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

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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 < 0.05.

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

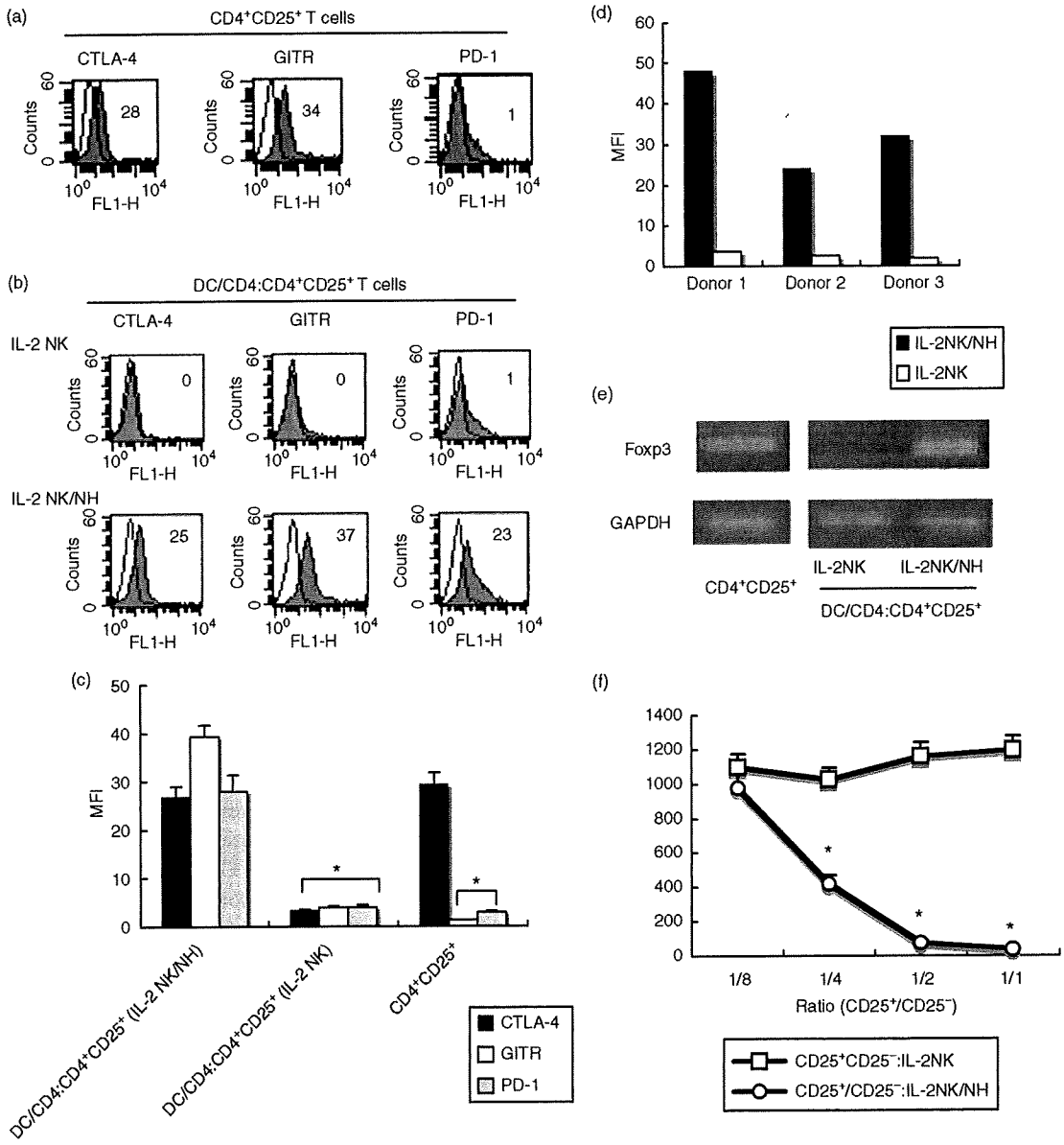

Figure 1. Human non-transformed hepatocyte (NH) modulation of activated natural killer (NK) cells endows dendritic cells (DCs) with the ability to induce $CD4^+$ $CD25^+$ regulatory T cells. (a) Freshly isolated $CD4^+$ $CD25^+$ T cells were cultured in the presence of plate-bound anti-CD3 antibody (Ab) 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. $^*P < 0.05$ 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. $^*P < 0.05$.

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.

