Allogeneic DCG Promote Lung NK Cell Activation and Antitumor Effect After Invariant NKT Cell Activation

HIROSHI HASEGAWA[1], KIMIHIRO YAMASHITA[1], DAI OTUBO[1], SHIN-ICHIRO FUJII[2], TAKASHI KAMIGAKI[1], DAISUKE KURODA[1] and YOSHIHIRO KAKEJI[1]

[1]*Department of Surgery, Division of Gastrointestinal Surgery, Kobe University Graduate School of Medicine, Kobe, Japan;*
[2]*Laboratory for Immunotherapy, RIKEN Center for integrative Medical Science (IMS-RCAI), Yokohama, Japan*

Abstract. *Aim: We aimed to investigate whether allogeneic dendritic cells pulsed with alpha-galactosylceramide (DCG) treatment induces activation of Natural Killer T (NKT) cells. Materials and Methods: C57BL/6 and BALB/c mice were injected with syngeneic and allogeneic DCG. We then examined NK and NKT cell activity in the lung and spleen and antitumor effect in various lung tumor metastatic models. Results: While splenic NKT activity was suppressed after allogeneic DCG treatment, allogeneic DCG treatment induced similar antitumor effect as well as lung NKT and NK cell activity compared to syngeneic DCG treatment. Furthermore, allogeneic DCG treatment prolonged survival in B16F10, LLC, Pan02, MethA, and CT26 tumor models in two strains of mice. Conclusion: This allogeneic DCG treatment could be substituted for syngeneic DCG treatment. The results obtained in the present study suggest that allogeneic DCG treatment may provide a new approach in cancer immunotherapy with NKT cells.*

Invariant Natural Killer T (NKT) cells constitute a unique population of T-cells with immunomodulatory properties that link innate and adaptive immune responses and play a role in immunity to infection, autoimmunity, atherosclerosis, allergy, airway hyperreactivity, and tumor rejection (1, 2). Numerous endogenous or exogenous ligands of the NKT cell T-Cell Receptor have been characterized, with alpha-galactosylceramide (αGalCer) being a well-studied exogenous ligand. Natural αGalCer is isolated from the marine sponge *Agelas mauritianus*, and its synthetic analog KRN7000 has demonstrated tumor growth inhibitory effects in mice subcutaneously inoculated with B16 melanoma cells (3), as well as in mice with liver metastases inoculated *via* the spleen, and in those with lung metastases inoculated *via* the tail vein (4, 5).

Dendritic cells pulsed with αGalCer (DCG) produce a CD1d–αGalCer complex. While administration of both free αGalCer and DCG induces *in vitro* tumor cytolytic activity (4), each of these treatments induces NKT cells with different properties: free αGalCer induces long-term NKT cell anergy (defined as unresponsiveness to subsequent stimuli), while DCG induces a prolonged Interferon (IFN)-γ-producing NKT cell response (6).

In this study, we examined allogeneic DCG treatment in the well-established intravenous injection model of B16 melanoma lung metastasis since a previous study demonstrated that subcutaneous injection of allogeneic DC induced stronger NK activation than did syngeneic DC (7). For a better understanding of the mechanism of allogeneic DCG treatment, we examined mice with both C57BL/6 and BALB/c background and evaluated the survival time for animals carrying different types of tumors.

Materials and Methods

Mice and cell lines. Pathogen-free C57BL/6 and BALB/c female mice, six to eight weeks old, were purchased from CLEA Japan (Tokyo, Japan). The mice were maintained under specific-pathogen-free conditions and studied in compliance with institutional guidelines of Kobe University (Approval number: P090113). The B16F10, 4T1, and CT26 cell lines were obtained from American Type Culture Collection (Manassas, VA , USA). The LLC cell line was from RIKEN Cell Bank (Ibaraki, Japan). MethA was from the Cell Resource Center, Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). Pan02 was kindly provided by Dr David C. Linehan (Washington University School of Medicine, United States of America).

Correspondence to: Dr. Kimihiro Yamashita, Department of Surgery, Division of Gastrointestinal Surgery, Kobe University Graduate School of Medicine, 7-5-2 Kusunoki-cho, Chuo-ku, Kobe, 650-0017, Japan. Tel: +81 783825925, Fax: +81 783825939, e-mail: kiyama@med.kobe-u.ac.jp

Key Words: NKT cells, NK cells, dendritic cells, tumor immunity, allogeneic cells.

