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Expansion of Lung Vα14 NKT Cells by Administration of α-Galactosylceramide-pulsed Dendritic Cells

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NKT cells, a novel murine lymphoid lineage bearing an invariant T cell receptor encoded by Vα14 and Jα281 gene segments, recognize a specific ligand glycolipid, α-galactosylceramide (α-GalCer) in a CD1d-dependent manner. Recent research has revealed that activated Vα14 NKT cells have dramatic antitumor effects against a wide variety of tumor cell lines *in vivo* and *in vitro*. Here, we demonstrate strong *in vivo* antitumor effects brought about by treatment with α-GalCer-pulsed dendritic cells in comparison with *in vitro*-activated Vα14 NKT cells. Furthermore, we show a significant expansion of endogenous Vα14 NKT cells in the lung following the administration of α-GalCer-pulsed dendritic cells. The feasibility of immunotherapy with α-GalCer-pulsed dendritic cells is discussed.

Key words: NKT cell — Dendritic cell — α-Galactosylceramide

A newly identified lymphocyte subset, NKT cells, has been recently characterized.[1)] Most murine NKT cells express an invariant T cell receptor encoded by Vα14 and Jα281 gene segments.[2–4)] Therefore, NKT cells are designated Vα14 NKT cells, and can be detected in various peripheral tissues with differing frequencies. Vα14 NKT cells play crucial roles in various immune responses *in vivo*, including the induction of autoimmune diseases such as type 1 diabetes mellitus.[5, 6)] Recently, these Vα14 NKT cells were reported to be activated by a specific ligand, α-GalCer in a CD1d-dependent fashion.[7–9)] After activation, strong antitumor effects mediated by perforin-dependent cytotoxicity were observed against a wide variety of tumor cells *in vivo* and *in vitro*.[10, 11)] Recently, Smyth *et al.* reported an important physiological function of Vα14 NKT cells in tumor surveillance.[12)] In addition, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been reported to play a critical role in the IFNγ-mediated antitumor effect of α-GalCer-activated Vα14 NKT cells.[13)] We have established activation cultures of mouse and human NKT cells in anti-CD3ε-antibody-coated plates in the presence of rIL-2 or α-GalCer, and these cells were found to express strong cytotoxicity.[14, 15)] Intravenous injection of α-GalCer-pulsed dendritic cells (DCs) produced a potent anti-tumor effect in B16 melanoma liver metastasis models, in which metastasized tumor nodules disappeared, and furthermore, intravenous injection of α-GalCer-pulsed DCs was more effective than administration of α-GalCer itself.[16)] On the other hand, the direct injection of α-GalCer also inhibited tumor metastasis in the liver and lung.[10)] However, up until now, there has been no direct evidence for the activation and expansion of endogenous Vα14 NKT cells by the administration of α-GalCer-pulsed DCs *in vivo*.

In this report, we present evidence of the specific expansion of Vα14 NKT cells *in vivo* following the administration of α-GalCer-pulsed DCs. The efficiency of α-GalCer-pulsed DCs compared with cultured Vα14 NKT cells activated *in vitro* was examined, using a mouse Lewis lung carcinoma (LLC) lung metastatic model. The feasibility of novel immunotherapy for lung cancer patients using α-GalCer-pulsed DCs is discussed.

MATERIALS AND METHODS

Mice Vα14 NKT mice (RAG-1$^{-/-}$Vα14 transgenic (t.g.) Vβ8.2tg mice) with a C57BL/6 (B6) background were established by mating with RAG-1$^{-/-}$Vβ8.2tg and RAG-1$^{-/-}$Vα14tg mice as described.[7)] In the Vα14 NKT mice, only transgenic TCRαβ (Vα14tg and Vβ8.2tg) was expressed, resulting in the preferential development of Vα14 NKT cells with undetectable levels of T cells, NK cells, or B cells. RAG-1$^{-/-}$ mice were kindly provided by Dr. P. Momberts, MIT, Boston, MA.[17)] B6 mice were purchased from Japan SLC (Shizuoka). All mice used in this

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study were 8–12 weeks old and were maintained in our facility under specific pathogen-free conditions. Animal care was in accordance with the guidelines of Chiba University.

