Introduction

Dendritic cell (DC)-based vaccines are attractive cancer modalities since DCs are competent to coordinately induce both tumor antigen-specific cytotoxic T lymphocytes (CTLs) and helper T cells [1–3]. In this regard, DCs pulsed with TAA derived peptides have proven clinically effective in eliciting protective and therapeutic anti-tumor immunity in the setting of a broad range of cancer types [3]. Recent studies have also suggested that DCs may effectively activate elements of innate immunity (NK cells [4–9] and NKT cells [10–12]) via IL-12 secretion and direct cellular interaction. The liver is an enriched source of innate immune cells such as NK cells and NKT cells compared with other organs, supporting the specialized role of this organ in the immune system [13–15]. Indeed, it has been shown that liver-associated innate immune cells play a critical role in the first-line defense against metastatic liver tumors [16, 17]. However, despite numerous reports supporting the efficacy of DC-based vaccines in murine s.c. tumor models [18–20], the efficacy of this approach in liver tumor models remains under developed. Given the possibility that DC-based vaccines may efficiently activate NK cells, NKT cells and specific T cells in the liver, they could offer a preferred immunotherapy for liver cancer.

The liver is the most common site of distal metastasis for tumors developing in distal organs, and physiologic status of this organ correlates with survival in patients with advanced disease, even if primary tumor site are resected curatively [21, 22]. Recently, adjuvant chemotherapies have been reported to yield significant improvement in disease (including liver metastasis) free interval and overall patient survival, however, dose-limiting toxicities were often observed and liver metastasis could not be completely prevented [23–25]. In contrast, several peptide-pulsed DC vaccines have been shown to be clinically capable of stimulating tumor-specific T cells in patients with tumor liver metastasis [26–28], suggesting that such treatments may represent a new strategy option in the setting of metastatic liver cancer. In this context, several tumor-associated antigen (TAA) peptides have been identified in various types of cancer that often metastasize to liver [29–31], however, this approach remains encumbered by the necessity to restrict patient accrual to those individuals harboring specific HLA types.

We previously demonstrated that the recently defined TAA EphA2 derived peptide-pulsed DC (Eph-DC) vaccine prevented the subcutaneous tumor growth in mice, but unpulsed DC vaccine did not [32]. In the present study, we examined the anti-tumor protection of Eph-DC vaccines in the liver tumor model, which is under the unique immunological environment. Unexpectedly, we observed that preventive vaccination with not only Eph-DCs but also unpulsed DCs provide anti-tumor protection as a result of the activation of both innate immune cells and specific T cells. This suggests that cultured autologous DC (alone or pulsed with TAA peptides) may represent an effective modality for patients with tumors localized to their livers.

Materials and methods

Mice

Female C57BL/6 mice and severe combined immuno-deficiency (SCID) mice were purchased from Clea Japan, Inc. (Tokyo, Japan) and were used at 6–8 weeks of age. 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 our study protocol complied with the institution's guideline.

Cell lines and culture

MC38, a mouse colon carcinoma cell derived from C57BL6/J mice, was generously provided by Dr. Kazumasa Hiroishi (Showa University School of Medicine, Tokyo, Japan). BL6, a melanoma cell line, and YAC-1, a sensitive cell line to NK cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). These cell lines were maintained in Complete Medium [RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin] at 37°C in 5% CO_2.

Generation of dendritic cells in vitro from bone marrow (BM) and DC-based peptide vaccines

The procedure used in this study was described previously [32]. Briefly, BM-DCs were separated by magnetic cell sorting using CD11c Micro Beads (Miltenyi Biotec) and typically represented >90% of the harvested population of the cells based on morphology and expression of the CD40, CD80, CD86 and MHC class II (data not shown). The H-2 K^b-binding $mEphA2_{682-689}$ epitope (VVSKYKPM) was kindly provided by Dr. Walter Storkus (University of Pittsburgh Cancer Institute). BM-DCs were incubated with the $mEph_{682-689}$ peptide at a concentration of 10 μg/ml/10^6 DC/ml CM for 2 h at 37°C. The cells were harvested and washed three times with phosphate-buffered saline (PBS) before use [20].

Animal experiments

C57BL/6 mice or SCID mice were immunized s.c. in the flank with 1×10^6 Eph-DCs or unpulsed DCs in a total

volume of 100 µl of PBS twice a week. On day 0, at the time of the second injection with Eph-DCs or unpulsed DCs, 2×10^6 MC38 cells (EphA2-positive) or 5×10^5 BL6 (EphA2-negative) tumor cells were inoculated intrahepatically. Mice were sacrificed 14 days after tumor inoculation and liver weight was measured. Data are reported as the average liver weight ± SD.

Cytolytic assays

Liver mononuclear cells (LMNCs) were isolated from the liver 1 day after tumor inoculation, and subjected to 4-h ^{51}Cr release assays against NK-sensitive YAC-1 target cells. In some experiments, whole splenocytes were harvested 14 days after tumor inoculation, with T cells stimulated in vitro using MC38 cells pre-treated with Mitomycin C (Kyowa Hakko, Tokyo, Japan) in the presence of 30 IU/ml murine IL-2 (Strathmann Biotech, Hannover, Germany) for 5 days. Lymphocytes (bulk, CD4-depleted, or CD8-depleted) were then harvested and analyzed for their ability to kill MC38 tumor cells in 4-h ^{51}Cr-release assays.

Flow cytometric analysis

Liver mononuclear cells were isolated from the liver prior to the first vaccination and on days 1, 3, 7 after tumor inoculation as previously described [16]. The phenotype of LMNCs from mice treated with Eph-DCs, unpulsed DCs or PBS was examined by flow cytometric analysis. NK and NKT cells in the liver were identified separately by using PE anti-mouse NK1.1 monoclonal antibody and FITC anti-mouse TCR (BD Pharmingen, San Diego, CA, USA). Furthermore, NK activation was examined by using FITC anti-mouse CD69, the early activation marker (BD Pharmingen). Analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA) with the resulting data analyzed using the CELLQuest program (Becton Dickinson). NK cells were identified as NK1.1+/TCR-lymphocytes and NKT cells as NK1.1+/TCR+ lymphocytes. Activated NK1.1+ cells were identified as NK1.1+/CD69+ lymphocytes.

In vivo depletion experiments

The procedure used in this study was described previously [32]. The efficiency of specific subset depletions (CD4+, CD8+ T cell or NK cell) was comfirmed by flow cytometric analysis. In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

Tumor rechallenge

C57BL/6 mice were immunized s.c. with 1×10^6 Eph-DCs or unpulsed DCs twice a week and then challenged intrahepatically with 2×10^6 MC38 cells, at the time of the second Eph-DC or unpulsed DC immunization. On day 14 after tumor inoculation, 2×10^5 MC38 or 5×10^4 BL6 cells were injected s.c. in the flank. As a control, 2×10^5 MC38 or 5×10^4 BL6 cells were injected s.c. into naïve C57BL/6 mice. Tumor size was assessed on a weekly basis and recorded in mm^2 by determining the product of the largest perpendicular diameters measured by vernier calipers. Data are reported as the average tumor area ± SD.

Statistical analyses. Statistical differences between the groups was determined by applying a Student's t test with Welch correction or one-way ANOVA after each group had been tested with equal variance and Fisher's exact probability test. Statistical significance was defined as $P < 0.05$.

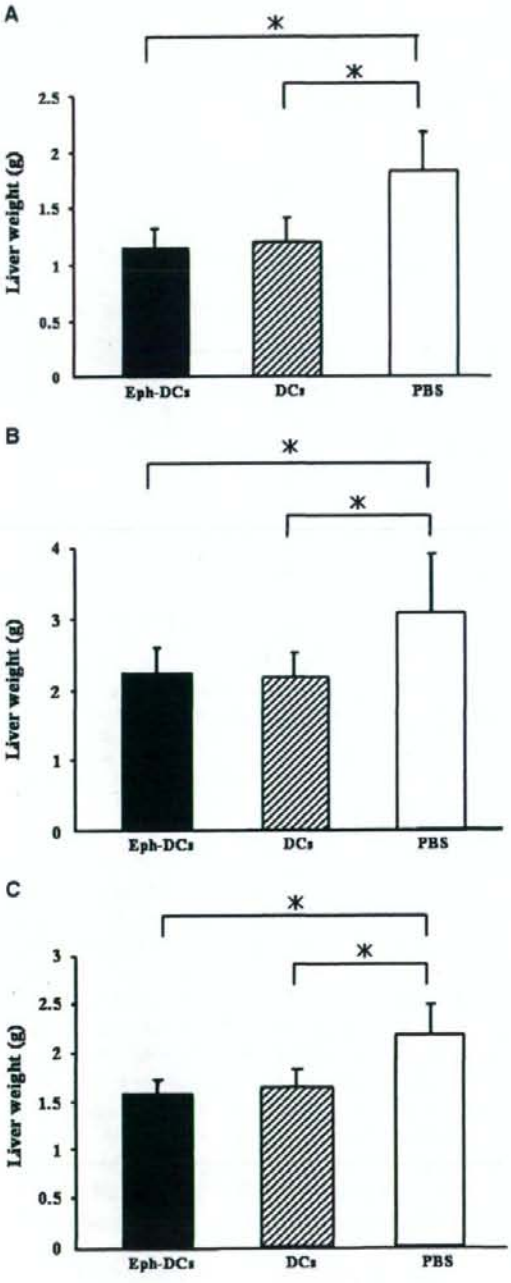
Results

Immunization with Eph-DCs or unpulsed DCs prevents progression of liver tumors in vivo

We examined whether immunization with Eph-DCs or unpulsed DCs would promote protective anti-tumor effects against the EphA2-positive MC38 or EphA2-negative BL6 liver tumors. MC38 liver tumor growth in mice immunized with either Eph-DCs or unpulsed DCs was significantly inhibited when compared to mice treated with PBS. Immunization with unpulsed DCs provided an equitable degree of anti-MC38 protection to that observed for immunization using Eph-DCs (Fig. 1a). BL6 tumor growth was also significantly inhibited by Eph-DCs or unpulsed DCs, to a comparable degree (Fig. 1b). These results suggest that immunization with DCs (whether pulsed with peptide or not) successfully inhibits the growth of two distinct $H\text{-}2^b$ tumors established in the liver. Moreover, MC38 liver tumor growth in SCID mice (T cell, B cell and NKT cell deficient mice) immunized with DCs (either Eph-DCs or unpulsed DCs) was also significantly inhibited when compared to PBS treated mice, with no significant difference between the Eph-DC and the unpulsed DC groups (Fig. 1c). These results suggest that hepatic NK cells play an important role in regulating tumor growth in the liver after being activated by DC-based vaccination.

