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Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFNα gene to mice

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Interferon (IFN) α is a pleiotropic cytokine acting as an antiviral substance, cell growth inhibitor and immunomodulator. To evaluate the therapeutic efficacy and mechanisms of IFNα on hepatic metastasis of tumor cells, we hydrodynamically injected naked plasmid DNA encoding IFNα1 (pCMV-IFNa1) into Balb/cA mice having 2 days hepatic metastasis of CT-26 cells. Single injection of pCMV-IFNa1 efficiently enhanced the natural killer (NK) activity of hepatic mononuclear cells, induced production of IFNγ in serum and led to complete rejection of tumors in the liver. Mice protected from hepatic metastasis by IFNα therapy displayed a tumor-specific cytotoxic T cell response and were resistant to subcutaneous challenge of CT-26 cells. NK cells were critically required for IFNα-mediated rejection of hepatic metastasis, because their depletion by injecting anti-asialo GM1 antibody completely abolished the antimetastatic effect. To find whether NK cells are directly activated by IFNα and are sufficient for the antimetastatic effect, the responses to IFNα were examined in SCID mice lacking T cells, B cells and NKT cells. IFNα completely rejected hepatic metastasis in SCID mice and efficiently activated SCID mononuclear cells, as evidenced by activation of STAT1 and a variety of genes, such as MHC class I, granzyme B, tumor necrosis factor-related apoptosis-inducing ligand and IFNγ, and also enhanced Yac1 lytic ability. Study of IFNγ knockout mice revealed that IFNγ was not necessary for IFNα-mediated NK cell activation and metastasis protection. In conclusion, IFNα efficiently activates both innate and adaptive immune responses, but NK cells are critically required and sufficient for IFNα-mediated initial rejection of hepatic metastasis of microdisseminated tumors.
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The liver is the most common site of metastatic malignancy and the status of this organ is an important determinant of survival in patients with advanced disease. The risk of hepatic metastasis remains high in many patients after potentially curative surgery at primary sites.[1] This suggests that the spread of tumor cells can occur in the liver even when they cannot be detected by current diagnostic modalities. To suppress the incidence of liver metastasis, whole liver therapy against microdisseminated tumors should be considered.[2] Since the liver contains an abundance of immune cells, the cytokine-meditated activation of these cells may be a promising approach toward this end.[3,4]

Interferon (IFN) α is a pleiotropic cytokine acting as an antiviral substance, cell growth inhibitor and immunomodulator. IFNα as well as IFNγ are primarily induced in response to viral infection of cells and ligate a cognate receptor for the Type 1 IFN expressed on target cells.[5] On the other hand, Type 2 IFN, IFNγ, is produced predominantly by T lymphocytes, natural killer (NK) cells and NKT cells and uses a distinct receptor. IFNα-mediated antiviral activity includes induction of 2′-5′ oligoadenylate synthetases, double-stranded RNA-activated protein kinase (PKR) and Mx proteins. IFNα can exert direct effects on tumor cells by inhibiting proliferation, inducing apoptosis and inhibiting the release of proangiogenic factors such as vascular endothelial growth factor.[6] IFNα-mediated immunomodulation includes dendritic cell maturation, NK cell activation, MHC Class I induction and cytokine production.[7] Most, if not all, of these actions are mediated by the Jak-STAT signaling pathway downstream of the Type 1 IFN receptor.[8–10] Type 1 IFN receptor upon ligand ligation phosphorylates Jack1 and then phosphorylates STAT1, which activates a variety of IFN-regulated genes. IFNα and IFNβ have been shown to elicit antitumor effects in various murine models of cancer.[11–14] IFNβ was also shown to be effective for retarding metastatic tumor growth in murine liver, but the underlying mechanisms have not been elucidated.[15]

In the present study, we investigated the efficacy of hydrodynamics-based expression of IFNα in the liver against a murine model of hepatic metastasis of CT-26 colon cancer cells and the mechanisms of an IFNα-mediated therapeutic effect of hepatic metastasis. Mice treated with IFNα completely rejected hepatic metastasis and became resistant to rechallenge by CT-26 cells. Although IFNα induced a variety of host responses including increased NK activity, increased IFNγ production and tumor-specific T cell responses, the initial rejection of hepatic metastasis was solely dependent on NK cells. Our study has shed light on NK cell activation as an important mechanism by which IFNα ablates microdisseminated tumors in the liver.

Material and methods

Mice

Specific pathogen-free female Balb/cA mice, SCID mice and their wild-type control mice were purchased from Clea Japan, Inc. (Tokyo, Japan). Rag2 knockout (Rag2 KO) mice were purchased from Taconic (Germantown, NY). IFNγ knockout (GKO) mice with a Balb/cA background was kindly provided by Dr. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo).[16] All mice were used at the age of 5 to 8 weeks. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care, and the study protocol complied with the institution's guidelines.

Tumor models

Intrasplenic injection of tumor cells was used to establish microdisseminated liver tumors in mice.[17] CT-26 colon cancer cells originating from Balb/cA mice were maintained in DMEM supplemented with 10% FCS. Syngeneic mice were anesthetized with pentobarbital and given a cut on the left side flank. CT-26 cells (1×10^5) were suspended in 150 μl of PBS and injected into the spleen. For subcutaneous tumor models, CT-26 cells (5×10^5) were injected into the back of the mice under light anesthesia.

NK cell depletion

For depletion of NK cells *in vivo*, anti-asialo GM1 antibody (Wako, Osaka, Japan) was intraperitoneally administered.[17] We

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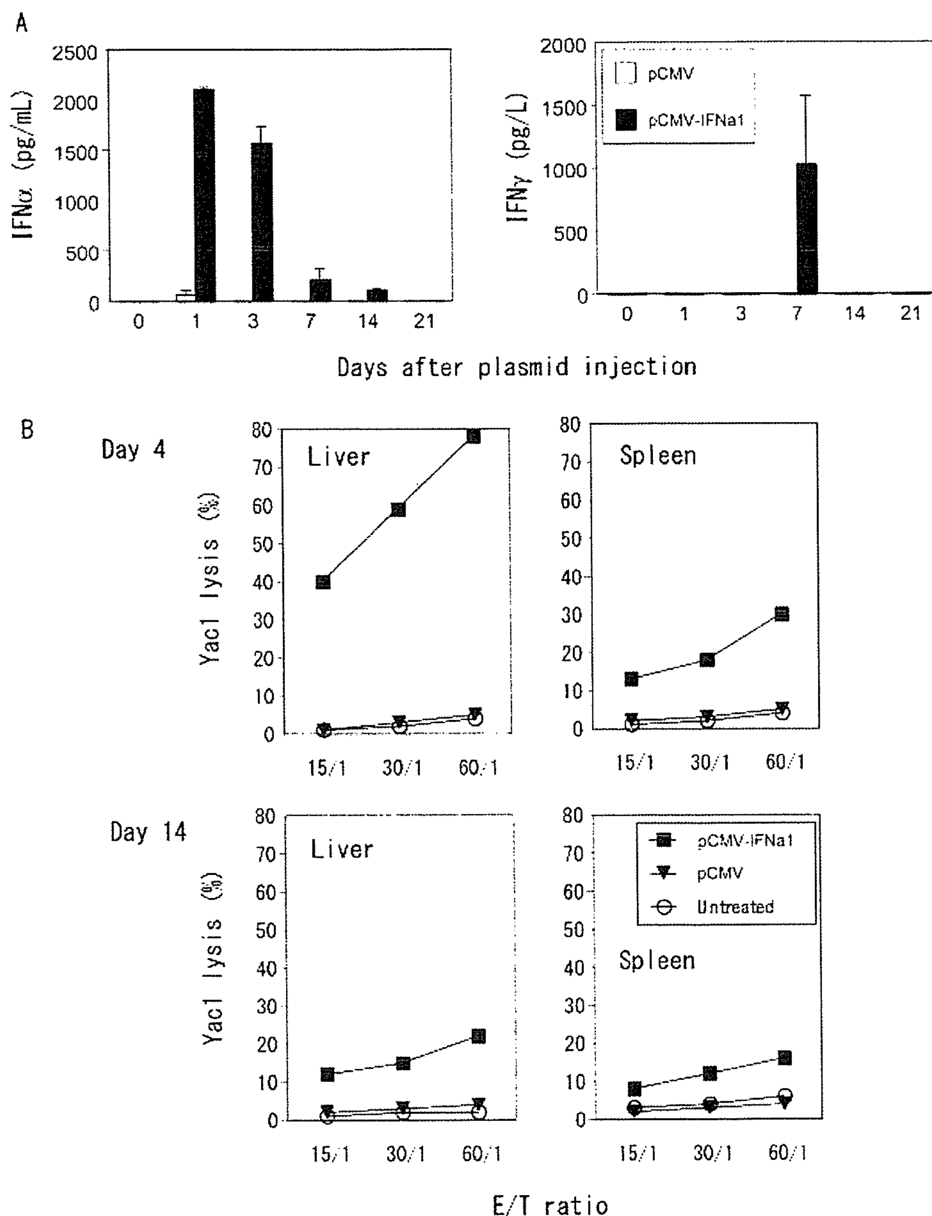