α-Galactosylceramide α-Galactosylceramide ((2S,3S, 4R)-1-O-(α-D-galactopyranosyl)-N-hexacosanoyl-2-amino-1,3,4-octadecanetriol; α-GalCer) was provided by Kirin Brewery (Gunma) and prepared as described previously.[7, 10]

Purification and preparation of DCs pulsed with α-GalCer or vehicle DCs were purified using the method described by Crowley *et al.* with some alterations.[7, 18] B6 spleens were treated with collagenase type 3 (400 U/ml; Worthington Biochemical, Freehold, NJ) for 20 min at 37°C in 5% CO_2, and then disrupted on a metal screen. The resulting single cell suspensions were loaded on dense BSA (Pentax Path-O-Cyte 4; Bayer, IL) and centrifuged at 1500*g* for 30 min at 4°C. The low-density fraction was applied to plastic culture dishes (Falcon, Franklin Lakes, NJ) for 90 min at 37°C in 5% CO_2. Adherent cells were pulsed with α-GalCer (100 ng/ml in 0.025% Polysolvate 20) or control vehicle (0.025% Polysolvate 20) overnight at 37°C. After extensive washing, the non-adherent cells were used as α-GalCer-pulsed or vehicle-pulsed DCs. The character of splenic DCs is broadly similar to that of monocyte-derived DCs.[19–22]

***In vitro* expansion of mouse Vα14 NKT cells** Vα14 NKT mouse spleens were homogenized on slide glasses and RBCs were lysed with red blood cell lysing buffer (Sigma Chemical Co., St. Louis, MO). Then, the cells (1×10^5 cells) were cultured in 96-well U-bottomed culture plates coated with anti-CD3ε mAb (10 μg/ml) in rIL-2 (50 U/ml) containing RPMI-1640 media for 4 days at 37°C in 5% CO_2. Next, the cells were transferred to new 96-well plates (1×10^4 cells/well) and cultured in rIL-2-containing medium for 3 days. Two cycles of this culture were carried out. Within 7 days, the cells had expanded 10 times, reaching about 100 times by the end of the second cycle of culture.[14] These cells (Vα14 NKT mouse origin) were used as cultured NKT cells.

^{51}Cr release cytotoxicity assay The ^{51}Cr release assay was conducted by the previously described method.[10] The cytotoxic activity of cultured Vα14 NKT cells was assessed for B16 melanoma cells, YAC-1 cells, FBL-3 cells, EL-4 cells, and LLC cells labeled with 100 μCi of sodium chromate (^{51}Cr; Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h at 37°C. Where indicated, B6 DCs were pulsed with α-GalCer or vehicle overnight at 37°C in the complete culture medium. Then various numbers of DCs were co-cultured overnight with 2×10^5 Vα14 NKT cells. The cytotoxic activity of these Vα14 NKT cells was evaluated against ^{51}Cr-labeled LLCs.

Tumor metastasis model LLC cells (4×10^5) were inoculated into B6 mice via the tail vein on day 0. The mice were sacrificed on day 21, and the numbers of lung metastatic LLC nodules were counted under a light microscope. Three mice were used for each group in each experiment.

Homing of cultured Vα14 NKT cells and DCs in the lung Two kinds of cells, DCs and cultured NKT cells, were labeled with 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) purchased from Molecular Probes, Inc. (Eugene, OR) for one min at room temperature, and injected into B6 mice via the tail vein. The mice were sacrificed 3, 6, 24, and 96 h later, and the lungs were slowly frozen in liquid N_2, and sliced into 8 μm sections with a microtome. Then, the sections were dried on slide glasses and observed with a laser scanning microscope, LSM510 (Carl Zeiss, Oberkochen, Germany).

