②電話対応の場合

②-1 試験担当医師へ電話にて問い合わせを行った場合、「症例登録票」（研究者作成様式-別紙 3）の余白に治験実施医療機関へ電話にて問い合わせた旨を赤字で記載する。その際、日時、対応者、問い合わせ先氏名、内容等を記録する。

②-2 軽微な訂正で「症例登録票」（研究者作成様式-別紙 3）への記載ですむ場合は、赤字にて修正、及び必要に応じて確認者の署名を入れる。

②-3 重大な訂正で「症例登録票」（研究者作成様式-別紙 3）の訂正が必要な場合は、速やかに、当該治験実施医療機関より訂正済みの「症例登録票」（研究者作成様式-別紙 3）を FAX 送信してもらう。

※「登録内容変更依頼書」の場合も、上記に準じる手順を実施する。

(3) 記載の不備の内容が明らかな場合、例えば治験実施医療機関名の略称での記載、単語の意味に影響を与えない誤字、患者プライバシー情報の記入（この場合はマスキング等して保存）等の場合は、登録センターは治験実施医療機関へ通知することなく受け入れることができる。

4.2.問い合せ後の処理

問い合せ内容について確認が完了した後、訂正・修正済の「症例登録票」（研究者作成様式-別紙 3）を用いて、登録処理を行う。

5. 不具合

登録センター業務において、不具合（エラー等）が発生した場合、登録担当者は速やかに登録責任者に報告する。登録責任者は不具合に対する改善を指示する。必要に応じて、本マニュアルも改善する。

6. 資料の保管

当該手続きにおいて発生した資料は、治験終了時まで登録センターにて保管する。原本はその後、治験調整事務局と確認の上、移管する。

7. 様式一覧

様式	送信者（作成者）	受信者（提出先）
登録内容変更依頼書	医療機関	登録センター
登録内容変更完了通知書	登録センター	医療機関
症例登録票	医療機関	症例適格性委員会／登録センター
症例登録結果確認票	登録センター	医療機関
症例登録内容ご確認のお願い	登録センター	医療機関
不適格のお知らせ	登録センター	医療機関
症例登録担当者の指名書	登録センター	—

Ⅲ. 研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nagato, K., Motohashi, S., Ishibashi, F., Okita, K., Yamasaki, K., Moriya, Y., Hoshino, H., Yoshida, S., Hanaoka, H., Fujii, S., Taniguchi, M., Yoshino, I. and Nakayama, T.	Accumulation of activated invariant natural killer T cells in the tumor microenvironment after α-Galactosylceramide-pulsed antigen presenting cells.	J Clin Immunol.	32	1071-1081	2012
Iwamura, C., Shinoda, K., Endo, Y., Watanabe, Y., Tumes, J. D., Motohashi, S., Kawahara, K., Kinjo, Y. and Nakayama, T.	Regulation of memory CD4 T-cell pool size and function by natural killer T cells in vivo.	Proc Natl Acad Sci U S A.	109	16992-16997	2012

J Clin Immunol (2012) 32:1071–1081
DOI 10.1007/s10875-012-9697-9

Accumulation of Activated Invariant Natural Killer T Cells in the Tumor Microenvironment after α-Galactosylceramide-Pulsed Antigen Presenting Cells

Kaoru Nagato · Shinichiro Motohashi · Fumihiro Ishibashi · Kohsuke Okita · Kazuki Yamasaki · Yasumitsu Moriya · Hidehisa Hoshino · Shigetoshi Yoshida · Hideki Hanaoka · Shin-ichiro Fujii · Masaru Taniguchi · Ichiro Yoshino · Toshinori Nakayama

Received: 31 January 2012 / Accepted: 10 April 2012 / Published online: 26 April 2012
© Springer Science+Business Media, LLC 2012

Abstract

Purpose The intravenous administration of α-Galactosylceramide (α-GalCer)-pulsed antigen presenting cells (APCs) is well tolerated and the increased IFN-γ producing cells in the peripheral blood after the treatment appeared to be associated with prolonged survival. An exploratory study protocol was designed with the preoperative administration of α-GalCer-pulsed APCs to clarify the mechanisms of these findings, while especially focusing on the precise tumor site.

Methods Patients with operable advanced lung cancer received an intravenous injection of α-GalCer-pulsed APCs before surgery. The resected lung and tumor infiltrating lymphocytes (TILs) as well as peripheral blood mononuclear cells were collected and the invariant NKT (iNKT) cell-specific immune responses were analyzed.

Results Four patients completed the study protocol. We observed a significant increase in iNKT cell numbers in the TILs and augmented IFN-γ production by the α-GalCer-stimulated TILs.

Conclusion The administration of α-GalCer-pulsed APCs successfully induced the dramatic infiltration and activation of iNKT cells in the tumor microenvironment.

Keywords Invariant NKT cell · antigen presenting cell · immunotherapy · tumor infiltrating lymphocyte · non-small cell lung cancer

K. Nagato · S. Motohashi · F. Ishibashi · K. Okita · K. Yamasaki · T. Nakayama
Department of Immunology, Graduate School of Medicine, Chiba University, Clinical Research Center, Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

K. Nagato · S. Motohashi (✉) · F. Ishibashi · Y. Moriya · H. Hoshino · S. Yoshida · I. Yoshino
General Thoracic Surgery, Graduate School of Medicine, Chiba University, Clinical Research Center, Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
e-mail: motohashi@faculty.chiba-u.jp

H. Hanaoka
Graduate School of Medicine, Chiba University, Clinical Research Center, Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

S.-i. Fujii
Research Unit for Cellular Immunotherapy, RIKEN Research Center for Allergy and Immunology, 1-7-22, Suehiro-cho, Tsurumi-ku, Yokohama, Japan

M. Taniguchi
Laboratory for Immune Regulation, RIKEN Research Center for Allergy and Immunology, 1-7-22, Suehiro-cho, Tsurumi-ku, Yokohama, Japan

Introduction

Vα24 invariant natural killer T (Vα24 iNKT) cells are a unique innate lymphocyte subpopulation characterized by the expression of a canonical invariant T cell receptor with a specific α-chain gene rearrangement (Vα24-Jα18) and pairing mostly with a Vβ11 β-chain in human. Synthetic glycolipid, α-Galactosylceramide (α-GalCer) is a mouse and human iNKT cell ligand, presented by a monomorphic class I-like antigen presenting molecule CD1d [1–3]. Ligand activated iNKT cells exhibit both direct and indirect potent anti-tumor activity.

Patients with malignant diseases show either a decreased number or functionally impaired Vα24 iNKT cells in human peripheral blood mononuclear cells (PBMCs) [4–9]. Head and neck cancer patients with poor circulating iNKT cell number show significantly worse clinical outcomes, suggesting an important contribution of iNKT cells to anti-tumor responses [10]. In addition, the ability to produce IFN-γ from circulating iNKT cells in cancer patients is preserved even though the absolute number of iNKT cells decreases, and thus, residual iNKT cells might still have a good competence to exert anti-tumor responses. Therefore, the expansion and activation of these cells in vivo may be therapeutically meaningful in patients with severely decreased or functionally deficient Vα24 iNKT cells. Clinical studies of α-GalCer-pulsed antigen presenting cells (APC)s have been conducted to recover a functionally sufficient number of Vα24 iNKT cells [11–14]. A phase I/II study ofα-GalCer-pulsed APCs in patients with advanced or recurrent non-small cell lung cancer (NSCLC) found that the treatment elicits Vα24 iNKT cell-dependent immune responses, which are correlated with prolonged overall survival time [13]. The mechanisms that underlie this positive clinical outcome are still unclear.

The current clinical trial focused on the iNKT cell-specific immunological responses in the tumor microenvironments to investigate further anti-tumor mechanisms of Vα24 iNKT cells after α-GalCer-pulsed APC treatment. Therefore, in this exploratory study, the preoperative administration of α-GalCer-pulsed APCs was performed to clarify the iNKT cell specific immune responses at the tumor site more precisely. The results indicated that α-GalCer-pulsed APCs successfully induced the activation of tumor infiltrating Vα24 iNKT cells in the lung.

Material and Methods

Patient Eligibility Criteria

The study included patients between 20 and 80 years of age, with a diagnosis of clinical stage IIB or IIIA NSCLC that was to be treated surgically. Further inclusion criteria were a performance status of 0, 1, or 2; normal or near normal renal, hepatic and hematopoietic function; and no chemotherapy or radiotherapy received for at least 4 weeks before enrollment. $V\alpha24^{+}V\beta11^{+}$ iNKT cells were detected by flow cytometry in the enrolled patients at a level of >10 cells in 1 ml peripheral blood. The exclusion criteria were a positive response to HIV, hepatitis C virus, or human T-cell lymphotrophic virus antibodies; positive for hepatitis B antigen; the presence of active inflammatory disease or active autoimmune disease; a history of hepatitis; pregnancy or lactation; concurrent corticosteroid therapy and evidence for another active malignant neoplasm. The α-GalCer-pulsed APC non-treatment cases were investigated as a control group to elucidate the effects of α-GalCer-pulsed APC treatment. The inclusion and exclusion criteria of the control group were the same as for the treatment group. The histological type, tumor-node-metastasis classification and the anti-tumor effect of treatment were classified according to the general rules for the clinical and pathologic recording of lung cancer as described by the Japan Lung Cancer Society.

Clinical Protocol and Study Design

The study was carried out in the Department of Chest Surgery, Chiba University Hospital, Japan, according to the standards of Good Clinical Practice for Trials on Medicinal Products in Japan. The protocol was approved by the Institutional Ethics Committee (No. 1972). In addition, this trial underwent ad hoc reviews by the Chiba University Quality Assurance Committee on Cell Therapy.

The study design is illustrated in Fig. 1. Written informed consent was obtained from all of the patients before undergoing a screening evaluation to determine eligibility. Clinical and laboratory assessments were conducted once a week, including of a complete physical examination and standard laboratory values. Any adverse events and changes in laboratory values were graded according to the National Cancer Institute Common Toxicity Criteria version 4.0.

Preparation of APCs from Peripheral Blood

All procedures were carried out according to the Good Manufacturing Practice standards. Eligible patients underwent peripheral blood leukapheresis (COBE Spectra, Gambro BCT,

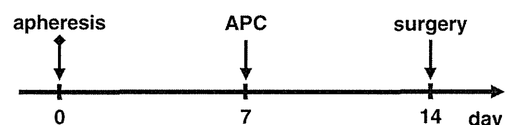

Fig. 1 Study design of α-GalCer-pulsed APC administration. The patients received α-GalCer-pulsed APCs. The timing for both apheresis and α-GalCer-pulsed APC administration are shown. APC, α-GalCer-pulsed APC administration

Inc., Lakewood, CO) and PBMCs were collected and further separated by density gradient centrifugation (OptiPrep, Nycomed Amersham, Oslo, Norway). Thereafter, whole PBMCs were cultured with GM-CSF and IL-2, as previously described [11, 15]. Briefly, PBMCs were washed three times and resuspended in AIM-V (Invitrogen Corp., Carlsbad, CA) with 800 units/ml of human granulocyte macrophage colony-stimulating factor (GeneTech Co., Ltd., China) and 100 Japanese reference units per milliliter of recombinant human IL-2 (Imunace, Shionogi, Osaka, Japan). The cultured cells were pulsed with 100 ng/ml of specific ligand, α-GalCer (KRN7000; Kirin Brewery, Gunma, Japan) on the day before administration. Whole cells were harvested after 7 days of cultivation, washed 3 times and resuspended in 100 ml of 2.5 % albumin in saline. The patients received an intravenous injection of the cultured cells once (Fig. 1). The criteria for α-GalCer-pulsed APC administration included a negative bacterial culture 48 h before APC injection, cell viability >70 % and an endotoxin test 48 h before APC injection with a result <0.7 Ehrlich units/ml. The patients were injected with 1×10^9 cells/m^2/injection of APCs.

Phenotype Evaluation of APCs

The phenotypes of α-GalCer-pulsed APCs were determined using a FACSCalibur flow cytometer (BD biosciences). The monoclonal antibodies (mAb) used were FITC-labeled anti-HLA-DR, CD83, CD14; phycoerythrin-labeled anti-CD86, CD1d; and allophycocyanin-labeled anti-CD11c, CD40 (Becton Dickinson, San Diego, CA). Isotype-matched control mAbs were used as negative controls.

Preparation of Tumor Infiltrating Lymphocytes, Tumor Cells, Normal Lung Mononuclear Cells and Lymph Nodes Mononuclear Cells

Fresh tumor tissue specimens were obtained from the surgical specimens and the tissue was cut into small pieces with scissors. The tissue specimen was placed in a flask with a mixture of 0.1 mg/ml DNase type I, 1 mg/ml collagenase type IV and 0.5 mg/ml hyaluronidase type V (all from Sigma, St. Louis, MO) in RPMI 1640 and stirred at room temperature for 1 h. The resultant cell suspension was washed in HBSS and subjected to two-layered (75 and 100 %) Ficoll-Hypaque discontinuous density gradient centrifugation at 1200 g for 20 min. The cells from the 100 % interface and 75 % interface were used as tumor infiltrating lymphocytes (TILs) and tumor cells, respectively. Normal lung tissue and lymph nodes were excised from the surgical specimen, cut with scissors in RPMI 1640 containing enzymes and passed through a gauze filter. The resultant cell suspension was washed in HBSS and subjected to Ficoll-Hypaque gradient centrifugation. The interface was collected and used as either a normal lung or lymph node.