FIGURE 1 – Effects of hydrodynamic injection of IFNα-expressing plasmid. (*a*) Serum IFNα and IFNγ concentration. Balb/cA mice were hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) and bled at indicated time points to measure the levels of serum IFNα and IFNγ. The results are indicated as mean and SD (n = 3/group). Shown are representative data for 2 independent experiments. (*b*) Yac1 lytic ability. Hepatic or splenic mononuclear cells were isolated from naive Balb/cA mice (open circles) and those injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles). Yac1 lytic ability was measured by a standard chromium-release assay at indicated effector and target ratios (E/T ratio). All experiments were performed at least 3 times and representative data are shown.

determined the appropriate dosing to be 500 μg/mouse (50 μl when dissolved according to the manufacturer's instructions) based on FACS analysis of hepatic mononuclear cells. Injection of this dose of anti-asialo GM1 antibody depleted more than 95% of DX-5 positive, TCRβ-negative cells (NK cells) in the liver. NKT cells were less affected than NK cells, because 40% of Cd1d-tetramer positive cells, which are invariant NKT cells, still remained in the liver after the treatment. Anti-asialo GM1 antibody was injected 1 day after tumor inoculation and then every 5 days. For the control, the same amount of normal rabbit immunoglobulin (DAKO, Copenhagen, Denmark) was intraperitoneally administered.

Injection of naked plasmid DNA

A plasmid coding the murine IFNa1 gene, pCMV-IFNa1, was generously provided by Dr. Daniel J. J. Carr (University of Oklahoma, Health Science Center).[18] Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Hydrodynamic injection of plasmid DNA was performed as previously described.[19] In brief, 25 μg of plasmid DNA was diluted with 2.0 ml of lactated Ringer's solution and injected into the tail vein, using a syringe with a 30-gauge needle. DNA injection was completed within 8 to 15 sec.

ELISA

Blood samples were serially obtained from the venous plexus in the retro-orbita under light anesthesia. The levels of serum IFNα and IFNγ were measured using commercially available ELISA kits (Biomedical Laboratories for murine IFNα; Endogen for murine IFNγ).

Mononuclear cells

Mononuclear cells were isolated from the liver or spleen as previously described.[20] The NK activity of mononuclear cells was assessed with standard 4-hr ^{51}Cr-releasing assay using Yac1 cells as targets. To examine CT-26-specific responses, splenocytes were stimulated with CT-26 cells for 5 days in the presence of 30 U/ml

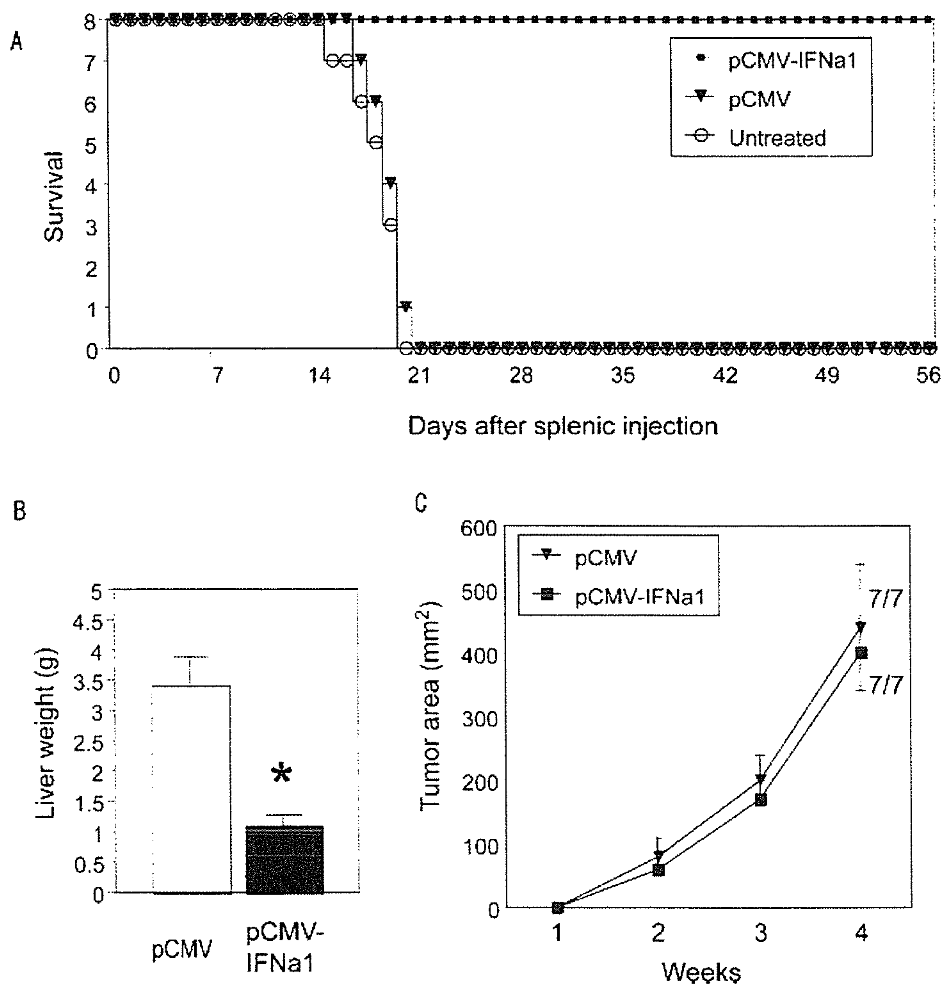

FIGURE 2 – Anti-tumor effects of IFNα therapy. (*a*) Survival. Balb/cA mice were intrasplenically injected with CT-26 cells. Two days later, the mice were randomly assigned to 3 groups and received hydrodynamic injection of either pCMV-IFNa1 (closed squares) or pCMV (closed triangles) or untreated (open circles). The number of survivors in each group was monitored. (*b*) Anti-metastatic effects. Balb/cA mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) 2 days later. At 14 days after the splenic injection, the mice were sacrificed to examine liver tumor development by measuring liver weight. All experiments were performed at least 3 times and representative data are shown. *, $p < 0.05$ *vs.* pCMV injection group. (*c*) Anti-tumor effects on subcutameous tumors. Balb/cA mice were subcutaneously injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles) 2 days later. Tumor growth was examined every week. Tumor size was expressed as the mean tumor size of only those mice bearing tumors. Each data point represents the mean tumor size and SD. The ratio of the number of mice bearing tumor/the number challenged for each treatment group at 4 weeks is shown in the figure.

of murine IL-2 and subjected to analysis for lytic activity against CT-26 cells or BNL A.7 murine hepatoma cells by 4-hr ^{51}Cr-releasing assay. In some experiments, mononuclear cells were separated into CD90-positive cells (T cells) and CD90-negative cells (non-T cells) using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Western blotting

Mouse recombinant IFNα was generously provided by Fujisaki Institute, Hayashibara Biochemical Laboratories (Okayama, Japan). Mononuclear cells were treated with or without IFNα. Whole cell lysate was prepared from mononuclear cells from mice, and 20 μg of protein was separated by SDS-PAGE and transferred to PVDF membrane. The membrane was stained with anti-STAT1 antibody (Upstate Biotechnology, Lake Placid, NY) or antiphospho-sepcific STAT1 (Y701) antibody (Upstate Biotechnology) and visualized by chemiluminescence. The specificities of STAT1 and phosphorylated STAT1 signals were confirmed by siRNA experiment using BNL A.7 cells in the presence or absence of IFNα treatment (data not shown). Anti-STAT antibody recognizes STAT1α, whereas antiphospho-STAT1 antibody recognizes phosphorylated form of both STAT1α and STAT1β.

Microarray analysis

Total RNA was isolated from cultured SCID splenocytes in the presence or absence of IFNα by ISOGEN. RNA was analyzed using the GeneChip Mouse Genome Array 430 2.0 (Affymetrix, Santa Clara, CA). Analysis of difference expression was performed by GeneChip Operating Software Ver. 1.1. Genes were considered to be significantly upregulated according to the following criteria: (*i*) the mean fold increase was more than 4-fold; (*ii*) the expression of a gene was significant in NK cells after IFNα treatment; (*iii*) a significant increase was registered based on the algorithm of the software.