Preparation of lung mononuclear cells (MNC) for RT-PCR α-GalCer-pulsed DCs and vehicle-pulsed DCs were injected intravenously into three B6 mice. Then, 40 h later, the lungs were removed, cut into small pieces, and incubated for 30 min in MEM containing 0.05% collagenase and 0.01% trypsin inhibitor in a shaking water bath at 37°C. The cells were passed through 200-gauge stainless steel mesh, and the mononuclear cells were separated by the Percoll centrifugation method as described.[23] The pellet was resuspended in the lysis solution for RBC, and then washed twice in MEM. These cells were used for RNA isolation.

RT-PCR Total RNA was eluted with TRIzol and chloroform from the cells derived from lungs as described above. The RNA was then incubated with oligo dt primer for 10 min at 70°C, chilled rapidly on ice and used to synthesize cDNA. The cDNA was used for PCR. The following specific primers were used: CCG AAT TCC CAA GTG GAG CAG AGT CCT, Vα14 forward; TCG AAT TCC TGT CCT GAG ACC, Vα14 reverse; TAC TGC CAC GGC ACA GTC ATT GAA, IFNγ forward; GCA GCG ACT CCT TTT CCG CTT CCT, IFNγ reverse; ACA TCT GCT GGA AGG TGG AC, β-actin forward; and GAG AGG GAA ATC GTG CGT GA, β-actin reverse.

Flow cytometry The proportion of Vα14 NKT cells in the lung of B6 mice was evaluated by flow cytometric analysis with anti-mouse CD3 mAb and α-GalCer-loaded CD1d tetramer-PE as described.[24]

RESULTS

***In vitro* and *in vivo* anti-tumor effects of cultured Vα14 NKT cells injected on day 0** Vα14 NKT cells from NKT mice were cultured with recombinant IL-2 and plate-bound anti-CD3ε antibody.[14] By this method, the Vα14 NKT cells were expanded up to 100 times in two weeks, and showed strong anti-tumor effects on various tumor cells *in vitro* (Fig. 1A). We used several tumor cell lines, including B16 melanoma, FBL-3, LLC, and EL-4. The cultured Vα14 NKT cells showed strong cytotoxicity

against B16 melanoma cells, FBL-3 cells, and LLC cells, but had no cytotoxic activity against EL-4 cells. To test whether the transfer of cultured Vα14 NKT cells induces antitumor effects *in vivo*, a lung metastatic model of LLC tumors was used. As can be seen in Fig. 1B, the simultaneous injection of LLC (4×10^5) and cultured Vα14 NKT cells (1×10^5, or 3×10^5) resulted in a decrease in the number of metastatic nodules. These results suggest that cultured Vα14 NKT cells exert strong anti-tumor effects in an LLC metastatic model.

Inhibition of LLC lung metastasis by injection of α-GalCer-pulsed DCs but not cultured Vα14 NKT cells on day 1 α-GalCer-pulsed DCs show a strong ability to eradicate liver metastasis.[16] We wanted to compare the *in vivo* antitumor effects of α-GalCer-pulsed DCs and cultured Vα14 NKT cells in the LLC lung metastasis model (Fig. 2). One day after mice were inoculated with 4×10^5 LLCs, 3×10^6 α-GalCer-pulsed DCs, vehicle-pulsed DCs, 3×10^6 cultured Vα14 NKT cells, or α-GalCer (2 μg) were injected. As can be seen in Fig. 2, only α-GalCer and α-GalCer-pulsed DCs suppressed lung metastasis. The antitumor effect of α-GalCer-pulsed DCs was as great as that of α-GalCer alone in the case of day 1 injection (data not shown). No significant antitumor effect of cultured Vα14 NKT cells was observed. Neither repeating the injection of cultured Vα14 NKT cells three times (on days 1, 2, and 3), nor injecting large numbers (1×10^8) of cells resulted in any significant antitumor activity (data not shown). These results indicate that the antitumor effect of treatment with cultured Vα14 NKT cells is weak.