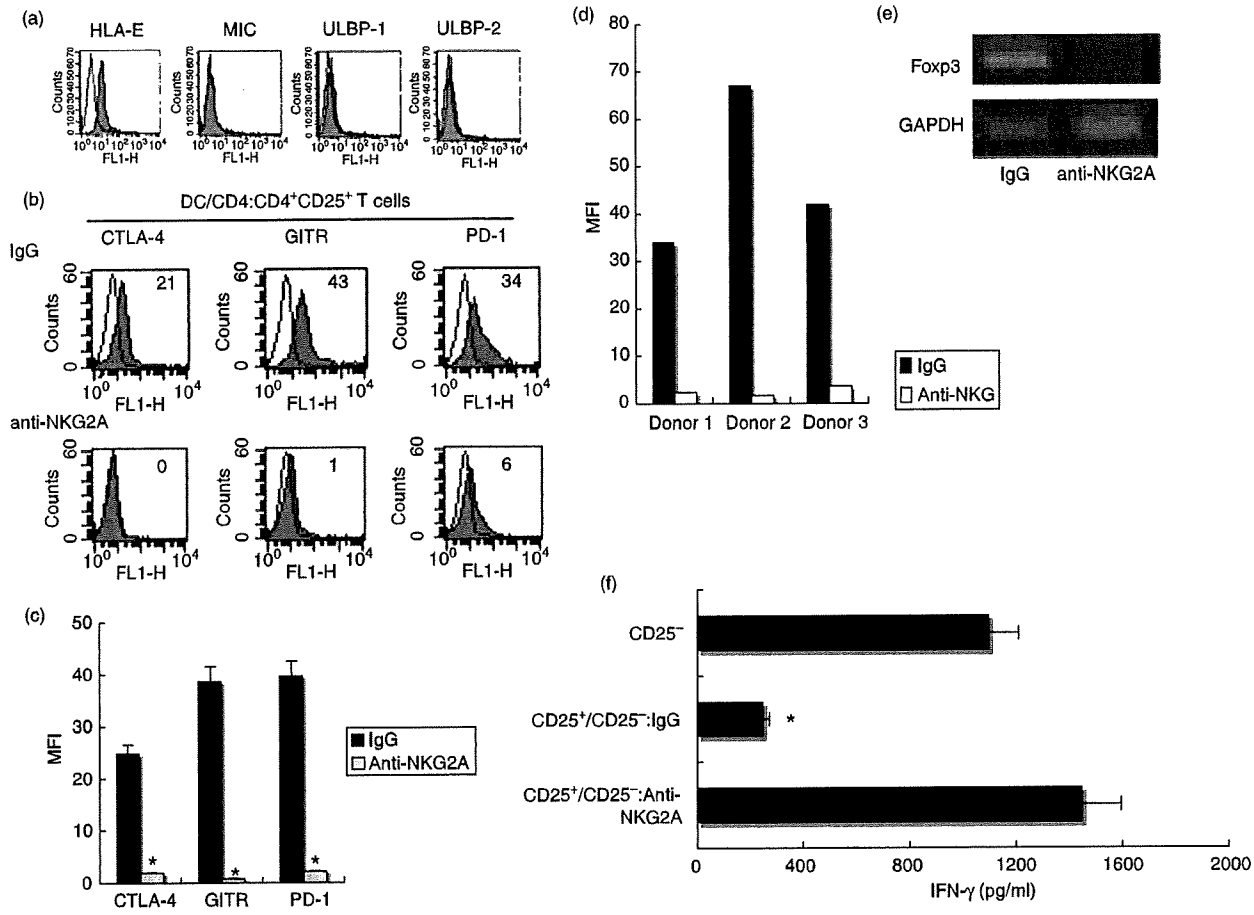

Figure 2. NKG2A signals of natural killer (NK) cells are required for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ T cells with the regulatory phenotype. (a) Surface expression of the ligands of NKG2A (HLA-E) as well as NKG2D (MIC, ULBP1 and ULBP2) in human non-transformed hepatocytes (NHs) 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

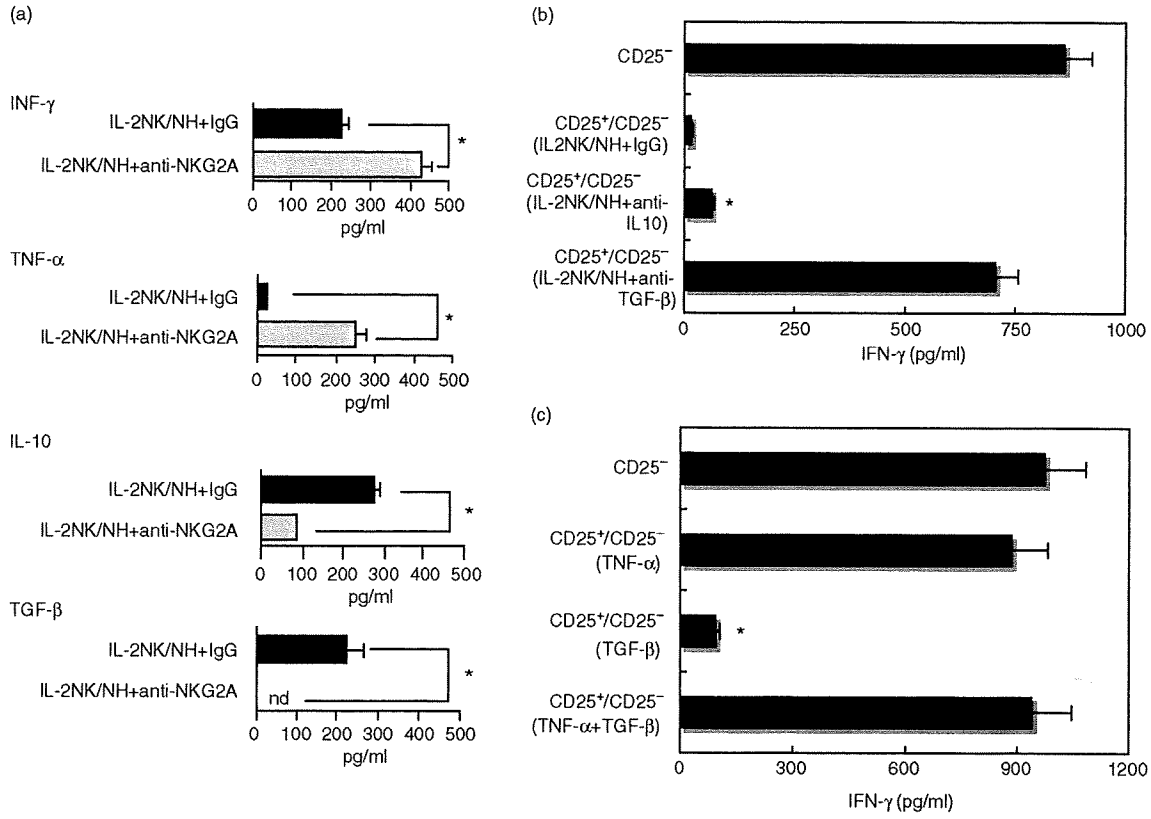

Figure 3. Change of cytokine production pattern of natural killer (NK) cells through NKG2A signals is responsible for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ Treg cells. (a) NK cells prestimulated with interleukin (IL)-2 were cultured with human non-transformed hepatocytes (NHs) in the 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