Reagents. Anti-asialo ganglio-N-tetraosylceramide (GM1) rabbit serum Lipopolysaccharide (LPS), and Granulocyte Macrophage colony-stimulating Factor (GM-CSF) were purchased from Wako Pure Chemicals (Osaka, Japan), α-GalCer (KRN7000) from Funakoshi (Tokyo, Japan), and chromium-51 (^{51}Cr) from MP Biomedicals (Tokyo, Japan).

Antibodies and flow cytometry. The following monoclonal antibodies (mAbs) were purchased from BD Biosciences (San Diego, CA, USA): anti-mouse Cluster of Differentiation (CD) 3e, Natural Killer (NK) 1.1, and IFN-γ. Anti-mouse CD1d–αGalCer tetramer was purchased from Proimmune Limited (Oxford, UK). Flow cytometric data were acquired with a FACS Calibur (Becton Dickinson, San Jose, CA, USA) or MofloXDP (Beckman Coulter, Fullerton, CA, USA) instrument, and analyzed using Flowjo software (Tree Star, Ashland, OR, USA).

Cell preparation. Bone marrow-derived DCG were generated in the presence of GM-CSF, LPS, and αGalCer as described previously (6). For cell sorting, pooled lung cells from three to four naïve or DCG-immunized mice were stained with anti-NK1.1 allophycocyanin and anti-CD3e Fluorescein isothiocyanate (FITC) mAb for 30 min at 4˚C and sorted by a Moflo XDP at 4˚C. For *in vitro* depletion of NK1.1$^+$ or CD3e$^+$ cells, pooled lung cells were stained with anti-NK1.1 Phycoerythrin (PE) or CD3e PE mAb, and NK1.1$^+$ or CD3e$^+$ cells were removed using anti-PE magnetic beads (Milltenyi Biotec, Bergisch Gladbach, Germany). For *in vivo* depletion of NK cells, mice were treated with 50 μl anti-asialo-GM1 1 d before injection of Phosphate buffered saline (PBS) or DCG.

Cytokine production. Samples of 4×10^5 spleen and lung cells from naïve Wild type (WT) and DCG-immunized mice were cultured in 200 μl RPMI-1640 medium containing 10% Fetal bovine serum (FBS) in 96-round-bottom-well plates with or without 100 ng αGalCer/ml for 16 h. The supernatants were collected, and IFN-γ production was measured using a CBA assay kit (BD Biosciences). Cytokine assays. The serum concentrations of IFN-γ and Interleukin (IL) -12p70 were measured using CBA assay kits. For intracellular cytokine staining of NK or NKT cells, splenocytes were incubated in GolgiPlug (BD Bioscience) for 2 h, and incubated with anti-NK1.1-Allophycocyanin (APC) and CD3e-FITC mAb. After the cell surface was labeled with mAb, cells were permeabilized in Cytofix-Cytoperm Plus (BD Bioscience) and stained with anti-IFN-γ PE.

^{51}Cr release assays. B16F10 target cells were labeled with ^{51}Cr for 1 h at 37˚C, washed three times and incubated in triplicate with effector cells at the E/T ratio 5, 10, 20, and 40. After a 5 h incubation, ^{51}Cr release into the supernatants was assayed with a scintillation counter (7). The percent specific lysis was calculated as: [(experimental release−spontaneous release)/(maximum release−spontaneous release)] ×100.

Statistical analysis. Differences were analyzed using the Student's t-test and log-rank test. A value of $p<0.05$ was considered statistically significant.

Results

Syngeneic and allogeneic DCG treatment induced similar survival benefits in B16F10 lung metastases models. We studied survival as a function of syngeneic DCG dosage. Mice were challenged with 5×10^5 B16F10 melanoma cells and then received PBS, 3×10^4, 3×10^5, or 3×10^6 syngeneic DCG. The maximum syngeneic DCG dose that could be studied was 3×10^6 DCG, because 1×10^7 DCG induced sudden death in 10-20% of mice due to lung thrombosis. The survival curves showed that the antitumor effect of syngeneic DCG treatment was dose-dependent (Figure 1A). We studied survival as a function of allogeneic DCG dosage and survival curves showed that the antitumor effect of allogeneic DCG treatment was also dose-dependent (data not shown). We then decided to use 3×10^6 syngeneic and allogeneic DCG.