Statistics

Data are represented as mean ± SD. Comparisons between groups were analyzed by unpaired *t*-test with Welch's correction or ANOVA for experiments with more than two subgroups. *Post hoc* tests were done using the Bonferroni's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Single intravenous injection of IFNa1 gene enhances NK activity and completely rejects hepatic metastasis of CT-26 cells

Hydrodynamics-based gene delivery establishes efficient foreign gene expression predominantly in the liver, especially in hepatocytes.[21,22] Serial measurement of serum IFNα demonstrated that pCMV-IFNa1 injection led to substantial IFNα production on Day 1. The levels of serum IFNα then declined but were still detectable at Day 14 (Fig. 1*a*). To examine biological effects of the produced IFNα, we evaluated the NK activity of mononuclear cells from the liver and spleen. pCMV-IFNa1 injection, but not

control pCMV injection, increased Yac1 lytic activity of hepatic mononuclear cells and, to a lesser extent, splenic mononuclear cells at 4 days. The levels of Yac1 lytic activity declined but were still higher at 14 days after the injection (Fig. 1*b*). We also measured IFNγ production in serum, since IFNα is known to activate IFNγ production.[23,24] pCMV-IFNa1 injection, but not pCMV injection, increased serum IFNγ at 7 days (Fig. 1*a*). Since serum IFNγ increased relatively at a later time point, it may be an indirect effect rather than a direct effect of IFNα. These data indicated that hydrodynamic injection of pCMV-IFNa1 efficiently produced biologically active IFNα for a while in mice.

To evaluate the therapeutic effects of IFNα, Balb/cA mice were intrasplenically injected with CT-26 cells. Two days later, the mice were randomized into 3 groups and intravenously injected with either pCMV-IFNa1 or pCMV or were not treated. All pCMV-injected mice or untreated mice died within 3 weeks (Fig. 2*a*). They exhibited massive liver tumor in the liver. In contrast, all mice receiving pCMV-IFNa1 survived more than 2 months. To evaluate tumor metastasis, we sacrificed another cohort of mice at 2 weeks after tumor inoculation. There were no macroscopic or microscopic liver tumors in the pCMV-IFNa1-injected mice. In contrast, livers from pCMV-injected mice had massive tumors and were significantly heavier than those from pCMV-injected mice (Fig. 2*b*).

These results clearly indicated the striking therapeutic effects of IFNα on hepatic metastasis of CT-26 cells. To examine this therapeutic effect at a site other than the liver, Balb/cA mice were subcutaneously injected on the back with CT-26 cells and hydrodynamically injected 2 days later with pCMV-IFNa1 or pCMV. No difference in tumor growth was noted between pCMV-IFNa1-injected mice and pCMV-injected mice (Fig. 2*c*).

Mice protected from hepatic metastasis by IFNα gene therapy were resistant to subcutaneous challenge of CT-26 cells and exhibited a tumor-specific T cell response

We next investigated the possibility of IFNα-mediated rejection of hepatic metastasis being followed by induction of an adaptive immune response to the original tumor. To this end, we subcutaneously injected CT-26 cells into the mice that had been protected from CT-26 hepatic metastasis by IFNα therapy. The mice were rechallenged with CT-26 cells 1 month after the initial splenic injection. The controls were naïve Balb/cA mice as well as those receiving pCMV-IFNa1 but not CT-26 splenic inoculation. The incidence of tumor formation was lower in mice that had rejected hepatic metastasis by IFNα therapy than in the control mice. Even if they developed subcutaneous tumors, tumor size was significantly smaller than in the control mice (Fig. 3*a*).

To examine the tumor-specific response, splenocytes were isolated 3 weeks after tumor inoculation and restimulated *in vitro* with CT-26 cells. Splenocytes isolated from CT-26 bearing mice treated with IFNα showed significant levels of killing ability against CT-26 cells, but not against BNL A.7 cells (Fig. 3*b*). When mice were intrasplenically injected with UV-irradiated CT-26 cells, the splenocytes did not show significant killing activity regardless of the subsequent IFNα therapy (Fig. 3*c*). Separation of effector cells into T cells and non-T cells based on CD90 expression revealed that this killing ability was mediated by T cells, but not by non-T cells (data not shown). Thus, a tumor-specific cytotoxic T cell response was established in mice that had rejected hepatic metastasis of CT-26 cells by IFNα therapy.

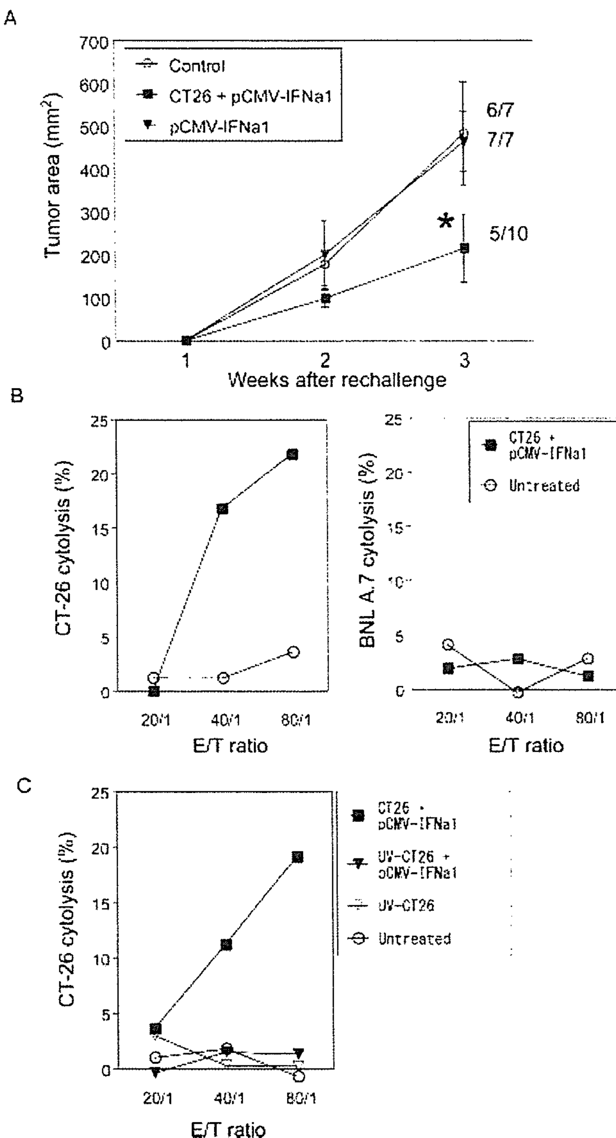

FIGURE 3 – Systemic immunity and tumor-specific T cell response. (*a*) Anti-tumor effects on rechallenged tumors. Balb/cA mice that had rejected hepatic metastasis of CT-26 cell by IFNα (closed squares), those treated with IFNα alone (closed triangles), and naïve mice (open circles) were challenged with subcutaneous injection of CT-26 cells 1 month after the previous treatment. Subcutaneous tumor growth was examined every week by measuring tumor area. Tumor size was expressed as the mean tumor size of only those mice bearing tumors. Each data point represents the mean tumor size and SD. The ratio of the number of mice bearing tumor/the number challenged for each treatment group at 3 weeks is shown in the figure. *, $p < 0.05$ *vs.* control or pCMV-IFNa1 injection only group. (*b*) *In vitro* tumor-specific killing ability. Balb/cA mice were intrasplenically injected with CT-26 cells and then treated with pCMV-IFNa1 2 days later. Splenocytes were isolated from CT-26 plus pCMV-IFNa1-injected mice at 3 weeks (closed squares) or naïve mice (open circles), restimulated with CT-26 cells for 5 days and then subjected to analysis for the lytic ability against CT-26 cells (left) or BNL A.7 cells (right). Shown are representative data for 3 independent experiments. (*c*) Requirement of CT-26 cells and IFNα on induction of tumor-specific killing ability. Balb/cA mice were intrasplenically injected with live CT-26 cells (squares) or UV-irradiated CT-26 cells (triangles) and then treated with (closed symbols) or without (open symbols) pCMV-IFNa1 2 days later. Splenocytes were isolated from mice at 3 weeks, restimulated with CT-26 cells for 5 days and then subjected to the analysis for the lytic ability against CT-26 cells. Mice injected with live CT-26 cells without following injection of pCMV-IFNa1 did not survive for 3 weeks naïve mice were included as controls (open circles). Shown are representative data for 3 independent experiments.

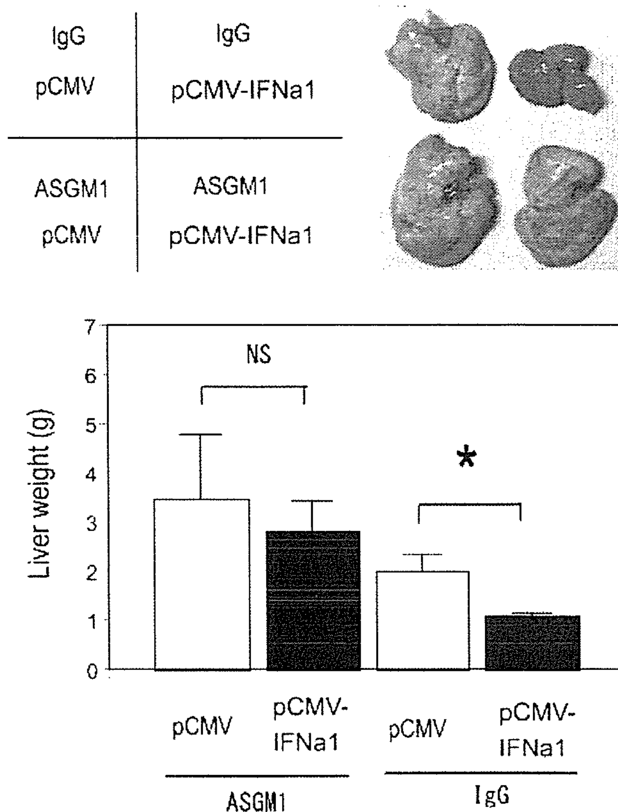

FIGURE 4 – Requirement of NK cells on IFNα-mediated anti-metastatic effects. Balb/cA mice were intrasplenically injected with CT-26 cells, intraperitoneally injected with either anti-ASGM1 or control IgG at 1 day, and hydrodynamically injected with either pCMV-IFNa1 (closed bars, n = 8/group) or pCMV (open bars, n = 7/group). Mice were sacrificed at 14 days to examine tumor growth in the liver. Top, a representative picture of the liver in each group. Bottom, comparison of liver weight among treatment groups. Experiments were performed at least 3 times and representative data are shown. *, $p < 0.05$. NS, not significant.

