

# Activation of V $\alpha$ 24NKT cells in malignant pleural effusion in patients with lung cancer

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**Abstract.** V $\alpha$ 24NKT cells are lymphocytes expressing both T-cell antigen receptors and NK-cell antigen receptors on their cell surface and are involved in tumor immunity. They exert their antitumor effects after being activated by a specific ligand,  $\alpha$ -galactosyl ceramide ( $\alpha$ -GalCer). Malignant pleural effusion, a frequently occurring complication in patients with lung cancer, contains numerous lymphocytes. In the present study, we examined the presence and functions of V $\alpha$ 24NKT cells in the lymphocytes in pleural effusion *in vitro*. The subjects were 13 untreated patients with primary lung cancer, who suffered malignant pleural effusion as a complication and who were treated between April 2004 and October 2007 at our hospital. Mononuclear cells were separated from the malignant pleural effusion and incubated with  $\alpha$ -GalCer and IL-12. The production of IFN- $\gamma$  and IL-4 after incubation and the proportion of the V $\alpha$ 24NKT cells before and after incubation were determined and compared. In the group cultured with  $\alpha$ -GalCer alone, no significant increase in IFN- $\gamma$  production was observed in comparison with the control group. In the group cultured with  $\alpha$ -GalCer + IL-12, IFN- $\gamma$  production increased significantly in comparison with the control group, and the proportion of V $\alpha$ 24NKT cells increased after incubation. IL-4 production was very much lower than IFN- $\gamma$  production. V $\alpha$ 24NKT cells were present in malignant pleural effusion in patients with lung cancer, but IFN- $\gamma$  production did not increase after addition of  $\alpha$ -GalCer alone. The V $\alpha$ 24NKT cells were activated by  $\alpha$ -GalCer in the presence of IL-12. The V $\alpha$ 24NKT cells in malignant pleural effusion were not activated by  $\alpha$ -GalCer alone, suggesting that V $\alpha$ 24NKT cell function is attenuated in malignant pleural effusion.

## Introduction

NKT cells are an immunocompetent cell population expressing both the T-cell antigen receptor and NK-cell antigen receptor on their cell surface. In humans, the chief component cells are V $\alpha$ 24NKT cells. Unlike T-cells, B-cells or NK-cells, the V $\alpha$ 24NKT cells are called the 'fourth lymphocytes.' The V $\alpha$ 24NKT cells are mainly engaged in prevention of infection (1) and tumor immunity (2); and are known to be involved in allergic diseases (3), transplantation immunity (4) and autoimmune diseases (5,6). In tumor immunity in particular, there have been numerous reports concerning anti-tumor effects in mice, including inhibition of hepatic metastasis of malignant tumors (7) and immunotherapy with NKT cells for patients with lung cancer (8,9). V $\alpha$ 24NKT cells express a T-cell receptor (TCR), but unlike T-cells, the TCR of V $\alpha$ 24NKT cells lack diversity, the  $\alpha$ -chain expresses the V $\alpha$ 24, and the  $\beta$ -chain mainly expresses the V $\beta$ 11 (10,11). The ligands to the V $\alpha$ 24NKT cells also lack diversity, and a type of glycolipid,  $\alpha$ -galactosyl ceramide ( $\alpha$ -GalCer), has been identified as a specific ligand of the V $\alpha$ 24NKT cells (12). The V $\alpha$ 24NKT cells actively produce cytokines, as well as IFN- $\gamma$  which induces Th1, and IL-4 which induces Th2 when activated (13), and have been indicated as being involved in controlling the differentiation of Th1 and Th2 of helper T-cells. Moreover, IL-12 is required for activation of V $\alpha$ 24NKT cells with  $\alpha$ -GalCer, and acts synergistically with  $\alpha$ -GalCer and induces IFN- $\gamma$  production from the V $\alpha$ 24NKT cells (14).