Liver NK cells are activated by DC vaccination

We examined whether LMNCs isolated from the liver 1 day after tumor inoculation displayed increased cytolytic activity against YAC-1 target cells in vitro. LMNCs harvested from mice treated with DCs (±peptide) were better killers of YAC1 cells than control LMNCs from PBS-treated or naïve mice (Fig. 2a). In contrast, splenocytes harvested from these same animals displayed only weak



◀ **Fig. 1** Anti-tumor effects with DC-based vaccination against liver and lung tumor. C57BL/6 mice were immunized on day-7 and 0 with 1×10^6 Eph-DCs, unpulsed DCs or PBS. On day 0, 2×10^6 MC38 cells (**a**) or 5×10^5 BL6 cells (**b**) were injected intrahepatically. Mice were sacrificed and liver weight was then determined 14 days after tumor inoculation, $^*P < 0.05$; $N = 10$/group. *Each data point* represents the mean liver weight ± SD. **c** SCID mice that lack T cells, B cells and NKT cells were immunized with 1×10^6 Eph-DCs, unpulsed DCs or PBS on day-7 and 0. On day 0, 2×10^6 MC38 cells were injected intrahepatically. Mice were sacrificed and liver weight was then determined 14 days after tumor inoculation, $^*P < 0.05$, $N = 8$/group. *Each data point* represents the mean liver weight ± SD

anti-YAC1 killing capacity (Fig. 2b). These results suggest the preferential activation of liver versus splenic NK effector cells by DC-based vaccination. We next examined the activation status (expression of CD69) of NK1.1+ cells by flow cytometry after DC-based vaccination. The frequency of hepatic NK1.1+ cells in mice immunized with Eph-DCs or unpulsed DCs were equal to those noted for mice treated with PBS alone (data not shown). The CD69 expression level on NK1.1+ cells in mice treated with either form of DC-based vaccine was significantly stronger than that of mice treated with PBS on day 1 after tumor inoculation, with this level of expression decreasing gradually on days 3, 7 after tumor inoculation (Fig. 2c). These results suggested that hepatic NK1.1+ cells were efficiently activated by DC vaccination versus PBS treatment. NK cells isolated from mice treated with Eph-DCs or unpulsed DCs displayed stronger cytolytic activity against MC38 targets when compared with PBS treatment, and there is no difference in anti-tumor killing by liver NK cells between Eph-DC group and unpulsed DC group (Fig. 2d).

Depletion of CD4+ T cells, CD8+ T cells or NK cells impairs the anti-tumor effects of immunization with DCs

To prove whether the therapeutic benefit associated with DC vaccination in the MC38 liver tumor model was dependent on CD4+, CD8+ T cells or NK cells in vivo, we performed selective cell subset depletion studies. The anti-tumor efficacy of DC-based immunization was significantly reduced in CD4+, CD8+ T cell or NK cell-depleted mice (Fig. 3). Notably, the liver weights of NK cell-depleted mice were significantly heavier than those of CD8+ T cell-depleted mice if the animals received unpulsed DC injections (Fig. 3a), while this was not observed for mice injected with Eph-DCs (Fig. 3b). These results suggest that not only NK cells, but also CD4+ T cells and CD8+ T cells are required for optimal anti-tumor effects associated with either DC vaccines, but that NK cells may play a greater role than CD8+ T cells in regulating tumor growth in mice receiving unpulsed DCs.

Induction of specific CTLs against MC38 cells after immunization with DCs

We next examined whether either Eph-DC or unpulsed DC immunization induced specific splenocyte (harvested 14 days after tumor inoculation) cytolytic activity against

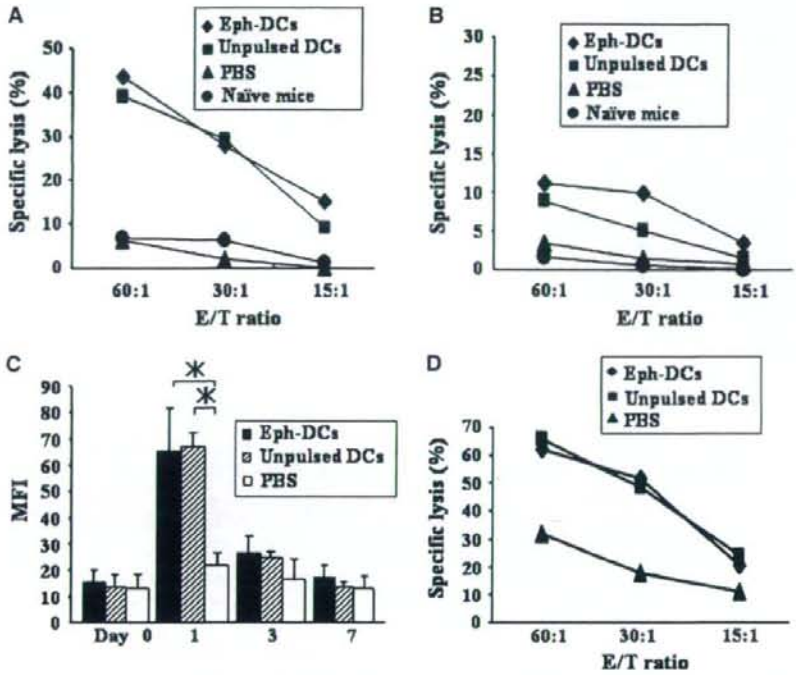


Fig. 2 Liver NK cells are activated by DC-based vaccination. LMNCs (a) or splenocytes (b) were isolated from the various treatment groups (Eph-DCs, unpulsed DCs or PBS) of mice or naïve mice 1 day after tumor inoculation, and subjected to 4-h ^{51}Cr release assays against YAC-1 targets at the indicated E:T ratios. Similar results were obtained in three experiments. c LMNCs were harvested before the first vaccination and on days 1, 3, 7 after tumor inoculation, and the phenotype of LMNCs from mice treated with Eph-DCs, unpulsed DCs or PBS was examined by flow cytometric analysis. Activated NK1.1 + cells were identified as NK1.1 + /CD69 + lymphocytes. *MFI* mean fluorescence intensity, $N = 3$/group. d LMNCs were isolated from the various treatment groups of mice 1 day after tumor inoculation, and liver NK cells were isolated from LMNCs by magnetic cell sorting using DX-5 MicroBeads (Miltenyi Biotec) and then subjected to 4-h ^{51}Cr release assays against MC38 target cells

MC38 or BL6 cells. Splenocytes isolated from mice treated with unpulsed DCs displayed stronger cytolytic activity against MC38 targets when compared with PBS treatment. Furthermore, splenocytes harvested from mice treated with Eph-DCs displayed stronger anti-MC38 cytolytic activity than unpulsed DC or PBS group (Fig. 4a), with this activity mediated by CD8+ T cells, but not CD4+ T cells (Fig. 4b). Cytolytic activity was not observed against EphA2-negative cells (i.e. BL6; Fig. 4c). These results suggest that either format of DC immunization induces MC38-specific CTLs in vivo, with somewhat greater levels of response observed in the case of peptide-specific vaccination.

Immunity to tumor rechallenge

We next determined whether DC vaccines that slow liver tumor progression protected mice against a consequent s.c. rechallenge with that same tumor. C57BL/6 mice were s.c. immunized with Eph-DCs or unpulsed DCs and MC38 liver tumors implanted. On day 14 post-intrahepaic tumor inoculation, 2×10^5 MC38 or 5×10^4 BL6 cells were injected s.c. into the flank of these mice and tumor growth monitored. We observed that s.c. MC38 (EphA2-positive) was inhibited by prior vaccination with Eph-DCs > unpulsed DCs versus naïve mice (Fig. 5a), and that the growth of BL6 (EphA2-negative) was not inhibited in any cohort analyzed versus control (Fig. 5b). These results suggested that immunization with either format of DCs elicited some degree of systemic anti-tumor effects against the EphA2-positive tumor, but that the specific immunization (Eph-DCs) was superior to unpulsed DCs in generating protective effects against EphA2-positive tumor outside the liver.

Discussion

DCs pulsed with TAA derived peptides (Peptide-DCs) have proven effective in eliciting protective and therapeutic anti-tumor immunity in patients against a diverse range of

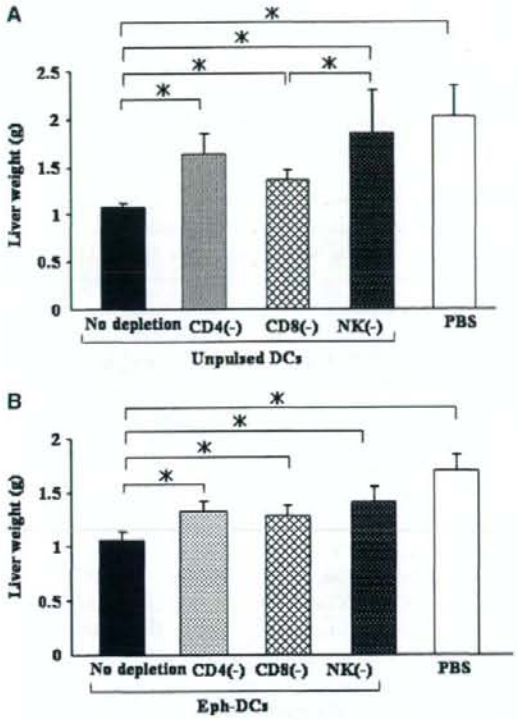


Fig. 3 DC-based vaccine efficacy is dependent upon T cells and NK cells. Ab-mediated in vivo depletion of CD4+, CD8+ T cells and NK cells were performed (as described in "Materials and methods"), with the depleted mice then receiving unpulsed DC (**a**) or Eph-DC vaccines (**b**) (on day-7 and 0) and intrahepatic 2×10^6 MC38 cell injection (day 0), $^*P < 0.05$, $N = 8$/group. *Each data point* represents the mean liver weight ± SD

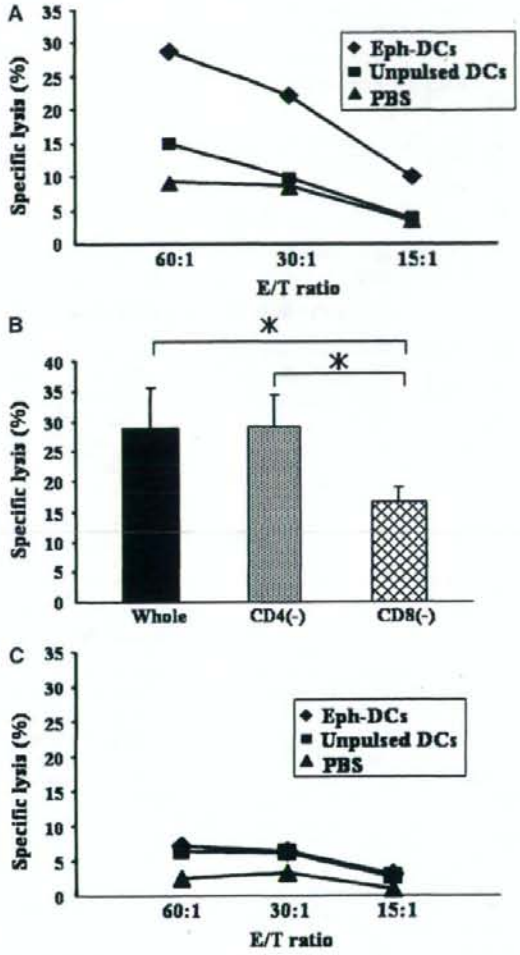


Fig. 4 DC-based vaccines induce anti-tumor T cells. Splenocytes were harvested from tumor-bearing mice 14 days after final treatment with Eph-DCs, unpulsed DCs or PBS. Splenocytes were stimulated in vitro with MMC-treated MC38 cells in the presence of low-dose recombinant human IL-2. After 5 days of culture, the cytolytic activity of the expanded T cells was evaluated using 4-h ^{51}Cr release assays against MC38 (**a**) or irrelevant BL6 (**c**) tumor target cells at the indicated E:T ratios. **b** Before performance of 4-h ^{51}Cr release cytolytic assays, CD4+ or CD8+ T cells were depleted from whole splenocytes of Eph-DC treated mice using specific MicroBeads. Similar results were obtained in three independent experiments

cancers [3]. However, at present, peptide-DC vaccines have not been comprehensively evaluated in clinical trials for treatment of metastatic liver cancers [25–27]. The liver uniquely contains an abundance of not only T cells, but also NK cells and NKT cells when compared with other organs [13–15]. Recently, DCs have been implicated as playing an important role in the activation of NK and NKT cells in both mice and humans [4–12, 33, 34], suggesting that DC-based therapies would be poised to activate an array of innate immune effector cells in the liver and might mediate clinical benefit within that organ. In this study, we demonstrated that administration of DCs prior to tumor implantation successfully promoted protective anti-tumor immunity against two distinct tumors in the liver (with little requirement for antigen-loading of DCs). Moreover, either Eph-DCs or unpulsed DCs have also proven effective in eliciting equally protective anti-tumor immunity against BL6 metastasis models in the lung that contains relatively high frequencies of innate immune effector cells (unpublished data).