NK cells are required for IFNa-mediated initial rejection of hepatic metastasis

To examine whether the observed increase in NK activity of hepatic mononuclear cells is involved in the complete rejection of hepatic metastasis, we induced depletion of NK cells by injecting anti-asialo GM1 antibody. pCMV-IFNa1 injection completely abrogated hepatic tumor formation in control immunoglobulin-injected mice. In sharp contrast, pCMV-IFNa1 injection did not offer antimetastatic effects in anti-aialo GM1 antibody-injected mice, suggesting the critical contribution of NK cells to the antimetastatic effects of IFNα (Fig. 4). We examined the possibility that hepatic mononuclear cells can serve as direct effectors cells for CT-26 eradication. Although CT-26 cells were more resistant to hepatic mononuclear cells than Yac1 cells, pCMV-IFNa1 injection clearly enhanced the killing ability of hepatic mononuclear cells against CT-26 cells (data not shown). This result indicated that CT-26 is potentially susceptible to hepatic mononuclear cells upon IFNα therapy.

IFNα directly activates NK cells

IFNα is known to be able to activate a variety of immune cells. To examine whether NK cells can be directly activated by IFNα, we analyzed SCID mice that lack T cells, B cell and NKT cells due to spontaneous DNA-dependent protein kinase point mutation.[25] SCID or wild-type splenocytes were cultured with IFNα and examined for STAT1 phosphorylation, which peaked at 30 min and declined at 6 hr after IFNα stimulation in both mice (Fig. 5*a*). However, the signals of STAT1 phosphorylation were weaker in SCID splenocytes than in wild-type cells. Of interest is the finding that STAT1 expression was reduced in SCID cells compared to wild-type cells. Similar data were also obtained from experiments on Rag2 KO mice, another model of deficiency for T cells, B cells and NKT cells. To examine the reasons for SCID or Rag2 KO cells expressing low levels of STAT1, we separated wild-type splenocytes into T cells and non-T cells based on CD90 expression. The levels of STAT1 expression were weaker in non-T cells than in T cells (Fig. 5*b*). Taken together, the difference in the levels of STAT1 expression among lymphocyte subsets could explain the reduced phosphorylation signals after IFNα treatment in SCID or Rag2 KO cells.

To examine the gene profiles activated by IFNα in NK cells, we used Affymetrix DNA array analysis on SCID hepatic mononuclear cells. Six hours treatment of IFNα (1,000 U/ml) upregulated 243 of 45,101 genes in SCID cells by more than 4-fold. They included well known IFNα-regulated genes such as H2, 2′-5′ oligoadenylate synthetases, Mx1, IRF and suppressor of cytokine signaling (SOCS). Among the effector molecules for cytotoxicity, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and granzyme B were activated. Various cytokines such as IL-15 and IFNγ were also upregulated. These data revealed that NK cells upon IFNα stimulation produced well-characterized IFN-inducible genes and others that are relatively specific to killer cells or immune cells.

pCMV-IFNa1 injection completely suppressed tumor formation in the liver in SCID mice

We examined the *in vivo* effects of IFNα in SCID mice. In agreement with SCID cell activation *in vitro*, pCMV-IFNa1 injection enhanced the Yac1 lytic ability of hepatic mononuclear cells in SCID mice (Fig. 5*c*). To examine whether NK cells are sufficient for IFNα-mediated rejection of hepatic metastasis, we injected pCMV-IFNa1 or pCMV into mice that had been intrasplenically injected with CT-26 cells 2 days earlier. pCMV-IFNa1 completely suppressed tumor formation in the liver (Fig. 5*d*). As described in the *Material and methods* section, anti-asialo GM1 injection reduces the number of NKT cells. However, this SCID experiment clearly showed that NKT cells are not required for NK cell activation by IFNα and its antimetastatic effects.

pCMV-IFNa1 injection completely suppressed tumor formation in the liver in GKO mice

IFNγ has been established as an endogenous inhibitor of tumor development and progression.[26] Exogenous administration of IFNγ suppresses tumor formation in a variety of models.[15,27] To examine the possibility of IFNγ being involved in antimetastatic effects on IFNα, we injected pCMV-IFNa1 or pCMV plasmid into GKO mice exposed to 2 days of metastasis of CT-26 cells. IFNα treatment led to complete rejection of CT-26 cells in GKO mice (Fig. 6*a*). pCMV-IFNa1 injection, but not pCMV injection, augmented the Yac1 lytic ability of mononuclear cells (Fig. 6*b*).

Discussion

Here we report that a single injection of pCMV-IFNa1 could lead to complete rejection of preexisting hepatic metastasis of colon cancer cells. This partly agrees with a previous report by Kobayashi et al.,[15] who hydrodynamically injected IFNβ- or IFNγ-expressing plasmid into CT-26 bearing mice and reported the antimetastatic effects of IFNβ or IFNγ. In contrast to our study, all mice died within 45 days due to metastasized tumor growth even if plasmid injection was began one day after tumor inoculation and repeated every other day. The complete protection against hepatic metastasis observed in the present study allowed