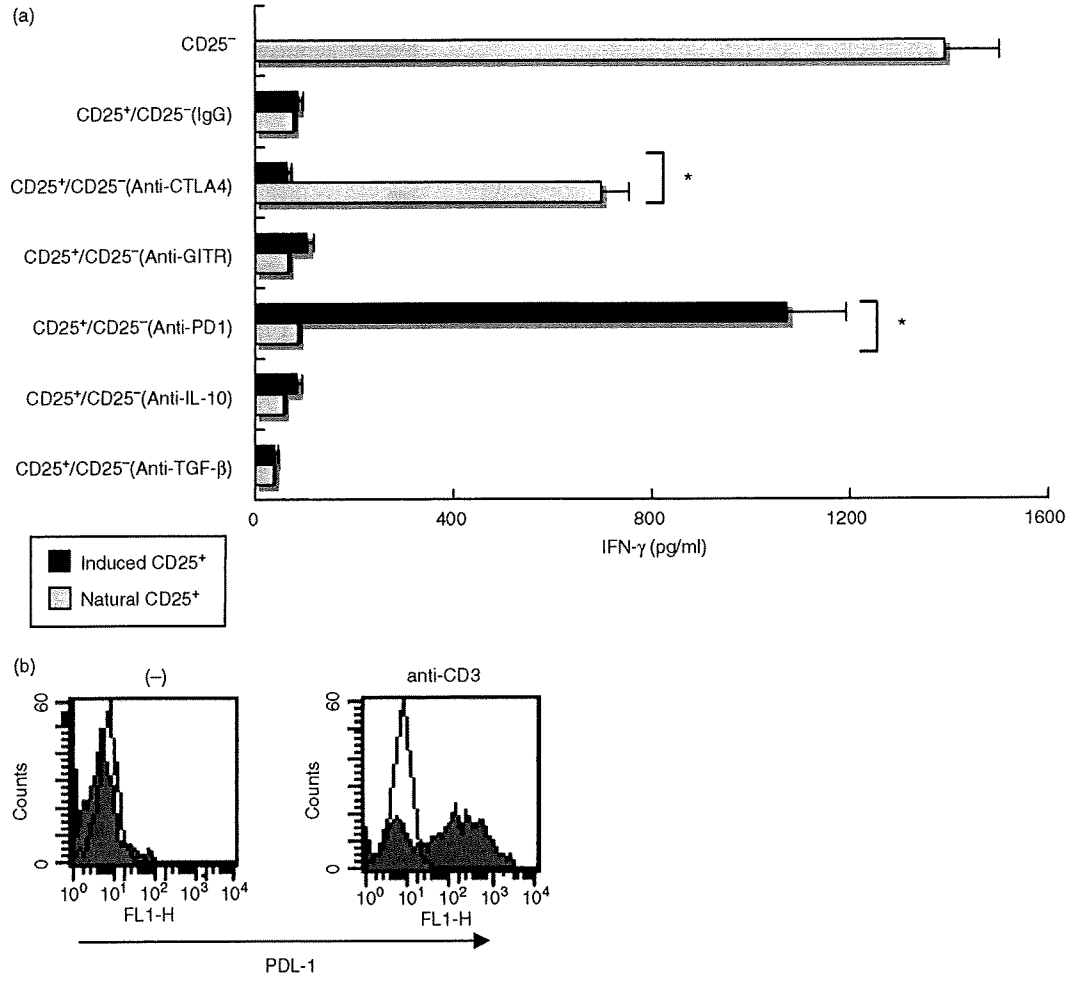

Figure 4. $CD4^+$ $CD25^+$ Treg cells induced by interleukin (IL)-2 natural killer (NK)/human non-transformed hepatocytes (NH)-treated dendritic cell (DC) suppressed T cell activation through programmed death-1 (PD-1)/programmed death ligand-1 (PDL-1) interactions. (a) DCs (1×10^5) were stimulated with 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. $^*P < 0.05$ 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.

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

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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.

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tion for novel antitumor therapy. *In vivo* animal studies have shown that systemic administration of α-GalCer can lead to anti-tumor effects against various tumors (including melanoma, sarcoma, colon carcinoma, and lymphoma) in hepatic and lung metastasis models.[20,21] Intravenous administration of α-GalCer pulsed DCs leads to more potent anti-tumor activities than direct administration of α-GalCer alone in mouse metastatic tumor models.[18,22] Based on the promising results of preclinical studies demonstrating the antitumor potential of α-GalCer, several phase I clinical studies have been done in cancer immunotherapy using intravenous administration of α-GalCer or α-GalCer-loaded DCs, but with limited clinical responses.[23-26] This might partly be because intravenous administered α-GalCer or α-GalCer-loaded DCs may not be delivered efficiently to the tumor site. Although the antitumor effect of α-GalCer has been demonstrated in murine metastatic liver tumor,[20-22,27] no clinical trial against liver cancer has been reported to date. For further development of liver cancer treatment, intrahepatic (i.h.) injection of α-GalCer-loaded DCs, expected to be the most efficient delivery system for tumor lesions, should be tested with respect to inducing effective antitumor therapy.

In this study, we evaluated the antitumor effect of i.h. injection of α-GalCer-pulsed DCs in murine liver tumor. Compared to the conventional peptide-pulsed DC vaccine, we observed effective antitumor effects against not only liver tumor but also disseminated tumor via more efficient activation of innate and acquired immunity in the liver.

Materials and Methods

Mice. Six- to eight-week-old female BALB/c mice were purchased from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan), and maintained in microisolator cages. The animals were handled under aseptic conditions. Procedures were performed according to approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

Cell Lines and $p53_{232-240}$ Peptide. CMS4 sarcomas ($H\text{-}2^d$) express mutated p53 and present the wild-type $p53_{232-240}$ epitope recognized by $H\text{-}2K^d$-resticted CTL.[28] Colon26, a mouse colon adenocarcinoma cell line, was kindly provided by Dr. Takashi Tsuruo (Institute of Molecular and Cellular Bioscience, University of Tokyo, Tokyo, Japan). These cell lines were maintained in complete media (CM), which is RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine (all reagents from GIBCO/Life Technologies, Grand Island, NY), in a humidified incubator at 5% CO_2 and 37°C.

α-GalCer. Alpha-GalCer was kindly provided by Kirin Brewery (Gunma, Japan) and prepared as described by Kawano et al.[9]

Preparation of α-GalCer-Pulsed DCs or p53 Peptide-Pulsed DCs. Bone marrow derived DCs were generated from BALB/c mice as previously described with minor modification.[29] Briefly, BALB/c bone marrow was cultured in CM supplemented with 1000 U/ml of rmGM-CSF and 1000 U/ml of rmIL-4 (PeproTech EC, London, UK) for 7 days. CD11c+ dendritic cells were isolated from whole bone marrow culture by magnetic cell sorting using MACS (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's protocol. DCs typically represented >90% of the harvested population of cells based on the morphology and expression of the CD11b, CD11c, CD40, CD80, CD86, and class II MHC antigens (data not shown). On day 7, DCs were added to α-GalCer (100 ng/ml) and cultured for 24 hours to prepare α-GalCer-pulsed DCs (αGCDC). To prepare p53 peptide-pulsed DCs (pepDC), the mouse $p53_{232-240}$ peptide was added to day 7 DCs as described.[28] Alpha-GCDC or pepDC were then washed twice with PBS before i.h. injection of these cells.

Animal Experiments. BALB/c mice were intrahepatically injected with 5×10^5 CMS4 cells and 1×10^6 αGCDC, pepDC, or DCs in a total volume of 100 μl of phosphate-buffered saline (PBS) on day 0. Two weeks after the tumor injection, the livers of the treated mice were removed, and the weight was measured to examine intrahepatic tumor growth. To assess the impact of systemic immunity from i.h. injection of αGCDC, BALB/c mice were injected intrahepatically with 5×10^5 CMS4 cells or Colon26 cells and 1×10^6 αGCDC. On day 28 after i.h. injection, 1×10^6 CMS4 cells or Colon26 cells were injected into the right flank of treated mice. Tumor size was assessed every 3 or 4 days and recorded in square millimeters by determining the product of the largest perpendicular diameters measured using vernier calipers. Data are reported as the average tumor area ± SD.