Next, we compared the survival benefit of syngeneic DCG and allogeneic DCG treatment and the contribution of NK cells. Mice were *i.v.* injected with B16F10 melanoma cells, and *i.p.* injected with anti-asialo GM1 or rabbit serum for NK depletion or control, respectively, one day later, and then given PBS, syngeneic DCG or allogeneic DCG by *i.v.* three days later. The antitumor effect of allogeneic DCG was similar to that of syngeneic DCG in both rabbit serum- and anti-asialo GM1-treated mice (Figure 1B).

Both syngeneic and allogeneic DCG elicited similar in vivo IFN-γ production and IFN-γ-producing ability in lung. We studied *in vivo* responses to allogeneic DCG. The serum concentrations of IFN-γ and IL-12p70 (Figure 2A) and IFN-γ expression in NK and NKT cells (Figure 2B) were not significantly different between syngeneic and allogeneic DCG-treated animals. Although IFN-γ expression by spleen cells from mice treated with allogeneic DCG was less than that from mice immunized with syngeneic DCG, IFN-γ production by lung cells from mice treated with allogeneic DCG was similar to that from mice treated with syngeneic DCG (Figure 2C).

Next, we investigated NKT and NK cell expansion in spleen and lungs. C57BL/6 mice were immunized with PBS, syngeneic DCG, or allogeneic DCG, and three days later the lungs and spleen were analyzed by cell count and determination of percentage of NKT and NK cells. Both syngeneic and allogeneic DCG induced similar NKT cell expansion in spleen and lungs (Figure 2D).

Allogeneic and syngeneic DCG induced similar in vitro cytolytic activity in which NK cells played a pivotal role against B16 melanoma cells. We measured tumor cytolytic activity in spleen and lungs. The lung cells from DCG-treated mice had higher cytolytic activity than did spleen cells at high E/T cell ratios (Figure 3A); consequently, we used lung cells for cytolytic activity assays. To characterize the main effector cells of *in vitro* cytolytic activity against B16 melanoma cells, pooled lung cells from PBS- or DCG-treated mice were sorted into NK1.1$^+$CD3e$^-$, NK1.1-CD3e$^-$, and CD3e$^+$ cells, and their cytolytic activities were measured. Only NK1.1$^+$CD3e$^-$ cells from DCG-treated mice

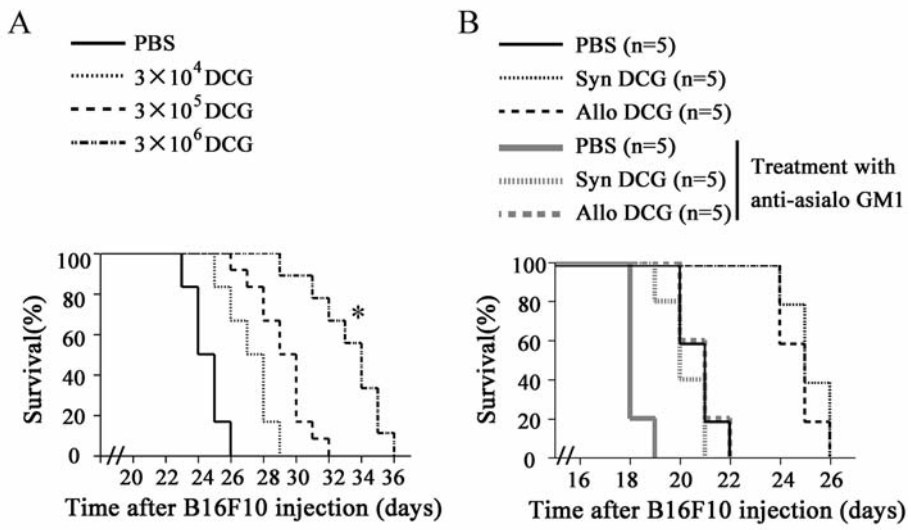

Figure 1. *Antitumor effect of allogeneic dendritic cells pulsed with alpha-galactosylceramide (DCG) treatment in murine B16 lung metastatic model. A: C57BL/6 mice were i.v. injected with 5×10^5 B16F10 melanoma cells, and three days later they were i.v. injected with Phosphate buffered saline (PBS) (n=6), 3×10^4 DCG (n=12), 3×10^5 DCG (n=12), or 3×10^6 DCG (n=9). Survival was dose-dependent: *$p<0.05$ for 3×10^6 DCG compared to the other DCG doses (log-rank test). B: C57BL/6 mice were i.v. injected with 7×10^5 B16F10 melanoma cells. Two days after tumor inoculation, rabbit serum or anti-asialo ganglio-N-tetraosylceramide (GM1) was i.p. injected. Three days after tumor inoculation, PBS, syngeneic DCG, or allogeneic DCG was i.v. injected, and survival was monitored.*