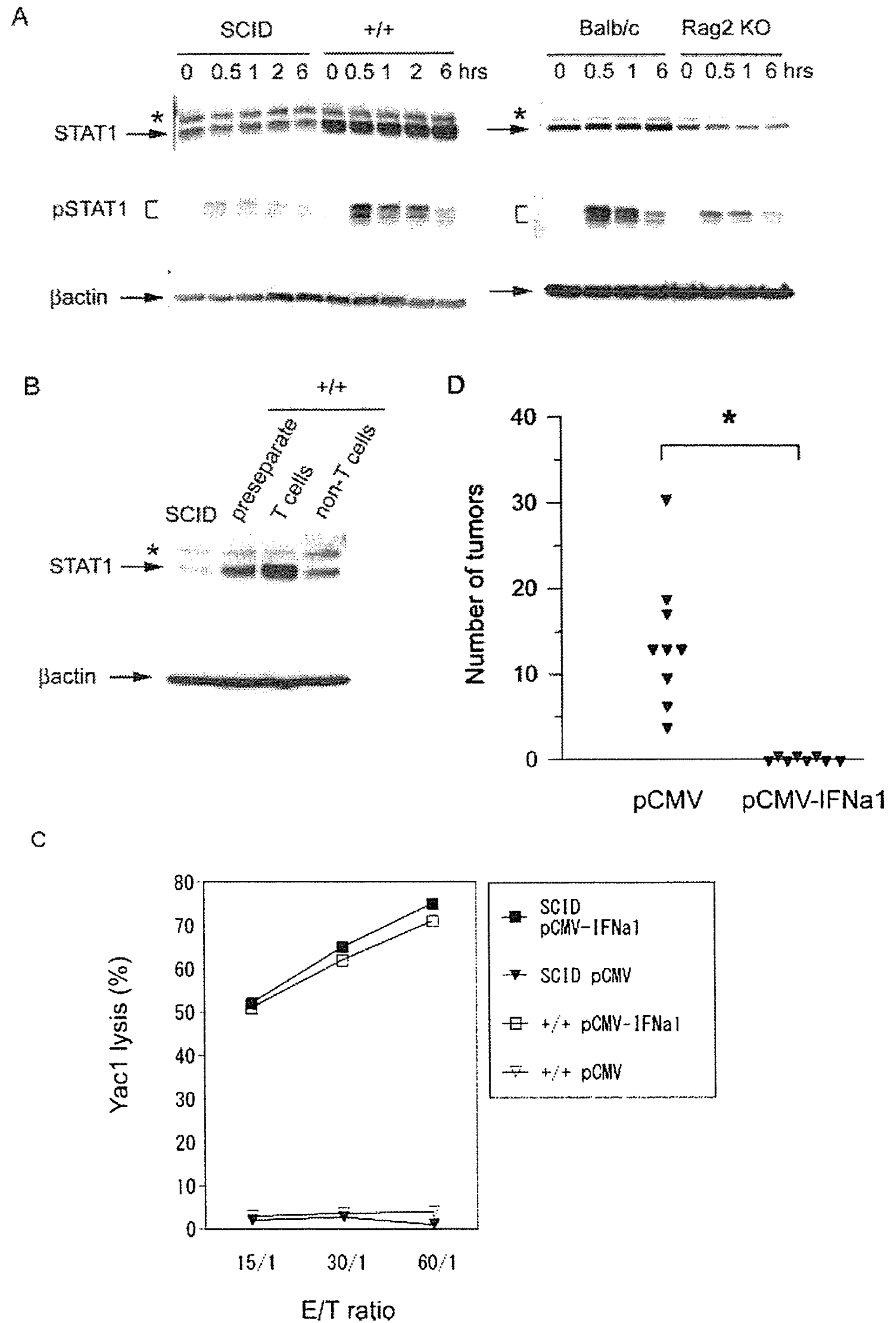

FIGURE 5 – IFNα-mediated NK cell activation and anti-metastatic effects in SCID mice. (*a*) STAT1 and phospho-STAT1 expression of splenocytes from SCID or Rag2 KO mice. Splenocytes were treated with 1,000 U/ml of IFNα and lysed at indicated time points (0 to 6 hr). Expression of STAT1 and phospho-STAT1 (pSTAT1) was analyzed by Western blot. +/+ and Balb/c indicate wild-type controls for SCID and Rag2 KO mice, respectively. *, non-specific band (see the *Material and methods* section). (*b*) STAT1 expression in T cells and non-T cells. Splenocytes from wild-type mice (+/+) were separated into T cells and non-T cells based on expression of CD90. Expression of STAT1 was analyzed by Western blot. SCID and preseparated wild-type splenocytes were included as controls. *, non-specific band (see the Material and methods section). (*c*) Yac1 lytic ability. SCID mice (closed symbols) or wild-type mice (open symbols) were hydrodynamically injected with either pCMV-IFNa1 (squares) or pCMV (triangles). Four days later, splenocytes isolated form the mice were examined for the lytic ability for Yac1 cells. Experiments were done at least 3 times and representative data are shown. (*d*) Anti-metastaic effects. SCID mice or wild-type mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 or pCMV 2 days later. After 14 days, mice were sacrificed to examine tumor development in the liver. The numbers of hepatic tumors were compared among the groups. Experiments were performed 3 times and representative data are shown. *, $p < 0.05$.

us to investigate the adaptive response after antimetastatic effects. Mice that had rejected CT-26 cells by IFNα showed a tumor-specific T cell response and suppressed tumor growth of rechallenged skin tumor. Therefore, pCMV-IFNa1 injection not only caused initial rejection of metastasized tumors but also induced durable and systemic adaptive immunity. Interestingly, splenic injection of UV-irradiated CT-26 cells, even if followed by pCMV-IFNa1 injection, did not elicit significant tumor-specific T cell responses. Therefore, the efficient induction of adaptive T cell responses requires IFNα-mediated rejection of live tumor cells and cannot be recapitulated by simple injection of dead tumor cells and IFNα.

NK cells are present in a high percentage in the liver.[28] In the present study, we focused on NK cells which were rapidly activated by IFNα to examine the cellular mechanisms of protection against hepatic metastasis. Critical requirement of NK cells was demonstrated by anti-asialo GM1 antibody-injected mice which did not show protection against CT-26 metastasis. In contrast, T cells, B cells or NKT cells were dispensable for IFNα-mediated antimetastatic effects since IFNα therapy did show antimetastatic

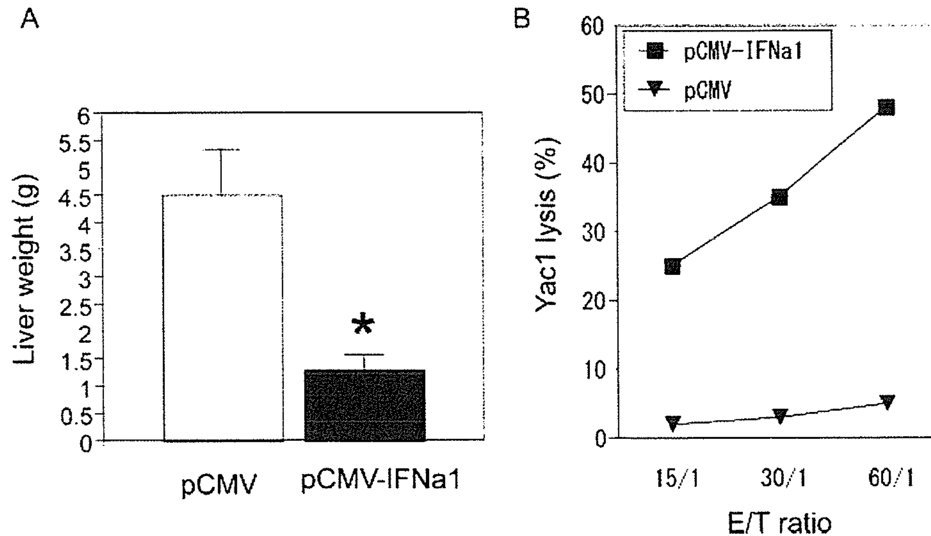

FIGURE 6 – NK cell activation and anti-metastatic effects in GKO mice. (*a*) Anti-metastatic effects. GKO mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) 2 days later. After 14 days, the mice were sacrificed to 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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References

1. Sasson AR, Sigurdson ER. Surgical treatment of liver metastases. Semin Oncol 2002;29:107–18.
2. Ruan DT, Warren RS. Liver-directed therapies in colorectal cancer. Semin Oncol 2005;32:85–94.
3. Seki S, Habu Y, Kawamura T, Takeda K, Dobashi H, Ohkawa T, Hiraide H. The liver as a crucial organ in the first line of defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag T+ cells in T helper 1 immune responses. Immun Rev 2000;174:35–46.
4. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer 2004;4:11–14.
5. Taniguchi T, Takaoka A. The interferon-α/β system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. Curr Opin Immunol 2002:14:111–6.
6. Marschall Z, Scholz A, Cramer T, Schafer G, Schirner M. Effects of interferon α on vascular endothelial growth factor gene transcription and tumor angiogenesis. J Natl Cancer Inst 2003;95:437–48.
7. Biron CA. Interferon α and β as immune regulators: a new look. Immunity 2001;14:661–4.
8. Durbin JE, Hackenmiller R, Simon MC, Levy DE. Targeted disruption of the mouse *Stat1* gene results in compromised innate immunity to viral disease. Cell 1996:84:443–50.
9. Meraz MA, White JM, Sheehan KCF, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, Carver-Moore K, DuBois RN et al. Targeted disruption of the *Stat1* gene in mice reveals unexpected physiologic specificity in the JAK-STA signaling pathway. Cell 1996;84:431–42.
10. Gil MP, Bohn E, O'Guin AK, Ramana CV, Levine B, Stark GR, Virgin HW, Schreiber RD. Biological consequences of STAT1-independent IFN signaling. Proc Natl Acad Sci USA 2001:98:6680–5.
11. Qin XQ, Tao N, Dergay A, Moy P, Fawell S, Davis A, Wilson JM, Barsoum J. Interferon-β gene therapy inhibits tumor formation and causes regression of established tumors in immune-deficient mice. Proc Natl Acad Sci USA 1998;95:14411–6.
12. Coleman M, Muller S, Quezada A, Mendiratta SK, Wang J, Thull NM, Bishop J, Matar M, Mester J, Pericle F. Nonviral interferon α gene therapy inhibits growth of established tumors by eliciting a systemic immune response. Hum Gene Ther 1998:9:2223–30.
13. Horton HM, Anderson D, Hernandez P, Barnhart KM, Norman JA, Parker SE. A gene therapy for cancer using intramuscular injection of plasmid DNA encoding interferon α. Proc Natl Acad Sci USA 1999; 96:1553–8.
14. Li S, Zhang X, Xia X, Zhou L, Breau R, Suen J, Hanna E. Intramuscular electroporation delivery of IFN-α gene therapy for inhibition of tumor growth located at a distant site. Gene Ther 2001;8:400–7.
15. Kobayashi N, Kuramoto T, Chen S, Watanabe Y, Takakura Y. Therapeutic effect of intravenous interferon gene delivery with naked plasmid DNA in murine metastasis models. Mol Ther 2002;6:737–44.
16. Yoshimoto T, Okamura H, Tagawa Y, Iwakura Y, Nakanishi K. Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-γ production from activated B cells. Proc Natl Acad Sci USA 1997;94:3948–53.
17. Miyagi T, Takehara T, Tatsumi T, Kanto T, Suzuki T, Jinushi M, Sugimoto Y, Sasaki Y, Hori M, Hayashi N. CD1d-mediated stimulation of natural killer T cells selectively activates hepatic natural killer cells to eliminate experimentally disseminated hepatoma cells in murine liver. Int J Cancer 2003;106:81–9.
18. Harle P, Noisakran S, Carr DJ. The application of a plasmid DNA encoding IFN-α 1 postinfection enhances cumulative survival of herpes simplex virus type 2 vaginally infected mice. J Immunol 2001;166: 1803–12.
19. Takehara T, Suzuki T, Ohkawa K, Housui A, Jinushi M, Miyagi T, Tatsumi T, Kanazawa Y, Hayashi N. Viral covalently closed circular DNA in a non-transgenic mouse model for chronic hepatitis B virus replication. J Hepatol 2006;44:267–74.
20. Miyagi T, Takehara T, Tatsumi T, Suzuki T, Jinushi M, Kanazawa Y, Hiramatsu N, Kanto T, Tsuji S, Hori M, Hayashi N. Concanavarin A injection activates intrahepatic innate immune cells to provoke an anti-tumor effect in murine liver. Hepatology 2004;40:1190–6.
21. Liu F, Song YK, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther 1999;6: 1258–66.
22. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injection of naked plasmid DNA. Hum Gene Ther 1999;10:1735–7.
23. Sareneva T, Matikainen S, Kurimoto M, Julkunen I. Influenza A virus-induced IFN-α/β and IL-18 synergistically enhance *IFN-γ* gene expression in human T cells. J Immunol 1998;160: 6032–8.
24. Cousens LP, Peterson R, Hsu S, Dorner A, Altman JD, Ahmed R, Biron CA. Two roads diverged: interferon α/β- and interleukin 12-mediated pathways in promoting T cell interferon γ responses during viral infection. J Exp Med 1999;189:1315–28.
25. Danska JS, Holland DP, Mariathasan S, Williams KM, Guidos CJ. Biochemical and genetic defects in the DNA-dependent protein kinase in murine acid lymphocytes. Mol Cell Biol 1996;16:5507–17.
26. Ikeda H, Old LJ, Schreiber RD. The roles of IFNγ in protection against tumor development and cancer immunoediting. Cytokine Growth Factors Rev 2002:13:95–109.
27. Sun WH, Burkholder JK, Sun J, Culp J, Turner J, Lu XG, Pugh TD, Ershler WB, Yang NS. In vivo cytokine gene transfer by gene gun reduces tumor growth in mice. Proc Natl Acad Sci USA 1995;92: 2889–93.
28. Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in the human liver. Immunol Rev 2000;174:5–20.
29. Lesinski GB, Anghelina M, Zimmerer J, Bakalakos T, Badgwell B, Parihar R, Hu Y, Becknell B, Abood G, Chaudhury AR, Magro C, Durbin J et al. The anti-tumor effects of IFN-α are abrogated in a STAT1-deficient mouse. J Clin Invest 2003;112:170–80.
30. Vermijlen D, Luo D, Froelich CJ, Medema JP, Kummer JA, Willems E, Braet F, Wisse E. Hepatic natural killer cells exclusively kill splenic/ blood natural killer-resistant tumor cells by the perforin/granzyme pathway. J Leukoc Biol 2002;72:668–76.
31. Lee C-K, Rao DT, Gertner R, Gimeno R, Frey AB, Levy DE. Distinct requirements for IFNs and STAT1 in NK cell function. J Immunol 2000;165:3571–4.
32. Nguyen KB, Watford WT, Salomon R, Hofmann SR, Pien GC, Morinobu A, Gadina M, O'Shea JJ, Biron CA. Critical role for STAT4 activation by type I interferons in the interferon-γ response to viral infection. Science 2002;297:2063–6.
33. Der SD, Zhou A, Williams BRG, Silverman RH. Identification of genes differentially regulated by interferon α, β, or γ using oligonucleotide arrays. Proc Natl Acad Sci USA 1998;95:15623–8.