Lung cancer, which has a poor prognosis, is on the increase worldwide. Since its initial symptoms are not clear, and it tends to be a relatively fast-progressing cancer, it is commonly found only at an advanced stage. Since the efficacy of chemotherapy is not very high, this disease remains a challenging type of cancer. Various complications appear in advanced lung cancer, among which malignant pleural effusion occurs frequently, at about 10% (15). In malignant pleural effusion, pleural fluid generally increases progressively, which induces dyspnea and markedly reduces the QOL of patients. Numerous lymphocytes are present in the cell component of malignant pleural effusion in addition to malignant cells. Lymphocytes commonly infiltrate into tumor tissues, and these lymphocytes are termed tumor-infiltrating lymphocytes (TILs) (16). They appear in response to tumor cells, and comprise mainly cytotoxic T-cells (17), but they remain little

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**Key words:** V $\alpha$ 24NKT cell,  $\alpha$ -GalCer, IL-12, IFN- $\gamma$ , IL-4, malignant pleural effusion

Table I. Patient characteristics.

Characteristics	Patients
No.	13
Age years	
Mean	61.2
Range	(43-77)
Gender	
Male	8
Female	5
Stage	
IIIb	6
IV	7
Histological classification	
adenocarcinoma	11
squamous cell carcinoma	1
small cell carcinoma	1
PS	
$\leq 2$	11
$\geq 3$	2
Number of cells	
Mean	2450
Range	(130-3850)
Lymphocyte rate	
Mean	41.1
Range	(0.2-95.0)

PS, performance status of Eastern Cooperative Oncology Group; number of cells, the number of nucleated cells in malignant pleural effusion; lymphocyte rate, the rate of lymphocytes in malignant pleural effusion.

understood. TILs are part of the key to elucidating the mechanism of tumor immunity, and the lymphocytes in malignant pleural effusion are thought to appear in response to malignant cells, and are thus a type of TIL. There have been numerous studies of TILs in tumor immunity, but the V $\alpha$ 24NKT cell components of the TILs have been rarely studied. V $\alpha$ 24NKT cells are non-uniformly located in organs and are commonly present in the lung and liver (18), but the involvement of V $\alpha$ 24NKT cells in malignant pleural effusion is unclear. In this study, we examined the presence of V $\alpha$ 24NKT cells and cytokine secretion in the lymphocytes in malignant pleural effusion using  $\alpha$ -GalCer and IL-12, specific ligands of the V $\alpha$ 24NKT cells. Moreover, there are various lymphocytes involved in tumor immunity, and CD4<sup>+</sup>CD25<sup>+</sup>-regulatory T-cells, which suppressively control immune reactions, have recently been discovered. The CD4<sup>+</sup>CD25<sup>+</sup>-regulatory T-cells are thought to act suppressively in tumor immunity (19), but their involvement in malignant pleural effusion and effects on the V $\alpha$ 24NKT cells are not clear.

The relationship between V $\alpha$ 24NKT cells and CD4<sup>+</sup>CD25<sup>+</sup>-regulatory T-cells in malignant pleural effusion was examined using anti-CTLA-4 antibody and anti-CD25 antibody, which inhibit the functions of the CD4<sup>+</sup>CD25<sup>+</sup>-regulatory T-cells. Since the objective of this study was to examine whether or not the V $\alpha$ 24NKT cells in malignant pleural effusion retain any antitumor immune capacity, we examined the functions of the V $\alpha$ 24NKT cells in malignant pleural effusion in patients with untreated lung cancer.

## Materials and methods

**Subjects.** The subjects were 13 untreated patients with primary lung cancer, treated between April 2004 and October 2007 at our hospital, who suffered malignant pleural effusion as a complication. The details of the study were explained to all the subjects, who gave their informed consent. The diagnosis of malignant pleural effusion was defined as patients showing cancer cells in cytological tests of pleural fluid. Patients with complications of diseases leading to immune abnormalities and those taking drugs affecting immunity were excluded as subjects. The patients' backgrounds are shown in Table I. As for systemic condition, there were 11 patients with performance status of Eastern Cooperative Oncology Group (PS) 2 or lower, commonly showing good PS, and the tissue type was adenocarcinoma in 11 subjects. The mean number of nucleated cells in malignant pleural effusion was 2450 cells/ml, and the mean percentage of lymphocytes occupying these nucleated cells was 41.1%.