The homing of cultured Vα14 NKT cells and DCs in the lung after intravenous administration In order to assess the homing of cultured Vα14 NKT cells and DCs after intravenous administration, cells were labeled with CFSE and fluorescent cells in the lung were monitored under a confocal microscope (Fig. 3). Green fluorescent Vα14 NKT cells were seen 3 h after injection, and were no longer detectable at 6 h. In contrast, CFSE-labeled DCs were seen in the lung at 3 h, 6 h and 24 h

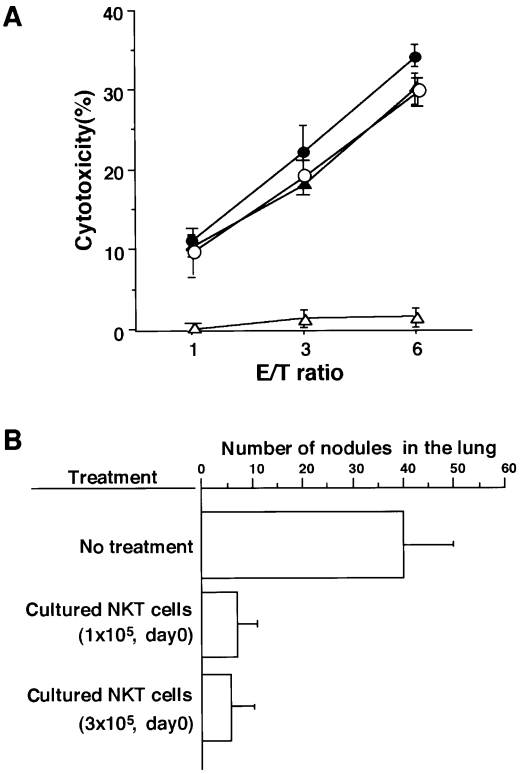

Fig. 1. Antitumor effects of cultured Vα14 NKT cells. A, Cytotoxicity of cultured Vα14 NKT cells from Vα14 NKT mice was evaluated using a standard ^{51}Cr releasing assay. Various target tumor cells were used: B16 (●), FBL-3 (▲), LLC (○), and EL-4 (△). Bars represent SDs. B, Antitumor effect of cultured Vα14 NKT cells from Vα14 NKT mice *in vivo*. LLC cells (4×10^5) were inoculated into B6 mice via the tail vein on day 0, and 1×10^5 or 3×10^5 cultured Vα14 NKT cells were administered simultaneously. On day 21, all mice were sacrificed and lung metastatic nodules were counted. Three mice were used in each group and mean values are shown with SDs.

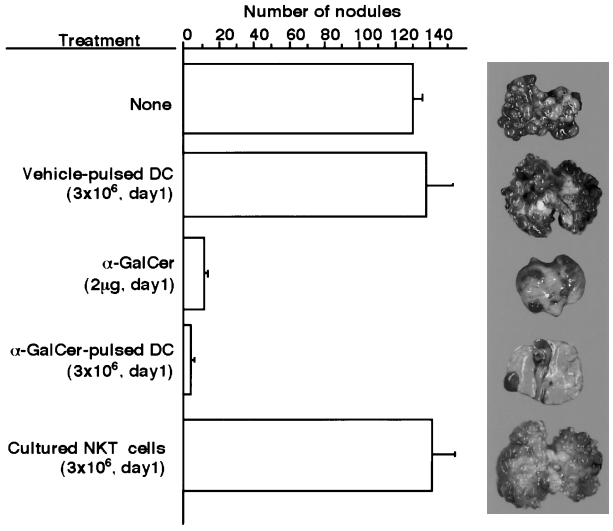

Fig. 2. Antitumor effect of vehicle-pulsed-DCs, α-GalCer-pulsed DCs, and cultured Vα14 NKT cells from Vα14 NKT mice *in vivo*. LLCs (4×10^5) were inoculated via the tail vain of B6 mice, and one day later 3×10^6 vehicle-pulsed DCs, α-GalCer-pulsed DCs (3×10^6), or 3×10^6 Vα14 NKT cells were administered. The numbers of metastatic nodules in the lung were counted on day 21. To compare the efficiency of these cells, the intraperitoneal injection of α-GalCer (2 μg/mouse) was done as a positive control. Representative photographic views are shown (right). Three mice were used in each group, and mean values with SDs are shown.