had evident cytolytic activity compared to cells from PBS-treated mice (Figure 3B). To confirm that NK cells were responsible for the observed cytolytic activity, $NK1.1^+$ cells were depleted *in vitro* by a magnetic bead system (Figure 3C), and NK cells were depleted *in vivo* by treatment with anti-asialo GM1 (Figure 3D). After *in vitro* depletion of $NK1.1^+$ cells, the cytolytic activity of the $NK1.1^+$-depleted lung cells from DCG-treated mice was reduced. After *in vivo* depletion of NK cells, the cytolytic activity of lung cells from NK-depleted mice was also suppressed. The tumor cytolytic ability of lung cells from C57BL/6 mice after allogeneic DCG treatment was similar to that after syngeneic DCG treatment (Figure 3E and F).

Allogeneic DCG treatment prolonged survival in various tumor models in two strains of mice. C57BL/6 mice were *i.v.* injected with Pan02 pancreas adenocarcinoma cells or LLC lung adenocarcinoma cells and then given *i.v.* PBS, syngeneic DCG, or allogeneic DCG three days later (Figure 4A). In addition, BALB/c mice were *i.v.* injected with MethA sarcoma cells, 4T1 breast adenocarcinoma cells, or CT26 colon adenocarcinoma cells and then treated by *i.v.* with PBS, syngeneic DCG, or allogeneic DCG three days later (Figure 4B). Neither syngeneic nor allogeneic DCG prolonged survival in 4T1 tumor models. Allogeneic DCG treatment conferred significantly better survival than did PBS treatment in LLC, Pan02, MethA, and CT26 tumor models ($p<0.01$). Allogeneic DCG treatment led to significantly better survival than did syngeneic DCG treatment in CT26 tumor models ($p<0.01$). These findings suggest that in both C57BL/6 and BALB/c mice, allogeneic DCG treatment improved survival in various tumor models except the 4T1 tumor model.

Discussion

In this study, we compared syngeneic and allogeneic DCG treatment. Although activation of NK cells is not the only resistance mechanism after DCG treatment, NK cells play a pivotal role in *in vivo* tumor rejection after αGalCer and DCG treatment (6). We carried-out an IFN-γ-producing assay (Figure 2D) to measure the function of NKT cells, and killing assay to measure the function of NK cells (Figure 3). We hypothesized that allogeneic DCG treatment would induce a more potent antitumor effect than syngeneic DCG treatment. However, allogeneic DCG treatment induced similar *in vivo* lung NK cell and NKT cell function compared to syngeneic DCG treatment.

We measured the IFN-γ-producing ability and tumor cytolytic activity by using mouse lung cells to evaluate prevention of lung metastasis by NKT cell activation more precisely. We adopted lung cells in the analysis because we thought that there might be tumor-inhibitory activity in the lungs, according to the *i.v.* tumor inoculation models. Therefore, we investigated the properties of lung cells after NKT cell activation. To our surprise, lung cells exhibited different properties in cell expansion, IFN-γ-producing ability, and tumor cytolytic activity compared to spleen cells.