**Separation of cells.** Thoracentesis was performed on untreated patients to collect malignant pleural effusion. The pleural fluid obtained was immediately centrifuged, and the cell components were suspended in PBS (Nissui Pharmaceutical Co., Tokyo, Japan). Mononuclear cells were separated from this cell suspension using a density separation medium (Lymphoprep: Fresenius Kabi Norge AS for Axis-Shield PoC AS, Oslo, Norway). The separated mononuclear cells were suspended in RPMI-1640 (Nissui Pharmaceutical Co.), the cell concentration was adjusted to  $1 \times 10^6$  cells/ml, and this suspension was used as the mononuclear cell suspension.

**Cell culture.** The lymphocytes contained in malignant pleural effusion were present in the fraction of the separated mononuclear cells. Cell culture was performed by addition of  $\alpha$ -GalCer, IL-12, and monoclonal antibody to these mononuclear cells. We prepared the mononuclear cell suspension with  $\alpha$ -GalCer (100 ng/ml, KRN7000; Kirin Brewery Co., Tokyo, Japan) only (GalCer group), with IL-12 (10 ng/ml, Recombinant Human Interleukin-12; Strathmann Biotec AG, Hamburg, Germany) only (IL-12 group), with  $\alpha$ -GalCer (100 ng/ml) and IL-12 (10 ng/ml) (GalCer + IL-12 group), with  $\alpha$ -GalCer (100 ng/ml) and anti-CTLA-4 (100 ng/ml, anti-CD152; Calbiochem, San Diego, CA) (GalCer + CTLA group), with  $\alpha$ -GalCer (100 ng/ml), IL-12 (10 ng/ml) and anti-CTLA-4 (100 ng/ml) (GalCer + IL-12 + CTLA group), with  $\alpha$ -GalCer (100 ng/ml) and anti-CD25 (100 ng/ml; BD Biosciences, San Diego, CA) (GalCer + CD25 group), with  $\alpha$ -GalCer (100 ng/ml), IL-12 (10 ng/ml) and anti-CD25



SPANDIDOS PUBLICATIONS Amount of IFN- $\gamma$  in culture medium.

	Amount of IFN- $\gamma$ $\pm$ SD (pg/ml)
Control group	25.952 $\pm$ 48.498
GalCer group	41.285 $\pm$ 109.216
IL-12 group	81.225 $\pm$ 127.582
GalCer + IL-12 group	120.559 $\pm$ 171.395

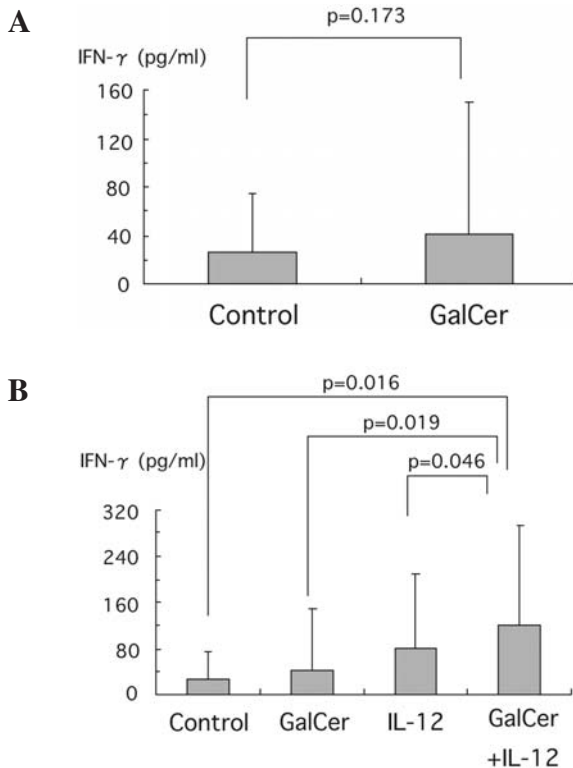


Figure 1. Amount of IFN- $\gamma$  in the culture supernatant fluid after culture of lymphocytes, (A) in the control group and the GalCer group, (B) among four groups.