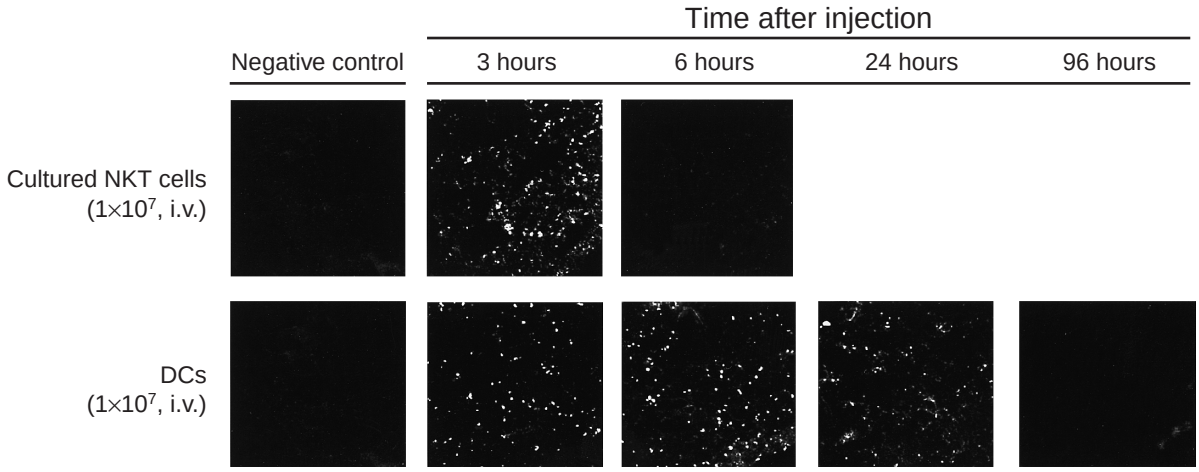

Fig. 3. The homing of cultured Vα14 NKT cells from Vα14 NKT mice and DCs in the lung. Cultured Vα14 NKT cells from Vα14 NKT mice and DCs (1×10^7) were labeled with CFSE and injected into B6 mice via the tail vein. The mice were sacrificed 3, 6, 24, 96 h later, and the lungs were frozen slowly in liquid N_2 and sliced (8 μm thickness) using a microtome. CFSE-labeled cells were visualized by a laser-scanning confocal microscope as fluorescent cells in the photographs.

after the injection. These results suggest that the DCs stay in the lung for at least 24 h. Thus, it appears that endogenous lung Vα14 NKT cells are efficiently stimulated by α-GalCer-pulsed DCs administered intravenously.

The ability of α-GalCer-pulsed DCs to activate quiescent Vα14 NKT cells *in vitro* The next experiment was designed to assess whether α-GalCer-pulsed DCs activate quiescent Vα14 NKT cells *in vitro*. α-GalCer-pulsed or vehicle-pulsed DCs and freshly prepared Vα14 NKT cells from NKT mice were co-cultured overnight in complete media. As shown in Fig. 4, Vα14 NKT cells cultured with α-GalCer-pulsed DCs showed strong cytotoxic activity against LLCs in a DC dosage-dependent manner. The cytotoxicity of Vα14 NKT cells cultured with vehicle-pulsed DCs was marginal. These results suggest that α-GalCer-pulsed DCs are able to activate quiescent Vα14 NKT cells *in vitro*.

Activation and expansion of endogenous lung Vα14 NKT cells of B6 mice Next, in order to assess whether lung Vα14 NKT cells are activated by α-GalCer-pulsed DCs *in situ*, lung mononuclear cells were isolated after the injection of α-GalCer-pulsed DCs, and levels of the mRNAs for Vα14, IFNγ, and β-actin were determined by RT-PCR. As shown in Fig. 5A, significantly increased levels of the mRNA for Vα14 were observed 40 h after the injection of α-GalCer-pulsed DCs (a), but not vehicle-pulsed DCs (b). Furthermore, significantly increased IFNγ mRNA (more than 7 times) was observed following the injection of α-GalCer-pulsed DCs. Marginal levels of IFNγ mRNA were observed following the injection of vehicle-pulsed DCs in comparison with the untreated group (c). These results suggest that the number of Vα14 NKT cells is increased by α-GalCer-pulsed DC injection, and that the number of IFNγ-producing cells increases.