In vitro cytotoxicity assays performed against the YAC-1 target cell line revealed that hepatic (and to a lesser extent splenic) NK cells were activated and mediated stronger killing function as a result of mice being treated with DCs (unpulsed or EphA2 peptide-pulsed) vaccination versus PBS controls. Consistent with this functional finding, hepatic NK 1.1+ cells (probably NK and NKT cells) in DC

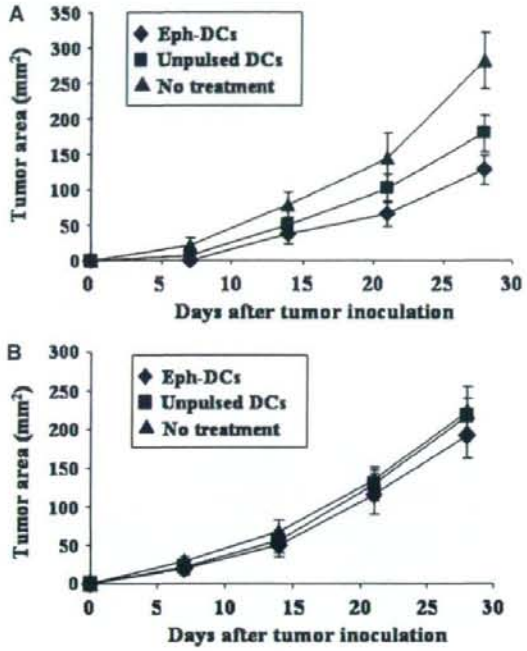


Fig. 5 DC vaccinated mice are partially protected against subcutaneous rechallenge with MC38 (EphA2-positive) but not BL6 (EphA2-negative) tumors. MC38-bearing mice that were successfully treated with DC-based vaccines were rechallenged s.c. in the flank with MC38 (a) or BL6 (b) tumor cells 14 days after the final DC injection. As controls, naïve mice were injected s.c. with MC38 or BL6 tumor cells. MC38 tumor growth in mice immunized with either format of DCs was significantly inhibited when compared to naive mice ($P < 0.05$ on day 28). In addition, tumor growth in the mice immunized with Eph- DCs was inhibited to a greater extent; i.e. $P < 0.05$ on day 28 versus unpulsed DCs, $P < 0.05$ on days 14, 21, 28 versus naive mice, $N = 8$/group. b Growth of the EphA2-negative BL6 tumor was not inhibited regardless of the treatment received, $N = 8$/group

treated mice also expressed elevated levels of the activation marker CD69 when compared to control treated animals.

Overall, these results suggest organ (i.e. Liver) focused anti-tumor "clinical" benefit derived from DC administration and that in conjunction with our results obtained in SCID models (lacking B cells, T cells, NKT cells) and in vitro cytotoxicity assays of liver NK cells performed against MC38 target cells, NK cells are major mediators of hepatic protection against tumor progression.

Recent studies have also suggested that DCs may effectively activate elements of innate immunity (NK cells [4–9] and NKT cells [10–12]) via direct cellular interaction and IL-12 secretion. In this study, the injected green fluorescence protein (GFP) mice-derived BM-DCs did not migrate to the liver following s.c. injection.in the flow cytometric analysis and IL-12 production in the serum harvested 1 day after either DC vaccination and tumor inoculation was not detected using IL-12 ELISA kits (unpublished data). Therefore, we would speculate that the injected DCs effectively enhance NK cell activity in the liver by some unknown humoral factors (cytokines except for IL-12, etc) or by secondary activated immune cells after DC immunization in vivo.

However, it is also clear that based on our in vivo lymphocyte depletion studies, CD4+ T cells and CD8+ T cells also contribute to suppression of tumor growth in the liver after DC vaccination. We would therefore speculate that hepatic NK cells may be a crucial early mediators of anti-tumor activity, with MC38 liver tumor-derived antigens then taken up by dedicated professional antigen-presenting cells in the liver that consequently cross-prime MC38 tumor-specific CD4+ or CD8+ T cells. In animals pre-vaccinated with Eph-DCs, the initial wave of tumor killing by NK cells may be boostered by EphA2-specific CD8+ T cells in the MC38 (EphA2-positive) model. This added degree of protection appeared to be dependent solely upon the presence of the loaded antigenic peptide on DCs, since we observed no other differences in Eph-DCs versus unpulsed DCs with regard to DC expression of a broad range of markers/parameters: CD40, CD80, CD86, MHC class II and IL12 production (S. Yamaguchi, unpublished data).

Data from our tumor rechallenge experiments indicate that unpulsed DC vaccines are not only effective in limiting MC38 liver tumor progression, but also offer protection against reintroduction of MC38 tumor at an extrahepatic site. However, Eph-DC immunizations were more effective in this endpoint, consistent with their superior capacity to promote tumor (i.e. EphA2)-specific CTLs. In contrast, in all cases when animals were rechallenged with the unrelated, EphA2-negative BL6 tumor, lesions grew progressively, suggesting that MC38-specific immunity was generated after liver tumor treatment. These results demonstrated that both unpulsed DC and peptide-pulsed DC vaccines generated systemic tumor-specific anti-tumor immunity against *metastatic liver tumor*, and that peptide-pulsed DC vaccination offered more optimal preventive treatment for extrahepatic tumor recurrence than unpulsed DC vaccines.

In spite of recent progress and early success reported for adjuvant chemotherapy trials in the prevention of liver metastasis, there remains a great need for developing novel and effective treatment modalities for this indication. In the current study, we have demonstrated that unpulsed DC vaccines, that are competent to activate both innate and acquired immunity within the liver, may represent a novel treatment option for metastatic liver tumors which expressed unknown TAAs. In cases where more disseminated disease is also present (or likely to occur) in non-liver

sites, the greatest degree of clinical efficacy may be expected of Peptide-DC vaccines, such as Eph-DCs.

Acknowledgments The authors wish to thank Dr. Walter J. Storkus (The University of Pittsburgh) for careful review and comments provided during the preparation of this manuscript. This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health, Labor and Welfare of Japan [N.H.].

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Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A in patients with chronic liver diseases and changes during transcatheter arterial embolization for hepatocellular carcinoma

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(Received January 16, 2008/Revised April 8, 2008/Accepted April 11, 2008/Online publication July 29, 2008)

Soluble forms of major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) are increased in the sera of patients with malignancy and impair the antitumor immune response by downregulating expression of their cognate immunoreceptor natural killer group 2, member D (NKG2D). Recently, soluble MICA/B were reported to appear even in some premalignant diseases, raising questions about the impact of soluble MICA/B produced from tumors on the expression of NKG2D. The present study examined soluble MICA/B in chronic liver disease and hepatocellular carcinoma (HCC) and their involvement in the immune-cell expression of NKG2D during transcatheter arterial embolization for HCC. The levels of soluble MICA/B were significantly higher in chronic liver disease and HCC patients than in healthy volunteers. The progression of liver disease and that of the tumor were independent determinants for soluble MICA/B levels. Immunohistochemistry revealed that MICA/B were expressed not only in HCC tissue but also on hepatocytes in cirrhotic livers. The transcatheter arterial emboli-zation therapy significantly decreased serum levels of soluble MICA, but not soluble MICB, and increased the NKG2D expression on natural killer cells and CD8-positive T cells; there was an inverse correlation between changes in soluble MICA levels and in NKG2D expression. In conclusion, although soluble MICA/B are produced from both HCC and premalignant cirrhotic livers, therapeutic intervention for HCC can reduce the levels of soluble MICA and thereby upregulate the expression of NKG2D. Cancer therapy may have a beneficial effect on NKG2D-mediated antitumor immunity. (*Cancer Sci* 2008; 99: 1643–1649)

MHC class I-related chain A and B, glycoproteins expressed on the cellular membrane, are ligands for NKG2D expressed on a variety of immune cells.[1] In contrast to classical MHC class I molecules, MICA/B are expressed rarely on normal cells but frequently on tumor cells, including colon cancer, prostate cancer, HCC, and brain tumors.[2–5] The engagement of MICA/B and NKG2D strongly activates NK cells and costimulates T cells, enhancing their cytolytic ability and cytokine production.[6] Thus, the MICA/B–NKG2D pathway is an important mechanism by which the host immune system recognizes and kills transformed cells.[7] In addition to those membrane-bound forms, MICA/B are also cleaved proteolytically from tumor cells and appear as soluble forms in sera of patients with malignancy.[8–10] The levels of NKG2D expression tend to be decreased in patients with high levels of soluble MICA/B.[4] In addition, sera from those patients can downregulate NKG2D expression *in vitro*.[5,11] These data suggest that soluble MICA/B in the circulation downregulate NKG2D expression and disturb NKG2D-mediated antitumor immunity, raising the possibility that cancer therapy might reduce the serum levels of soluble MICA/B and thereby improve the NKG2D-related immune environment. However, this possibility has not been addressed directly by examining soluble MICA/B and NKG2D expression in a cohort of patients before and after cancer therapy. Furthermore, recent reports by Holdenrieder *et al.* demonstrating that soluble MICA/B are increased not only in malignant disease but also in some benign diseases, such as of the gastrointestinal tract, gynecologic organs, and lungs, raise questions about the impact of cancer therapy on modulating soluble MICA/B levels.[12,13]

Hepatocellular carcinoma is one of the leading causes of cancer death worldwide. Chronic liver disease caused by hepatitis virus infection and non-alcoholic steatohepatitis leads to a predisposition for HCC; liver cirrhosis, in particular, is considered to be a premalignant condition.[14,15] With regard to treatment, surgical resection or percutaneous techniques such as ethanol injection and radiofrequency ablation are considered to be choices for curable treatment of localized HCC, whereas TAE is a well-established technique for unresectable HCC.[16] We reported previously that soluble MICA could be detected in sera of HCC patients.[17] However, the clinical significance of the soluble forms of NKG2D ligands in liver disease has not yet been established in a comprehensive manner, because the previous study was conducted on a small number of patients, did not include patients with premalignant conditions such as liver cirrhosis, and did not analyze its closely related molecule MICB. Furthermore, influences of therapeutic intervention on soluble NKG2D ligands in patients have been unclear. In the present study, we examined soluble MICA and soluble MICB in sera from a large number of patients with chronic liver diseases and HCC and their impact on NKG2D expression on immune cells during TAE therapy for HCC.