IFN-γ ELISA. Mice sera were harvested 2 weeks after intrahepatic tumor injection and αGCDC or pepDC treatment, and subjected to mouse IFN-γ ELISA (BD-Pharmingen, San Diego, CA), with lower levels of detection of 31.3 pg/ml.

Cytotoxic Assay. BALB/c mice were treated with an i.h. injection of 1×10^6 αGCDC, pepDC or DCs. After 48 hours, hepatic mononuclear cells (MNCs) were prepared as previously described.[20] To evaluate the cytotoxicity of hepatic NK cells, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-

2H-tetrazolium, monosodium, salt] assay (Nacalai Tesque, Kyoto, Japan) was performed as described with minor modification.[29] After 24 hours of coculture of hepatic MNC and NK-susceptible YAC-1 target cells at the 5:1 ratio (hepatic MNC:YAC-1 cells) in 96-well plates, 10 μl WST-8 was added to each well and the cells were incubated for another 1 hour. The 450 nm absorbance was measured using a microplate reader (Benchmark, Bio-Rad Laboratories, CA). NK cell cytotoxicity was calculated as described.[29]

CD8+ T Cell Response Against $p53_{232-240}$ Peptide. On day 14 after i.h. injection of CMS4 cells and αGCDC or pepDC, CD8+ T cells were isolated from the spleen cells of treated mice bearing CMS4 liver tumor by using magnetic beads (MACS) and then were cocultured (1×10^5 cells/well) with syngeneic DCs (2×10^4 cells/well) pulsed with or without $p53_{232-240}$ peptide in 96-well tissue culture plate. After 48 hours incubation, the culture supernatant was collected and analyzed for IFN-γ release using specific ELISA kit (BD-Pharmingen).

T Cell and NK Cell Depletion Experiments. On days −6, −1, 5, and 10 after tumor inoculation, mice were injected intraperitoneally with anti-CD4 [GK1.5 hybridoma, American Type Culture Collection (ATCC), Manassas, VA] or anti-CD8 (53-6.72 hybridoma, ATCC) as described.[29] The efficiency of specific subset depletions was validated by flow cytometry analysis of splenocytes using PE-conjugated anti-CD4 and anti-CD8 mAbs (Pharmingen). For NK cell depletion, mice were injected with anti-asialo GM-1 (Wako, Osaka, Japan) on day −1, 5, and 10 after tumor inoculation. The efficiency of NK cell depletion was validated by flow cytometry analysis of splenocytes using PE-conjugated anti-DX5 mAbs (Pharmingen). In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

Statistical Analysis. The statistical significance of differences between the groups was determined by applying the Student t test or 2-sample *t* test with Welch correction after each group had been tested with equal variance and Fisher's exact probability test. The statistical significance of the differences in more than 2 groups was determined by applying one-way ANOVA. Survival reliability was estimated from the Kaplan-Meier curve, and statistics were analyzed by the log rank test. We defined statistical significance as $P < 0.05$.

Results

Intrahepatic Delivery of αGCDC Is More Therapeutic Than pepDC in the CMS4 Liver Tumor Model. We examined the therapeutic potential of αGCDC or pepDC against CMS4 liver tumor. DCs were generated from bone marrow cells and pulsed with α-GalCer or $p53_{232-240}$ peptide. BALB/c mice were injected intrahepatically with 5×10^5 CMS4 cells and 1×10^6 αGCDC, pepDC, DCs only or PBS. Two weeks after the tumor injection, the livers of the treated mice were removed, and the weight was measured to examine intrahepatic tumor growth. Large CMS4 tumors formed in all mice treated with either PBS or DCs (Fig. 1A). Small CMS4 tumors formed in the liver of pepDC treated mice, with the exception of 1 mouse among the 6 pepDC-treated mice. No tumor formation was observed in the liver of any of the αGCDC-treated mice. The liver weight of the pepDC treatment group tended to be lighter than that of the PBS treatment group, but not with statistical significance. The liver weight of the αGCDC treatment group was significantly lighter than that of the PBS treatment groups (Fig. 1B). The survival rate of the pepDC-treated group was significantly higher than those of the DC-treated or PBS-treated groups ($P < 0.05$) whereas that of the DC-treated group was not significantly different from that of the PBS-treated group. All αGCDC-treated mice survived at 70 days after tumor inoculation, but all pepDC-treated mice died within 35 days (Fig. 1C). These results suggested that the αGCDC treatment has more therapeutic potential against CMS4 liver tumor than conventional pepDC treatment. Therapeutic potential of αGCDC is not unique to CMS4 liver tumor model, because no Colon26 liver tumor was observed in mice treated with αGCDC and the liver weight of αGCDC-treated mice was significantly lighter than that of PBS-treated mice (Fig. 2).

Serum IFN-γ Level and Hepatic NK Cell Activation Are Associated with the Degree of Therapeutic Effect. We next evaluated whether the therapeutic benefits observed in our DC-based treatment regimens were associated with the degree of serum IFN-γ in treated animals. Mice serum was harvested 2 weeks after intrahepatic tumor injection and αGCDC or pepDC treatment, and subjected to mouse IFN-γ ELISA. Serum IFN-γ levels were elevated in αGCDC-treated and pepDC-treated mice, whereas no IFN-γ was detected in PBS-treated mice, mice treated with DCs only, or normal nontreated mice (Fig. 3A). The serum IFN-γ of αGCDC-treated mice was significantly higher than that of pepDC-treated mice,

To examine whether hepatic NK cells were actually activated by i.h. injection of αGCDC or pepDC, we examined the cytotoxic activity of hepatic MNC against YAC-1 cells after i.h. injection of αGCDC or pepDC. The cytotoxic activity of αGCDC-treated mice was significantly stronger than those of pepDC-treated, DC-treated, or PBS-treated mice (Fig. 3B).