Next, the proportion of Vα14 NKT cells in the lung was assessed by flow cytometric analysis with the α-GalCer/CD1d tetramer. Lung mononuclear cells were prepared from B6 mice treated with α-GalCer-pulsed DCs (a) or vehicle-pulsed DCs (b), or untreated B6 mice (c). Then, the cells were stained with α-GalCer/CD1d tetramer and anti-CD3 mAb. As shown in Fig. 5B, the percentage of Vα14 NKT cells was increased more than 5 times in the α-GalCer-pulsed DC treatment group. This result suggests

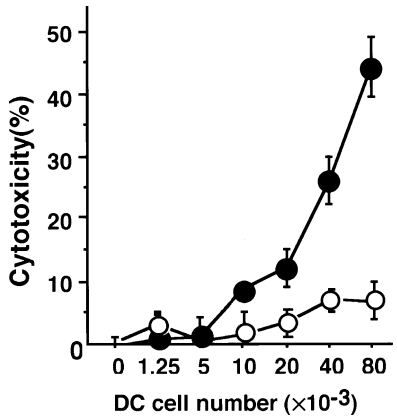

Fig. 4. Activation of Vα14 NKT cells with α-GalCer-pulsed DCs *in vitro*. Various numbers of α-GalCer-pulsed DCs (●) or vehicle-pulsed DCs (○) were co-cultured with freshly prepared Vα14 NKT cells (2×10^5 cells/well). The cytotoxic activity of the activated Vα14 NKT cells against LLC cells was assessed by the standard ^{51}Cr-releasing assay. NKT cell: 2×10^5 cells/well, target: LLC.

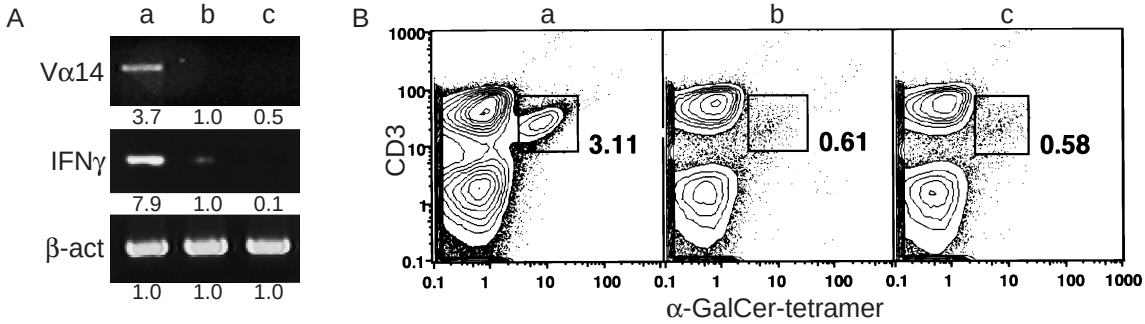

Fig. 5. Assessment of the expansion and activation of Vα14 NKT cells in the lung. A, 3×10^6 α-GalCer-pulsed DCs (a) and vehicle-pulsed-DCs (b) were administered to B6 mice via the tail vein, and the mice were sacrificed 40 h later. (c) no treatment. The lungs were homogenized, and then the mononuclear cells were purified by the Percoll density-gradient method, and total RNA was prepared. Ten nanograms of the RNA for Vα14, 20 ng for IFNγ, and 20 ng for β-actin were used. A representative RT-PCR pattern is shown. B, Representative flow cytometric profiles and frequency of Vα14 NKT cells in mice injected with α-GalCer-pulsed DCs (a) or vehicle-pulsed-DCs (b). (c) no treatment. The percentages of α-GalCer/CD1d-tetramer positive and CD3-positive cells are shown in each panel. The yield of lung mononuclear cells was similar in the treated mice. Three independent mice were used, and similar results were obtained.

that endogenous lung Vα14 NKT cells expand *in situ* following treatment with α-GalCer-pulsed DCs.