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Abbreviations: APC, allophycocyanin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; MFI, mean fluorescence intensity; MICA/B, major histocompatibility complex (MHC) class I-related chain A and B; NK, natural killer; NKG2D, natural killer group 2, member D; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

doi: 10.1111/j.1349-7006.2008.00859.x
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Table 1. Control and patient characteristics

Characteristic	Healthy control	Chronic hepatitis	Liver cirrhosis	HCC
Number	104	141	104	232
Sex (male/female)	49/55	78/63	60/44	177/55*
Age (years)	62 ± 15	55 ± 13**	61 ± 12	68 ± 9***
Etiology				
HBV/HCV	–	27/107	12/78	37/187
Alcohol/NASH		0/5/	2/1/	4/0/
AIH/PBC/others		2/0/0	1/6/4	0/0/3
Child–Pugh (A/B/C)	–	–	34/27/26	131/84/17****
TNM stage (I/II/III/IV)	–	–	–	59/68/64/39

AIH, autoimmune hepatitis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cirrhosis; TNM, tumor node metastasis.
*$P < 0.05$ vs control, hepatitis, and cirrhosis by χ^2-test; **$P < 0.05$ vs control, cirrhosis, and HCC by ANOVA and post hoc Bonferroni test; ***$P < 0.05$ vs control, hepatitis, and cirrhosis by ANOVA and post hoc Bonferroni test; ****$P < 0.05$ vs cirrhosis by χ^2-test.

Materials and Methods

Stock sera from patients with chronic liver disease and HCC. We used frozen stock sera obtained from consecutive patients with chronic liver disease who had been registered at our institute from February 2002 to April 2006. They included 141 patients with chronic hepatitis, 104 patients with liver cirrhosis, and 232 patients with HCC. The differential diagnosis between chronic hepatitis and liver cirrhosis was basically from liver biopsy ($n = 98$), but for those who had not undergone biopsy the diagnosis was based on clinical findings from the aspartate aminotransferase/platelet ratio index (APRI) score.[18] Diagnosis of HCC was based on unequivocal clinical and imaging data. The control group consisted of 104 healthy volunteers of an age range similar to the liver cirrhosis group. Table 1 summarizes the control and patient characteristics of age, sex, etiology of liver disease, Child–Pugh classification, and TNM staging of HCC. Child–Pugh classification is a well-established index for progression of liver disease in cirrhotic patients where A, B, and C indicate compensated cirrhosis, mildly decompensated cirrhosis, and severely decompensated cirrhosis, respectively. The TNM staging adopted in the present study was that modified by the Liver Cancer Study Group of Japan.[16]

Detection of soluble MICA/B by ELISA. Serum levels of soluble MICA and soluble MICB were determined differentially by commercially available ELISA kits (R & D Systems, Minneapolis, MN, USA). In preliminary experiments, we determined the median intra-assay variation ($n = 5$) to be between 3.5 and 5.6% for soluble MICA and between 2.4 and 7.8% for soluble MICB, and the median interassay variation ($n = 5$) to be between 12.8 and 18.9% for soluble MICA and between 15.2 and 18.7% for soluble MICB.

Detection of MICA/B on liver tissues by immunohistochemistry. The human liver tissues examined were one normal liver, three from those at fibrosis stages 1 and 2 of chronic hepatitis, five from liver cirrhosis (fibrosis stage 4) patients, and five from HCC patients. Paraffin-embedded liver sections were deparafinized, heat-inactivated by a microwave oven and then subjected to immunohistochemical staining using the ABC procedure (Vector Laboratories, Burlingame, CA, USA). The primary antibody used was 6D4 monoclonal antibody, which recognizes the α1 and α2 domains of MIC molecules shared by both MICA and MICB.[2] To confirm the specificity of the staining, the 6D4 antibody was incubated with recombinant MICA (R & D Systems) for 2 h and then applied to liver sections in parallel with staining of the primary antibody as the absorption test.

Table 2. Characteristics of hepatocellular carcinoma patients

Characteristic	TAE-treated group	Non-treated group
Number	38	21
Sex (male/female)	28/10	17/4
Age (years)	75 ± 11	74 ± 8
Etiology (HBV/HCV)	2/36	1/21
Child–Pugh (A/B/C)	29/9/0	16/5/0
TNM stage (I/II/III/IV)	4/20/14/0	2/11/8/0

HBV, hepatitis B virus; HCV, hepatitis C virus; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

Detection of membrane-bound and soluble forms of MICA/B on cultured cells. HepG2 hepatoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human non-transformed hepatocytes were purchased from Cambrex Bio Science (Charles City, IA, USA) and cultured according to the manufacturer's instructions. For detection of membrane-bound MICA/B, a single-cell suspension was stained with PE-labeled 6D4 monoclonal (R & D Systems) antibody, fixed with 2% paraformaldehyde, and then subjected to flow cytometric analysis. The culture supernatants were subjected to analysis of soluble forms of MICA and MICB using the above-mentioned ELISA assay.

Patients with HCC and TAE therapy. Thirty-eight patients with HCC admitted to our institution for TAE therapy were enrolled prospectively in the present study. TAE was carried out by the standard procedure using an emulsion of farmorubicin and lipiodol followed by gelatin sponge particles. Blood samples were collected before and 2 weeks after TAE therapy. Twenty-one patients with HCC, matching the TAE group with respect to TNM stage and Child–Pugh score, were also enrolled as controls (Table 2). Blood samples were collected twice at a 2-week interval. Written informed consent was received from all patients and the study protocol was approved by the Ethical Committee of Clinical Research at Osaka University Hospital.

Natural killer cell analysis. PBMC were isolated from heparinized venous blood by a standard procedure. PBMC were stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD56 antibody, and PE-labeled anti-NKG2D antibody. They were also stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD8 antibody, and PE-labeled anti-NKG2D antibody. All antibodies were purchased from Becton Dickinson (San Jose, CA, USA). NKG2D expression on NK cells (defined as CD56-positive and CD3-negative cells) and CD8-positive T cells (defined as CD3-positive and CD8-positive cells) were analyzed by flow cytometry. As a control, corresponding fluorescence-labeled irrelevant antibodies were used. As most NK and CD8-positive T cells express NKG2D, the levels of expression were evaluated by the mean fluorescence intensity of the stained cells.

Statistics. Values were expressed as the median and interquartile range as a box plot, and the 10th and 90th percentiles as a horizontal bar. For comparison of more than two groups, the Kruskal–Wallis rank sum test was used. If the Kruskal–Wallis test was significant, post hoc multiple comparisons were carried out using the Steel–Dwass procedure. Differences between pretreatment and post-treatment values were tested by paired t-test. $P < 0.05$ was considered statistically significant.

Results

Soluble MICA and soluble MICB in chronic liver disease and HCC. Soluble MICA and soluble MICB were assessed in sera from patients with chronic hepatitis, liver cirrhosis, and HCC as well as healthy volunteers. There was a stepwise increase in the levels of both soluble MICA and soluble MICB from hepatitis

doi: 10.1111/j.1349-7006.2008.00859.x
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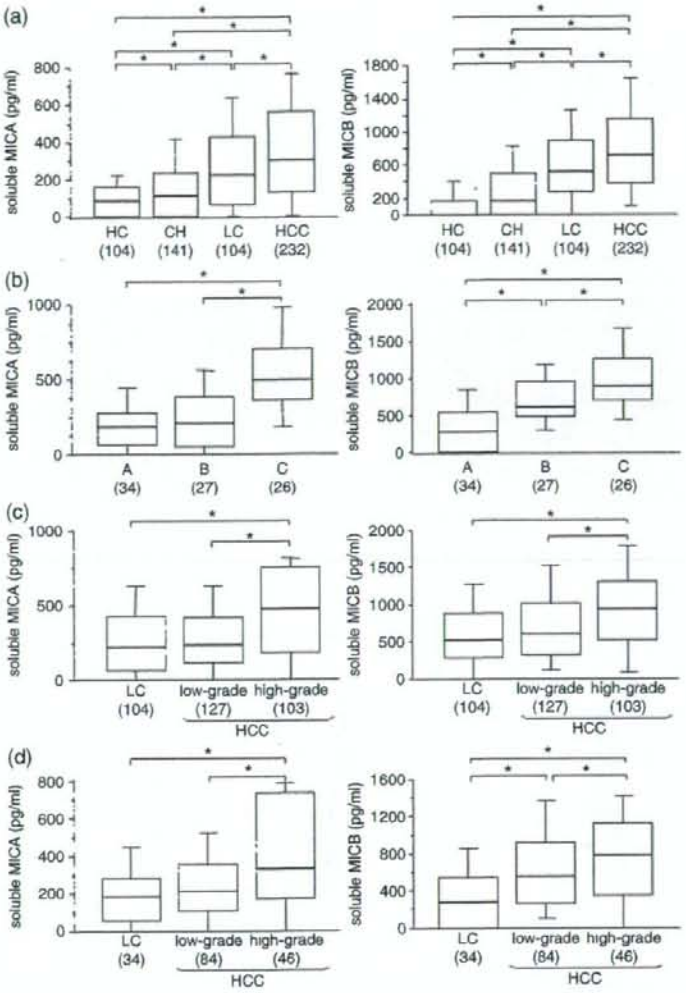

Fig. 1. Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) in chronic liver disease and hepatocellular carcinoma (HCC). (a) Soluble MICA and soluble MICB levels in serum samples of healthy controls (HC), chronic hepatitis (CH), liver cirrhosis (LC), and HCC. (b) Soluble MICA and soluble MICB are associated with the progression of liver disease. Data on cirrhotic patients were stratified based on Child–Pugh classification. (c,d) Soluble MICA and soluble MICB are associated with the progression of tumors. (c) Data on cirrhosis and HCC patients were classified into three groups: patients with absence of HCC (cirrhosis), patients with low-grade HCC (tumor node metastasis [TNM] stage I and II), and patients with high-grade HCC (TNM stage III and IV). (d) To exclude the possibility of progression of liver disease being involved in increase in soluble MICA/B, soluble MICA/B levels were compared among the three groups of Child–Pugh classification A. Data are represented as box plots (median values, 10th, 25th, 75th, and 90th percentiles). The number in parentheses indicates the number of patients in each group. $^*P < 0.05$ by Kruskal–Wallis test and post hoc Steel–Dwass test.

to HCC (Fig. 1a). Although the difference between hepatitis patients and healthy volunteers was modest, both of the levels were clearly higher in patients with liver cirrhosis and HCC than in normal volunteers or hepatitis patients. To examine whether the progression of liver disease in cirrhotic patients affects the levels of soluble MICA/B, cirrhosis patients were stratified based on Child–Pugh classification. The levels of both soluble MICA and MICB were increased significantly with the progression of liver disease (Fig. 1b).