(GalCer + IL-12 + CD25 group), with nothing (the control group). Aliquots (1 ml) of the mononuclear cell suspension were dispensed into 24-well plates ( $1 \times 10^6$  cells/well) and cultured at 37°C under 5% CO<sub>2</sub> for 14 days.

**Determination of IFN- $\gamma$  and IL-4.** The culture supernatant fluid after culture was collected, and the IFN- $\gamma$  and IL-4 present in the culture supernatant fluid were determined by ELISA (human IFN- $\gamma$  Instant ELISA, human IL-4 Instant ELISA; Bender MedSystems, Vienna, Austria).

**Flow cytometric analysis.** The proportion of V $\alpha$ 24NKT cells contained in the mononuclear cell suspension was determined by flow cytometry before and after cell culture. Cells expressing both V $\alpha$ 24 and V $\beta$ 11 were identified as V $\alpha$ 24NKT cells. The mononuclear cell suspension was cultured with  $\alpha$ -GalCer and IL-12 for 14 days.

**Statistical analysis.** The levels of IFN- $\gamma$  and IL-4 present in the culture supernatant fluid were compared using Wilcoxon's signed rank test. The level of significance was set at  $p < 0.05$ . Statistical analysis was performed using SPSS 11.0.1J.

## Results

**Level of IFN- $\gamma$  present in the culture supernatant fluid after culture of lymphocytes (Table II, Fig. 1).** The mean level of IFN- $\gamma$  present in the culture supernatant fluid after culture in the control group was 25.952 pg/ml. The mean level of IFN- $\gamma$  present in the culture supernatant fluid after culture in the GalCer group was 41.285 pg/ml. When the level of IFN- $\gamma$  in the culture supernatant fluid was compared between these two groups, no statistically significant difference was observed. The mean level of IFN- $\gamma$  present in the culture supernatant fluid after culture in the IL-12 group was 81.225 pg/ml. When the level of IFN- $\gamma$  in the culture supernatant fluid was compared between the control group and IL-12 group, no statistically significant difference was observed. The mean level of IFN- $\gamma$  present in the culture supernatant fluid after culture in the GalCer + IL-12 group was 120.559 pg/ml. When the IFN- $\gamma$  level in the culture supernatant fluid was compared between the control group and GalCer + IL-12 group, the level of IFN- $\gamma$  in the GalCer + IL-12 group was significantly higher than the control group. The level of IFN- $\gamma$  in the GalCer + IL-12 group was significantly higher than that in the  $\alpha$ -GalCer group or IL-12 group.

**Level of IL-4 present in the culture supernatant fluid after culture of lymphocytes (Table III, Fig. 2).** The mean level of IL-4 present in the culture supernatant fluid after culture in the control group was 1.742 pg/ml. The level of IL-4 present in the culture supernatant fluid after culture in the GalCer group was 4.105 pg/ml. When the level of IL-4 in the culture supernatant fluid was compared between these two groups, the level of IL-4 in the GalCer group was significantly higher than the control group. The mean level of IL-4 present in the culture supernatant fluid after culture in the GalCer + IL-12 group was 4.399 pg/ml. The level of IL-4 in the GalCer + IL-2 group was significantly higher than the control group. In the GalCer + IL-12 group, however, there was no significant increase in the level of IL-4 in comparison with the GalCer group or the IL-12 group.

