導することが可能である．このようにして誘導したDCは未成熟な表現型を示すが，これをLPSやTNFαで刺激することにより成熟させることができる．一般に未成熟DCは貪食能が高いが抗原提示能や遊走能が低く，また成熟DCは貪食能は低いが抗原提示・遊走能が高い．DCのex vivo誘導には時間がかかり，またヒトでの安全性が必ずしも保障されていない薬剤を用いる必要があるなどの問題があったが，最近，我々はこれらの問題を克服した新規の誘導法を開発し，進行癌に対する免疫治療に用いている[19]．

肝癌に対するDC治療としてはすでに3つのパイロットスタディの結果が報告されている．Ladhamsら[20]は，未成熟DCを自己の腫瘍細胞と混合刺激し，これを2例の肝癌患者に皮下接種した．重篤な副作用の出現はなく1例において，臨床経過が改善されたと報告している．Iwashitaら[21]は未成熟DCを腫瘍細胞lysateでパルスし，その後TNFαを用いて成熟誘導し，これを10例の切除不能肝癌患者の鼠径リンパ節に投与した．2例に軽度の腫瘍マーカーの低下と，1例に腫瘍の縮小を認め，重篤な合併症を認めた症例はなかったと報告している．彼らは同時にDCをマーカーとしてKLHで刺激し，7例においてDTH陽性になったことを示し，進行肝癌患者においてDCが明らかに特異免疫応答を誘導し得ることを示した．Stiftら[22]はTNFαで分化誘導したDCに腫瘍lysateをパルスし，これを2例の進行肝癌症例に投与している．明確な抗腫瘍効果は認められなかったと報告している．

DCを用いた抗腫瘍効果の限界を克服する1つの方法として，Tatsumiら[23]はIL-12の併用効果について検討しており，IL-12存在下においてDCの抗腫瘍効果が増強することを報告している．このような動物実験の結果を受けてIL-12で遺伝子改変を行ったDCの抗腫瘍効果についての検討がなされている．Mazzoliniら[24]はIL-12発現アデノウイルスをトランスフェクションしたDCを肝腫瘍に直接注入し，その安全性と効果について検討した．それによると，安全性に関しては問題がなかったが，期待された抗腫瘍効果は認められなかった．この1つの機序として腫瘍細胞がIL-8を分泌することにより，投与したDCが腫瘍局所にトラップされてしまう可能性が示唆されている[25]．DCは2次リンパ節においてT細胞を活性化することから，DCをリンパ節に直接注入する方法が検討される必要がある．

以上のように，DCを用いた肝癌に対する免疫治療法は進行肝癌を対象としたパイロットスタディが中心であるが，明確な治療効果は示されていない．今後，投与経路の工夫やより強力なDCの使用などの検討が必要である．また，進行癌症例に対する検討だけでなく，根治後の補助療法における意義などの検討も必要である．

D. 腫瘍ペプチドワクチン治療

肝癌は種々の腫瘍抗原を発現しており，患者末梢血中にはAFP[26]，NY-ESO-1[27]，MAGE-Aファミリー[28]，Glypican-3[29]などに対して特異的に反応するT細胞が存在することが知られている．このような知見を背景に，不活化した腫瘍細

胞や腫瘍抗原特異ペプチドを用いた肝癌に対する獲得免疫誘導の治療効果について臨床的な検討が行われている.

Kuangら[30]は, 肝癌治癒切除54例を自己フォルマリン固定腫瘍ワクチン投与群と非投与群に無作為に割付けた. 観察期間中央値15カ月の時点で, 無再発生存は投与群が対照群に比べ有意に良好であった. 再発リスクが81%低下したとしている.

Butterfieldら[31]はAFPを標的としたペプチド療法の可能性について検討している. 彼らはAFPに対する特異的T細胞応答を誘導するHLA-A*0201拘束性のペプチド領域を4カ所同定し, これをアジュバントに溶解しAFP高値を示す進行肝癌6症例に2週間毎に皮内接種した. AFPの低下や抗腫瘍効果は認められなかったが, テトラマー陽性細胞が増加し, IFNγ ELISPOTが増加したことから, AFPに対する特異的な免疫応答が誘導されることが確認された. 彼らはまた10例の進行肝癌症例に, これらのペプチドをパルスした自己DCを2週間毎に接種した[32]. やはり半数以上の症例においてAFP反応性のT細胞の増加とIFNγ ELISPOTの増加をみたと報告している. 現時点ではペプチドを用いたこのような治療で明らかな抗腫瘍効果は観察されていない.