Hepatocellular carcinoma often develops from cirrhotic liver and most patients with HCC included in the present study had complications from cirrhosis. To examine whether the development and progression of HCC contributes to increasing soluble MICA/B, patients with liver cirrhosis and those with HCC were classified into three groups: those with an absence of HCC, low-grade HCC (TNM stage I/II) and high-grade HCC (TNM stage III/IV). There was no significant difference in soluble MICA or soluble MICB between patients without HCC and low-grade HCC patients. However, the high-grade HCC patients showed significantly higher levels of soluble MICA or soluble MICB than patients without HCC or the low-grade HCC patients (Fig. 1c). To exclude the possibility of the progression of liver disease affecting the increases in soluble MICA/B in high-grade HCC, we selected and analyzed only the Child–Pugh A patients. In this subgroup of patients, the levels of soluble MICA/B were also significantly higher with high-grade HCC than with low-grade HCC or the absence of HCC (Fig. 1d). Thus, the progression of liver disease and that of the tumor independently affects the levels of soluble MICA or soluble MICB.

MICA/B expression in liver tissues and production of soluble MICA/B. The increase in soluble MICA/B in cirrhotic patients suggests that MICA/B may be expressed in cirrhotic livers. We therefore examined MICA/B expression by immunohistochemistry in various human tissues including normal liver, chronic hepatitis (F1 and F2 stage), liver cirrhosis, and HCC (Fig. 2a). MICA was detected clearly in four of five HCC tissues, agreeing with a

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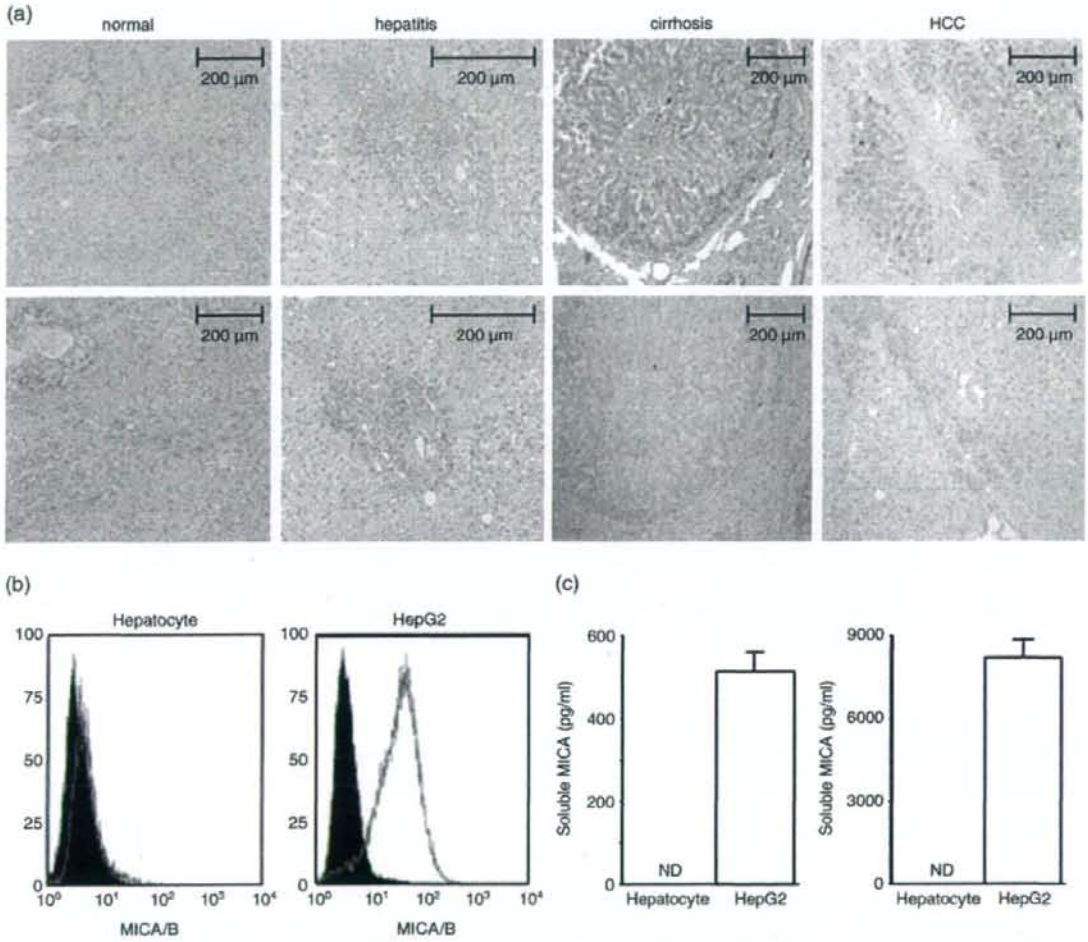

Fig. 2. Expression of major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) and production of their soluble forms. (a) Immunohistochemical detection of MICA/B in liver tissues. Representative staining with anti-MICA/B monoclonal antibody (6D4) is shown for normal liver, chronic hepatitis (F1 stage), liver cirrhosis (F4 stage), and hepatocellular carcinoma (HCC) (upper panel). As a control, 6D4 monoclonal antibody was preabsorbed with recombinant MICA and applied to the neighboring corresponding sections (lower panel). (b) Flow cytometric analysis of surface expression of MICA/B on HepG2 hepatoma cells and non-transformed hepatocytes. Open and closed histograms represent the staining of anti-MICA/B antibody (6D4) and control antibody, respectively. (c) Soluble MICA and soluble MIC8 released from HepG2 hepatoma cells and non-transformed hepatocytes. Cells were seeded in a subconfluent condition and cultured for 48 h. The culture supernatants were applied for analysis of soluble MICA and soluble MICB by enzyme-linked immunosorbent assay. ND, not detected.

previous report.[3] Importantly, hepatocytes in four of five cirrhotic livers were positive for MICA/B, whereas MICA/B were not detected in hepatocytes from normal liver or liver at the early stage of chronic hepatitis.

We also examined the expression of MICA/B on normal hepatocytes and HepG2 hepatoma cells. Flow cytometric analysis revealed that HepG2 cells expressed MICA/B on the cell surface (Fig. 2b). Both soluble forms of MICA and MICB were detected in the supernatant of HepG2 cells cultured for 48 h (Fig. 2c). In contrast, non-transformed hepatocytes expressed MICA/B faintly and soluble MICA/B could not be detected in their culture supernatant. This observation supported the idea that both soluble MICA and soluble MICB are produced from MICA/B-expressing hepatic cells.

Downregulation of soluble MICA levels by TAE. The above findings suggest that soluble MICA/B are produced from cirrhotic livers as well as HCC. In addition, the progression of the tumor is an important determinant of soluble MICA/B independent of the progression of liver disease. We then asked the question of whether therapeutic intervention of HCC would reduce the levels of soluble MICA or soluble MICB and affect the levels of NKG2D expression on immune cells. We prospectively analyzed the levels of soluble MICA/B and NKG2D expression in 38 HCC patients before and 2 weeks after TAE therapy. As a control, 21 HCC patients who did not receive TAE therapy but were matched to the TAE group with respect to clinical characteristics were analyzed over a 2-week interval.

doi: 10.1111/j.1349-7006.2008.00859.x
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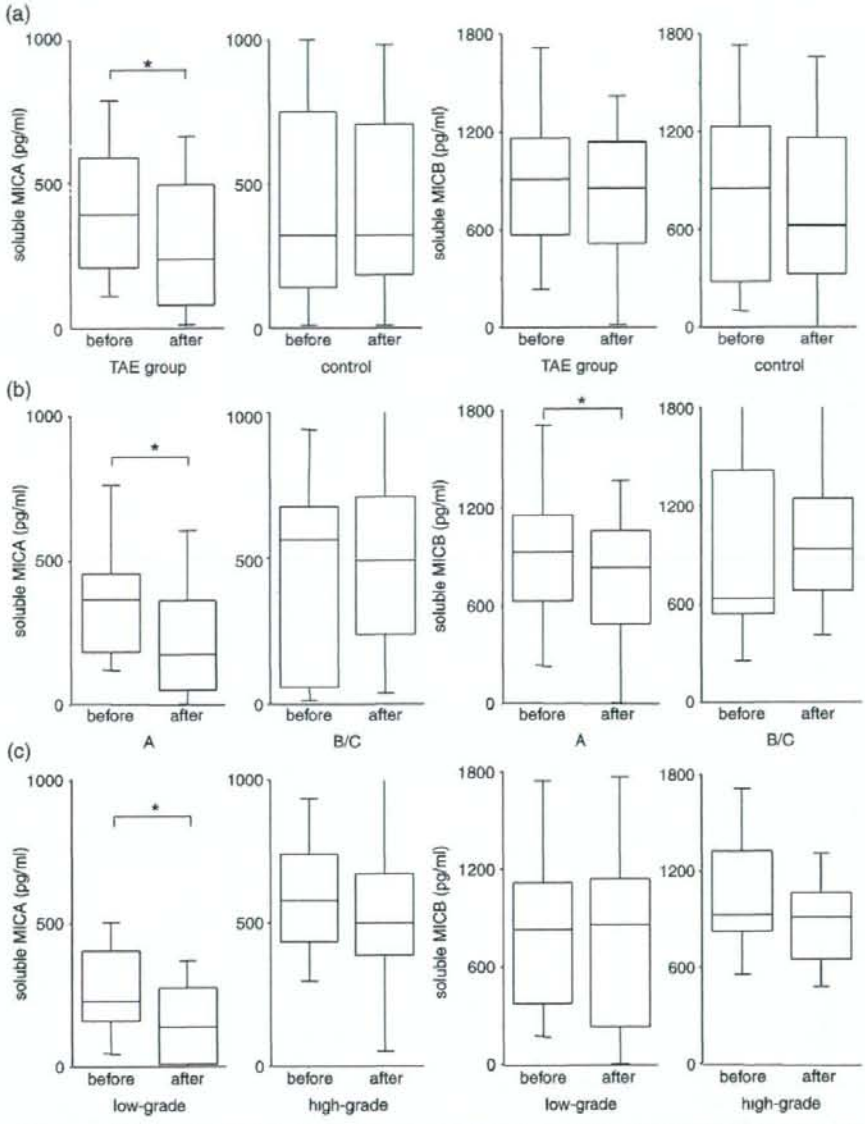

Fig. 3. Soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) during transcatheter arterial embolization (TAE) therapy. (a) Soluble MICA and soluble MICB were measured for 38 patients before and 2 weeks after TAE therapy. Twenty-one patients who did not receive TAE therapy served as controls, with soluble MICA/B being measured twice with a 2-week interval. (b) TAE-treated patients were divide into two groups: Child–Pugh A ($n = 29$) and Child–Pugh B and C ($n = 9$). (c) TAE-treated patients were divided into two groups: low-grade hepatocellular carcinoma (HCC) ($n = 24$) and high-grade HCC ($n = 14$). *$P < 0.05$ by paired t-test.