Immunological Monitoring

PBMC samples were obtained at least twice before APC administration and 1 week after APC injection.

Flow Cytometric Analysis of $V\alpha24^+V\beta11^+$ Inkt Cells in the Peripheral Blood and TILs

The cell concentrations of $V\alpha24^+V\beta11^+$ iNKT cells in PBMCs, TILs and mononuclear cells from normal lung tissue or lymph node were assessed by flow cytometry. Mononuclear cells were three-color stained with FITC-conjugated anti–T-cell receptor (TCR) Vα24 mAb (C15; Immunotech, Marseilles, France), phycoerythrin-conjugated anti-TCR Vβ11 mAb (C21, Immunotech) and APC-conjugated anti-CD3 mAb (UCTH1; BD Bioscience). The stained cells were subjected to flow cytometry and the percentages of $V\alpha24^+V\beta11^+CD3^+$ cells among mononuclear cells were calculated. Thereafter, the number of iNKT cells (counts/ml) was estimated based on the PBMC counts.

Single-Cell Enzyme-Linked Immunospot Assay

PBMCs, TILs and cells from normal lung tissue or lymph nodes were washed 3 times with PBS and then were stored in liquid nitrogen until use. IFN-γ-secreting cells were assayed in 96-well filtration plates (Millipore, Bedford, MA) coated with mouse anti-human IFN-γ (10 μg/ml; Mabtech, Nacka Strand, Sweden). The cells (5×10^5 per well) were incubated for 16 h with or without α-GalCer (100 ng/ml) in 10%FCS containing RPMI. Phorbol 12-myristate 13-acetate (10 μg/ml) plus ionomycin (10 nmol/l) was used as a positive control. After culture, the plates were washed and incubated with biotinylated anti-IFN-γ (1 μg/ml; Mabtech). Spot-forming cells were quantified by microscopy.

Quantitative Real Time PCR of Vα24 Invariant TCR and CD1d Expression

Total RNA was extracted from the tumors, normal lung tissue and lymph nodes using TRIzol Reagent (Sigma Aldrich) and reverse transcribed using Superscript II RT (Invitrogen Life Technologies) and oligo (dT12–18) primers (Invitrogen Life Technologies). The primers specific for the constant region of TCR α chain (Cα) (sense, CGCCTTCAA CAACAGCATTA; antisense, ACCAGCTTGACATCA CAGGA), TCR Vα24 (sense, GCAAAGCTCTCT GCACATCA; antisense, CCAGGGTTGAGCCTCTGTC), CD1d (sense,gtcagggaagtcggaactga; antisense, atcctgaga-catggcacacc) were used with 5 μg of sample cDNA and

amplified with *Taq* polymerase (Promega). Quantitative real-time PCR was performed using real-time Taq-Man technology and an ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, CA). The expression was normalized using the Cα signal for Vα24 and GAPDH for CD1d.

Statistical Methods

Statistical analyses were performed using Student's *t*-test.

Results

Patient Characteristics

A total of 4 patients met the inclusion criteria and were enrolled in the study. The patient characteristics are summarized in Table I. The study included one patient with adenocarcinoma and three patients with squamous cell carcinoma. Two patients were stage IIb and two were stage IIIa primary lung cancer. No patients had received any previous treatments.

In addition, a total of 6 patients who had not received α-GalCer-pulsed APC injection were enrolled as the control group. Fresh tumor tissue, normal lung tissue and lymph nodes were excised from the surgical specimens. The patient characteristics of the control group are also listed in Table I.

Phenotypes of αGalCer-Pulsed APCs

The phenotypes of αGalCer-pulsed APCs prepared for administration were analyzed by flow cytometry. All profiles for each patient are shown in Fig. 2. The percentages of HLA-DR$^+$, CD11c$^+$, CD86$^+$, CD40$^+$, CD83$^+$ and CD1d$^+$ cells were determined by the overtone subtraction test using the population comparison platform in the FlowJo software package. More than 50 % of the cultured cells were HLA-DR$^+$ cells, 10 % to 50 % were CD11c$^+$ cells and 50 % to 80 % were CD86$^+$ cells. Interestingly, the majority of the cultured cells were CD3$^+$ T cells or CD56$^+$CD3$^-$ NK cells, indicating the expression of HLA-DR$^+$, CD11c$^+$ or CD86$^+$ on human T cells or NK cells (data not shown). Some variations were observed in the expression of CD83 (23.9–55.4 %), CD40 (8.5–17.1 %) and CD1d (23.1–69.9 %; Fig. 2).

Adverse Events

No serous (grade >2) toxicity or severe side effects were observed in any patients.

Immunological Monitoring of PBMCs and Resected Specimens

Immunological assays were conducted for all patients. The frequency of peripheral blood Vα24 iNKT cells in all patients was measured by FACS. Figure 3 shows that two patients (cases 002 and 004) showed an increased number of circulating Vα24 iNKT cells after the α-GalCer-pulsed APC administration. No clear relationship was found between the number of circulating Vα24 iNKT cells and the α-GalCer-pulsed APC administration in the remaining two patients (cases 001 and 003).

Immunological assays were also performed for TILs and mononuclear cells (MNC)s from normal lung and lymph node tissues. TILs from all 4 cases in the α-GalCer-pulsed APC administration group contained a high percentage of Vα24 iNKT cells in comparison to the normal lung MNCs (TILs; 1.86 %, 0.32 %, 0.15 % and 0.39 % vs. lung MNCs; 0.031 %, 0.013 %, 0.003 % and 0.01 %, Fig. 4a). The frequency of Vα24 iNKT cells in the TILs in case 001 was 60 times higher than the normal lung MNCs. Though the content of Vα24 iNKT in the normal lung MNCs was extremely low in case 003, the Vα24 iNKT cells were found to have accumulated in the TILs. The average percentage of Vα24 iNKT cells in the TILs was 50 times higher than that

Table I Patient characteristics of α-GalCer-pulsed APC group and control group

Case	Treat[a]	Age/Sex	Histology	c-stage	Operation method
001	APC[b]	75/M	Ad[d]	T2N1M0 (stage IIB)	Lobectomy+LND[g]
002	APC	76/M	Sq[e]	T2N1M0 (stage IIB)	Lobectomy+LND
003	APC	74/M	Sq	T1N2M0 (stage IIIA)	Lobectomy+LND
004	APC	68/M	Sq	T3N1M0 (stage IIIA)	Pneumonectomy+LND
c-01	cont[c]	71 M	Sq	T2N1M0 (stage IIB)	Lobectomy+LND
c-02	cont	55 M	large[f]	T2N1M0 (stage IIB)	Lobectomy+LND
c-03	cont	70 M	Sq	T3N0M0 (stage IIB)	Lobectomy+LND
c-04	cont	72 M	Sq	T3N1M0 (stage IIIA)	Bilobectomy+LND
c-05	cont	56/M	Sq	T2N1M0 (stage IIB)	Lobectomy+LND
c-06	cont	63/M	Ad	T2N2M0 (stage IIIA)	Lobectomy+LND

[a]Treat, Treatment; [b]APC, α-GalCer-pulsed APC administration; [c] cont, control; [d]Ad, Adenocarcinoma; [e] Sq, Squamous cell carcinoma; [f] large, large cell carcinoma; [g] LND, Lymph Node dissection

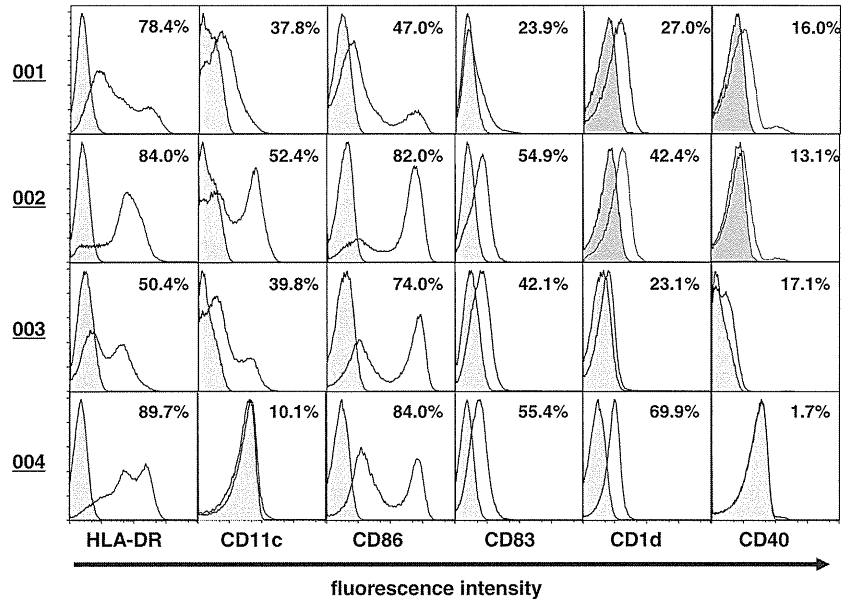

Fig. 2 Flow cytometric analysis of α-GalCer-pulsed APCs. The expression levels of HLA-DR, CD11c, CD86, CD83, CD1d and CD40 were assessed by flow cytometry. Shaded areas: background staining with an iso-type control. Solid lines: staining profiles of the indicated molecules. Values represent the percentages of positive cells

in normal lung MNCs. The Vα24 iNKT cell frequency in the draining lymph nodes of each case was almost the same as that in the normal lung MNCs (Fig. 4a).

The proportion of Vα24 iNKT cells in the control group showed a relatively high percentage of TILs in comparison to the normal lung MNCs (TILs; 0.031 %, 0.058 %, 0.13 %, 0.47 %, 0.18 % and 0.12 % vs. lung MNCs; 0.034 %, 0.011 %, 0.004 %, 0.039 %, 0.014 % and 0.02 %, Fig. 4b). The average percentage of Vα24 iNKT cells in the TILs was only 8 times higher than that in the normal lung MNCs.

Normal lung MNCs in the control group demonstrated a trend toward a higher Vα24 iNKT cell rate in comparison to the treatment group (Fig. 4c). On the other hand, the proportion of Vα24 iNKT cells in TILs tended to increase in the α-GalCer-pulsed APC injected group in comparison to the

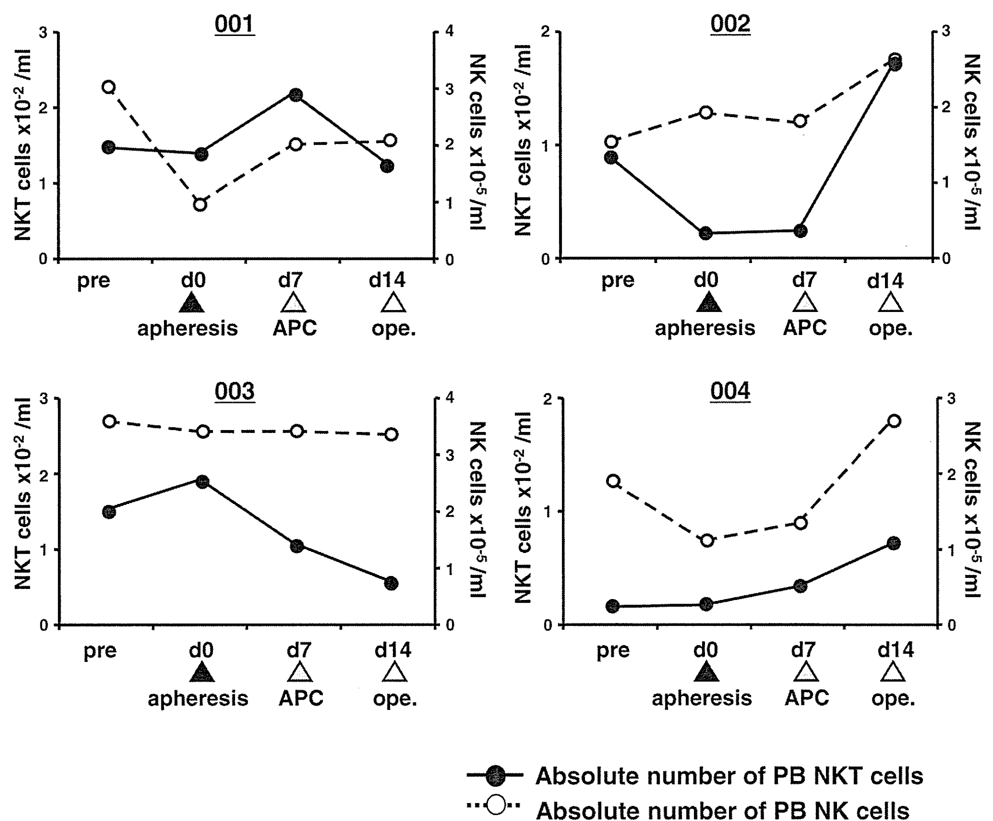

Fig. 3 Immunological monitoring of PBMCs of patients with α-GalCer-pulsed APC administration. The absolute number of peripheral blood iNKT cells (Vα24$^+$Vβ11$^+$ cells) and NK cells (CD56$^+$CD3$^-$ cells). Flow cytometric analysis and automated full blood counts (Chiba University Hospital) indicated the absolute number of Vα24 iNKT cells and NK cells. APC, α-GalCer-pulsed APC administration; ope., operation