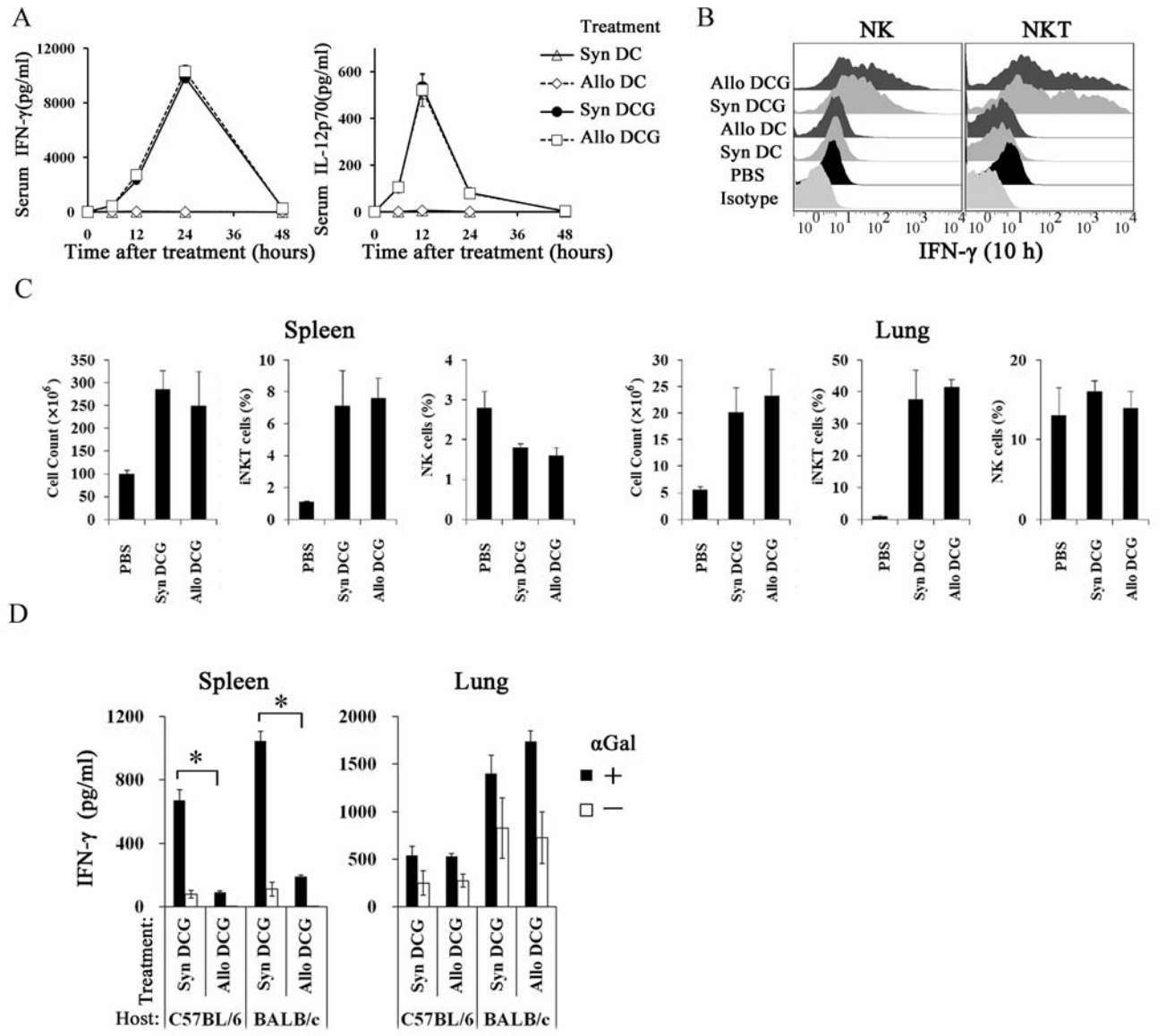

Figure 2. *Allogeneic DCG activates lung Natural Killer T (NKT) cells. A: C57BL/6 mice were i.v. injected with syngeneic Dendritic Cells (DC), allogeneic DC, syngeneic DCG, or allogeneic DCG, and the serum concentrations of Interferon (IFN)-γ and Interleukin (IL)-12p70 were measured 6, 12, 24, and 48 h later. Each data point is the mean±SD of the measurements from two mice. B: This experiment was carried-out as in A, but the spleens were analyzed for IFN-γ production in NK1.1$^+$CD3e$^-$ Natural Killer (NK) cells and NK1.1$^+$CD3e$^+$ NKT cells 10 h after treatment. C: C57BL/6 and BALB/c mice were i.v. injected with syngeneic DCG or allogeneic DCG, and two days later, 4×10^5 spleen and lung cells were cultured in 200 μl with or without 100 ng alpha-galactosylceramide (αGalCer)/ml for 16 h. The IFN-γ concentration in each medium was then measured. Each data point is the mean±SD of triplicate cultures. Syngeneic DCG treatment induced more IFN-γ prediction in spleen cells than did allogeneic DCG treatment (*p<0.05). D: C57BL/6 mice were i.v. injected with syngeneic or allogeneic DCG and three days later, the lungs and spleen were analyzed for NKT and NK cell count and percentages. Each data point is the mean±SD of measurements from four mice.*

NKT cell expansion and cytolytic activity was more evident in lungs than in spleen after NKT cell activation. Interestingly, both syngeneic and allogeneic DCG elicited similar lung NKT IFN-γ-producing ability, while splenic NKT cell function was suppressed after allogeneic DCG treatment compared to syngeneic DCG treatment (Figure 2D).