These results indicated that i.h. injection of αGCDC in the liver could induce IFN-γ production in treated

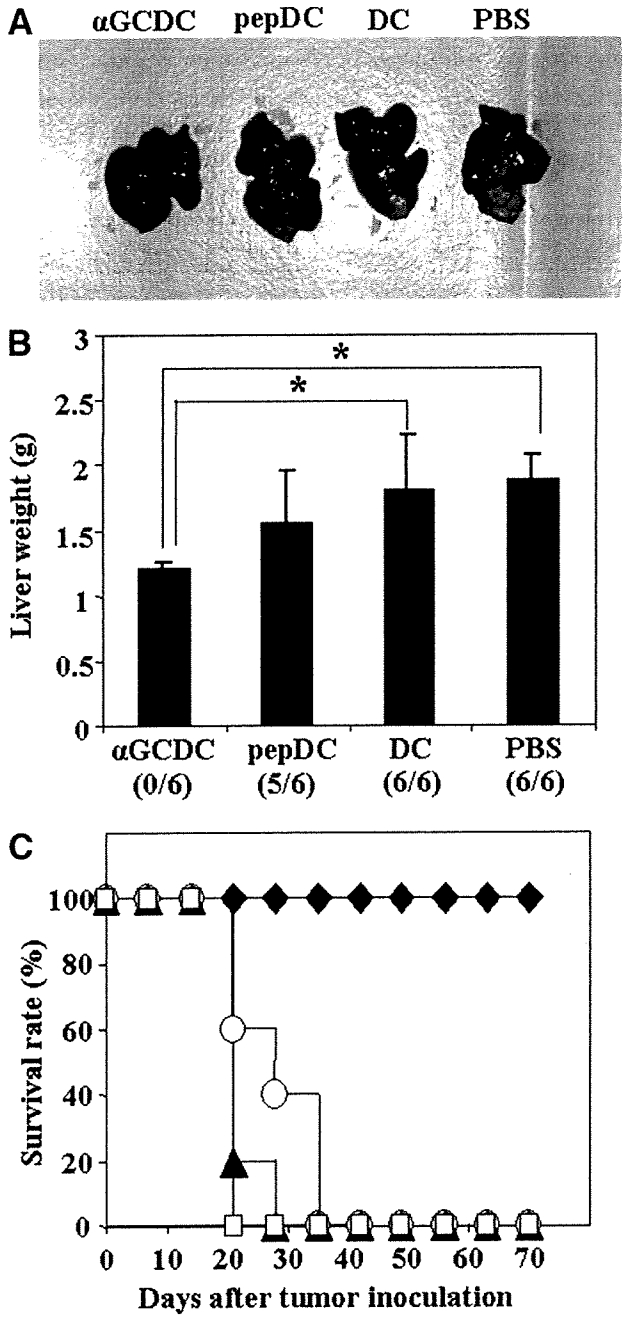

Fig. 1. Improved therapeutic effectiveness of i.h. delivered α-GalCer-pulsed DCs in CMS4 liver tumor model. BALB/c mice were injected intrahepatically with 5×10^5 CMS4 cells and 1×10^6 α-GalCer-pulsed DCs (αGCDC), $p53_{232\text{-}240}$ peptide-pulsed DCs (pepDC), DCs only (DC), or PBS (n = 6 in each treatment group). Two weeks after the CMS4 tumor injection, the livers were removed from all treated mice. (A) Representative liver macroscopic view of each group. (B) Comparison of liver weight of each group. As a control, the mean liver weights of untreated normal mice were 1.11 ± 0.05 g. *$P < 0.05$. In all cases, the fraction of mice bearing liver tumor in each treatment group at 14 days is given in parentheses. (C) Survival curve of mice intrahepatically inoculated with CMS4 cells. CMS4 liver tumor bearing mice were treated with αGCDC (solid diamonds), pepDC (empty circles), DC (solid triangles), or PBS (empty squares) (n = 10 in each treatment group).

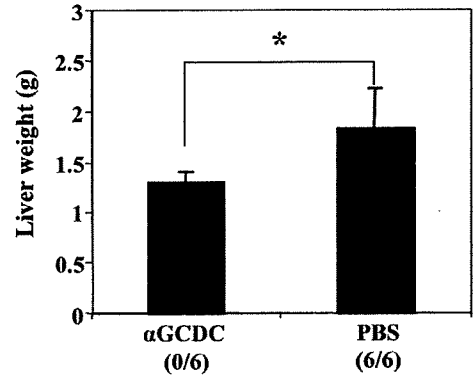

Fig. 2. Improved therapeutic effectiveness of i.h. delivered α-GalCer-pulsed DCs in Colon26 liver tumor model. BALB/c mice were injected intrahepatically with 5×10^5 Colon26 cells and 1×10^6 α-GalCer-pulsed DCs (αGCDC) or PBS (n = 6 in each treatment group). Two weeks after the Colon26 tumor injection, the livers were removed from all treated mice. As a control, the mean liver weights of untreated normal mice were 1.11 ± 0.05 g. Comparison of liver weight of each group. *$P < 0.05$. In all cases, the fraction of mice bearing liver tumor in each treatment group at 14 days is given in parentheses.

mice and efficiently activate hepatic NK cells, suggesting that there may be an association between IFN-γ production or NK cell activation and the degree of therapeutic effects observed in this system.

Depletion of NK Cells Abolishes the Antitumor Effect of αGCDC. To verify that the therapeutic benefit of αGCDC-based regimen in the CMS4 liver tumor model was T cell-dependent and NK cell-dependent, we performed T cell subset depletion and NK cell depletion studies (Fig. 4). NK cell depletions significantly inhibited the therapeutic efficacy of i.h. injections with αGCDC ($P < 0.05$ versus NK cell-depleted mice). In contrast, neither CD4+ nor CD8+ T cell depletions inhibited the therapeutic efficacy of i.h. injection with αGCDC. These results suggested that NK cells, but neither CD8+ nor CD4+ T cells, play critical roles in the antitumor effect against mouse liver tumor.

$p53_{232\text{-}240}$ Peptide-Specific CTLs Are Generated After DC Treatment of Liver Tumor. We next evaluated whether $p53_{232\text{-}240}$ peptide-specific CTLs were generated after treatment of liver tumor by DC treatment. CD8+ T cells were isolated from the spleen cells of treated mice and then cocultured with syngeneic DCs pulsed with $p53_{232\text{-}240}$ peptide strongly expressed on CMS4 cells. The $p53_{232\text{-}240}$ peptide-specific IFN-γ production of CD8+ T cells differed significantly among the treatment groups (Fig. 5). CD8+ T cells from mice treated with αGCDC produced higher levels of the Th-1 associated cytokine IFN-γ in response to $p53_{232\text{-}240}$ peptide than CD8+ T cells obtained from mice treated with any other DC-based vaccine or with