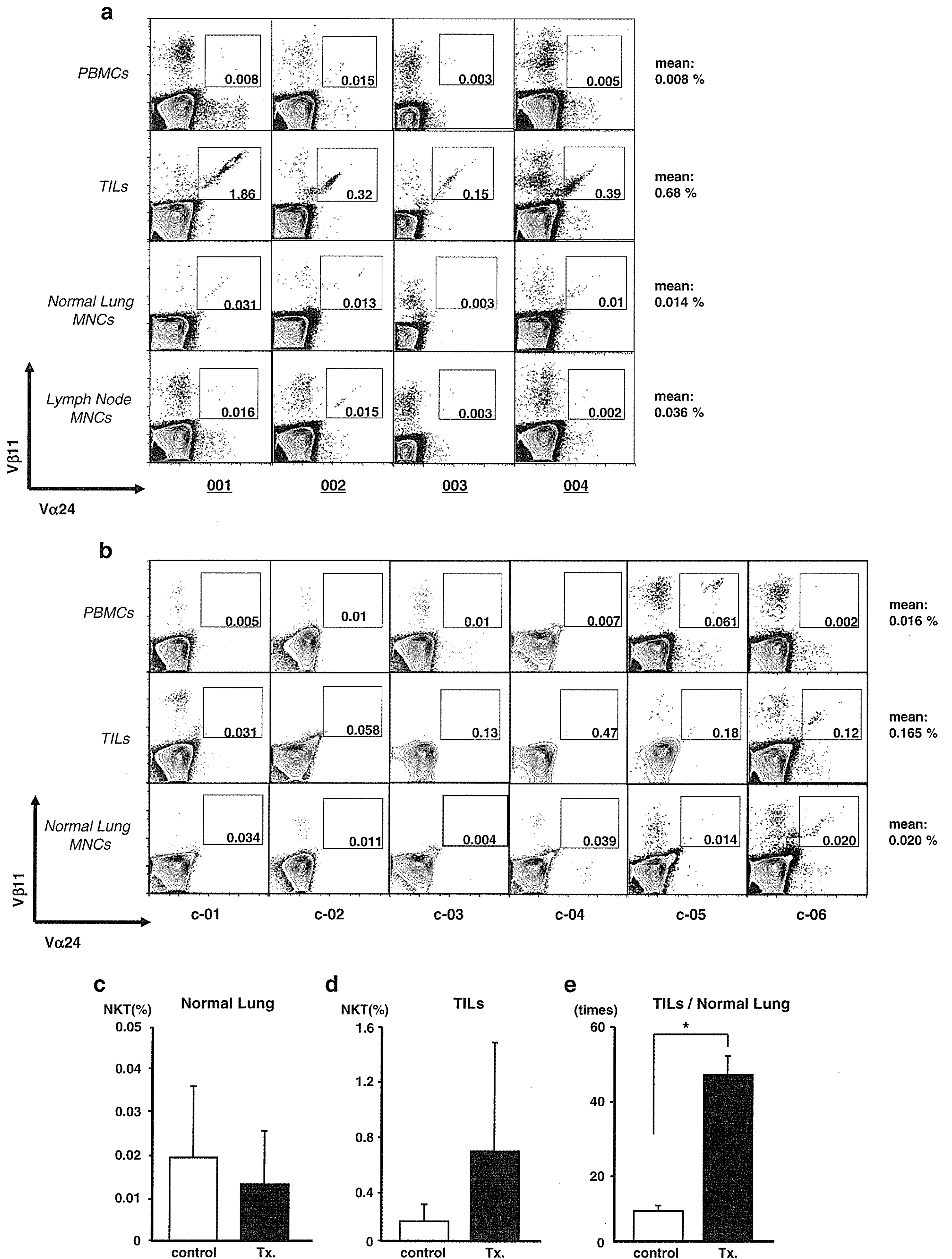
a
PBMCs
TILs
Normal Lung MNCs
Lymph Node MNCs
0.008
0.015
0.003
0.005
mean: 0.008 %
1.86
0.32
0.15
0.39
mean: 0.68 %
0.031
0.013
0.003
0.01
mean: 0.014 %
0.016
0.015
0.003
0.002
mean: 0.036 %
Vβ11
Vα24
001
002
003
004
b
PBMCs
TILs
Normal Lung MNCs
0.005
0.01
0.01
0.007
0.061
0.002
mean: 0.016 %
0.031
0.058
0.13
0.47
0.18
0.12
mean: 0.165 %
0.034
0.011
0.004
0.039
0.014
0.020
mean: 0.020 %
Vβ11
Vα24
c-01
c-02
c-03
c-04
c-05
c-06
c
Normal Lung
NKT(%)
control
Tx.
d
TILs
NKT(%)
control
Tx.
e
TILs / Normal Lung
(times)
*
control
Tx.

Fig. 4 The frequencies of Vα24 iNKT cells in TILs and mononuclear cells from normal lung and lymph node tissues. **a**, The proportion of Vα24 iNKT cells (Vα24⁺Vβ11⁺ cells) in PBMCs on day 14, TILs, normal lung MNCs and lymph node MNCs in theα-GalCer-pulsed APC administration group were assessed by flow cytometry. The lymph node MNCs column depicts one representative MNC profile in the draining lymph nodes including hilar (#10, 11 and 12) and mediastinal (#1, 3, 4 and 7) nodes. **b**, The proportion of Vα24 iNKT cells in PBMCs, TILs and normal lung MNCs in the control group were assessed by flow cytometry. **c-d**, The comparison between the Vα24 iNKT cell contents in normal lung MNCs **c** and TILs **d** in the α-GalCer-pulsed APC treatment group and the control group. **e** The TIL/Normal Lung MNC ratio of Vα24 iNKT cell proportion. control, control group; Tx, α-GalCer-pulsed APC administration group; $*p=0.0008$

control group (mean percentage, 0.68 % and 0.165 %, Fig. 4d). The Vα24 iNKT cell ratio of TILs/normal lung in the α-GalCer-pulsed APC administration group was significantly higher than that of the control group ($p=0.0008$, Fig. 4e).

The number of IFN-γ-producing cells after restimulation with α-GalCer in vitro was concurrently monitored in PBMCs, TILs, normal lung MNCs and lymph node MNCs using an ELISPOT assay. An analysis of the PBMCs and resected specimen showed the highest value of α-GalCer-responsive IFN-γ-producing cell number in the TILs of the α-GalCer-pulsed APC treated group (Fig. 5a). The absolute number of α-GalCer-responsive IFN-γ-producing cells in the TILs was apparently high in cases 001 and 002, whereas a relatively low value was seen in cases 003 and 004. This observation was not detected with the use of control group specimens (Fig. 5b). Together with the results in Fig. 4, the administration of α-GalCer-pulsed APCs induced the mobilization of endogenous Vα24 iNKT cells into the primary site of the lung cancer and augmented the IFN-γ-producing ability of tumor infiltrating Vα24 iNKT cells.

In addition, the number of IFN-γ-producing cells in PBMCs was determined after restimulation with α-GalCer

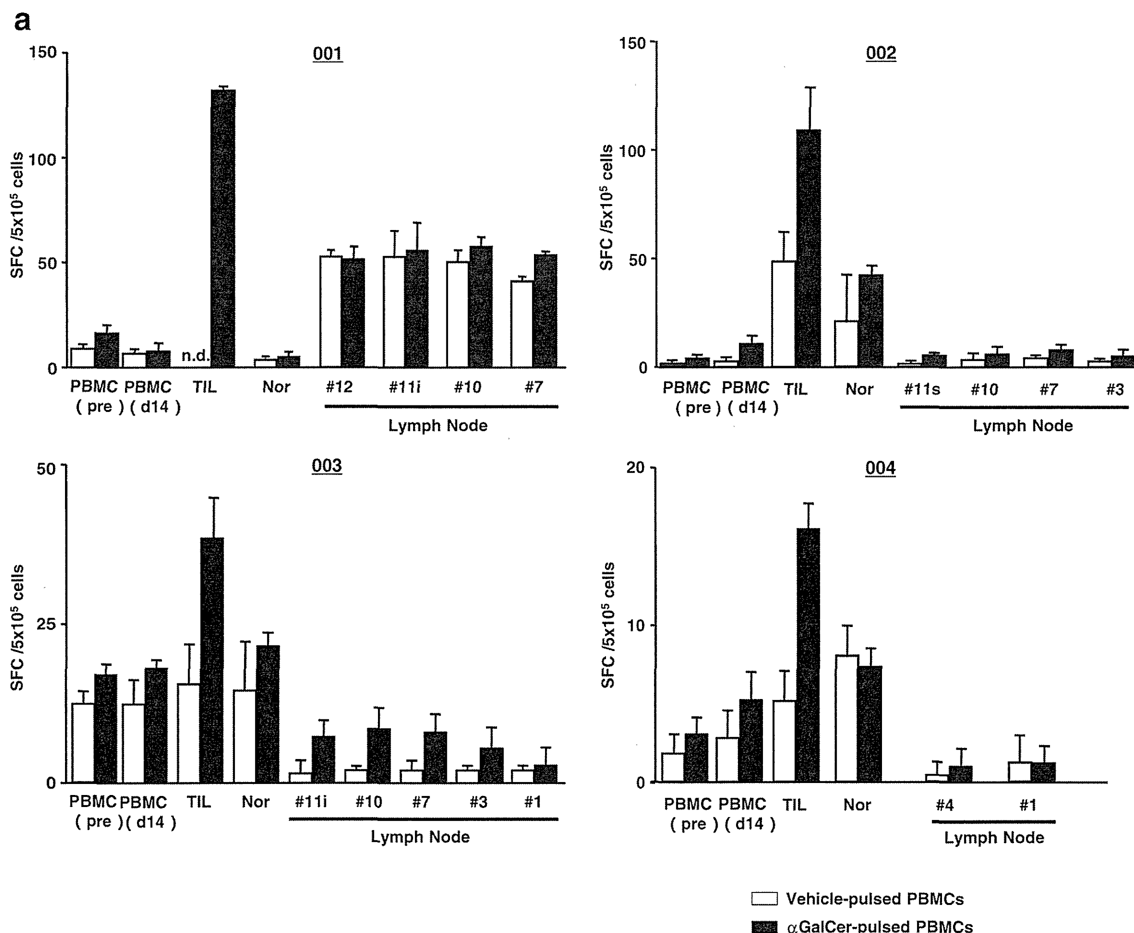

Fig. 5 Detection of α-GalCer-reactive IFN-γ-producing cells by enzyme-linked immunospot assay. **a** Cryopreserved PBMCs, TILs, normal lung MNCs and lymph node MNCs of the α-GalCer-pulsed APC treated group were thawed and cultured overnight with either α-GalCer or vehicle. The presence of IFN-γ-producing cells was quantified by an enzyme-linked immunospot assay. The resected draining lymph nodes including hilar (#10, 11 and 12) and mediastinal (#1, 3, 4 and 7) nodes are shown. Spot number of IFN-γ with standard deviation for triplicate culture of 4 cases are shown. **b** Spot-forming cell number in the control group. SFC, Spot Forming Cell; pre, pretreatment; d14, day 14; Nor, normal lung MNCs; n.d., not done; #11 s, lymph node #11 superior; #11i, lymph node #11 inferior

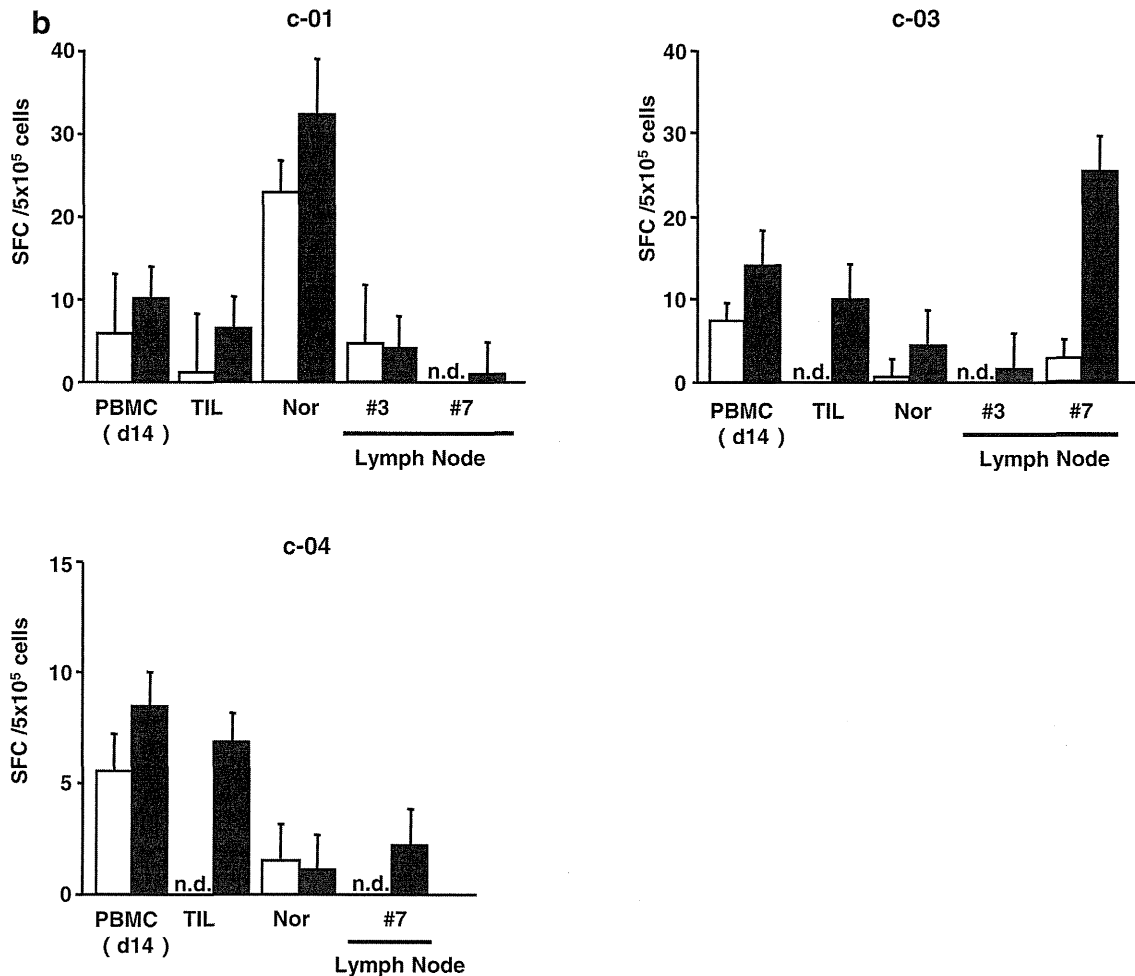

Fig. 5 (continued)

in vitro. In Fig. 5a, the number of IFN-γ producing cells in PMBC increased 14 days after treatment in case 002 and 004, indicating that global NKT cell activation in these patients.