To determine the antitumor effect of allogeneic DCG treatment, we investigated six tumor models. Neither syngeneic nor allogeneic DCG prolonged survival in 4T1 tumor models because intravenously-injected 4T1 tumor cells metastasize to brain (8). In five tumor models, the efficacy of allogeneic DCG treatment was almost equivalent

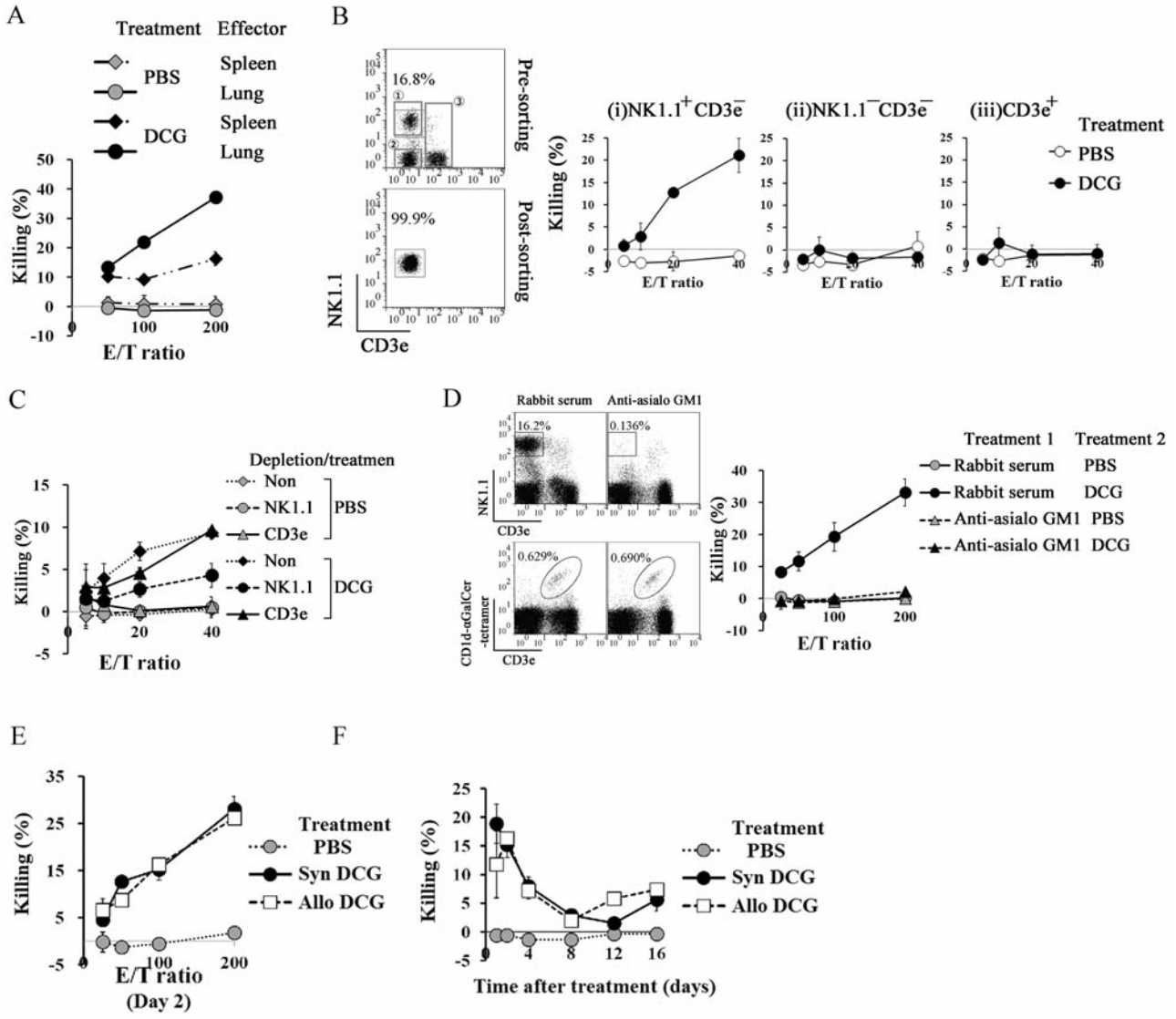

Figure 3. *Lung Natural Killer (NK) cells were main effector cells of in vitro cytolytic activity against B16F10 melanoma cells, and allogeneic dendritic cells pulsed with alpha-galactosylceramide (DCG) treatment induced sufficient cytolytic activity. A: C57BL/6 mice were i.v. injected with syngeneic DCG and assayed for cytotoxicity two days later by incubating the spleen or lung cells with 51Cr-labeled target B16F10 cells for 5 h at the indicated Effector/Target ratios and then measuring ^{51}Cr-release. B: This experiment was carried-out as in A, but the effector lung cells were sorted into (i) NK1.1$^+$CD3e$^-$, (ii) NK1.1$^-$CD3e$^-$, and (iii) CD3e$^+$ cells by a cell-sorter and assayed for cytotoxicity two days after i.v. injections. C; C57BL/6 mice were i.v. injected with Phosphate buffered saline (PBS) or DCG, and two days later lung NK1.1$^+$ and CD3e$^+$ cells were depleted in vitro by a magnetic bead system and assayed for cytotoxicity. D: C57BL/6 