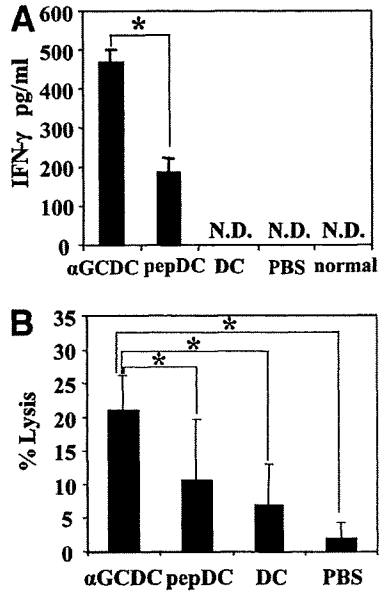

Fig. 3. Increase in serum IFN-γ levels and activation of hepatic NK cells in mice treated with αGCDC. (A) Mice sera were harvested two weeks after intrahepatic CMS4 tumor injection and treatments of αGCDC, pepDC treatment, DC only, or PBS, and subjected to IFN-γ ELISA. Naïve mice (normal) were used as controls. Cytokine levels are reported in picograms per milliliter (mean ± SD of triplicate samples). Similar results were obtained in 2 independent experiments. N.D., not detected. $*P < 0.05$. (B) BALB/c mice were treated with an i.h. injection of 1×10^6 αGCDC, pepDC, DC only, or PBS. After 48 hours, hepatic mononuclear cells were isolated from the liver to evaluate the cytotoxicity against YAC-1 cells at 5:1 effector/target cells ratio. Similar results were obtained in 2 independent experiments. $*P < 0.05$.

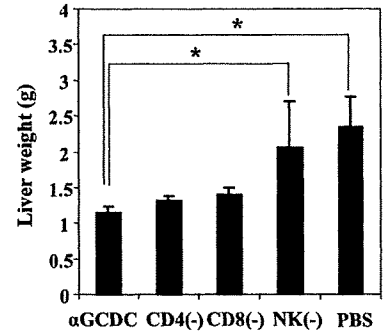

Fig. 4. Dependence of antitumor efficacy of i.h. αGCDC delivery on NK cells, not on CD4+ or CD8+ T cells. To prove that the therapeutic benefit of αGCDC-based regimen in the CMS4 liver tumor model is T cell-dependent and NK cell-dependent, the liver weights of αGCDC treated mice with $CD4^+$ (CD4(-)) and $CD8^+$ (CD8(-)) T cell subset or NK cell (ASGM1) depletion or without depletion (αGCDC) were shown. The liver weights of mice treated with PBS were also shown. As a control, the mean liver weights of untreated normal mice were 1.11 ± 0.05 g. Ab-mediated *in situ* depletion of NK cells, but not $CD4^+$ or $CD8^+$ T cells, markedly reduced the therapeutic efficacy of αGCDC therapy (n = 6 in each treatment group). $*P < 0.05$ versus αGCDC.

PBS only, suggesting that strong $p53_{232\text{-}240}$ peptide-specific CTLs were generated by αGCDC treatment of the liver tumor.

Systemic Therapeutic Antitumor Immunity Is Induced by i.h. Injection with αGCDC. Because strong anti-CMS4 CTL response was generated in αGCDC-treated animals, we next chose to analyze whether treatment of a CMS4 lesion in the liver would affect the growth of rechallenged CMS4 tumors. BALB/c mice were intrahepatically injected with CMS4 tumors and αGCDC. After 28 days, 1×10^6 CMS4 cells or Colon26 cells were injected subcutaneously in the right flank. The subcutaneous CMS4 tumors in mice receiving the αGCDC regimen were completely rejected in all mice (Fig. 6A). The growth of subcutaneous Colon26 tumor in αGCDC-treated mice was not inhibited, suggesting that CMS4 specific antitumor immunity could be induced (Fig. 6B) by αGCDC treatment. Acquired immune responses induced by αGCDC is not unique to CMS4 tumor model, because the subcutaneous tumor growth of Colon26 cells (Fig. 6D), but not CMS4 cells (Fig. 6C), were also significantly inhibited in mice that had been protected Colon26 liver tumor by i.h. injection of αGCDC.

Discussion

Tumor associated antigen derived peptide-pulsed DCs-based vaccine have been reported in various mouse tumor models,[30,31] and clinical applications of peptide-pulsed DCs have been tried with various cancer patients.[3,32-34] However, although tumor-specific T cells were promoted by vaccination in most patients, objective clinical responses have thus far only been observed in a

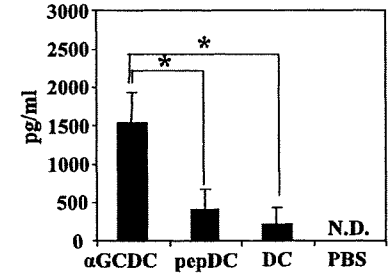

Fig. 5. Evaluation of $p53_{232\text{-}240}$ peptide specific CD8+ CTL in responder mice. CD8+ T cells were isolated from the spleen of mice 14 days after i.h. injection with DCs (αGCDC, α-GalCer pulsed DCs; pepDC, $p53_{232\text{-}240}$ peptide pulsed DCs; DC, DCs only; PBS, control) and CMS4 cells. IFN-γ production from CD8+ T cells against $p53_{232\text{-}240}$ peptide was measured by ELISA (results in picograms per milliliter; mean ± SD of triplicate samples). Syngeneic DCs pulsed with $p53_{232\text{-}240}$ peptide served as the antigen-presenting cells. IFN-γ production from CD8+ T cells against peptide-unpulsed syngeneic DCs served as negative control, and this value was subtracted from all experimental determinations to determine $p53_{232\text{-}240}$ peptide specific IFN-γ production. Similar results were obtained in two independent experiments. N.D., not detected. $*P < 0.05$.