The mRNA Expression Level of Vα24 Inkt Cell Receptor and CD1d

Quantitative RT-PCR was performed to further confirm the increase in the number of Vα24 iNKT cells. The mRNA from the primary tumor, normal lung tissue and lymph node samples was obtained from the α-GalCer-pulsed APC treated patients and the relative expression level of Vα24 invariant TCR and the constant region of TCR (Cα) mRNA were evaluated. The mRNA for Vα24 TCR was highly expressed in the tumor (Fig. 6a). The Vα24-Jα18 invariant TCR mRNA could not be detected in the normal lung tissue since the lung parenchyma included mainly alveolar epithelial cells and only a low copy number of Cα mRNA was detected. The relative gene expression of CD1d was also evaluated by quantitative RT-PCR. CD1d expression was ascertained both in normal lung tissue and tumor tissue in all 4 cases and the expression level appeared to be higher in normal lung tissue (Fig. 6b).

Discussion

The major aim of this study was to investigate the Vα24 iNKT cell-specific immune responses in the primary tumor site after the intravenous injection of α-GalCer-pulsed APCs in patients with advanced NSCLC. The development of these immunotherapeutic approaches with the administration of α-GalCer-pulsed APCs requires a thorough knowledge of local immune responses. Vα24 iNKT cells accumulate in lung cancer lesions [5], as observed in this report (Fig. 4e). A significant increase in the tumor infiltrating Vα24 iNKT cell population was detected after the administration of α-GalCer-pulsed APCs in comparison to the non-injected control group. This observation is quite reasonable for the iNKT cell-targeted therapy aimed at activation of Vα24 iNKT cells in the tumor located site in vivo. The activation status of CTLs, rather than just the existence

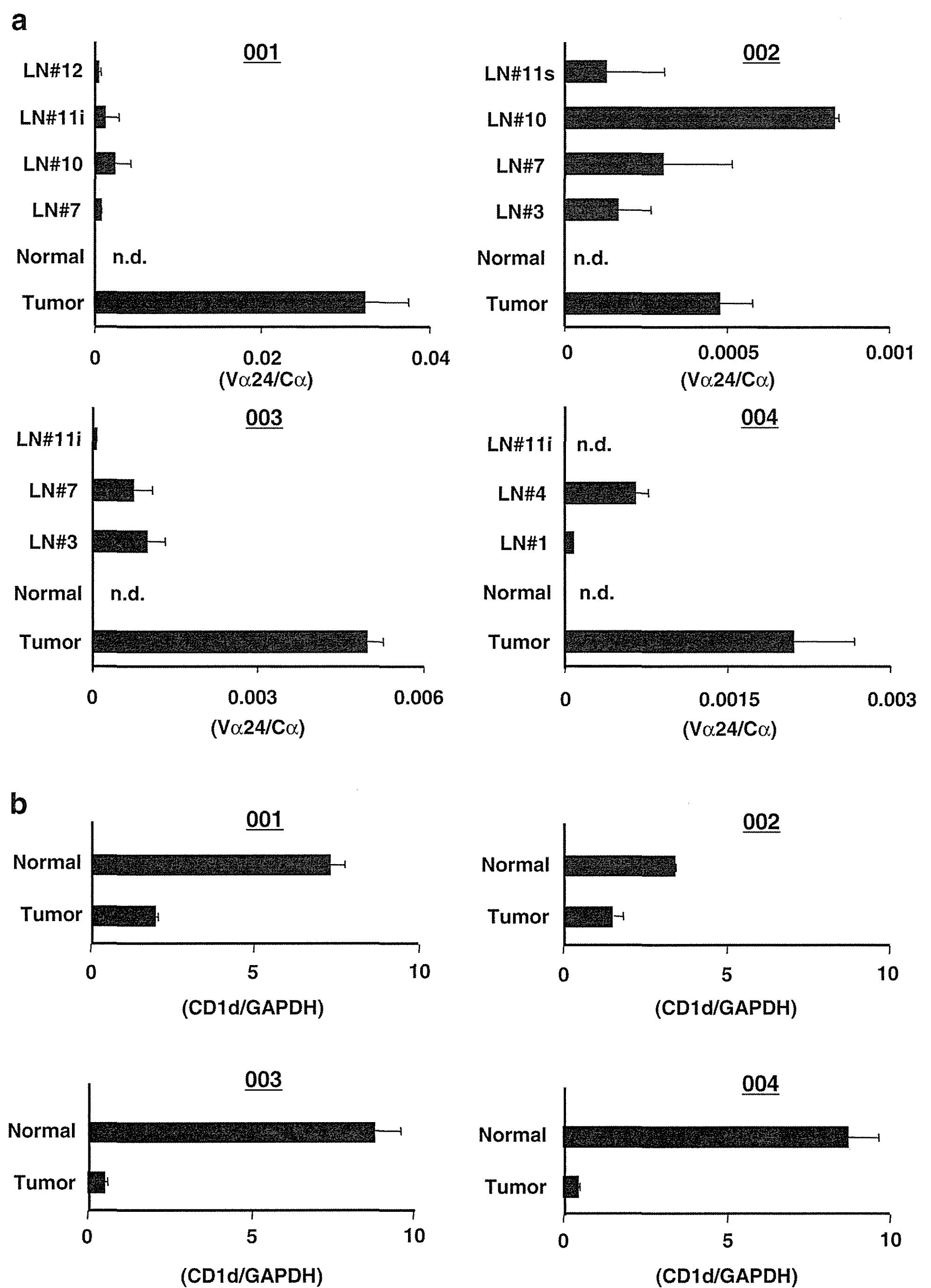

Fig. 6 The relative mRNA expression of the Vα24^{+} TCR in tumor, normal lungs and lymph nodes. Cancer tissue, non-cancerous lung tissue and lymph nodes were obtained from α-GalCer-pulsed APC treated patients. **a** The expression level of Vα24 TCR mRNA in each sample was analyzed by quantitative RT-PCR. Each mRNA was quantified by the standard curve method and copy numbers of Vα24 TCR were normalized by the copy number of the constant region of the TCR α chain (Cα mRNA. **b** The expression level of CD1d mRNA in each sample was analyzed by quantitative RT-PCR. Each mRNA was quantified by the standard curve method and copy numbers of CD1d were normalized by the copy number of the GAPDH mRNA. LN, lymph node; Normal, normal lung tissue; Tumor, tumor tissue; n.d., not detected; #11 s, lymph node #11 superior; #11i, lymph node #11 inferior

of CTLs, has great prognostic significance [16–18]. Therefore, IFN-γ-producing cells were also monitored in PBMCs, TILs and MNCs from normal lung tissue and lymph nodes using an ELISPOT assay. After starting the protocol, standard preoperative chemo-radiotherapy was introduced to treat locally advanced NSCLC, and such a change of treatment strategy hindered the entry of patients into this protocol. In spite of the limited number of patients analyzed, the results obtained by ELISPOT assay indicated that tumor infiltrating mononuclear cells had augmented IFN-γ producing capacity, which may have a positive impact on the tumor microenvironment.

Tumor infiltrating lymphocytes are found in a variety of cancers and they are thought to be a result of a host immune response directed against tumor cells. Several reports have shown that the presence of large numbers of tumor-infiltrating CD8^{+} T cells are associated with a favorable prognosis in esophageal carcinoma [18, 19], colorectal cancer [20, 21], ovarian cancer [22], and pancreatic carcinoma [23], while the infiltration of CD4^{+} T cells that posses regulatory function,

such as Foxp3$^+$ regulatory T cells (Treg) are associated with the poor prognosis of ovarian cancer [24, 25]. The balance between CD8$^+$ CTLs and Tregs in tumors is critical for disease progression and survival [24, 26, 27]. These diverse results indicate that the functional roles of TILs are complicated and uncertain and the effects of TILs might vary with the type and stage of cancers. In addition to CD8$^+$ cytotoxic T cells, tumor infiltrating Vα24 iNKT cells have been reported to be a positive prognostic factor for colorectal carcinoma [28]. The current results indicated that the injection of α-GalCer-pulsed APCs could induce the accumulation of Vα24 iNKT cells in TILs, which would therefore lead to a good prognosis after a complete surgical resection.

Although a complete surgical resection is regarded as the optimal treatment for NSCLC, only around 25 % of NSCLC are suitable for potentially curative resection. Despite optimal surgical management, the 5-year survival rate of resected NSCLC ranges between 85.9 % for pathological stage Ia and 41 % for pathological stage IIIa [29]. Approximately 50 % or more of patients with NSCLC who undergo surgery experience relapse due to the existence of microscopic lesions that could not be detected by preoperative screening. Recently, adjuvant chemotherapy given after surgery has been shown to improve survival [30–32]. A meta-analysis suggested that cisplatin-based adjuvant chemotherapy could yield an absolute overall survival advantage of 5 % at 5 years [33]. At the same time, chemotherapeutic agents often show severe toxic effects and it was reported that in patients with early-stage disease have deleterious effects on long-term survival. This emphasizes the importance of development of less-invasive preoperative or postoperative therapy to suppress the growth of micrometastases. Therefore, immune cells for tumor surveillance, such as NK and iNKT cells, which possess anti-tumor activity, should be beneficial and post-surgical adjuvant immunotherapy by the use of these cells may be favorable since the residual tumor is quite small after a complete resection.

Conclusions

α-GalCer-pulsed APC administration successfully induced the dramatic infiltration and activation of Vα24 iNKT cells in the tumor lesion. This report is the first clinical trial of Vα24 iNKT cell targeted immunotherapy that shows a functional Vα24 iNKT cell accumulation in the tumor microenvironment. These results encourage the further development of immunotherapy aimed at the activation of endogenous Vα24 iNKT cells in the lung.

Acknowledgements We thank Kyowa Hakko Kirin Co. Ltd. for providing clinical grade α-GalCer (KRN7000) for these studies. We also thank all the nurses and staff surgeons in the Department of the Thoracic Surgery, Chiba University Hospital, Chiba, Japan, for their excellent help with patient care and continuous support.

This work was supported by Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment), City Area Program (Kazusa / Chiba Area) MEXT (Japan), and by grants from the Ministry of Education, Culture, Sports, Science and Technology (Japan) (Grants-in-Aid: for Scientific Research on Priority Areas #17016010; Scientific Research [B] #21390147, and Scientific Research [C] #21591808, and Cancer Translational Research Project), the Ministry of Health, Labor and Welfare (Japan), Uehara Memorial Foundation, Mochida Foundation, Chiba Foundation for Health Promotion and Disease Prevention and Mitsui Life Social Welfare Foundation.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. Science. 1997;278:1626–9.
2. Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, Dellabona P, et al. CD1d-mediated recognition of an α-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. J Exp Med. 1998;188:1521–8.
3. Spada FM, Koezuka Y, Porcelli SA. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. J Exp Med. 1998;188:1529–34.
4. Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB, et al. Loss of IFN-γ production by invariant NK T cells in advanced cancer. J Immunol. 2001;167:4046–50.
5. Motohashi S, Kobayashi S, Ito T, Magara KK, Mikuni O, Kamada N, et al. Preserved IFN-γ production of circulating Vα24 NKT cells in primary lung cancer patients. Int J Cancer. 2002;102:159–65.
6. Molling JW, Kolgen W, van der Vliet HJ, Boomsma MF, Kruizenga H, Smorenburg CH, et al. Peripheral blood IFN-g-secreting Vα24$^+$Vβ11$^+$ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. Int J Cancer. 2005;116:87–93.
7. van der Vliet HJ, Nishi N, Koezuka Y, von Blomberg BM, van den Eertwegh AJ, Porcelli SA, et al. Potent expansion of human natural killer T cells using α-galactosylceramide (KRN7000)-loaded monocyte-derived dendritic cells, cultured in the presence of IL-7 and IL-15. J Immunol Methods. 2001;247:61–72.
8. Yanagisawa K, Seino K, Ishikawa Y, Nozue M, Todoroki T, Fukao K. Impaired proliferative response of Vα24 NKT cells from cancer patients against α-galactosylceramide. J Immunol. 2002;168:6494–9.
9. Dhodapkar MV, Geller MD, Chang DH, Shimizu K, Fujii S, Dhodapkar KM, et al. A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. J Exp Med. 2003;197:1667–76.
10. Molling JW, Langius JA, Langendijk JA, Leemans CR, Bontkes HJ, van der Vliet HJ, et al. Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. J Clin Oncol. 2007;25:862–8.
11. Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M, et al. A phase I study of α-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. Clin Cancer Res. 2005;11:1910–7.
12. Motohashi S, Ishikawa A, Ishikawa E, Otsuji M, Iizasa T, Hanaoka H, et al. A phase I study of in vitro expanded natural killer T cells