mice were i.p. injected with rabbit serum or anti-asialo ganglio-N-tetraosylceramide (GM-1) for in vivo depletion of NK cells, and one day later they were injected with PBS or DCG. The lung cells were assayed for cytotoxicity one day later. Each data point is the mean of triplicate experiments. The in vivo depletion of NK1.1$^+$CD3e$^-$ NK cells was verified by flow cytometry analyses (left). E: This experiment was carried-out as in A, but C57BL/6 mice were i.v. injected with PBS, syngeneic DCG, or allogeneic DCG, and two days later lung cells were assayed for cytolytic activity. F: This experiment was as in E, but 1, 2, 4, 8, 12, and 16 days after treatment, lung cells were assayed for cytolytic activity at an E/T ratio of 100.*

to that of syngeneic DCG treatment, and in the CT26 tumor model, allogeneic DCG treatment led to significantly better survival than did syngeneic DCG treatment. Although further research is required to understand the efficacy of allogeneic DCG, to the best of our knowledge, this is the first time that the efficacy of allogeneic DCG treatment has been studied.

A phase I clinical trial using αGalCer (9) and phase I-II studies using antigen-presenting cells pulsed with αGalCer (10-13) have been carried-out. It has been reported that it is difficult to generate a sufficient number of dendritic cells