In the TAE-treated group, the levels of soluble MICA were decreased significantly 2 weeks after TAE therapy compared with those before TAE (Fig. 3a). In contrast, TAE did not affect the levels of soluble MICB. Neither the levels of soluble MICA nor those of soluble MICB changed during the 2-week interval in HCC patients not receiving TAE therapy. As the progression of liver disease and that of the tumor affects the levels of soluble MICA/B, TAE-treated patients were divided according to their Child–Pugh stage or tumor stage. The levels of soluble MICA decreased significantly after TAE therapy in Child–Pugh A patients but not in Child–Pugh B and C patients (Fig. 3b). Interestingly, Child–Pugh A patients showed a significant decrease even in soluble MICB levels after TAE therapy but Child–Pugh B and C patients did not. As for tumor stage, a significant decrease in

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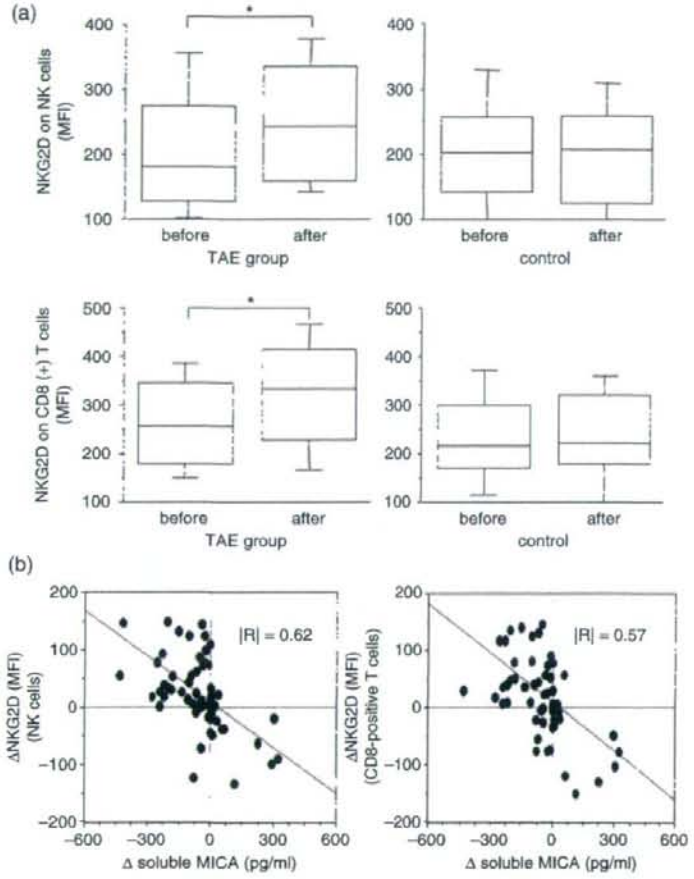

Fig. 4. Natural killer group 2, member D (NKG2D) expression during transcatheter arterial embolization (TAE) therapy. (a) NKG2D expression on natural killer (NK) cells and CD8-positive T cells. NKG2D expression on immune cells was analyzed in 38 patients before and 2 weeks after TAE therapy. Twenty-one patients who did not receive TAE therapy served as a control by measuring NKG2D expression for 2-week interval. NKG2D expression on each cell type was evaluated by mean fluorescence intensity (MFI). *$P < 0.05$ by paired t-test. (b) Correlation between change of soluble MICA and that of NKG2D expression on NK cells or CD8-positive T cells.

soluble MICA levels after TAE therapy was found in low-grade HCC but not in high-grade HCC (Fig. 3c). The levels of MICB did not change in the low-grade or high-grade HCC groups.

Upregulation of NKG2D expression by TAE. The number of PBMC as well as NK and T-cell subsets did not change over the 2-week interval in both the control and TAE-treated patients (data not shown). However, the levels of NKG2D expression on NK and CD8-positive T cells increased significantly upon TAE therapy, but not in the control group (Fig. 4a). To examine the involvement of soluble MICA in NKG2D expression, we analyzed the relationship of changes between soluble MICA and NKG2D expression in HCC patients. Change in soluble MICA was correlated inversely with changes in NKG2D expression on NK and CD8-positive T cells (Fig. 4b). There was no significant correlation between changes in soluble MICB and NKG2D expression (data not shown).

Discussion

In the present study, we demonstrated that soluble MICA/B increases with the progression of chronic liver disease as well as the progression of HCC. Increases in soluble MICA/B in advanced stages of tumors have been reported in some malignancies.[12] However, little is known about soluble MICA/B in the premalignant condition. Recently, Holdenrieder *et al.* examined soluble MICA/B levels in benign as well as malignant diseases from heterogeneous organs.[12,13] They found that benign diseases, such as gastrointestinal tract adenoma, pulmonary infectious disease, and gynecologic benign tumors, showed intermediate levels of soluble MICA/B between healthy controls and malignant disease. Our present findings not only agree with theirs, but also provide evidence that soluble MICA/B increases in premalignant conditions such as liver cirrhosis.

Malignant disease is known to lead frequently to the expression of MICA/B.[2] In contrast, their expression in premalignant tissues has not been fully elucidated. In the present study, MICA/B were found to be expressed in liver cirrhosis as well as HCC tissues, but not in the early stages of chronic hepatitis or in normal liver. This finding is consistent with the tendencies observed for serum-soluble MICA/B levels in chronic liver disease and HCC. Analysis of cultured cells also revealed that MICA/B expressed on hepatoma cells is released spontaneously into the culture supernatant as soluble forms, supporting the idea that MICA/B expressed in the liver may be released into the circulation. In contrast, MICA/B were not expressed on nor released from cultured non-transformed hepatocytes, which is consistent with the *in vivo* immunohistochemical finding. An issue to be resolved is the underlying mechanism by which non-transformed

doi: 10.1111/j.1349-7006.2008.00859.x
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hepatocytes express and release MICA/B in pathological conditions such as liver cirrhosis. Recently, it was reported that non-transformed pulmonary epithelial cells can express MICA/B under oxidative stress-inducing conditions.[19] It was also reported that MICA/B are upregulated in non-tumor cell lines by genotoxic stress.[20] It has been speculated that oxidative and genotoxic stresses may accumulate in hepatocytes in chronic diseased liver. Thus, it is possible that those stresses may contribute to MICA/B expression in chronic diseased liver. Further study is needed to clarify this issue.

MICA/B expression in the premalignant condition raises the question of which contributes more to the production of soluble MICA/B, malignant tissues or non-malignant tissues. To address this question we analyzed the levels of soluble MICA/B in HCC patients before and after therapeutic intervention. Among treatments for HCC, TAE is a well-established technique for unresectable, advanced HCC.[16] To include HCC patients who show relatively high levels of soluble MICA/B, we chose a cohort of patients who received the TAE therapy in the present study. The data indicated that the levels of soluble MICA, but not those of soluble MICB, decreased after TAE therapy. It is not clear why soluble MICB did not change during TAE therapy. One possibility is that soluble MICB production from non-tumor livers may be relatively high compared with that of soluble MICA. In our subpopulation analysis, Child–Pugh A patients showed a significant decrease in soluble MICB levels after TAE therapy. In general, TAE therapy is more effective for Child–Pugh A patients than Child–Pugh B or C patients because the former is better able to tolerate the large dose of lipiodol emulsion and gelatin sponge that is necessary for efficient antitumor effect. Indeed, Child–Pugh A patients in our cohort showed a larger decease in α-fetoprotein levels after TAE therapy than Child–Pugh B and C patients, although the difference did not reach a significant level (our unpublished data). Thus, TAE therapy might reduce the levels of soluble MICB when it achieves substantial antitumor effect. Most importantly, the data also indicated that NKG2D expression on immune cells was clearly ameliorated with TAE therapy. Furthermore, there was an inverse correlation between a reduction in soluble MICA and upregulation of NKG2D, suggesting the link between soluble MICA and NKG2D expression in cancer patients.

It is generally speculated that soluble MICA/B produced from tumors may deactivate NKG2D-mediated immune responses.[8,9] *In vitro* experiment indicates that soluble MICA could down-regulate NKG2D expression and effector cell function. However, the regulation by soluble forms of NKG2D ligands would be more complicated *in vivo*. First, soluble forms of NKG2D ligands could be produced not only from malignant tissues but also from non-malignant tissues, as shown in the present study. Second, MHC-encoded MICA/B may not be the sole family of proteins serving as NKG2D ligands. Non-MHC-encoded UL16-binding proteins also act as NKG2D ligands and were very recently found to be cleaved proteolytically from tumor cells.[21] The present study provides evidence that soluble MICA is derived, at least in part, from HCC and regulates NKG2D expression on NK and CD8-positive T cells. Although several species of soluble NKG2D ligands may exist in the circulation, the present study suggests that soluble MICA regulates NKG2D expression directly in cancer patients.

In conclusion, soluble MICA and MICB are significantly increased in the sera of patients not only with HCC but also with chronic liver disease. Soluble MICA/B increases together with the progression of liver disease as well as the tumor. Therapeutic intervention for HCC leads to reduction of soluble MICA levels in association with upregulation of NKG2D on immune cells, offering *in vivo* evidence of soluble MICA regulating NKG2D expression. Thus, cancer therapy may have a beneficial effect on the NKG2D-mediated immune response even if some of the soluble NKG2D ligands are produced from non-cancerous premalignant tissues.

Acknowledgments

We sincerely thank Dr Veronika Groh and Dr Thomas Spies (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) for providing 6D4 antibody and Dr Alexander Steinle (University Tübingen, Tübingen, Baden-Württemberg, Germany) for providing the RSV-MICA*04 and control plasmids. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan and a Grant-in-Aid for Research on hepatitis and bovine spongiform encephalopathy (BSE) from the Ministry of Health, Labor and Welfare, Japan.