in patients with advanced and recurrent non-small cell lung cancer. Clin Cancer Res. 2006;12:6079–86.
13. Motohashi S, Nagato K, Kunii N, Yamamoto H, Yamasaki K, Okita K, et al. A phase I-II study of α-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. J Immunol. 2009;182:2492–501.
14. Motohashi S, Nakayama T. Invariant natural killer T cell-based immunotherapy for cancer. Immunotherapy. 2009;1:73–82.
15. Ishikawa E, Motohashi S, Ishikawa A, Ito T, Uchida T, Kaneko T, et al. Dendritic cell maturation by $CD11c^-$ T cells and $V\alpha24^+$ natural killer T-cell activation by α-galactosylceramide. Int J Cancer. 2005;117:265–73.
16. Grabenbauer GG, Lahmer G, Distel L, Niedobitek G. Tumor-infiltrating cytotoxic T cells but not regulatory T cells predict outcome in anal squamous cell carcinoma. Clin Cancer Res. 2006;12:3355–60.
17. van Beek J, zur Hausen A, Snel SN, Berkhof J, Kranenbarg EK, van de Velde CJ, et al. Morphological evidence of an activated cytotoxic T-cell infiltrate in EBV-positive gastric carcinoma preventing lymph node metastases. Am J Surg Pathol. 2006;30:59–65.
18. Schumacher K, Haensch W, Roefzaad C, Schlag PM. Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. Cancer Res. 2001;61:3932–6.
19. Cho Y, Miyamoto M, Kato K, Fukunaga A, Shichinohe T, Kawarada Y, et al. $CD4^+$ and $CD8^+$ T cells cooperate to improve prognosis of patients with esophageal squamous cell carcinoma. Cancer Res. 2003;63:1555–9.
20. Menon AG, Janssen-van Rhijn CM, Morreau H, Putter H, Tollenaar RA, van de Velde CJ, et al. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. Lab Invest. 2004;84:493–501.
21. Prall F, Duhrkop T, Weirich V, Ostwald C, Lenz P, Nizze H, et al. Prognostic role of $CD8^+$ tumor-infiltrating lymphocytes in stage III colorectal cancer with and without microsatellite instability. Hum Pathol. 2004;35:808–16.
22. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med. 2003;348:203–13.
23. Ryschich E, Notzel T, Hinz U, Autschbach F, Ferguson J, Simon I, et al. Control of T-cell-mediated immune response by HLA class I in human pancreatic carcinoma. Clin Cancer Res. 2005;11:498–504.
24. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial $CD8^+$ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A. 2005;102:18538–43.
25. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004;10:942–9.
26. Alvaro T, Lejeune M, Salvado MT, Bosch R, Garcia JF, Jaen J, et al. Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. Clin Cancer Res. 2005;11:1467–73.
27. Gao Q, Qiu SJ, Fan J, Zhou J, Wang XY, Xiao YS, et al. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. J Clin Oncol. 2007;25:2586–93.
28. Tachibana T, Onodera H, Tsuruyama T, Mori A, Nagayama S, Hiai H, et al. Increased intratumor Vα24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. Clin Cancer Res. 2005;11:7322–7.
29. Sawabata N, Miyaoka E, Asamura H, Nakanishi Y, Eguchi K, Mori M, et al. Japanese lung cancer registry study of 11,663 surgical cases in 2004: demographic and prognosis changes over decade. J Thorac Oncol. 2011;6:1229–35.
30. Arriagada R, Bergman B, Dunant A, Le Chevalier T, Pignon JP, Vansteenkiste J. Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small-cell lung cancer. N Engl J Med. 2004;350:351–60.
31. Winton T, Livingston R, Johnson D, Rigas J, Johnston M, Butts C, et al. Vinorelbine plus cisplatin vs. observation in resected non-small-cell lung cancer. N Engl J Med. 2005;352:2589–97.
32. Douillard JY, Rosell R, De Lena M, Carpagnano F, Ramlau R, Gonzales-Larriba JL, et al. Adjuvant vinorelbine plus cisplatin versus observation in patients with completely resected stage IB-IIIA non-small-cell lung cancer (Adjuvant Navelbine International Trialist Association [ANITA]): a randomised controlled trial. Lancet Oncol. 2006;7:719–27.
33. Pignon JP, Tribodet H, Scagliotti GV, Douillard JY, Shepherd FA, Stephens RJ, et al. Lung adjuvant cisplatin evaluation: a pooled analysis by the LACE Collaborative Group. J Clin Oncol. 2008;26:3552–9.

Regulation of memory CD4 T-cell pool size and function by natural killer T cells in vivo

Chiaki Iwamura[a], Kenta Shinoda[a], Yusuke Endo[a], Yukiko Watanabe[a], Damon John Tumes[a], Shinichiro Motohashi[a], Kazuyoshi Kawahara[b], Yuki Kinjo[c], and Toshinori Nakayama[a,d,1]

[a]Department of Immunology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan; [b]Department of Applied Material and Life Science, College of Engineering, Kanto Gakuin University, Kanagawa, 236-8501, Japan; [c]Department of Chemotherapy and Mycoses, National Institute of Infectious Diseases, Tokyo 162-8640, Japan; and [d]Core Research for Evolutionary Science and Technology, Japan Science and Technology Agency, Chiba 260-8670, Japan

Edited by Albert Bendelac, Howard Hughes Medical Institute, University of Chicago, Chicago, IL, and accepted by the Editorial Board August 30, 2012 (received for review March 1, 2012)

To develop more effective vaccines and strategies to regulate chronic inflammatory diseases, it is important to understand the mechanisms of immunological memory. Factors regulating memory CD4+ T helper (Th)-cell pool size and function remain unclear, however. We show that activation of type I invariant natural killer T (iNKT) cells with glycolipid ligands and activation of type II natural killer T (NKT) cells with the endogenous ligand sulfatide induced dramatic proliferation and expansion of memory, but not naïve, CD4 T cells. NKT cell-induced proliferation of memory Th1 and Th2 cells was dependent largely on the production of IL-2, with Th2-cell proliferation also affected by loss of IL-4. Type II NKT cells were also required for efficient maintenance of memory CD4 T cells in vivo. Activation of iNKT cells resulted in up-regulation of IFN-γ expression by memory Th2 cells. These IFN-γ–producing memory Th2 cells showed a decreased capability to induce Th2 cytokines and eosinophilic airway inflammation. Thus, activated NKT cells directly regulate memory CD4 T-cell pool size and function via the production of cytokines in vivo.

α-galactosylceramide | CD1d KO mice | Jα18 KO mice | STAT5 | allergy

Immunologic memory plays a central role in the immune system, in which memory CD4 T cells may provide protection against infections or cancer and provide the basis for successful vaccines (1, 2). On the other hand, memory CD4 T cells induce or prolong inflammatory diseases, such as allergic disorders (3, 4) and autoimmune diseases (5). After antigen recognition by the T-cell receptor (TCR), naïve CD4 T cells undergo clonal expansion and become functionally polarized effector T helper (Th) cells (e.g., Th1, Th2, and Th17 cells) within 1 or 2 wk (6, 7). After antigen clearance, most effector Th cells are thought to undergo apoptotic cell death during a period known as the contraction phase (8); however, some effector CD4 T cells escape cell death, differentiate into memory CD4 T cells, and survive for long periods in vivo. Once long-lived memory T cells are established, these cells persist for months and years, accompanied by slow basal turnover and homeostasis (9–12), while maintaining the ability to proliferate and produce polarized cytokines on antigen reencounter (13). In addition, heterogeneity in cytokine production potential is suggested in memory CD4 T cells (3, 13–15). However, once memory cells are established, factors that influence memory CD4+ Th-cell pool size and function remain poorly defined.

Natural killer T (NKT) cells belong to a unique lymphoid lineage distinct from T, B, and NK cells. Invariant NKT (iNKT) cells, or type I NKT cells, are characterized by the expression of a restricted TCR repertoire consisting of Vα14-Jα18 in mice and Vα24-Jα18 in humans, with a highly skewed set of Vβs, mainly Vβ8.2 in mice and Vβ11 in humans (16, 17). The most potent and well-analyzed ligand for the iNKT antigen receptor is a glycolipid, α-galactosylceramide (α-GalCer), which is presented exclusively by CD1d, a monomorphic class Ib molecule (18). Recently, several investigators have shown that iNKT cells are activated by microbial glycolipids from *Shingomonas* spp, *Borrelia burgdorferi*, *Mycobacterium bovis*, *Helicobacter pylori*, and *Streptococcus pneumonie* (19–22). Activated iNKT cells play critical roles in the regulation of various immune responses, including infection, allergic inflammation, antitumor immunity, and autoimmune responses, and thus represent a potential immunotherapeutic target with clinical potential (23, 24). In addition to iNKT cells, other CD1d-restricted, lipid antigen-reactive NKT cells, known as type II NKT cells, are present in humans and mice (25). Type II NKT cells express biased TCR repertoires and recognize a range of hydrophobic antigens, sulfatide, lysophosphatidylcholine, and even small aromatic molecules (26). Sulfatide is considered an endogenous ligand for type II NKT cells. Type II NKT cells have an activated or memory-like phenotype and the ability to modulate immune responses, including suppression of autoimmunity and inhibition of tumor rejection (27).

Given that the effects of NKT cells on T-cell memory remain to be fully defined, we examined the interplay between NKT cells and the memory CD4 Th-cell pool using an experimental system called "memory Th1/Th2 mouse," in which antigen-specific memory CD4 T cells are efficiently generated and maintained in vivo (28).

Results

Activation of iNKT Cells with α-GalCer–Induced Proliferation of Memory CD4 T Cells, but Not Naïve CD4 T Cells, in Vivo. To examine whether iNKT cells control the generation and maintenance of memory Th2 cells, we used WT and Jα18-deficient (Jα18 KO) mice that lack iNKT cells and produced memory Th2 mice in which ovalbumin (OVA)-specific DO11.10 transgenic (Tg) memory Th2 cells are efficiently generated 1 mo after effector Th2-cell transfer (28). We administered α-GalCer i.p. to these memory Th2 mice at 30 d after cell transfer (Fig. S1*A*). The absolute numbers of memory Th2 cells (KJ1+ donor-derived cells) in the livers of these mice at 3 d after α-GalCer administration are shown in Fig. 1*A*. We found a dramatic increase in the number of memory Th2 cells in WT mice, but no increase in Jα18 KO mice, indicating iNKT cell-dependent increases in memory Th2 cells. We assessed cell division in memory Th1 and Th2 cells after α-GalCer administration using a carboxyfluorescein succinimidyl ester (CFSE)-labeling method (Fig. S1*B*). We found multiple rounds of cell division in memory Th1 and Th2 cells, but not in naïve CD4 T cells, in each organ tested (Fig. 1*B*); however, the magnitude of increase was smaller than that seen in memory Th2 cells. Cell division was observed in memory Th2 cells even at 3 d after α-GalCer administration (Fig. S1*C*). These results indicate that activation of iNKT cells with α-GalCer induced proliferation of memory Th1 and Th2 cells, but not of naïve CD4 T cells.

We next monitored the number of memory Th2 cells in various organs of WT mice after α-GalCer administration, and found

Author contributions: C.I., S.M., and T.N. designed research; C.I., K.S., Y.E., Y.W., D.J.T., and S.M. performed research; K.K. and Y.K. contributed new reagents/analytic tools; K.S., Y.E., Y.W., and D.J.T. analyzed data; and C.I., D.J.T., and T.N. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. A.B. is a guest editor invited by the Editorial Board.

[1]To whom correspondence should be addressed. E-mail: tnakayama@faculty.chiba-u.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1203494109/-/DCSupplemental.