DISCUSSION

In this report, the activation and expansion of Vα14 NKT cells in the lung following the intravenous administration of α-GalCer-pulsed DCs is demonstrated. This observation may bridge the previous two experimental results showing that (1) the administration of α-GalCer-pulsed DCs inhibits tumor metastasis in the lung,[16] and (2) Vα14 NKT cells activated with α-GalCer-pulsed DCs exert strong anti-tumor effects on a variety of tumor cell lines *in vitro*.[7, 10, 25–27]

In vitro-activated Vα14 NKT cells, which show strong anti-tumor cytotoxic activity against various tumor lines *in vitro* (Fig. 1), have a less potent antitumor effect on LLC lung metastasis than α-GalCer-pulsed DCs (Fig. 2). The administration of α-GalCer-pulsed DCs was effective even on day 1, but cultured Vα14 NKT cells were just as effective when administered simultaneously with tumor cells. The reason why cultured Vα14 NKT cells were not effective when administered 1 day after the tumor cell inoculation, may be the depletion of IL-2 in the environment. IL-2 may be necessary for the long-term survival of cultured Vα14 NKT cells.[28] In fact, Vα14 NKT cells infected with the retrovirus-mediated IL-2 gene survive significantly longer *in vitro* than Vα14 NKT cells infected with control retrovirus (unpublished observation). Patients undergoing LAK therapy sometimes receive IL-2,[29–32] but administration of IL-2 to cancer patients can produce serious side effects including febricula, pleural effusion, gastrointestinal perforation, insanity, etc.; therefore, the clinical usage of cultured Vα14 NKT cells with IL-2 might be difficult.

Unlike cultured Vα14 NKT cells, Vα14 NKT cells containing spleen cells prepared from α-GalCer-injected Vα14 NKT mice were effective until day 3 after the injection of tumor cells (data not shown). Human Vα24 NKT cells bearing an invariant Vα24JαQ antigen receptor, which are considered to be the counterpart of murine Vα14 NKT cells, have been found to be activated by α-GalCer in a CD1d-dependent fashion.[33–35] However, as far as realistic clinical treatment is concerned, the proportion of the Vα24 NKT cell population in human peripheral blood is very low (0.1−0.001%) compared to that in mouse spleen (up to 1.0%). Therefore, the use of human peripheral blood cells as a source of Vα24 NKT cells might be difficult. We also observed that human cord blood contains plentiful Vα24 NKT cells.[15] Although a protocol for the use of allogenic Vα24 NKT cells has not yet been established, cord blood cells would be a good source for so-called Vα24 NKT cell-based immunotherapy in the near future.

We compared the homing of DCs, cultured Vα14 NKT cells, and spleen cells from α-GalCer-injected Vα14 NKT mice, and found that DCs stay the longest of these three, for at least 24 h (Fig. 3). Spleen cells from α-GalCer-injected Vα14 NKT mice showed a similar pattern to cultured Vα14 NKT cells (unpublished observation). Thus, at this time, the administration of DCs is more effective than that of cultured Vα14 NKT cells as far as clinical treatment is concerned.

Recent advances in medicine have improved the long-term survival of cancer patients. But micrometastasis of carcinoma cells following radical surgery remains a major problem, although many efforts are being made to resolve this issue.[36, 37] Using α-GalCer-pulsed DCs, either alone or together with *in vitro*-activated Vα24 NKT cells, may

make it possible to prevent micrometastasis in patients who have undergone radical surgery. Similar to the mouse system,[24] the administration of α-GalCer induced disappearance of NKT cells in the peripheral blood in some patients. However, no severe toxic effect was observed in a phase I study (Giaccone *et al.*, *Proc. Am. Soc. Clin. Oncol.*, **19**, 1871 (2000)). Although various conditions for clinical treatment remain to be evaluated, e.g. the culture conditions for the DCs and the conditions for pulsing patient's DCs with α-GalCer *in vitro*, α-GalCer-pulsed DC therapy may eventually provide a novel immunotherapy for certain cancer patients.

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