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Journal of Hepatology 49 (2008) 779–786

Journal of Hepatology

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Decreased expressions of CD1d molecule on liver dendritic cells in subcutaneous tumor bearing mice☆

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***Background/Aims*: α-Galactosylceramide (α-GalCer) has been attracting attention as a novel approach to treat metastatic liver cancer. However, the activation of liver innate immunity by α-GalCer should be examined because clinical trials of α-GalCer resulted in limited clinical responses.**

***Methods*: We examined the activation of liver innate immunity by α-GalCer in subcutaneous Colon26 tumor bearing-mice (C26s.c.TB-mice).**

***Results*: The expressions of CD1d molecule on liver dendritic cells (DCs) were significantly lower in C26s.c.TB-mice than those in tumor-unbearing normal mice. Although liver NK cells and NKT cells activated in normal mice after α-GalCer treatment, the activation of these cells were significantly inhibited in C26s.c.TB-mice. α-GalCer treatment resulted in significant antitumor effect against Colon26 metastatic liver tumor in normal mice, but not in C26s.c.TB-mice. The serum levels of TGF-β, known to suppress the CD1d expressions on DCs, in C26s.c.TB-mice were significantly higher than those in normal mice. Surgical subcutaneous tumor mass reduction resulted in the reduction of serum TGF-β, the recovery of CD1d expressions on liver DCs and the improvement of antitumor effect of α-GalCer against metastatic liver tumor.**

***Conclusions*: These results suggested that tumor burden reduces CD1d expressions on liver DCs, thus impeding α-GalCer-mediated NK cell activation and antitumor activity in the liver.**

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***Keywords*: α-Galactosylceramide; CD1d; Liver dendritic cells; Antitumor immunity**

1. Introduction

The glycolipid antigen α-galactosylceramide (α-GalCer) induces activation of NKT cells in a CD1d-dependent manner [1]. α-GalCer presented by DCs efficiently stimulates NKT cells implicated in the innate immunity [2,3]. Recently α-GalCer has been attracting attention for novel anti-tumor therapy. *In vivo* animal studies have shown that systemic administration of α-GalCer can lead to anti-tumor effects against metastatic liver tumor [4,5], suggesting that α-GalCer treatment might be promising for clinical application against liver tumor. Metastatic liver tumors, one of the most common types of advanced malignancy, resist conventional chemotherapy and radiotherapy, and present with a poor prognosis. Thus novel and more effective immunotherapy is needed, especially for metastatic liver cancer. Several phase I clinical studies have been carried

Received 29 February 2008; received in revised form 11 June 2008; accepted 14 June 2008; available online 2 July 2008
Associate Editor: V Barnaba

☆ The authors declare that they do not have anything to disclose regarding funding from industries or conflict of interest with respect to this manuscript.

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Abbreviations: DC, dendritic cell; APC, antigen-presenting cells; CTL, cytotoxic T lymphocytes; α-GalCer, α-galactosylceramide; M-NC, mononuclear cells; TB, tumor bearing.

0168-8278/$34.00 © 2008 Published by Elsevier B.V. on behalf of the European Association for the Study of the Liver.
doi:10.1016/j.jhep.2008.06.011

out in cancer immunotherapy using intravenous administration of α-GalCer, but with limited clinical responses [6,7]. Most clinical trials of cancer immunotherapy have been conducted with patients at advanced stages of cancer. Thus, for further development of α-GalCer treatment in such patients, the antitumor effect of α-GalCer should be examined in hosts with an advanced tumor burden.

In the current study, we evaluated the anti-tumor effect of administration of α-GalCer against liver tumor in subcutaneous tumor bearing animals. Both the antitumor effect of α-GalCer against liver tumor and liver NK cell and NKT cells activation were impaired in subcutaneous tumor bearing mice (s.c.TB-mice). The liver DCs were poorly activated by α-GalCer administration with lower expression of CD1d, NKT-activating molecules. However, the CD1d expression increased and the antitumor effect of α-GalCer against liver tumor was improved after surgical resection of the subcutaneous tumor mass. Our study has shed light toward understanding of the antitumor effect of α-GalCer in metasatic liver cancer patients.

2. Materials and methods

2.1. Mice

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

2.2. Cell lines

Colon26, a mouse colon adenocarcinoma cell line was kindly provided by Dr. Takashi Tsuruo (Institute of Molecular and Cellular Bioscience, The University of Tokyo, Tokyo, Japan). This cell line was maintained in complete medium (CM, 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, New York) in a humidified incubator at 5% CO_2 and 37 °C.

2.3. α-GalCer

α-GalCer was kindly provided by Kirin Pharma Co. Ltd. (Gunma, Japan) and prepared as previously described [8].

2.4. Animal experiments

To establish Colon26 s.c.TB-mice (C26s.c.TB-mice), BALB/c mice were subcutaneously injected with 3×10^6 Colon26. On day 42, when the tumor size reached approximately 200 mm^2, bone marrow-derived DCs (BM-DCs) and liver DCs were prepared to evaluate the CD1d expression in C26s.c.TB-mice. BM-DC were generated as previously described [8]. Hepatic mononuclear cells (MNC) were prepared as previously described [8]. CD11c+ dendritic cells were isolated from hepatic MNC by magnetic cell sorting using MACS (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's protocol.

Hepatic metastasis of Colon26 cells was established as previously described [9]. To examine antitumor effect of α-GalCer in the liver of C26s.c.TB-mice, C26s.c.TB-mice or normal mice were injected with 5×10^5 Colon26 cells into the spleen 42 days after mice were subcutaneously injected with 3×10^6 Colon26 cells. Twenty-four hours later, α-GalCer (2 μg/100 μl) or 100 μl of the vehicle was administered intraperitoneally to each mouse. Ten days after tumor injection, the livers of the treated mice were removed, and the liver weight was measured to examine intrahepatic tumor growth.

2.5. Flow cytometry

For phenotypic analysis of BM-DCs and liver DCs, PE- or FITC-conjugated monoclonal antibodies (Ab) against mouse cell surface molecules [CD1d, CD80, CD86 CD11c (all from BD-Pharmingen, San Diego, CA), MHC class II (Miltenyi Biotec)], and appropriate isotype controls were used. We defined DCs with CD11c+ MHC class II+ cells by flow cytometry. To detect the NK cell and NKT cell population in liver MNCs, MNC were stained with PE-conjugated DX5 Ab and FITC-conjugated TCRβ (all from BD-Pharmingen). C26s.c.TB-mice and normal mice were injected intraperitoneally with α-GalCer (2 μg/100 μl) or 100 μl of vehicle. Hepatic MNC were prepared on day 0, 1, 3 and 7 after α-GalCer injection, and both NK cell and NKT cell populations in hepatic MNC were evaluated by flow cytometry. Flow cytometric analysis was performed using a FACscan (Becton Dickinson, San Jose, CA) flow cytometer. The results of flow cytometric analysis are reported in arbitrary mean fluorescence intensity (MFI) units.

2.6. TGF-β and IL-10 ELISA

Mice sera from C26s.c.TB-mice were harvested 42 days after intrahepatic tumor injection. Mice sera and the culture supernatants of Colon26 cells were subjected to mouse TGF-β ELISA (R&D systems, Minneapolis, MN) and mouse IL-10 ELISA (BD-Pharmingen), with lower levels of detection of 31.2 and 31.3 pg/ml, respectively.

2.7. Cytotoxic assay

To evaluate the activation of liver NK cells in C26s.c.TB-mice treated with α-GalCer, liver MNC were isolated 48 h after α-GalCer injection and subjected to ^{51}Cr release assay against NK-susceptible YAC-1 target as previously described [4]. Assays were performed in triplicate, with spontaneous release of all assays not exceeding 25% of the maximum release.

2.8. Surgical resection of subcutaneous tumor

To assess the impact of subcutaneous tumor on the CD1d expression of liver DCs, subcutaneous Colon26 tumors were surgically resected on day 42 after subcutaneous injection of Colon26 cells (C26s.c.TB-ope mice). Fourteen days after subcutaneous tumor resection, liver DCs were isolated and subjected to flow cytometry to evaluate the CD1d expression. To examine antitumor effect of α-GalCer in the liver of C26s.c.TB-ope mice, C26s.c.TB-mice or C26s.c.TB-ope mice were injected with 5×10^5 Colon26 cells into the spleen 10 days after subcutaneous tumor resection. Twenty-four hours later, α-GalCer (2 μg/100 μl) was administered intraperitoneally as above. Ten days later, the livers of the treated mice were removed, and the liver weights were measured to examine intrahepatic tumor growth.

2.9. Statistical analysis

The statistical significance of differences between the groups was determined by applying compared t test with Welch correction or Mann–Whitney U test. The statistical significance of the differences in more than three groups was determined by applying one-way ANOVA. We defined statistical significance as $p < 0.05$.

3. Results

3.1. Expressions of CD1d on DCs in C26s.c.TB-mice were lower than those in normal mice

Since α-GalCer induces activation of NKT cells in a CD1d-dependent manner [1], the expression of CD1d plays an important role in the activation of NKT cells. We examined the CD1d expressions on DCs in C26s.c.TB-mice. The expressions of CD1d on BM-DCs were similar in both normal and C26s.c.TB-mice (Fig. 1A and B). In contrast, those on liver DCs from C26s.c.TB-mice were significantly lower than those from normal mice (Fig. 1A and C). Spleen DCs from C26s.c.TB-mice were also significantly lower than those from normal mice (Fig. 1A and D). These results demonstrated that systemic decrease of CD1d expressions on DCs in each organ is observed in C26s.c.TB-mice, but the potential of differentiation of CD1d expressing DCs from precursor cells in bone marrow was similar between in C26s.c.TB-mice and normal mice.

3.2. The activation of liver NK cells, liver NKT cells and liver DCs was impaired in C26s.c.TB-mice

We next examined the activation of liver NK cells and liver NKT cells in C26s.c.TB-mice after administration of α-GalCer. The cytolytic activity of liver NK cells in α-GalCer-treated mice was stronger than that in vehicle-treated mice in normal mice. In marked contrast, the cytolytic activities in both α-GalCer and vehicle-treated mice were very weak in C26s.c.TB-mice (Fig. 2A). In normal mice, the liver NK cell proportions in whole liver MNCs increased with the peak at 1 day after α-

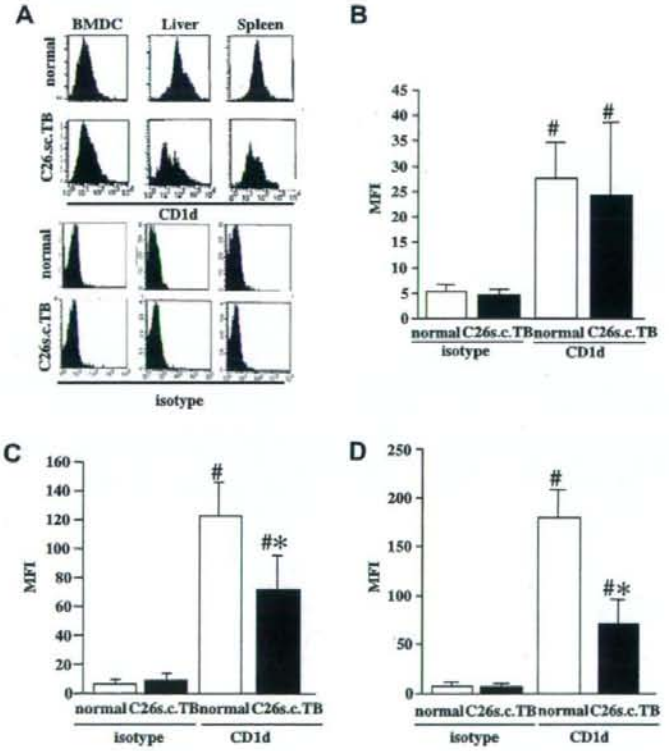

Fig. 1. CD1d expression on DCs in C26s.c.TB-mice. BM-DCs, liver and spleen DCs were prepared from C26s.c.TB-mice or normal mice ($N = 3$ in each group), and the expressions of CD1d molecules on DCs were evaluated by flow cytometry. The representative flow cytometry data of CD1d expressions on BM-DCs, liver DCs and spleen DCs were shown in Fig. 1A. The expression levels of CD1d molecules are reported in arbitrary MFI (mean ± SD). Normal: MFI of DCs from normal mice stained with anti-CD1d or isotype control antibody. C26s.c.TB: MFI of DCs from C26s.c.TB-mice stained with anti-CD1d or isotype control antibody. The CD1d expression on BM-DCs (B), on liver DCs (C), on spleen DCs (D). $^{\#}p < 0.05$ vs. respective isotype control $^{*}p < 0.05$ vs. CD1d expression in normal mice.