34. Belardelli F, Ferrantini M, Proietti E, Kirkwood JM. Interferon-α in tumor immunity and immunotherapy. Cytokine Growth Factor Rev 2002;13:119–34.
35. Kirkwood J. Cancer immunotherapy: the interferon-α experience. Semin Oncol 2002;29:18–26.
36. Grem JL, van Groeningen CJ, Ismail AA, Johnston PG, Alexander HR, Allegra CJ. The role of interferon-α as a modulator of fluorouracil and leucovorin. Eur J Cancer 1995;31A:1316–20.
37. Wadler S, Lembersky B, Atkins M, Kirkwood J, Petrelli N. Phase II trial of fluorouracil and recombinant interferon α-2a in patients with advanced colorectal carcinoma: an Eastern Cooperative Oncology Group study. J Clin Oncol 1991;9:1806–10.
38. Dufour P, Husseini F, Dreyfus B, Cure H, Martin C, Prevost G, Olivier JP, Dumas F, Duclos B, Olivares R, Leszler A, Bergerat JP et al. 5-fluorouracil versus 5-fluorouracil plus α-interferon as treatment of metastatic colorectal carcinoma. a randomized study. Ann Oncol 1996;7:575–9.
39. Link KH, Kornmann M, Staib L, Redenbacher M, Kron M, Beger HG. Increase of survival benefit in advanced resectable colon cancer by extent of adjuvant treatment: results of a randomized trial comparing modulation of 5-FU + levamisole with folinic acid or with interferon-α. Ann Surg 2005;242:178–87.

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

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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.

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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

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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

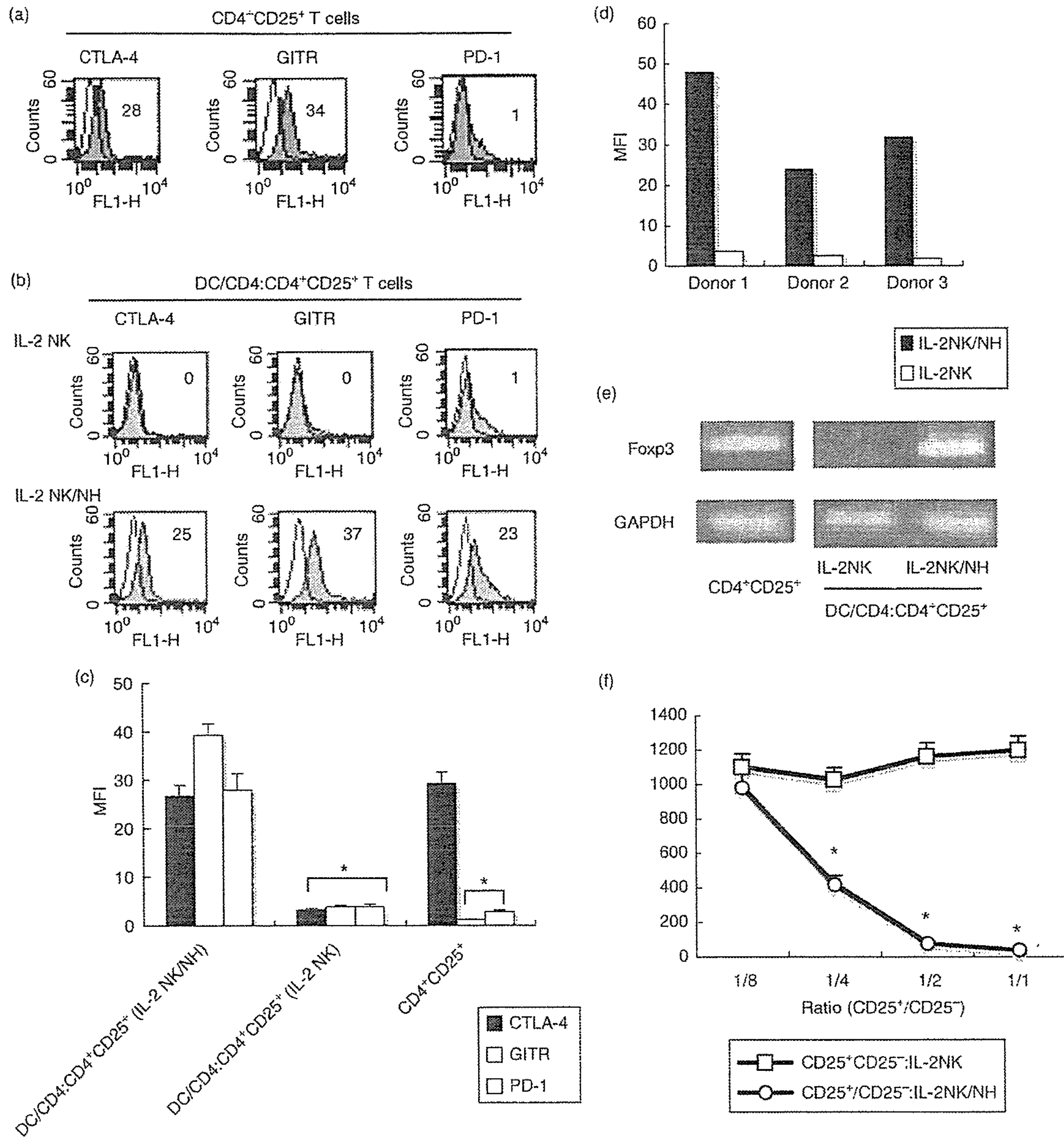

Figure 1. Human non-transformed hepatocyte (NH) modulation of activated natural killer (NK) cells endows dendritic cells (DCs) with the ability to induce $CD4^+$ $CD25^+$ regulatory T cells. (a) Freshly isolated $CD4^+$ $CD25^+$ T cells were cultured in the presence of plate-bound anti-CD3 antibody (Ab) 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. $^*P < 0{\cdot}05$.