**Proportion of V $\alpha$ 24NKT cells in the mononuclear suspension before and after culture.** The proportion of the V $\alpha$ 24NKT cells in the mononuclear cell suspension was determined by flow cytometry. We found that the proportion of the V $\alpha$ 24NKT cells tended to increase after culture. Typical data are shown in Fig. 3. The proportion of the V $\alpha$ 24NKT cells before culture was 0.05%. After culture along with both  $\alpha$ -GalCer and IL-12, the proportion of the V $\alpha$ 24NKT cells increased to 0.07%.

**Effects of anti-CTLA-4 antibody and anti-CD25 antibody on activation of the V $\alpha$ 24NKT cells (Fig. 4).** In five of the

Table III. Amount of IL-4 in culture medium.

	Amount of IL-4 $\pm$ SD (pg/ml)
Control group	1.742 $\pm$ 1.886
GalCer group	4.105 $\pm$ 2.607
IL-12 group	3.519 $\pm$ 3.343
GalCer + IL-12 group	4.399 $\pm$ 1.685

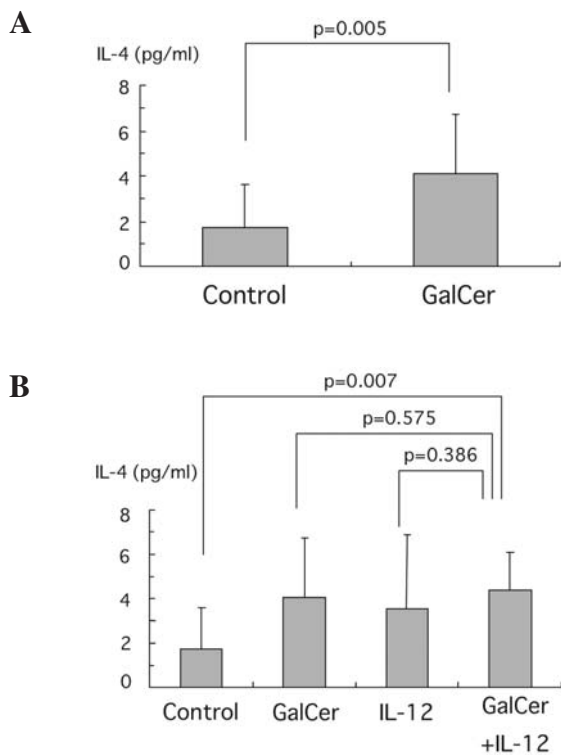


Figure 2. Amount of IL-4 in the culture supernatant fluid after culture of lymphocytes, (A) in the control group and the GalCer group, (B) among four groups.

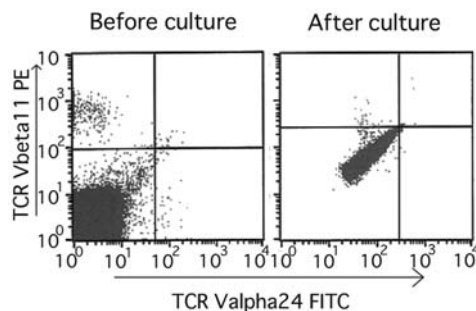


Figure 3. Flow cytometric profiles from one lung cancer patient. The frequency of V $\alpha$ 24NKT cells before and after culture is shown.

subjects, the lymphocyte culture fluid added anti-CTLA-4 antibody was cultured to examine its effects on activation of the V $\alpha$ 24NKT cells. The mean level of IFN- $\gamma$  present in the culture supernatant fluid after culture in the GalCer + CTLA group was 6.585 pg/ml. The mean level of IFN- $\gamma$  in the GalCer