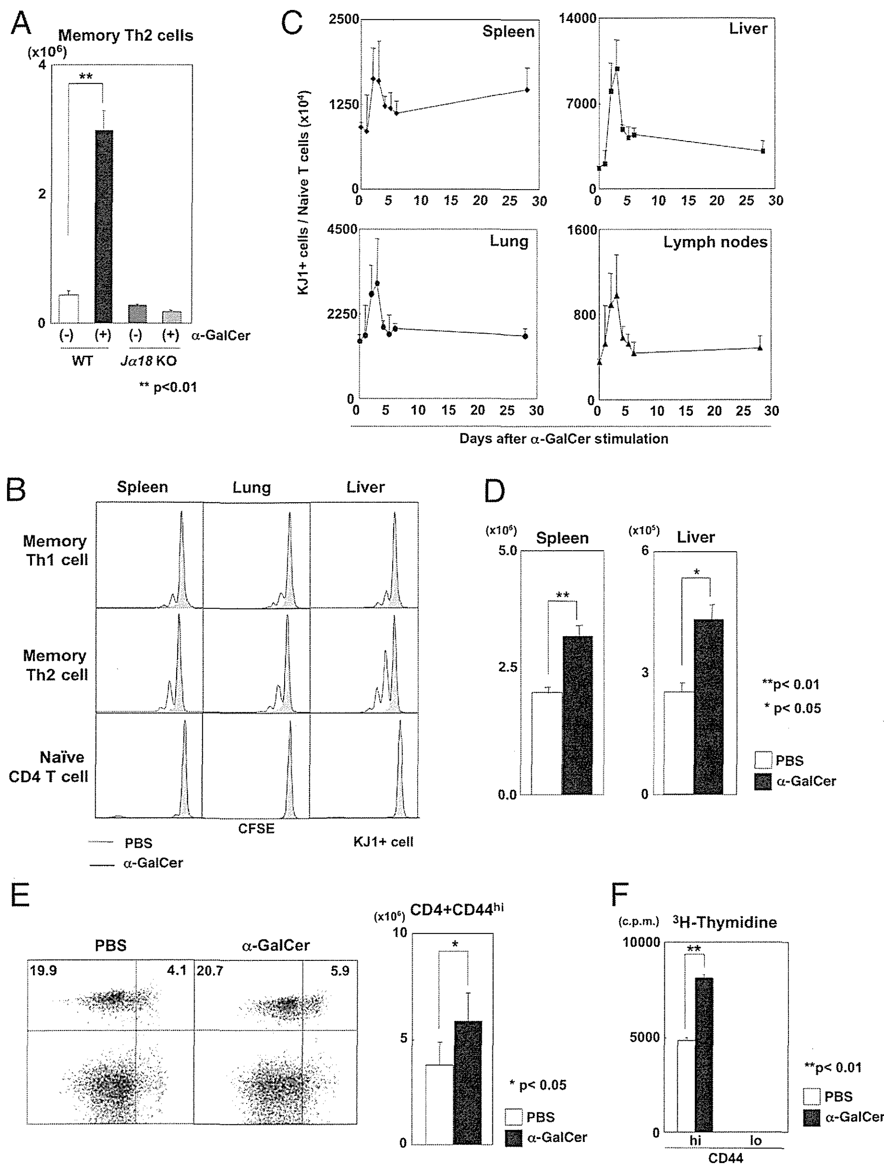

Fig. 1. Activation of iNKT cells induced the proliferation of memory CD4 T cells in vivo. (*A*) Numbers of memory Th2 cells in the livers of WT and Jα18 KO mice at 3 d after α-GalCer administration. Values are mean ± SEM ($n = 5$). $^{**}P < 0.01$. (*B*) CFSE analysis of memory Th2 cells. Memory Th2 cells (5×10^6) purified from Th2 memory mice and $CD44^{lo}CD62L^{hi}$ naïve CD4 T cells purified from normal DO11.10 Tg mice were labeled with CFSE and then transferred into syngenic mice. One day later, α-GalCer was injected. On day 7, cell division of transferred cells in the liver was assessed by flow cytometry. (*C*) Monitoring of memory Th2 cells in various organs after α-GalCer administration into memory Th2 mice. Values are mean ± SEM ($n = 3$). (*D*) Numbers of memory Th2 cells in the spleens and livers at 4 wk after the last stimulation. (*E*) Percentages and absolute numbers of memory CD4 T cells ($CD44^{hi}$) in the spleens of mice immunized with KLH and CFA. (*F*) ^{3}H-thymidine uptake in $CD44^{hi}$ and $CD44^{lo}$ CD4 T cells purified from the spleens of mice immunized with KLH and CFA. The donor cells and recipient mice used in Fig.1 were BALB/c background. Similar data were obtained from at least three (*A* and *B*) or two (*C–F*) independent experiments. Values are mean ± SEM ($n = 5$). $^*P < 0.05$; $^{**}P < 0.01$.

that memory Th2 cells increased and peaked in each organ at 3 d after α-GalCer administration (Fig. 1*C*). To determine whether the steady-state number of memory Th2 cells is maintained at high levels after iNKT-cell activation, we administered four injections of α-GalCer–pulsed bone marrow-derived dendritic cells (BMDCs) to memory Th2 mice, and 1 mo later assessed the number of memory Th2 cells (Fig. S1*D*). To minimize the induction of anergic changes in NKT cells by repeated stimulation (29), we used α-GalCer–pulsed BMDCs for NKT-cell stimulation in this experiment. We found significantly increased numbers of memory Th2 cells in the spleen and liver, indicating expansion of the memory Th2-cell pool (Fig. 1*D*).

We observed that the numbers of memory CD4 T cells generated from naïve cells by immunization in vivo are also increased by iNKT-cell activation (Fig. S1*E*). Furthermore, we found an increase in naturally occurring $CD44^{hi}$ phenotypic memory CD4 T cells (Fig. S1*F*). We assessed the percentages of $CD44^{hi}$ and $CD44^{lo}$ CD4 T cells in the spleen and liver and their cell division at 7 d after α-GalCer administration and found increased percentages of naturally occurring memory CD4 T ($CD44^{hi}$) cells (Fig. S1*G*), and several cell divisions in these $CD44^{hi}$ cells (Fig. S1*H*). To study the effect of activated iNKT cells on endogenous polyclonal memory CD4 T cells, we immunized normal BALB/c mice with keyhole limpet hemocyanin (KLH) and complete Freund's adjuvant (CFA) (Fig. S1*I*). The number of $CD44^{hi}$ CD4 T cells was increased slightly but significantly after α-GalCer administration (Fig. 1*E*). In addition, proliferation of memory phenotype $CD44^{hi}$ CD4 T cells in response to specific antigen was significantly enhanced by the activation of iNKT cells, whereas proliferation of $CD44^{lo}$ CD4 T cells was not detected (Fig. 1*F*). Taken together, these results indicate that the activation of iNKT cells with α-GalCer induces memory Th1/Th2-cell proliferation and expansion of the memory CD4 T-cell pool.

IL-2 Produced by Activated iNKT Cells Induced Proliferation of Memory Th1/Th2 Cells. We next sought to identify functional molecules involved in the activated iNKT-cell–mediated proliferation of memory Th2 cells. Activated iNKT cells are known to regulate other immune cells by secreting various cytokines (16). We found that culture supernatant of splenocytes stimulated with α-GalCer was sufficient to induce proliferation of memory Th2 cells (Fig. S2*A*). We assessed the mRNA expression of NKT-cell– or memory T-cell–related cytokines in the liver at 3 d after α-GalCer administration (Fig. S2*B*) and found dramatically increased expression of IL-2 and IL-21 and significantly increased expression of IL-4, IL-7, IL-10, IL-12 (p35), IL-15, IFN-β, and IFN-γ. We conducted several

IMMUNOLOGY

in vitro experiments to examine whether α-GalCer or any of these cytokines can induce proliferation of memory Th2 cells. Memory Th2 cells purified from memory Th2 mice were cocultured with syngeneic whole splenocytes as a source of iNKT cells in the presence of α-GalCer. Neutralizing antibodies against these cytokines were added to the α-GalCer stimulation culture (Fig. 2*A*). Anti–IL-2 and –IL-4 mAbs inhibited the proliferation of memory Th2 cells, whereas anti–IL-7, –IL-15, and –IL-21 mAbs had no apparent effect. These results indicate that IL-2 and IL-4 predominantly induced the proliferation of memory Th2 cells in the α-GalCer stimulation culture.

We next purified memory Th2 cells by cell sorting and directly stimulated these cells with IL-2, IL-4, IL-7, IL-15, and IL-21, and then evaluated their proliferation by [^{3}H]-thymidine incorporation (Fig. 2*B*). IL-2 and IL-4 induced substantial memory Th2-cell proliferation, IL-7 and IL-15 induced slight proliferation, and IFN-β, IL-10, TNF-α, and IFN-γ induced no proliferation (Fig. S2*C*). IL-2 induced memory Th1-cell proliferation. Naïve CD4 T cells did not proliferate in response to these cytokines. IL-2–induced [^{3}H]-thymidine incorporation by CD62L^{hi} memory Th1/Th2 cells was greater than that observed for CD62L^{lo} memory Th2 cells (Fig. S2*D*). We labeled the sorted CD62L^{hi} memory Th2 cells with CFSE and assessed cell division in the presence of IL-2, IL-4, and IL-7. CD62L^{hi} memory Th2 cells underwent several rounds of cell division after culture with IL-2 and IL-4, accompanied by reduced CD62L expression; IL-7 also had a slight effect in this culture (Fig. 2*C*). The α-GalCer–induced up-regulation of IL-2Rα expression was inhibited by anti–IL-2 and anti–IL-4 mAbs, and was almost completely inhibited in the presence of both mAbs (Fig. 2*D*). The α-GalCer–induced up-regulation of IL-2Rβ appeared to be more dependent on IL-4. These results indicate that IL-2Rα and IL-2Rβ expression by memory Th2 cells is up-regulated by IL-2 and/or IL-4. IL-2 stimulation did not induce cytokine production (IL-4, IFN-γ, or IL-2) from memory Th2 cells (Fig. S2 *E* and *F*), indicating that autocrine stimulation via IL-2 produced by activated memory Th2 cells is unlikely.

To confirm the importance of IL-2 and IL-4 produced from activated iNKT cells in vivo, we examined cell division of memory Th2 cells after α-GalCer administration in IL-2–deficient mice and IL-4–deficient mice (Fig. 2*E*). We found no cell division of memory Th2 cells in IL-2 KO and Jα18 KO mice, but moderate cell division in IL-4 KO mice. These results indicate that IL-2 is the major cytokine responsible for the induction of α-GalCer–mediated proliferation of memory Th2 cells. The proliferation of memory Th1 cells after α-GalCer administration also appeared to be dependent largely on IL-2 produced by activated iNKT cells (Fig. S3). Memory CD8 T cells, such as memory Tc1 and Tc2 cells generated in our effector T-cell transfer system (28), also showed a dramatic α-GalCer–induced proliferation that was also largely dependent on IL-2 (Fig. S4).

We also found that another iNKT-cell ligand, α-glucoronsylceramide (GSL-1), a glycosphingolipid derived from *Sphingomonas* (22), induced similar effects on the memory T-cell population. After splenocyte coculture with GSL-1′–pulsed BMDCs, IL-2 and IFN-γ production was lower than that induced by α-GalCer, but IL-4 production was comparable (Fig. S5*A*). GSL-1′ also induced proliferation of memory Th1 and Th2 cells, which was inhibited by anti–IL-2 (Fig. S5*B*). GSL-1′–pulsed CD1d KO BMDCs did not induce the proliferation of memory Th2 cells (Fig. S5*C*), whereas both α-GalCer– and GSL-1′–pulsed BMDCs induced cell division of memory Th1 and Th2 cells in the liver (Fig. S5*D*).

IL-21 Enhanced IL-2–Mediated Memory CD4 T-Cell Proliferation and STAT5 Signaling. Although IL-21 alone did not induce the proliferation of memory Th1 or Th2 cells, IL-21 significantly augmented IL-2–induced proliferation of purified memory Th1 and Th2 cells (Fig. S6*A*). In fact, IL-21R expression was up-regulated on memory Th1 and Th2 cells by IL-2 (Fig. S6*B*). Stimulation of memory Th1 and

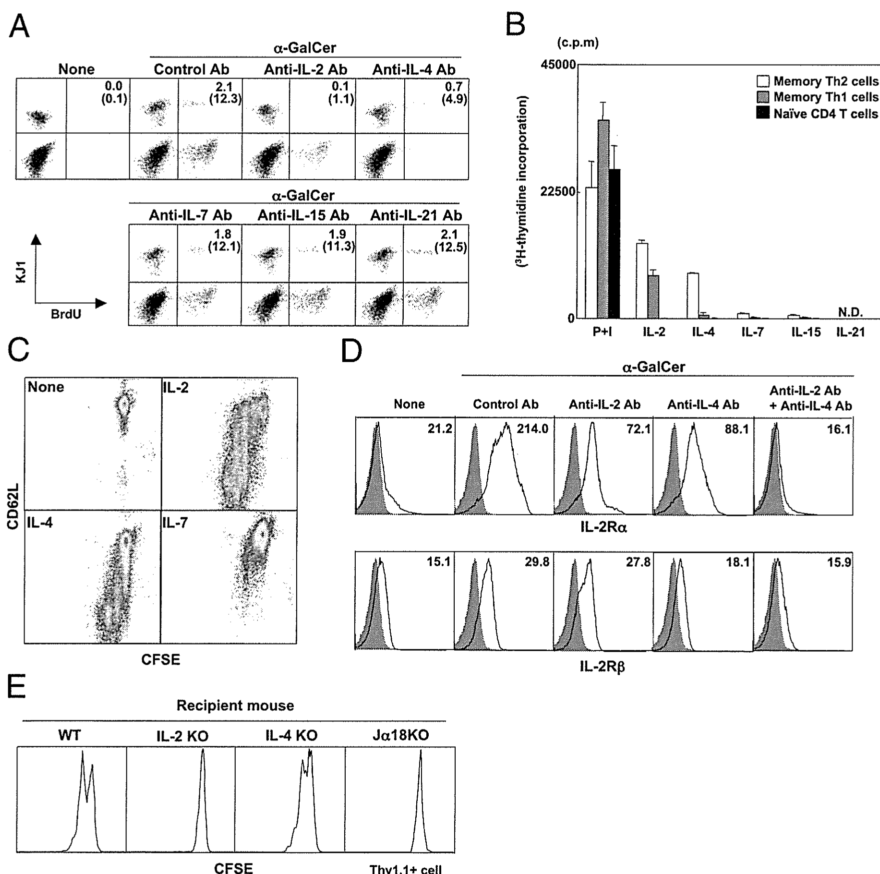

Fig. 2. IL-2 produced by activated iNKT cells induced the proliferation of memory Th1 and Th2 cells. (*A*) BrdU incorporation by memory Th2 cells in vitro. Memory Th2 cells (2 × 10^5) were purified from the spleens of memory Th2 mice and cocultured with BALB/c splenocytes (2 × 10^6) in the presence of α-GalCer (100 ng/mL) or absence of neutralizing antibodies (10 μg/mL) for 3 d. Values represent the number of cells as a percentage of total cells (in parentheses, as a percentage of KJ1$^+$ cells). (*B*) [^{3}H]-thymidine incorporation in memory Th cells and naïve CD4 T cells. Purified memory Th1 and Th2 cells (2 × 10^5) and naïve CD4 T cells (2 × 10^5) from DO11.10 Tg mice were stimulated with phorbol myristate acetate (50 ng/mL) plus ionomycin (500 nM) or each cytokine in the presence of IL-2 (25 U/mL), IL-4 (100 U/mL), IL-7 (100 U/mL), IL-15 (100 ng/mL), or IL-21 (100 ng/mL). (*C*) Purified CD62L^{hi} memory Th2 cells were labeled with CFSE and stimulated with each cytokine for 48 h with the same concentration of cytokines used in *B*. (*D*) The expression of IL-2Rα and IL-2Rβ on memory Th2 cells stimulated with splenocytes (2 × 10^6) and α-GalCer (100 ng/mL) in presence of anti–IL-2 and/or anti–IL-4 antibody (10 μg/mL). (*E*) Memory Th2 cells (Thy1.1$^+$; C57BL/c background) cells (5 × 10^6) were labeled with CFSE and transferred into WT, IL-2 KO, IL-4 KO, or Jα18 KO mice (Thy1.2$^+$; C57BL/c background). On day 1, the mice were given α-GalCer. The divisions of memory Th2 cells in the liver were determined by flow cytometry on day 7. The donor cells and recipient mice used in these experiments were BALB/c background except for those shown in Fig. 1*E*. Similar data were obtained from at least three (*A*–*D*) or two (*E*) independent experiments.