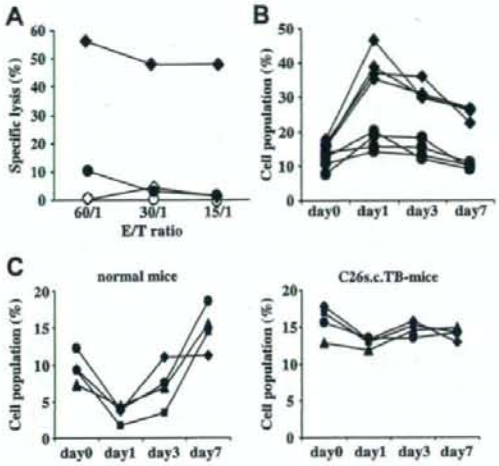

Fig. 2. Impaired activation of liver NK cells and NKT cells in C26s.c.TB-mice. (A) To evaluate the activation of liver NK cells in C26s.c.TB-mice treated by α-GalCer, liver MNC were isolated 48 h after α-GalCer injection and were subjected to ^{51}Cr release assay against NK-susceptible YAC-1 target. (♦) α-GalCer-treated normal mice, (◇) vehicle-treated normal mice, (•) α-GalCer-treated C26s.c.TB-mice, (○) vehicle-treated C26s.c.TB-mice. Representative data shown here is from three independent experiments. (B, C) BALB/c normal mice or C26s.c.TB-mice were injected intraperitoneally with α-GalCer. Hepatic MNC were prepared on day 0, 1, 3 and 7 days after α-GalCer injection. Liver NK cell and NKT cell populations in hepatic MNC were evaluated by flow cytometry. (B) Liver NK cell populations (DX5+/TCRβ− cells) in hepatic MNC after α-GalCer treatment. (♦) NK cell in each normal mice, (•) NK cell in each C26s.c.TB-mice ($N = 4$ in each group). (C) Liver NKT cell populations (DX5+/TCRβ+ cells) in hepatic MNC after α-GalCer treatment in normal mice and C26s.c.TB-mice ($N = 4$ in each group).

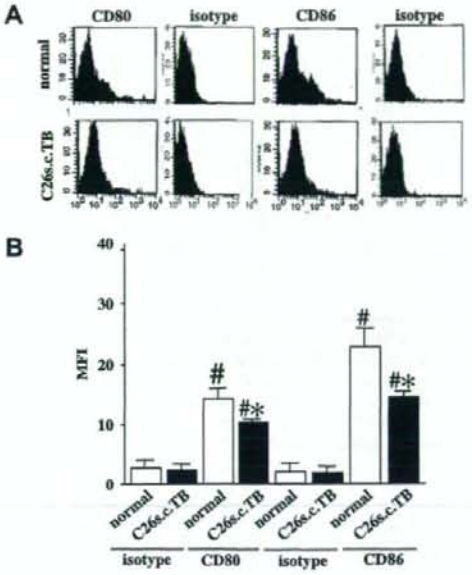

Fig. 3. The CD80 and CD86 expressions of liver DCs in C26s.c.TB-mice and normal mice. The expressions of CD80 and CD86 on liver DCs from both normal mice and C26s.c.TB-mice were evaluated by flow cytometry ($N = 3$ in each group). The representative flow cytometry data of CD80 and CD86 expressions on liver DC were shown in Fig. 3A. The expression levels of CD80 and CD86 molecules are reported as arbitrary MFI (mean ± SD of triplicate samples, Fig. 3B). $^{\#}p < 0.05$ vs. respective isotype control $^{*}p < 0.05$ vs. CD80 or CD86 expressions in normal mice.

GalCer administration, and the liver NK cell proportion at 7 days gradually decreased (Fig. 2B). C26s.c.TB-mice showed weaker increase of liver NK cell proportions in whole liver MNCs than normal mice (Fig. 2B). The liver NKT cell proportion decreased on day 1 and increased again on day 3 and day 7 after α-GalCer administration in normal mice. In marked contrast, those did not change on day 1, day 3 and day 7 after α-GalCer administration in C26s.c.TB-mice (Fig. 2C). The liver NK cell and NKT cell proportion in vehicle-treated mice exhibited no change in both mice groups (data not shown). These results demonstrated that the activation of liver NK cells and NKT cells by α-GalCer was impaired in C26s.c.TB-mice.

We also examined the CD80 and CD86 expressions of liver DCs in both C26s.c.TB-mice and normal mice, which are indicators of the antigen-presenting function of DCs. The expressions of CD80 and CD86 molecules on liver DCs from C26s.c.TB-mice were significantly lower than those from normal mice after α-GalCer administration (Fig. 3), suggesting that the antigen-presenting function of liver DC in C26s.c.TB-mice was also impaired compared with normal mice.

3.3. The antitumor effect of α-GalCer administration against metastatic liver tumor was impaired in C26s.c.TB-mice

We examined the antitumor effect of α-GalCer administration against metastatic liver tumor in both normal and C26s.c.TB-mice. With normal mice, no tumor formation was observed in the liver of any of the α-GalCer-treated mice although large Colon26 liver tumors had formed in all vehicle-treated mice. In contrast, with the C26s.c.TB-mice, large Colon26 liver tumors had formed in both α-GalCer-treated and vehicle-treated mice. The liver weights of the α-GalCer treatment group were significantly lighter than those of the vehicle treatment group for normal mice, while they were similar for both groups of the C26s.c.TB-mice (Fig. 4). These results demonstrated that the antitumor effect of α-GalCer against metastatic liver tumor was impaired in C26s.c.TB-mice.

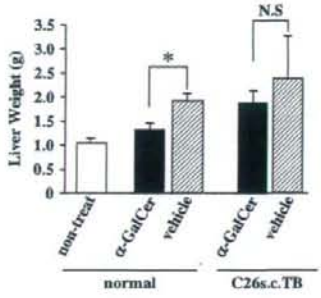

Fig. 4. Impaired antitumor effect of α-GalCer treatment against Colon26 liver tumor in C26s.c.TB-mice. To establish C26s.c.TB-mice, BALB/c mice were subcutaneously injected with 3×10^6 Colon26 cells 42 days before intrasplenic injection of tumor cells. BALB/c normal mice or C26s.c.TB-mice were injected into spleen with 5×10^5 Colon26 cells, and 24 h later either α-GalCer or vehicle was administered intraperitoneally ($N = 6$ in each treatment group). Ten days after treatment, the livers were removed from all treated mice and the liver weights of the groups were compared. As a control, the mean liver weights of untreated normal mice were 1.08 ± 0.09 g. $^*p < 0.05$. α-GalCer treatment group vs. vehicle treatment group in normal mice. N.S. α-GalCer treatment group vs. vehicle treatment group in C26s.c.TB-mice.

3.4. Serum TGF-β levels in C26s.c.TB-mice were increased compared with those in normal mice

Previous reports demonstrated that CD1d expressions on DCs decreased after co-culture with either TGF-β [10] or IL-10 [11]. The supernatants of 24 h cultures of Colon26 cells were subjected to TGF-β and IL-10 ELISA. The production of TGF-β in the supernatants of Colon26 was significantly higher than the control medium (Fig. 5A). No production of IL-10 was detected in the supernatants of Colon26 cells (data not shown). We next evaluate the serum TGF-β and IL-10 levels in C26s.c.TB-mice. The levels of TGF-β in C26s.c.TB-mice were significantly higher than that in normal mice (Fig. 5B). IL-10 was not detected in all mice sera from C26s.c.TB-mice and normal mice (data not shown).

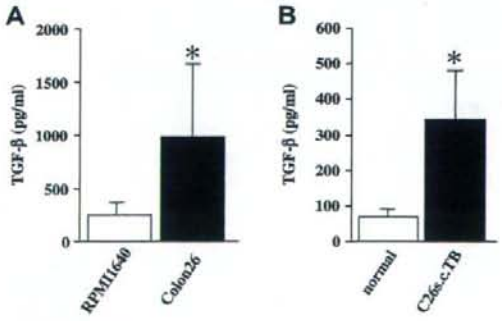

Fig. 5. The TGF-β production from Colon26 cells and the increase in serum TGF-β levels in C26s.c.TB-mice. (A) The culture supernatants of Colon26 cells or culture medium only (RPMI1640) were subjected to mouse TGF-β ELISA. (B) Mice sera from C26s.c.TB-mice were harvested 42 days after subcutaneous tumor injection and were subjected to mouse TGF-β ELISA. Mice sera from normal mice were used as controls. Cytokine levels are reported in pg/ml (mean ± SD of triplicate samples). Similar results were obtained in two independent experiments. $^*p < 0.05$.

3.5. Serum TGF-β levels decreased, the expression of CD1d molecules on liver DCs increased and the antitumor effect of α-GalCer was improved after tumor mass reduction

We next examined serum TGF-β levels and the CD1d expressions on liver DCs after surgical mass reduction in C26s.c.TB-mice. BALB/c mice were subcutaneously injected with 3×10^6 Colon26. On day 42, most Colon26 subcutaneous tumors were surgically excised (C26s.cTB-ope mice). Fourteen days later, serum TGF-β levels were evaluated, and liver DCs from C26s.c.TB-ope mice were prepared to evaluating the CD1d expression in comparison with those from C26s.c.TB-mice. The serum TGF-β levels in C26s.c.TB-ope mice were significantly lower than those in C26s.c.TB-mice (Fig. 6A). The expressions of CD1d on liver DCs from C26s.c.TB-ope mice were significantly higher than those from C26s.c.TB-mice and were similar to those from normal mice (Fig. 6B and C). These results demonstrated that surgical tumor mass reduction might lead to recovery of the impaired immune circumstances in the liver of C26s.c.TB-mice. We examined the antitumor effect of α-GalCer administration against metastatic liver tumor in both C26s.c.TB-mice and C26s.c.TB-ope mice. The liver weights of α-GalCer treated C26s.c.TB-ope mice were significantly lighter than those of α-GalCer treated C26s.c.TB-mice (Fig. 6D). These results demonstrated that the antitumor effect of α-GalCer against metastatic liver tumor was improved after subcutaneous tumor mass resection.

4. Discussion

A previous study showed that administration of α-GalCer resulted in complete rejection of Colon26 metastatic liver cancer in normal mice [5]. In the current study, we evaluated the antitumor effect of α-GalCer against the same Colon26 metastatic liver tumor model in C26s.c.TB-mice. α-GalCer treatment resulted in complete rejection of metasatic Colon26 liver tumor in normal mice, but the antitumor effect of α-GalCer against metastatic liver tumor was significantly impaired in C26s.c.TB-mice. These results were consistent with the clinical data of α-GalCer treatment in