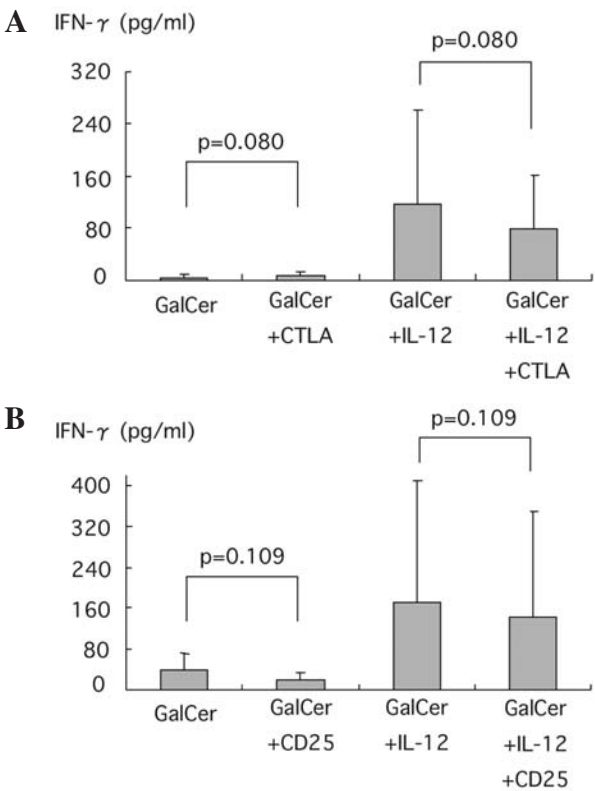


Figure 4. Amount of IFN- $\gamma$  in the culture supernatant fluid after culture of lymphocytes in the GalCer group and the GalCer+IL-12 group, (A) with anti-CTLA-4, (B) with anti-CD25.

group was 3.554 pg/ml, with no statistically significant difference observed between these two groups. The mean level of IFN- $\gamma$  present in the culture supernatant fluid after culture in the GalCer + IL-12 + CTLA group was 79.330 pg/ml. The mean level of IFN- $\gamma$  in the GalCer + IL-12 group was 116.669 pg/ml, with no statistically significant difference observed between these two groups.

In three of the subjects, moreover, the lymphocyte culture fluid added anti-CD25 antibody was cultured to examine its effects on activation of V $\alpha$ 24NKT cells. The mean level of IFN- $\gamma$  present in the culture supernatant fluid after culture in the GalCer + CD25 group was 19.107 pg/ml. The mean level of IFN- $\gamma$  in the GalCer group was 39.777 pg/ml, with no statistically significant difference observed between these two groups. The mean level of IFN- $\gamma$  present in the culture supernatant fluid after culture in the GalCer + IL-12 + CD25 group was 143.653 pg/ml. The mean level of IFN- $\gamma$  in the GalCer + IL-12 group was 171.602 pg/ml, with no statistically significant difference observed between these two groups.

Discussion

A specific ligand of the V $\alpha$ 24NKT cells,  $\alpha$ -GalCer, is a relatively recently-discovered glycolipid. Microorganism-derived glycosylphosphatidylinositol (GPI) has been identified as another ligand of the V $\alpha$ 24NKT cells (20). The structure of  $\alpha$ -GalCer is a glycolipid in which a ceramide, consisting of a fatty acid, C26 cerotic acid and a long chain base, C18 phytosphingosine, is bound to galactose at the  $\alpha$ -position (21). Dendritic cells (DCs) are required for activation of





NKT cells with  $\alpha$ -GalCer. As the mechanism of action of the  $V_{\alpha}24$ NKT cells,  $\alpha$ -GalCer first binds to the CD1d present on the surface of the DCs. Next, the  $V_{\alpha}24$ NKT cells recognize the  $\alpha$ -GalCer bound to the CD1d via the TCR and react with the DCs. As a result, the DCs produce IL-12, and this endogenously-produced IL-12 stimulates the  $V_{\alpha}24$ NKT cells to produce IFN- $\gamma$  and IL-4. The IFN- $\gamma$  produced by the  $V_{\alpha}24$ NKT cells increases the number of IL-12 receptors on the surface of the  $V_{\alpha}24$ NKT cells via an autocrine action (14). IL-12 is a key cytokine in the activation of the  $V_{\alpha}24$ NKT cells with  $\alpha$ -GalCer, which is produced mainly from DCs or macrophages, and which activates NK cells and T-cells in addition to  $V_{\alpha}24$ NKT cells (22).  $\alpha$ -GalCer does not act on NK cells or T-cells, since  $V_{\alpha}24$ NKT cells are the only target cells of  $\alpha$ -GalCer (14). In the present study, we cultured *in vitro* the lymphocytes in malignant pleural effusion from patients with lung cancer along with  $\alpha$ -GalCer and IL-12 to examine the functions of the  $V_{\alpha}24$ NKT cells in malignant pleural effusion.