Th2 cells with IL-2, IL-21, and IL-2 plus IL-21 for 30 min revealed that STAT5 phosphorylation was up-regulated by IL-2 or IL-21 and was greatly enhanced by IL-2 and IL-21 together (Fig. S6*C*). STAT3 phosphorylation was induced by IL-21 in memory Th2 cells, but was not enhanced by costimulation with IL-2 (Fig. S6*D*). Thus, IL-2 appears to up-regulate the IL-21R on memory Th1 and Th2 cells, and IL-21 enhances IL-2–induced proliferation accompanied by enhanced activation of the STAT5 signaling pathway.

Type II NKT Cells Contributed to the Maintenance of Memory Th1 and Th2 Cells. We next examined whether type II NKT cells also contribute to the proliferation of memory Th1 and Th2 cells when activated with an endogenous ligand, sulfatide (30). Memory Th2 cells were cultured with sulfatide-pulsed BMDCs and splenocytes as a source of type II NKT cells. Increased numbers of BrdU-incorporated memory Th2 cells were observed in the culture with sulfatide-pulsed WT BMDCs, but not in the culture with sulfatide-pulsed CD1d KO BMDCs (Fig. 3*A*). Sulfatide stimulation induced the production of IL-2 and IL-4, but at lower levels than those induced by α-GalCer (Fig. 3*B*). The sulfatide-induced proliferation of memory Th1 and Th2 cells was also dependent on IL-2, as demonstrated by the finding that the addition of anti–IL-2 mAbs to the culture inhibited proliferation (Fig. 3*C*). Moreover, sulfatide-pulsed BMDCs induced cell division of both memory Th1 and Th2 cells in vivo (Fig. 3*D*).

To examine the physiological role of NKT cells in the maintenance of memory Th1 and Th2 cells, we transferred memory CD4 T cells into NKT-cell–deficient mice. Given the unavailability of an appropriate specific marker for type II NKT cells, the most reliable way to study the in vivo function of type II NKT cells is by comparing the results obtained using CD1d KO mice (which lack both iNKT cells and type II NKT cells), Jα18 KO mice (which lack only iNKT cells), and WT mice. In this experiment, we generated memory Th1 or Th2 cells in WT mice; transferred these cells into WT, Jα18 KO, or CD1d KO mice; and then evaluated the numbers of memory Th1 or Th2 cells maintained in the liver after 2 mo. The percentages of memory Th1 and Th2 cells were lower in CD1d KO mice compared with WT mice, whereas the percentages of memory Th1 and Th2 cells in Jα18 KO mice were similar to that of WTmice (Fig. 3*E*). The absolute numbers of memory Th1 and Th2 cells were significantly reduced in CD1d KO mice compared with WT and Jα18 KO mice, but the reduced numbers of these cells in Jα18 KO mice was not significant compared with WT mice (Fig. 3*F*). The decreases in memory Th1 and Th2 cells in the CD1d KO mice might be related to rejection, given that memory Th1 and Th2 cells express CD1d molecules (Fig. S7*A*), and CD1d KO mice might not be tolerant of CD1d. However, the maintenance of CD1d KO memory Th2 cells was also significantly impaired in CD1d KO mice, which excludes the possibility of rejection of CD1d-expressing memory Th2 cells in CD1d KO mice (Fig. S7*B*). Moreover, CD1d KO memory Th2 cells were normally generated in WT mice. Therefore, NKT cells play an important role in the maintenance of memory Th2 cells. Taken together, these data indicate that type II NKT cells may be activated by endogenous ligands presented on CD1d and contribute to the maintenance of memory Th1 and Th2 cells.

α-GalCer Administration Altered the Function of Memory Th2 Cells and Attenuated Memory Th2-Cell–Dependent Allergic Airway Inflammation. To assess the function of memory Th2 cells after iNKT-cell activation, we sorted memory Th2 cells from the memory Th2 mice at

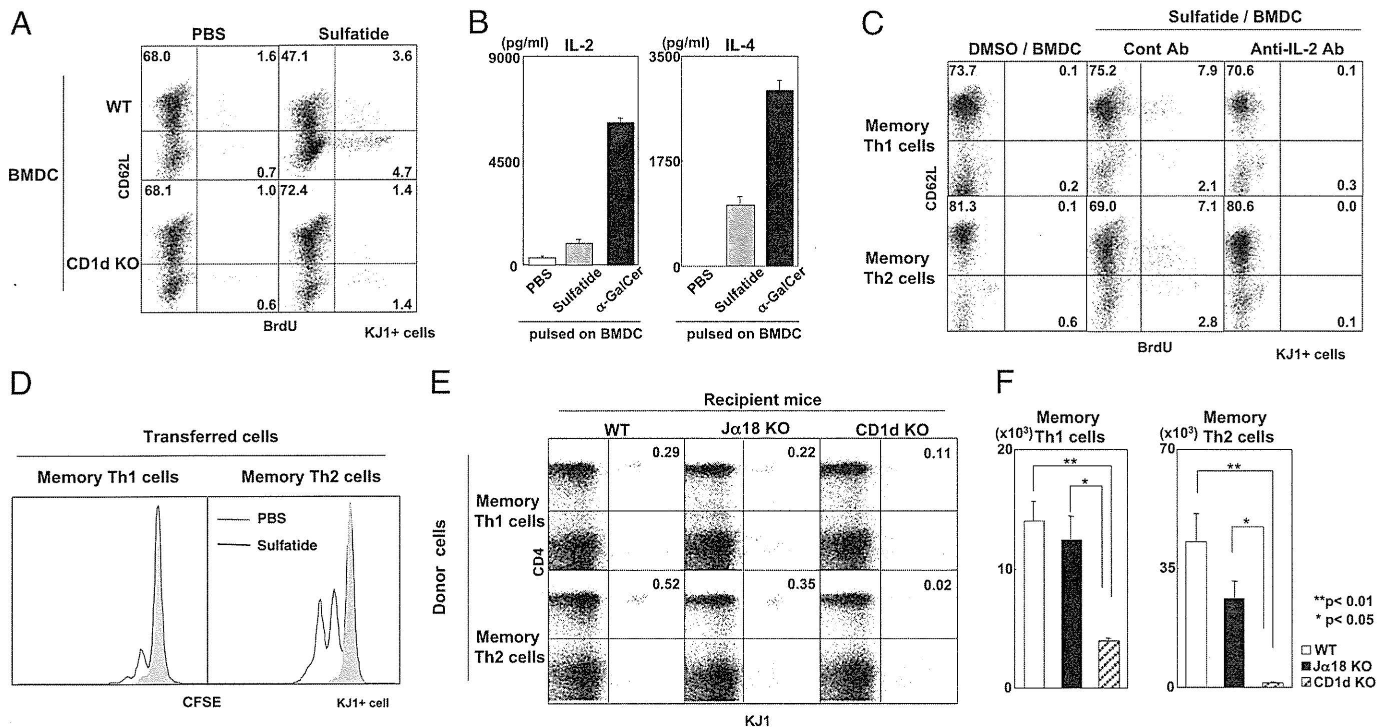

Fig. 3. Type II NKT cells contributed to the maintenance of memory Th1 and Th2 cells. (*A*) BrdU incorporation by memory Th2 cells stimulated with sulfatide was examined by flow cytometry. Memory Th2 cells (2×10^5) prepared from memory Th2 mice were cultured with BALB/c splenocytes (2×10^6) and sulfatide-pulsed WT or CD1d KO BMDCs (4×10^5) for 72 h. (*B*) Splenocytes from BALB/c mice were cultured with sulfatide or α-GalCer–pulsed BMDCs for 72 h. Cytokine concentrations in the culture supernatants were determined by ELISA. (*C*) Memory Th1 and Th2 cells were prepared and stimulated with sulfatide-pulsed BMDCs in the presence of BALB/c splenocytes and anti–IL-2 mAb (10 μg/mL). (*D*) Memory Th1 or Th2 cells (5×10^6) were labeled with CFSE and adoptively transferred into naïve syngenic mice. Sulfatide-pulsed BMDCs (5×10^5) were transferred the next day. On day 7, cell division of memory Th1 or Th2 cells in the liver was assessed. (*E* and *F*) Memory Th1 or Th2 cells (5×10^6) prepared from the spleens of memory Th1 and Th2 mice were transferred into syngeneic WT, Jα18 KO, and CD1d KO mice. Sixty days later, percentages and absolute number of memory Th1 and Th2 cells in the liver were assessed. No specific stimulation, such as with sulfatide/BMDCs, was used for this experiment. The donor cells and recipient mice used in this experiments were BALB/c background. Similar data were obtained from at least three independent experiments. Values are mean ± SEM ($n = 5$). $*P < 0.05$; $**P < 0.01$.

30 d after α-GalCer treatment and stimulated these cells with OVA peptide and irradiated allophycocyanin. After iNKT-cell activation in vivo, IL-4, IL-5, and IL-13 production was reduced, but IFN-γ production was increased (Fig. 4*A*), indicating acquisition of the Th1 phenotype by memory Th2 cells. The production of IL-2 and IFN-γ by memory Th1 cells was not obviously altered by the α-GalCer treatment (Fig. S8*A*).

To assess the effect of activation of iNKT cells on memory Th2-cell function in vivo, we examined memory Th2 cell-dependent allergic airway inflammation (Fig. S8*B*). Airway hyperreactivity (AHR) and eosinophillic infiltration were reduced in the α-GalCer–injected OVA-challenged memory Th2 mice compared with PBS-injected OVA-challenged control mice (Fig. 4 *B* and *C*). The leukocytes accumulated in the peribronchiolar regions of the lungs were greatly decreased in the memory Th2 mice treated with α-GalCer (Fig. S8*C*). In addition, reduced periodic acid-Schiff staining was evident in the bronchial epithelium of the asthmatic lungs (Fig. S8*D*). Furthermore, reduced IL-4, IL-5, and IL-13 production and increased IFN-γ production in bronchoalveolar lavage fluid were detected in samples from α-GalCer–treated memory Th2 mice (Fig. S8*E*). Finally, we transferred memory Th2 cells into normal mice and then exposed these mice to OVA to induce allergic responses (Fig. S8*F*). AHR and eosinophil infiltration into the airways were reduced in the mice that received memory Th2 cells prepared from mice treated with α-GalCer compared with the mice that received cells from mice treated with PBS (Fig. 4 *D* and *E*). These results indicate that activation of iNKT cells alters memory Th2-cell function and attenuates memory Th2 cell-dependent allergic airway inflammation.