むすび

肝癌に対する免疫治療への期待は高いが, 明確な治療効果をもつ治療法はいまだ確立されていない. 進行肝癌に対してはIFNαを併用した化学療法が有用である可能性があるが, 単独で有用性が示された免疫治療法はない. 免疫モニタリングシステムを用いて, 特異的な免疫応答がどの程度誘導されるかなどの検討を加えて, 今後治療法の改良を図っていく必要がある. 治療後のアジュバントとしてはIFN治療をはじめ, 腫瘍ワクチン治療や養子免疫治療がある一定の効果をもつ可能性があると考えられる.

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Intrahepatic Delivery of α-Galactosylceramide-Pulsed Dendritic Cells Suppresses Liver Tumor

Tomohide Tatsumi, Tetsuo Takehara, Shinjiro Yamaguchi, Akira Sasakawa, Ryotaro Sakamori, Kazuyoshi Ohkawa, Keisuke Kohga, Akio Uemura, and Norio Hayashi

Alpha-galactosylceramide, a glycosphingolipid, mediates interaction of dendritic cells (DCs) and NKT cells, leading to activation of both innate and acquired immunity. For cancer treatment, conventional DC-based vaccine has been tried, but its clinical efficacy is limited against liver cancer. Intrahepatic injection of α-Galactosylceramide-pulsed DCs (αGCDC) has not yet been tested in the liver that contains abundant immune cells such as NK, NKT, and T cells. In the present study, we examined the efficacy of αGCDC administration in comparison with p53 peptide-pulsed DCs using a well-established murine CMS4 tumor model. Injection of αGCDC into CMS4 liver tumors resulted in complete tumor rejection and established long-term survival of the animals, while injection of $p53_{232-240}$ peptide-pulsed DCs (pepDC) only partially suppressed tumor growth in the liver. The levels of IFN-γ in sera of αGCDC-treated mice were significantly higher than those of pepDC-treated mice. Hepatic NK cells were efficiently activated by αGCDC injection and played a critical role in liver tumor rejection as evidenced by an in vivo antibody-mediated NK cell depletion study. Injection of αGCDC into liver tumor led to higher $p53_{232-240}$ peptide-specific CD8+ T cell response than that of pepDC. The mice that had been protected from CMS4 liver tumor by αGCDC injection became resistant to subcutaneous CMS4 rechallenge, but not to Colon26 rechallenge. *Conclusion:* These results demonstrate that αGCDC injection into the liver can efficiently activate NK cells that in turn reject liver tumors to establish potent acquired immunity against the original tumor. (HEPATOLOGY 2007;45:22-30.)

Dendritic cells (DCs) effectively elicit immune responses to self and foreign antigens.[1,2] These specialized antigen-presenting cells (APCs) can induce the generation of both antigen-specific cytotoxic T lymphocytes (CTLs) and T helper cells. In this regard, conventional DCs pulsed with tumor-associated antigens in various forms, including peptide or tumor cell lysates, have been applied to human cancer treatment.[3] Recent research in DC biology has revealed that DCs also contribute to innate immune responses by activating NK cells[4-8] and NKT cells[9-11] via IL-12 secretion and direct cellular interaction. As the liver contains both a large compartment of innate immune cells (NK cells and NKT cells) and acquired immune cells (T cells),[12,13] if DCs can efficiently activate both T cells, NK cells and NKT cells in the liver, DC-based vaccines could offer attractive strategies for treating liver cancer. Primary and metastatic liver tumors are common malignancies that resist conventional chemotherapy and radiotherapy with poor prognosis. Recently, several conventional DC-based vaccine trials against liver cancer have been reported.[14-17] Although tumor-specific T cells were promoted by vaccination in most patients, clinical benefits have thus far only been observed in only a minority of treated individuals. Therefore, there is a great need to improve this therapeutic strategy, especially for advanced liver cancer.

The glycolipid antigen α-Galactosylceramide (α-GalCer) induces activation of NKT cells in a CD1d-dependent manner.[9] α-GalCer presented by DCs efficiently stimulates NKT cells implicated in the innate immunity.[18,19] Recently α-GalCer has been attracting atten-

Abbreviations: APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; α-GalCer, α-galactosylceramide; MNC, mononuclear cell; PBS, phosphate-buffered saline.

From the Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka, Japan.

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Address reprints request to: Norio Hayashi, M.D., Ph.D., Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: hayashin@gh.med.osaka-u.ac.jp; fax: (81) 6-6879-3629.

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