The level of IFN- $\gamma$  in the culture supernatant fluid in lymphocytes in malignant pleural effusion did not increase significantly in the GalCer group in comparison with the control group. The lymphocytes in malignant pleural effusion were not activated by  $\alpha$ -GalCer alone. In the GalCer + IL-12 group, IFN- $\gamma$  was significantly higher than the control group, indicating that the lymphocytes in malignant pleural effusion were activated by  $\alpha$ -GalCer in the presence of IL-12. Production of IFN- $\gamma$  also increased in the IL-12 group, but since it is impossible to activate the  $V_{\alpha}24$ NKT cells with IL-12 alone, this event was considered attributable to the fact that the NK cells or T-cells contained in the malignant pleural fluid were activated. The production of IFN- $\gamma$  significantly increased in the GalCer + IL-12 group compared with the GalCer group and IL-12 group. We believe that, since  $\alpha$ -GalCer activates only  $V_{\alpha}24$ NKT cells and since the proportion of the  $V_{\alpha}24$ NKT cells contained in the lymphocytes in malignant pleural effusion in the GalCer + IL-12 group increased after culture compared with that before culture, the  $V_{\alpha}24$ NKT cells in malignant pleural effusion were activated by  $\alpha$ -GalCer in the presence of IL-12 and produced IFN- $\gamma$  as a result. Generally, the  $V_{\alpha}24$ NKT cells are activated by administration of  $\alpha$ -GalCer alone plus the endogenous IL-12 secreted from the DCs, but in the present experiment, they were not activated by administration of  $\alpha$ -GalCer alone but required the presence of exogenous IL-12. It has been reported that DCs are present in malignant pleural effusion and have antigen-presentation capacity (23). The reason for the  $V_{\alpha}24$ NKT cells in malignant pleural effusion not being activated by  $\alpha$ -GalCer alone may be that the local tumor immunity functions of DC decreased and that the production of IL-12 from the DCs also decreased.  $V_{\alpha}24$ NKT cells are commonly present in the liver and lung, but in our results, they are present in very low proportion, as only 0.05% of lymphocytes in malignant pleural effusion. In an examination by Yanagisawa *et al.*, of  $V_{\alpha}24$ NKT cells using peripheral blood mononuclear cells in tumor-bearing patients moreover, it was reported that IFN- $\gamma$  production from the  $V_{\alpha}24$ NKT cells and the increase in the  $V_{\alpha}24$ NKT cells resulting from

administration of  $\alpha$ -GalCer surpassed those in our results (24). It is impossible to compare these results directly, however, because the subjects and methods were not identical; but since it is possible that the functions of the  $V_{\alpha}24$ NKT cells decrease in TILs in the tumor region, compared with those in the peripheral blood in tumor-bearing patients, it was considered necessary to compare the functions of the  $V_{\alpha}24$ NKT cells in the peripheral blood with those of the  $V_{\alpha}24$ NKT cells contained in the TIL in the same tumor-bearing patients.