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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.

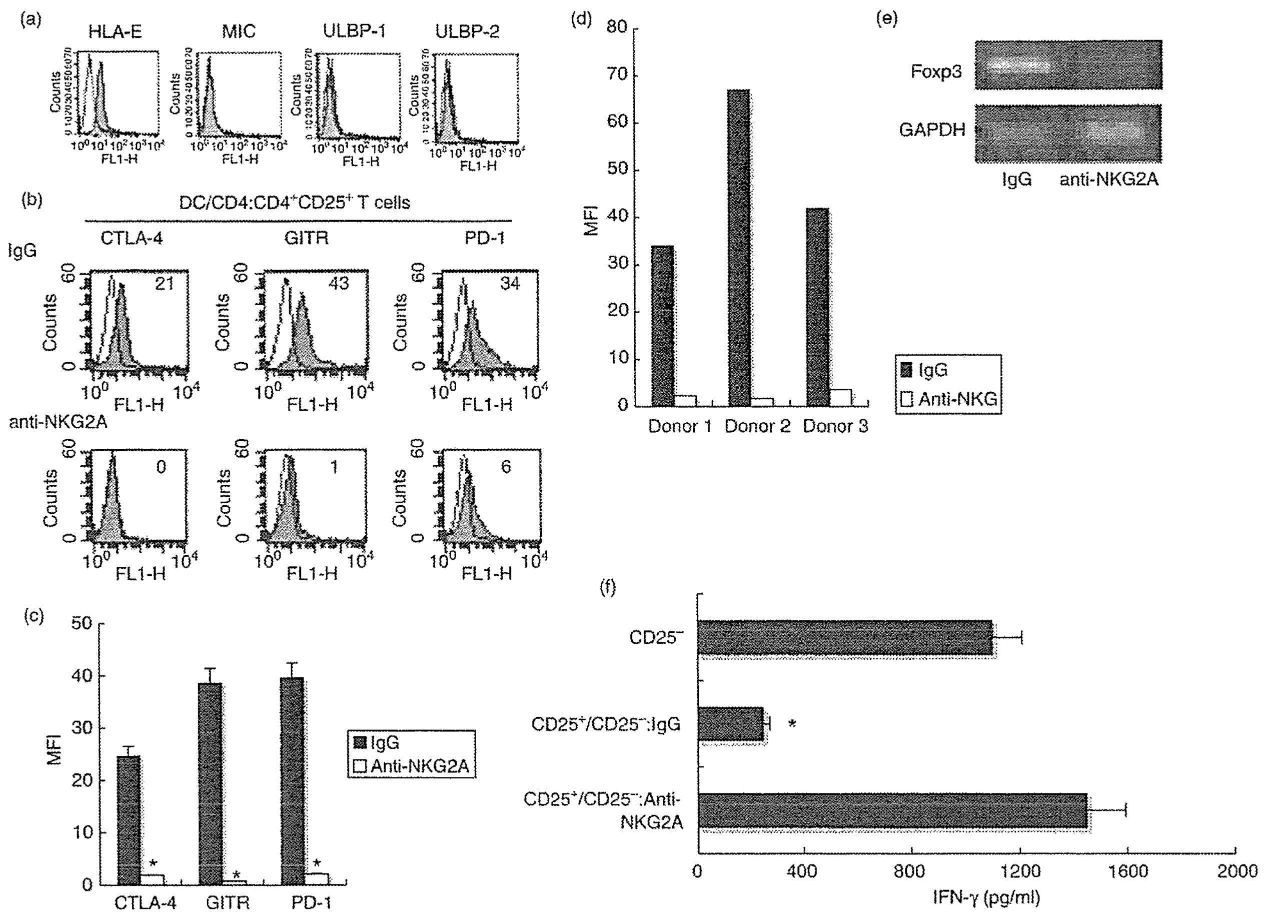

Figure 2. NKG2A signals of natural killer (NK) cells are required for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ T cells with the regulatory phenotype. (a) Surface expression of the ligands of NKG2A (HLA-E) as well as NKG2D (MIC, ULBP1 and ULBP2) in human non-transformed hepatocytes (NHs) 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. $*P < 0{\cdot}05$ 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). $*P < 0{\cdot}05$. 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

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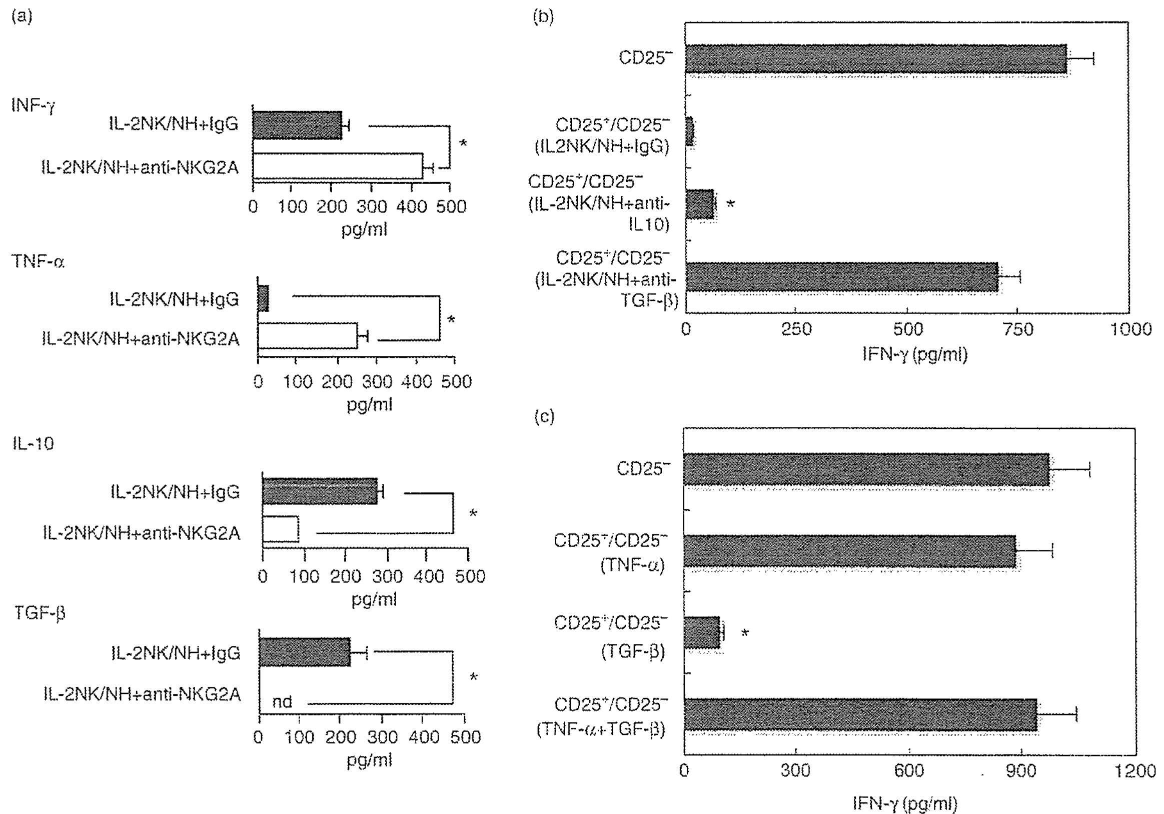

Figure 3. Change of cytokine production pattern of natural killer (NK) cells through NKG2A signals is responsible for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ Treg cells. (a) NK cells prestimulated with interleukin (IL)-2 were cultured with human non-transformed hepatocytes (NHs) in the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