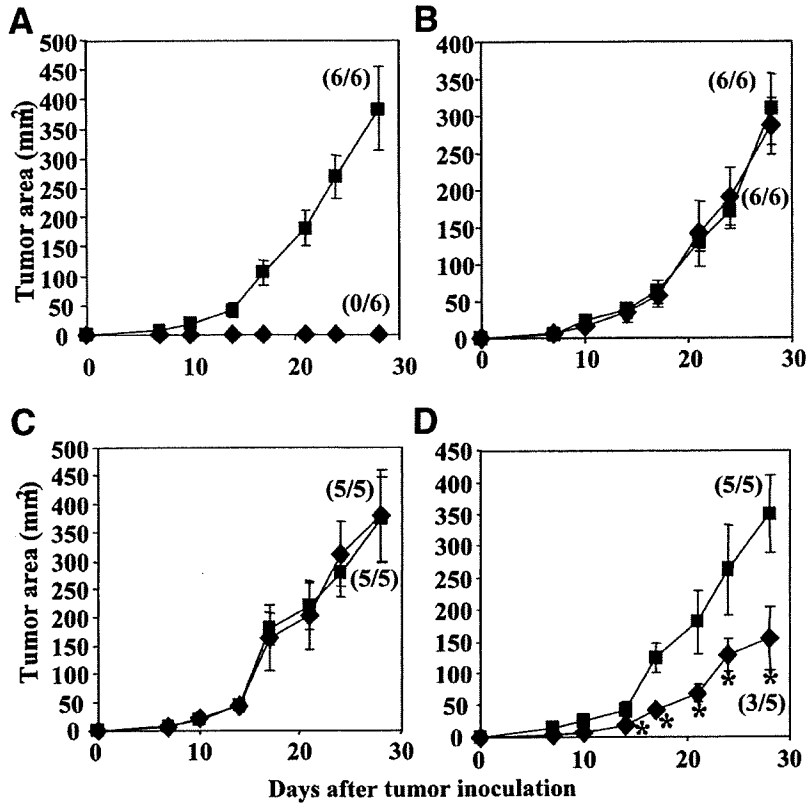

Fig. 6. Alpha-GCDC-based intrahepatic therapy results in the development of systemic anti-tumor immunity that protects distal tumor rechallenge. On day 0, BALB/c mice were injected intrahepatically with αGCDC and either CMS4 tumors (A, B, n = 6 in each treatment group)or Colon26 tumors (C, D, n = 5 in each treatment group). Twenty-eight days after treatment, mice were challenged subcutaneously with 1×10^6 CMS4 cells (A, C) or Colon26 cells (B, D) injected into the right flank. As a control, naïve mice were challenged with 1×10^6 CMS4 cells (A, C) or Colon26 cells (B, D).The fraction of mice bearing a tumor in each treatment group at day 28 is indicated in parentheses. Tumor size was expressed as the mean tumor size of only those mice bearing a tumor. αGCDC treated mice (solid diamonds); naive mice (solid squares). Each data point represents the mean tumor size ± SD. $^*P < 0.05$. versus naive mice.

minority of treated individuals. A normal liver contains lymphocytes that are usually enriched with NK and NKT cells; i.e., 25% NK cells and 30% NKT cells in contrast to peripheral blood that contains only 10% NK and 5% NKT cells.[12,13] Recently, DCs have been implicated in the activation of NKT and NK cells in both mice and humans,[4-6,9-11,23,35] suggesting that activation of many innate immune cells in the liver by a DC-based vaccine would be promising for treating liver tumor. We hypothesize that i.h. injection of α-GalCer-pulsed DC can efficiently activate abundant innate immune cells in the liver and elicit effective innate and acquired immunity against liver tumor. Our results demonstrated that i.h. injection with αGCDC resulted in complete rejection of CMS4 liver tumors with prolonged survival of tumor-bearing mice whereas injection with pepDC did not. These results suggested the efficacy of i.h. injection of αGCDC for treating liver cancer and the superiority of αGCDC treatment over conventional pepDC treatment. Intrahepatic injection of αGCDC also revealed a strong antitumor effect in the Colon26 liver tumor model. Although DCs pulsed with both α-GalCer and peptide (αGC-pepDC) would be expected to provide a better therapy index, the therapeutic potential of αGC-pepDC was found to be similar with that of αGCDC in the CMS4 liver tumor model (Tatsumi T et al., unpublished data). These findings suggest that i.h. delivery of αGCDC may optimally promote antitumor effects in the mouse liver tumor microenvironment.

We have shown that serum IFN-γ was detected in αGCDC-treated and pepDC-treated mice and that serum IFN-γ levels in αGCDC-treated mice were significantly higher than in pepDC-treated mice. Zitvogel et al. reported that the antitumor effects of DC-based vaccination were dependent on the production of Th1-associated cytokines such as IFN-γ, tumor necrosis factor-α, and IL-12.[36] Therefore, enhanced IFN-γ production resulting from injection of αGCDC or pepDC into liver tumor may also play an important role in the antitumor activity in vivo. Our results also suggested that αGCDC treat-

ment in the liver could induce stronger antitumor immunity than pepDC treatment.

Efficient activation of abundant NKT cells and NK cells in the liver might be important in an antitumor effect against liver tumor. We and others have previously reported that sequential activation of NKT cells and NK cells could be observed in the liver after α-GalCer administration and that most NKT cells had disappeared from the liver within 12 hours of α-GalCer administration.[20,37] Thus, the activated NK cells mainly play critical roles in the antitumor effect of disseminated liver tumor.[20] We found that the NK cell activity of αGCDC-treated mice were significantly stronger than those of pepDC-treated, DC-treated, or PBS-treated mice. These findings offer evidence that intrahepatic injection of DCs activate hepatic NK cells and that αGCDC may activate liver abundant NK cells more efficiently than pepDC, which might be associated with the therapeutic outcomes of these treatments in liver tumors.

In this study, the depletion of NK cells, but not CD4+ or CD8+ T cells, diminished the antitumor effect against liver tumor by α-GalCer pulsed DCs. These results suggested that only NK cells play a critical role in eradication of mouse liver tumor by i.h. injection of αGCDC and that neither CD4+ nor CD8+ T cells play critical roles in the early phase of the eradication of liver tumor cells. However, our following results demonstrated that strong systemic acquired immunity could be generated after early eradication of liver tumor by treatment with αGCDC. These results suggested that NK cells activated by αGCDC might be the main effector cells in the early eradication of liver tumor cells and that liver tumor-derived tumor antigens are taken up by dedicated professional antigen-presenting cells in the liver, which might generate prolonged liver tumor antigen-specific acquired immunity.