The level of IL-4 in the culture supernatant fluid in the lymphocytes in malignant pleural effusion was significantly higher in the GalCer group than in the control group. However, the level of IL-4 produced from the  $V_{\alpha}24$ NKT cells in malignant pleural effusion was very low, at 4.104 pg/ml. The GalCer + IL-2 group showed no significant increase in IL-4 compared with the GalCer group, and unlike the result for IFN- $\gamma$ , there was no increase in IL-4 after the addition of IL-12. We conclude that the IL-4 production did not increase because IL-12 induces Th1 but inhibits Th2. The balance between Th1 and Th2 in the helper T-cells is increasingly being thought to have a major influence on immunological control (25,26), and is also regarded as playing an important role in tumor immunity. In tumor immunity, the Th1 type cytokine, including IFN- $\gamma$ , is chiefly involved in antitumor effects, and the superiority of Th1 in the Th1/Th2 balance in local tumors promotes their elimination. It is not known whether the  $V_{\alpha}24$ NKT cells producing the cytokines of Th1 and Th2 types act locally on tumors predominantly with the Th1 type or Th2 type. Our results indicate that when the  $V_{\alpha}24$ NKT cells in malignant pleural effusion are activated by  $\alpha$ -GalCer and IL-12, more IFN- $\gamma$  is produced than IL-4, suggesting that local tumors can be induced predominantly with Th1. Based on studies of TILs, the Th1/Th2 balance in local tumors varies among tumor-bearing patients, but it appears useful for elimination of cancer to make the Th1 dominant in this balance.

The CD4<sup>+</sup>CD25<sup>+</sup>-regulatory T-cells are involved in maintenance of immunological self-tolerance, and their association with autoimmune diseases, allergy, transplantation immunity and tumor immunity has been observed. In an experiment in mice, anti-tumor immunity was enhanced by removal of the CD4<sup>+</sup>CD25<sup>+</sup>T-cells (27), and there is a possibility that immune responses to tumor cells may be attenuated by the CD4<sup>+</sup>CD25<sup>+</sup>-regulatory T-cells. Anti-CTLA-4 antibody and anti-CD25 antibody reduce the functions of the CD4<sup>+</sup>CD25<sup>+</sup>-regulatory T-cells and provoke antitumor immune responses (28,29). The presence of CD4<sup>+</sup>CD25<sup>+</sup>-regulatory T-cells in malignant pleural effusion has been confirmed (data not shown), but it is not known if CD4<sup>+</sup>CD25<sup>+</sup>-regulatory T-cells exert an influence on antitumor immunity with  $V_{\alpha}24$ NKT cells. We reduced the function of the CD4<sup>+</sup>CD25<sup>+</sup>-regulatory T-cells by addition of anti-CTLA-4 antibody and anti-CD25 antibody to the lymphocytes in pleural effusion and examined the resultant effects on activation of the  $V_{\alpha}24$ NKT cells. When the cell suspensions in the GalCer group and the GalCer + IL-12 group were cultured along with anti-CTLA-4 antibody, there was no significant increase in IFN- $\gamma$  production. With anti-CD25 antibody, moreover, no significant

increase in IFN- $\gamma$  production was observed. Anti-CTLA-4 antibody or anti-CTLA-4 antibody showed no enhancement of activation of the V $\alpha$ 24NKT cells in malignant pleural effusion. There have been few studies of CD4<sup>+</sup>CD25<sup>+</sup>-regulatory T-cells in malignant pleural effusion, and until now, no relationship with the V $\alpha$ 24NKT cells has been reported. In the present results, there was no enhancement of the functions of the V $\alpha$ 24NKT cells due to suppression of the CD4<sup>+</sup>CD25<sup>+</sup>-regulatory T-cells in the lymphocytes in malignant pleural effusion.

In this study, V $\alpha$ 24NKT cells were present in malignant pleural effusion in patients with lung cancer, but their proportion was low, and no obvious activation occurred with  $\alpha$ -GalCer alone. This may be because the functions of the V $\alpha$ 24NKT are attenuated in malignant pleural effusion in patients with lung cancer. We suspect that factors that suppress antitumor immunity are involved in the decreased functions of the V $\alpha$ 24NKT cells, but this attenuation was corrected by the addition of IL-12. To confer antitumor effects on V $\alpha$ 24NKT cells in malignant pleural effusion, we believe it will be necessary to elucidate, in further studies, the mechanism that suppresses these functions.

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