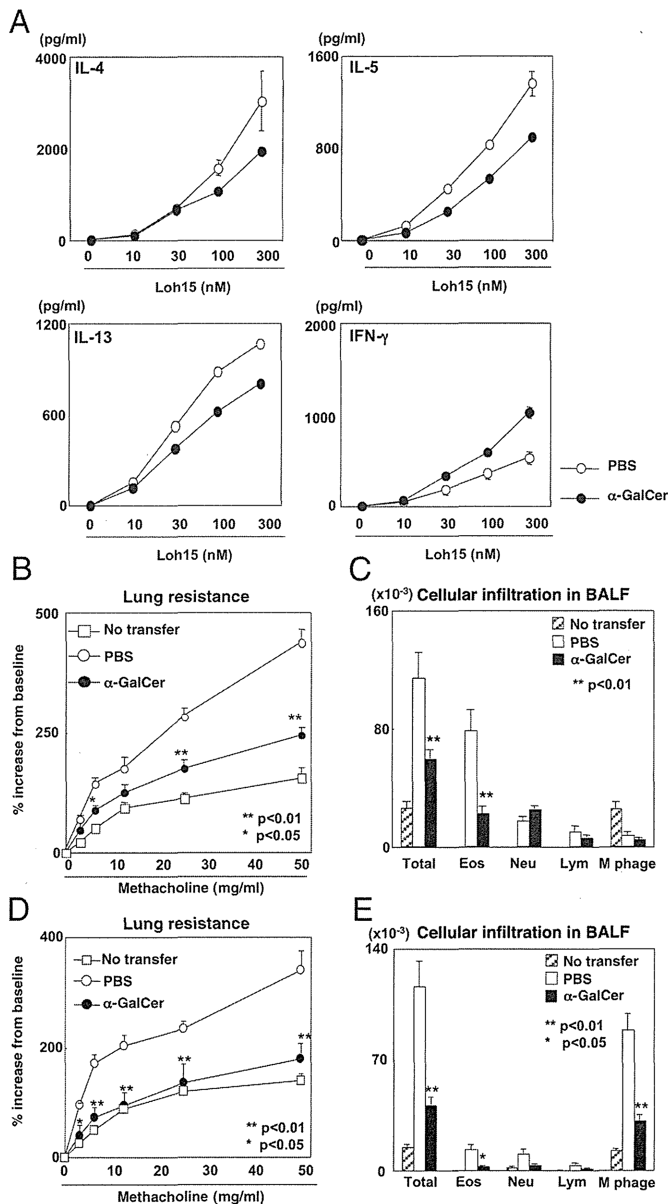

Fig. 4. α-GalCer administration altered the function of memory Th2 cells and attenuated memory Th2-dependent allergic airway inflammation. (*A*) Memory Th2 cells were purified from memory Th2 mice treated with or without α-GalCer (100 μg/kg) at 30 d before analysis. Memory Th2 cells (4×10^4) were purified from the spleens of these mice and then stimulated with irradiated splenocytes (2×10^5) and antigenic peptide (Loh15) at the indicated concentrations. Three days later, culture supernatants were collected, and cytokine levels were determined by ELISA. (*B* and *C*) Thirty days after α-GalCer administration, memory Th2 mice were exposed to OVA aerosol to induce airway inflammation. One day after the last OVA challenge, AHR was assessed by measuring lung resistance. The absolute numbers of leukocytes in the bronchoalveolar lavage fluid are shown. (*D* and *E*) Memory Th2 cells were transferred as shown in Fig. S8*F*, and AHR and leukocyte infiltration were assessed as *B* and *C*. (*B–E*) Values are mean ± SEM ($n = 5$). The donor cells and recipient mice used in these experiments were BALB/c background. Similar data were obtained from at least three independent experiments. $*P < 0.05$; $**P < 0.01$.

Discussion

Here we report that iNKT cells activated with glycolipid ligands selectively induced proliferation of memory Th1 and Th2 cells and increased the steady-state numbers of memory Th1 and Th2 cells in vivo. IL-2 produced by activated iNKT cells plays a major role in inducing the proliferation of both memory Th1 and Th2 cells, with IL-4 also playing a role in the proliferation of Th2 cells. Type II NKT cells appear to be required to maintain the steady-state population of memory Th1 and Th2 cells, possibly in response to recognition of an endogenous ligand. Furthermore, activated iNKT cells alter the function of memory Th2 cells, resulting in the attenuation of memory Th2-dependent allergic airway inflammation. Thus, NKT cells appear to control memory CD4 T-cell pool size and also function in vivo.

We found that IL-2 produced by activated NKT cells induced the proliferation of memory Th1 and Th2 cells, but not of naïve CD4 T cells (Fig. 1). Because this proliferation is independent of antigen recognition by TCRs, exposure to cytokines such as IL-2 appears to be sufficient to induce memory CD4 T-cell proliferation. IL-2 upregulated IL-2R components, particularly IL-2Rα, on memory Th2 cells (Fig. 2) and facilitated the IL-2/STAT5–mediated proliferation of these cells (Fig. S6*C*). We found that memory Th1 and Th2 cells proliferated in response to IL-2 produced by NKT cells activated with GSL-1′, a component of the cell wall of the Gram-negative bacteria *Sphingomonas* (22) (Fig. S5). Therefore, iNKT cells likely are activated during bacterial infection, leading to the bystander proliferation of memory Th1 and Th2 cells. In addition to IL-2, both IL-4 and IL-21, which are produced by activated iNKT cells, contributed to the proliferation of memory CD4 T cells (Fig. S6). IL-4 alone induced the proliferation of memory Th2 cells (Fig. 2*B*), but the level was lower than that induced by IL-2. α-GalCer–induced upregulation of IL-2Rα and IL-2Rβ expression was inhibited by anti-IL-4 (Fig. 2*D*), indicating that IL-4 may facilitate the IL-2–mediated proliferation of memory Th2 cells. IL-4 had only a marginal role in the induction of memory Th1-cell proliferation, probably because of the low expression of IL-4R on these cells. IL-21 augmented the IL-2–mediated proliferation of memory Th1 and Th2 cells, accompanied by increased phosphorylation of STAT5 (Fig. S6). Thus, although IL-2/STAT5–mediated proliferation appears to be of central importance, several cytokines produced by activated iNKT cells may cooperate to induce the proliferation of memory Th1 and Th2 cells.

The maintenance of memory Th1 and Th2 cells was significantly impaired in CD1d KO mice lacking both type I and type II NKT cells, whereas no obvious effect was observed in Jα18 KO mice lacking only iNKT cells (Fig. 3). This finding indicates that type II NKT cells play an important role in maintaining the

steady-state pool size of memory CD4 T cells independent of activation by infectious agents. It is possible that type II NKT cells are activated by endogenous ligands in vivo, thereby controlling the steady-state numbers of memory Th1 and Th2 cells. In fact, sulfatide, an endogenous ligand for type II NKT cells, induced the proliferation of memory Th1 and Th2 cells, again via IL-2 (Fig. 3). These data suggest a possible role for sulfatide in the maintenance of memory CD4 T cells in vivo. Although further specific studies are needed to identify the most important endogenous ligands recognized by NKT cells to maintain the memory CD4 T-cell pool, our data indicate an implicit role for NKT cells in the maintenance of T-cell memory.

TCR stimulation in concert with type I and type II IFNs and IL-12 during lymphocytic choriomeningitis virus infection has been found to reprogram effector Th2 cells to Th2+1 phenotype cells by the induction of T-bet (31), indicating that polarized Th cells display a greater degree of functional plasticity than previously believed (6). Our findings also may indicate that the Th-cell function can be modulated in the microenvironment in which memory T cells reside, and that a mixed cytokine secretion phenotype in memory Th cells can be induced particularly in the peripheral tissues, where various microorganisms interact with the regional immune system under physiological conditions. It will be interesting to study the factors induced in the microenvironment that can modulate the function of memory Th cells in various in vivo infectious and disease models.

In summary, we have demonstrated that activation of NKT cells induces the proliferation of memory $CD4^+$ Th1 and Th2 cells through the production of IL-2. This may occur during infection and also even in steady state to control maintenance of the memory CD4 T-cell pool in the body. In addition, activated NKT cells alter the function of memory Th2 cells. Therefore, NKT cells control the quantity and quality of memory CD4 T cells in vivo, and thus the current study may provide insight into the immunoregulatory role of NKT cells in vaccine development for infectious diseases, as well as the pathogenesis of chronic allergic disorders and autoimmune diseases.

Materials and Methods

Generation of Memory Th1 and Th2 Cells in Vivo. Memory Th1 and Th2 cells were generated as described previously (28). In brief, CD4 T cells from DO11.10 OVA-specific TCR Tg mice or OT-II Tg mice were stimulated with specific OVA peptides (Loh15) plus allophycocyanin for 6 d in vitro under either Th1 or Th2 conditions. These effector Th1 or Th2 cells (3×10^7) were transferred i.v. into syngeneic recipient mice (BLAB/c, C57BL/6, BALB/c *nu*/*nu*, or TCR-βδ KO mice).

Cell Division Assay. For analysis of cell division by CFSE staining, donor memory Th1 or Th2 cells were generated in BALB/c *nu*/*nu* or TCR-βδ KO mice, isolated with a CD4 T-cell isolation kit and AutoMACS separator (Miltenyi Biotec), and then labeled with CFSE (Invitrogen). The day after adoptive transfer into syngenic mice, recipient mice were injected i.p. with α-GalCer (100 μg/kg) or PBS. Six days later, donor T cells were analyzed by flow cytometry.

ACKNOWLEDGMENTS. We thank Chizuka Obara, Kaoru Sugaya, Hikari Asou, Miki Kato, and Toshihiro Ito for their excellent technical assistance. This work was supported by the Global Center for Education and Research in Immune System Regulation and Treatment Program and by the City Area Program (Kazusa/Chiba Area) of the Ministry of Education, Culture, Sports, Science, and Technology of Japan; and by grants from Ministry of Education, Culture, Sports, Science, and Technology of JapanGrants-in-Aid for Scientific Research (B) 21390147 and for Young Scientists (B) 22790452; the Ministry of Health, Labor and Welfare of Japan; the Uehara Memorial Foundation; the Mochida Foundation; and the Naito Foundation.

1. Palucka K, Ueno H, Banchereau J (2011) Recent developments in cancer vaccines. *J Immunol* 186:1325–1331.
2. Pulendran B, Ahmed R (2011) Immunological mechanisms of vaccination. *Nat Immunol* 12:509–517.
3. Endo Y, et al. (2011) Eomesodermin controls interleukin-5 production in memory T helper 2 cells through inhibition of activity of the transcription factor GATA3. *Immunity* 35:733–745.
4. Woodfolk JA (2007) T-cell responses to allergens. *J Allergy Clin Immunol* 119:280–294.
5. Hiepe F, et al. (2011) Long-lived autoreactive plasma cells drive persistent autoimmune inflammation. *Nat Rev Rheumatol* 7:170–178.
6. O'Shea JJ, Paul WE (2010) Mechanisms underlying lineage commitment and plasticity of helper $CD4^+$ T cells. *Science* 327:1098–1102.
7. Reiner SL (2007) Development in motion: Helper T cells at work. *Cell* 129:33–36.
8. Schluns KS, Lefrançois L (2003) Cytokine control of memory T-cell development and survival. *Nat Rev Immunol* 3:269–279.
9. Kalia V, Sarkar S, Gourley TS, Rouse BT, Ahmed R (2006) Differentiation of memory B and T cells. *Curr Opin Immunol* 18:255–264.
10. Lefrançois L (2006) Development, trafficking, and function of memory T-cell subsets. *Immunol Rev* 211:93–103.
11. Stockinger B, Bourgeois C, Kassiotis G (2006) $CD4^+$ memory T cells: Functional differentiation and homeostasis. *Immunol Rev* 211:39–48.
12. Williams MA, Bevan MJ (2007) Effector and memory CTL differentiation. *Annu Rev Immunol* 25:171–192.
13. Nakayama T, Yamashita M (2008) Initiation and maintenance of Th2 cell identity. *Curr Opin Immunol* 20:265–271.
14. MacLeod MK, Clambey ET, Kappler JW, Marrack P (2009) CD4 memory T cells: What are they and what can they do? *Semin Immunol* 21:53–61.
15. McKinstry KK, Strutt TM, Swain SL (2010) The potential of CD4 T-cell memory. *Immunology* 130:1–9.
16. Bendelac A, Savage PB, Teyton L (2007) The biology of NKT cells. *Annu Rev Immunol* 25:297–336.
17. Cohen NR, Garg S, Brenner MB (2009) Antigen presentation by CD1 lipids, T cells, and NKT cells in microbial immunity. *Adv Immunol* 102:1–94.
18. Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H (2003) The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol* 21: 483–513.
19. Chang YJ, et al. (2011) Influenza infection in suckling mice expands an NKT cell subset that protects against airway hyperreactivity. *J Clin Invest* 121:57–69.
20. Godfrey DI, Rossjohn J (2011) New ways to turn on NKT cells. *J Exp Med* 208: 1121–1125.
21. Kinjo Y, et al. (2011) Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. *Nat Immunol* 12:966–974.
22. Tupin E, Kinjo Y, Kronenberg M (2007) The unique role of natural killer T cells in the response to microorganisms. *Nat Rev Microbiol* 5:405–417.
23. Cerundolo V, Silk JD, Masri SH, Salio M (2009) Harnessing invariant NKT cells in vaccination strategies. *Nat Rev Immunol* 9:28–38.
24. Motohashi S, Okamoto Y, Yoshino I, Nakayama T (2011) Anti-tumor immune responses induced by iNKT cell-based immunotherapy for lung cancer and head and neck cancer. *Clin Immunol* 140:167–176.
25. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L (2004) NKT cells: What's in a name? *Nat Rev Immunol* 4:231–237.
26. Blomqvist M, et al. (2009) Multiple tissue-specific isoforms of sulfatide activate CD1d-restricted type II NKT cells. *Eur J Immunol* 39:1726–1735.
27. Godfrey DI, Stankovic S, Baxter AG (2010) Raising the NKT cell family. *Nat Immunol* 11:197–206.
28. Nakayama T, Yamashita M (2009) Critical role of the Polycomb and Trithorax complexes in the maintenance of CD4 T cell memory. *Semin Immunol* 21:78–83.
29. Parekh VV, et al. (2005) Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* 115:2572–2583.
30. Jahng A, et al. (2004) Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. *J Exp Med* 199: 947–957.
31. Hegazy AN, et al. (2010) Interferons direct Th2 cell reprogramming to generate a stable GATA-$3^{(+)}$T-$bet^{(+)}$ cell subset with combined Th2 and Th1 cell functions. *Immunity* 32:116–128.