Subsequent analyses revealed that CD8+ T cells isolated from mice treated with i.h. injection of αGCDC or pepDC in liver tumors secrete IFN-γ in response to $p53_{232\text{-}240}$ peptide strongly expressed on CMS4 cells when presented by syngeneic DCs *in vitro*. The IFN-γ level of CD8+ T cells from αGCDC-treated mice was much higher than that from pepDC-treated mice. Mayordomo et al. reported that immunization of $p53_{232\text{-}240}$ peptide-pulsed DCs induced peptide-specific CTL in immunized mice that showed cytolytic activity against CMS4, p53 overexpressing cells, and that $p53_{232\text{-}240}$ peptide-pulsed DC vaccine offered protection against CMS4 tumor challenge in mice.[28] These results suggested the therapeutic potential of $p53_{232\text{-}240}$ peptide-based DCs vaccine in the CMS4 tumor model. Our current data revealed that i.h. injection of αGCDC to liver tumors generated $p53_{232\text{-}240}$ peptide-specific CTL more efficiently than that of pepDC. The activation of NKT cells was associated with an expansion of antigen-specific CTL, as might be expected if the DCs that matured *in vivo* in response to NKT cells were capturing antigens.[38-41] In a clinical study, Chang et al. reported that increases of antigen-specific memory T cells were observed in cancer patients treated with α-GalCer-pulsed dendritic cells.[26] Our results suggested that the activation of hepatic NK cells in the liver might be associated with the efficiency of generation of tumor antigen-specific CTL. We believe that injection of αGCDC into the liver more efficiently activates innate immune cells, NKT cells and NK cells, followed by generation of tumor antigen-specific CTL than injection of pepDC.

Additional experiments using subcutaneous rechallenge with tumor demonstrated that i.h. αGCDC treatment of liver tumors not only blocked treated CMS4 liver tumor progression but offered complete protection against "recurrence" of the same tumor at a distant site. In contrast, Colon26 rechallenge tumor was not inhibited in the treated mice, suggesting that CMS4-specific immunity was generated after liver tumor treatment. These results were consistent with the activation of acquired immunity evaluated by IFN-γ secretion from CD8+ T cell in response to $p53_{232\text{-}240}$ peptide. Intrahepatic αGCDC treatment of Colon26 liver tumor also led to resistance to subsequent Colon26 challenge but not to CMS4 challenge. These data supported that i.h. αGCDC injection generally induced acquired immunity after rejection of original liver tumors. Taken together, we believe that i.h. αGCDC treatment of liver tumors offers the optimal therapeutic treatment for both local liver tumor and distant metastatic tumor.

In spite of recent progress and early successes reported for DC-based cancer immunotherapies, there remains significant room for improvement in these regimens, especially with respect to advanced liver cancer. The liver is the most common site of metastasis of gastrointestinal cancers (i.e. colorectal cancer, gastric cancer and pancreatic cancer). Thus, new therapeutic approaches of DC-based immunotherapy for advanced liver tumor need to be developed. Recently, percutaneous liver tumor ablation methods, radiofrequency ablation (RFA) therapy, and ethanol injection therapy (PEIT) have become well-established in hepatocellular carcinoma treatment. This encourages gastroenterologists to apply i.h. injection-immunotherapy to liver tumor treatment. We show here that i.h. injection of α-GalCer-pulsed DCs has greater antitumor efficacy than that of tumor antigen-derived peptide-pulsed DCs in liver cancer treatment and that i.h. injection of α-GalCer pulsed DCs into liver tumor results

in the coordinated activation of both innate and acquired immunity in the liver, leading to superior antitumor efficacy. These findings indicate that the use of i.h. delivery of α-GalCer-pulsed DCs might represent a particularly promising approach to suppressing tumor growth and promoting regression of metastatic lesions in liver cancer patients.

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Impaired ability of interferon-alpha-primed dendritic cells to stimulate Th1-type CD4 T-cell response in chronic hepatitis C virus infection

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SUMMARY. In interferon-alpha (IFN-α)/ribavirin combination therapy for chronic hepatitis C (CHC), an enhanced T helper 1 (Th1) response is essential for the eradication of hepatitis C virus (HCV). We aimed to elucidate the role of IFN-α or IFN-α/ribavirin in dendritic cell (DC) ability to induce Th1 response in HCV infection. We generated monocyte-derived DC from 20 CHC patients and 15 normal subjects driven by granulocyte–macrophage colony-stimulating factor and interleukin 4 (IL-4) without IFN-α (GM/4-DC), with IFN-α (IFN-DC), with ribavirin (R-DC) or with IFN-α/ribavirin (IFN/R-DC) and compared their phenotypes and functions between the groups. We also compared them in 14 CHC patients between who subsequently attained sustained virological response (SVR) and who did not (non-SVR) by 24 weeks of IFN-α/ribavirin therapy. Compared with GM/4-DC, IFN-DC displayed higher CD86 expression, but lesser ability to secrete IL-10 and were more potent to prime $CD4^+$ T cells to secrete IFN-γ and IL-2. Such differences were more significant in healthy subjects than in CHC patients. No additive effect of ribavirin was observed in DC phenotypes and functions *in vitro* either which was used alone or in combined with IFN-α. However, in the SVR patients, an ability of IFN/R-DC to prime T cells to secrete IFN-γ and IL-2 was higher than those of IFN-DC and those of IFN/R-DC in the non-SVR group, respectively. In conclusion, DC from CHC patients are impaired in the ability to drive Th1 in response to IFN-α. Such DC impairment is restored *in vitro* by the addition of ribavirin in not all but some patients who cleared HCV by the combination therapy.

Keywords: chronic hepatitis C, dendritic cells, hepatitis C virus, interferon-alpha, ribavirin, Th1.

INTRODUCTION

The prevalence of hepatitis C virus (HCV) infection is evident with 170–200 million being affected worldwide [1,2]. Approximately 30% of those exposed to HCV are able to eradicate it after the initial exposure, while the remaining 70% cannot, subsequently developing to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [3]. In the early phase of acute HCV infection, HCV continues to replicate in the liver, where interferon-alpha (IFN-α) and IFN-inducible genes are significantly induced, suggesting that HCV hampers the execution of IFN-α-mediated anti-virus or immune response [4,5]. In order to eradicate HCV from chronically infected patients, IFN-α has been used. However, IFN-α monotherapy successfully eradicates HCV in only 10–20% of treated patients [6], the efficacy being lower in patients infected with HCV genotype 1 than those with other genotypes [7]. Pegylated IFN-α in combination with ribavirin has been widely used as the first-line anti-HCV therapy, as the rate of HCV clearance has been improved to be 46–56% of the treated patients [8]. These clinical results show that IFN-α alone is not sufficient to initiate anti-HCV activity in some chronically infected patients.

Both IFN-α and ribavirin have an immunomodulatory effect on immune cells in addition to their direct antiviral effects; however, the mechanisms of action of these drugs during the therapy are poorly understood. IFN-α directly or indirectly stimulates T helper 1 (Th1) cell development and

Abbreviations: CHC, chronic hepatitis C; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; GM-CSF, granulocyte–macrophage colony-stimulating factor; HCV, hepatitis C virus; IFN-α, interferon-alpha; IL, interleukin; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MoDC, monocyte-derived DC; PBMC, peripheral blood mononuclear cells; SVR, sustained virological response; Th1, T helper 1

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