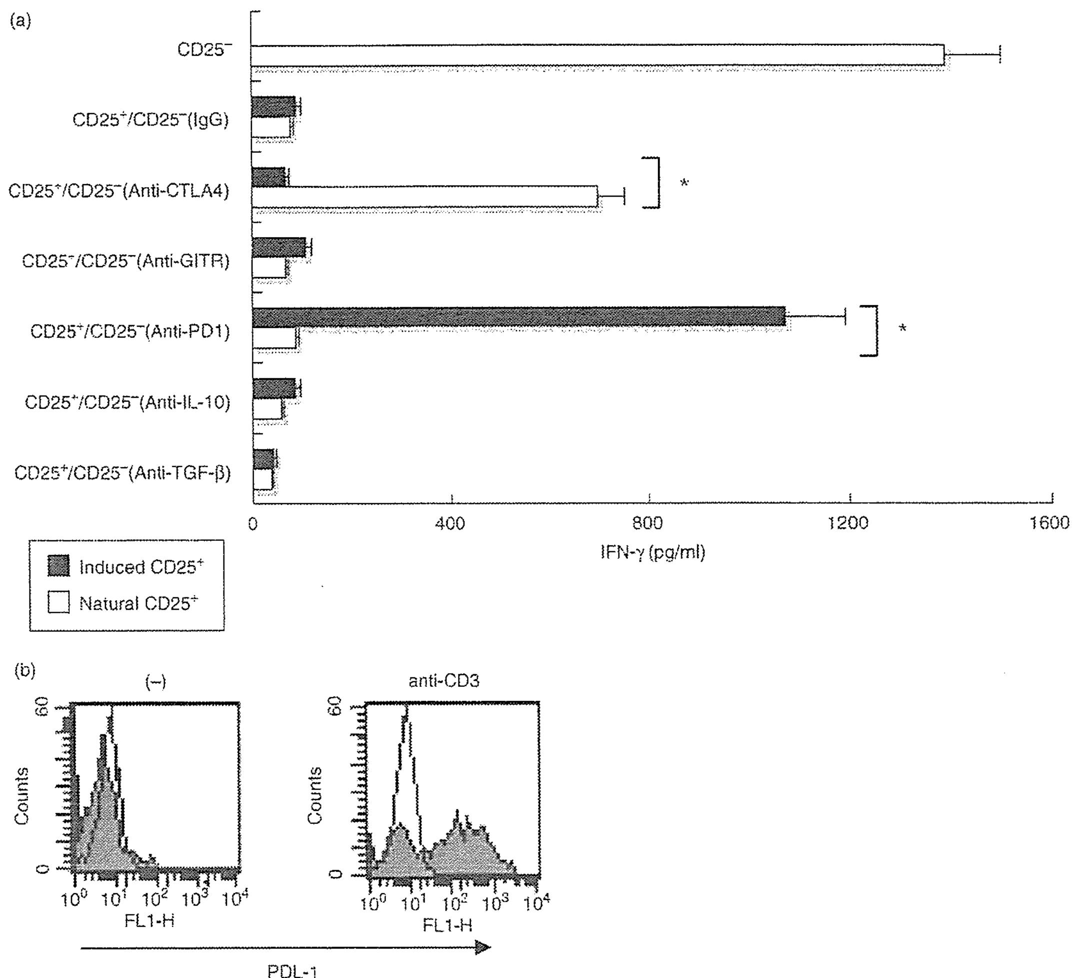

Figure 4. CD4$^+$ CD25$^+$ Treg cells induced by interleukin (IL)-2 natural killer (NK)/human non-transformed hepatocytes (NH)-treated dendritic cell (DC) suppressed T cell activation through programmed death-1 (PD-1)/programmed death ligand-1 (PDL-1) interactions. (a) DCs (1×10^5) were stimulated with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

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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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References

1 Shevach EM. $CD4^+$ $CD25^+$ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2002; 2:389–400.

2 Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification of functional characterization of human $CD4^+$ $CD25^+$ T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; **193**:1285–94.

3 Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. *Ex vivo* isolation and characterization of $CD4^+$ $CD25^+$ T cells with regulatory properties from human blood. *J Exp Med* 2001; **193**:1303–10.

4 Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. $CD4^+$ $CD25^+$ regulatory T cells control Leishmania major persistence and immunity. *Nature* 2002; **420**:502–7.

5 Wang HY, Lee DA, Peng G, Guo Z, Li Y, Kiniwa Y, Shevach EM, Wang RF. Tumour-specific human $CD4^+$ regulatory T cells

and their ligands: implication for immunotherapy. *Immunity* 2004; **20**:107–18.

6 Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD4$^+$ CD25$^+$ regulatory cells that control intestinal inflammation. *J Exp Med* 2002; **192**:295–302.

7 Herman AE, Freeman GJ, Mathis D, Benoist C. CD4$^+$ CD25$^+$ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *J Exp Med* 2004; **199**:1479–89.

8 Khoury SJ, Sayegh MH. The roles of the new negative T cell costimulatory pathways in regulating autoimmunity. *Immunity* 2004; **20**:529–38.

9 Gavin MA, Clarke SR, Negrou E, Gallegos A, Rudensky A. Homeostasis and anergy of CD4 ($^+$) CD25 ($^+$) suppressor T cells *in vivo*. *Nat Immunol* 2004; **3**:33–41.

10 Steinman RM, Hawiger D, Nussenzweig D. Tolerogenic dendritic cells. *Annu Rev Immunol* 2003; **21**:685–711.

11 Mahnke K, Quan Y, Knop J, Enk AH. Induction of CD4$^+$ CD25$^+$ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood* 2003; **101**:4862–9.

12 Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, Bandeira A. CD4$^+$ CD25$^+$ T cells regulate the expansion of peripheral CD4$^+$ T cells through the production of IL-10. *J Immunol* 2001; **166**:3008–18.

13 Yamagiwa S, Gray JD, Hashimoto H, Horwitz DA. A role of TGF-β in the generation and expansion of CD4$^+$ CD25$^+$ regulatory T cells from human peripheral blood. *J Immunol* 2001; **166**:7282–9.

14 Peng Y, Laouar Y, Li MO, Green EA, Flavell RA. TGF-β regulates *in vivo* expansion of Foxp3-expressing CD4$^+$ CD25$^+$ regulatory T cells responsible for protection against diabetes. *Proc Natl Acad Sci USA* 2004; **101**:4572–7.

15 Moretta A. The dialogue between human natural killer cells and dendritic cells. *Curr Opin Immunol* 2005; **17**:306–11.

16 Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural killer cells and dendritic cells: 'L'union fait la force'. *Blood* 2005; **106**:2252–8.

17 Mocikat R, Braumuller H, Gumy A *et al.* Natural killer cells activated by MHC^{LOW} targets prime dendritic cells to induce protective CD8 T cell responses. *Immunity* 2003; **19**:561–9.

18 Van den Broeke LT, Daschbach E, Thomas EK, Andringa G, Berzofsky JA. Dendritic cell-induced activation of adaptive and innate antitumour immunity. *J Immunol* 2003; **171**:5842–52.

19 Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. *Nat Rev Immunol* 2001; **1**:41–9.

20 Jinushi M, Takehara T, Tatsumi T *et al.* Negative regulation of NK cell activities by inhibitory receptor CD94/NKG2A leads to the altered NK cell-induced modulation of dendritic cell functions in chronic hepatitis C virus infection. *J Immunol* 2004; **173**:6072–81.

21 Lee N, Goodlett DR, Ishitani A, Marquardt H, Geraghty DE. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J Immunol* 1998; **160**:4951–60.

22 Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 1999; **285**:727–9.

23 Pende D, Sivori S, Accame L *et al.* HLA-G recognition by human natural killer cells. Involvement of CD94 both as inhibitory and as activating receptor complex. *Eur J Immunol* 1997; **27**:1875–80.

24 Valerie V, Vosters O, Beuneu C, Nicaise C, Stordeur P, Goldman M. Induction of FOXP3-expressing regulatory CD4$^+$ T cells by human mature autologous dendritic cells. *Eur J Immunol* 2003; **34**:762–72.

25 Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD4$^+$ CD25$^+$ regulatory T cells through GITR break immunological self-tolerance. *Nat Immunol* 2002; **3**:135–42.

26 Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**:1057–61.

27 Piccioli D, Sbrana S, Melandri E, Valiante NM. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J Exp Med* 2002; **195**:335–41.

28 Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, Mauri C. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNF-α therapy. *J Exp Med* 2004; **200**:277–85.

29 Wu AJ, Hua H, Munson SH, McDevitt HO. Tumour necrosis factor-α regulation of CD4$^+$ CD25$^+$ T cell levels in NOD mice. *Proc Natl Acad Sci USA* 2002; **99**:12287–92.

30 Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 2005; **6**:1219–27.

31 Bluestone JA, Abbas AK. Natural and adaptive regulatory T cells. *Nat Rev Immunol* 2003; **3**:253–7.

32 Paust S, Lu L, McCarty N, Cantor H. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc Natl Acad Sci USA* 2004; **101**:10398–403.

33 Ahmad A, Alvarez F. Role of NK and NKT cells in the immunopathogenesis of HCV-induced hepatitis. *J Leukoc Biol* 2004; **76**:743–59.

34 Golden-Mason L, Rosen HR. Natural killer cells: primary target for hepatitis C virus immune evasion strategies. *Liver Transplant* 2006; **12**:363–72.

35 Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* 2005; **5**:215–29.

36 Lauer GM, Barnes E, Lucas M *et al.* High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. *Gastroenterology* 2004; **127**:924–36.

37 Cox AL, Mosbruger T, Lauer GM, Pardoll D, Thomas DL, Ray S. Comprehensive analysis of CD8$^+$ T cell responses during longitudinal study of acute human hepatitis C. *Hepatology* 2005; **42**:104–12.

38 Cabrera R, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C, Nelson DR. An immunomodulatory role for CD4$^+$ CD25$^+$ regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 2004; **40**:1062–71.

39 Rushbrook SM, Ward SM, Unitt E, Vowler M, Lucas M, Klenerman P, Alexander GJ. Regulatory T cells suppress *in vitro* proliferation of virus-specific CD8$^+$ T cells during persistent hepatitis C virus infection. *J Virol* 2005; **79**:7852–9.

40 Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; **439**:682–7.

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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 $p53_{232\text{-}240}$ 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 $p53_{232\text{-}240}$ 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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