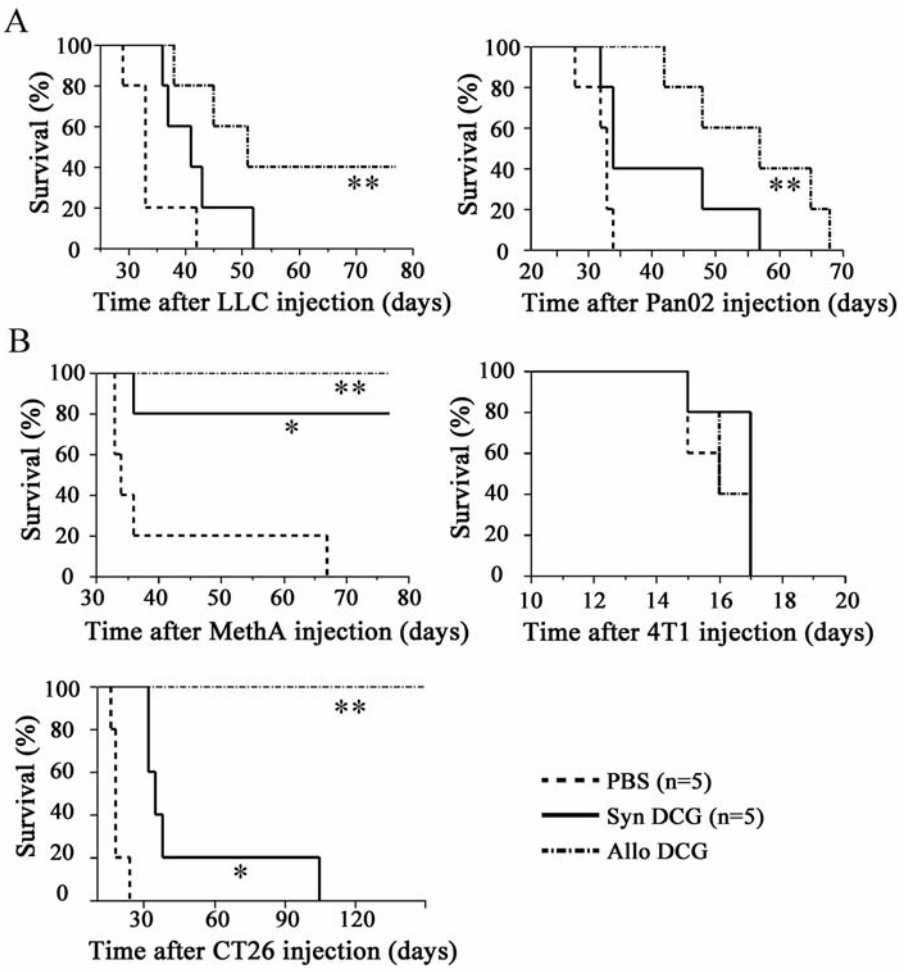

Figure 4. *Antitumor effect of allogeneic dendritic cells pulsed with alpha-galactosylceramide (DCG) treatment in various murine tumor i.v. injection models. A: C57BL/6 mice were i.v. injected with 5×10^5 LLC cells or Pan02 cells. At three days after tumor inoculation, Phosphate buffered saline (PBS), syngeneic DCG, or allogeneic DCG was i.v. injected, and survival was monitored. B: This experiment was carried-out as in A, but BALB/c mice were i.v. injected with MethA cells, 4T1 cells, or CT26 cells. Survival was greater with allogeneic DCG treatment than with PBS (**p<0.01), and greater with syngeneic DCG than with PBS (*p<0.05) (log-rank test).*

from human CD34+ and CD14+ progenitors in patients with cancer (14-15). In practice, insufficient quality and quantity of syngeneic dendritic cells might restrict the clinical use of DCG treatment, because a sufficient number of functional dendritic cells were needed in order to produce an acceptable antitumor effect in this murine model, as we showed. We can expect that allogeneic cells might provide a stable supply of high quality cells from healthy donors; we therefore hope that allogeneic DCG treatment can provide a new approach in cancer immunotherapy with NKT cells.

Disclosure Statement

The Authors declare no financial or commercial conflict of interest.

Acknowledgements

This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1 Cerundolo V, Silk JD, Masri SH and Salio M: Harnessing invariant NKT cells in vaccination strategies. Nat Rev Immunol *9*: 28-38, 2009.

2 Godfrey DI and Rossjohn J: New ways to turn on NKT cells. J Exp Med *208*: 1121-1125, 2011.

3 Morita M, Motoki K, Akimoto K, Natori T, Sakai T, Sawa E, Yamaji K, Koezuka Y, Kobayashi E and Fukushima H Structure–activity relationship of alpha-galactosylceramides against B16-bearing mice. J Med Chem *38*: 2176-2187, 1995.

4 Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, Kondo E, Harada M, Koseki H, Nakayama T, Tanaka Y and Taniguch M: Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Vα14 NKT cells. Proc Natl Acad Sci USA *95*: 5690-5693, 1998.
5 Chiodoni C, Stoppacciaro A, Sangaletti S, Gri G, Cappetti B, Koezuka Y and Colombo MP: Different requirements for alpha-galactosylceramide and recombinant IL-12 antitumor activity in the treatment of C-26 colon carcinoma hepatic metastases. Eur J Immunol *31*: 3101-3110, 2001.
6 Fujii S, Shimizu K, Kronenberg M and Steinman RM: Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. Nat immunol *3*: 867-874, 2002.
7 Yasuda T, Kamigaki T, Kawasaki K, Nakamura T, Yamamoto M, Kanemitsu K, Takase S, Kuroda D, Kim Y, Ajiki T and Kuroda Y: Superior anti-tumor protection and therapeutic efficacy of vaccination with allogeneic and semiallogeneic dendritic cell/tumor cell fusion hybrids for murine colon adenocarcinoma. Cancer Immunol Immunother *56*: 1025-1036, 2007.
8 Liu Y, Kosaka A, Ikeura M, Kohanbash G, Fellows-Mayle W, Snyder LA and Okada H: Premetastatic soli and prevention of breast cancer brain metastasis. Neuro Oncol *15*: 891-903, 2013.
9 Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, von Blomberg BM, Scheper RJ, van der Vliet HJ, van den Eertwegh AJ, Roelvink M, Beijnen J, Zwierzina H and Pinedo HM: A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. Clin Cancer Res *8*: 3702-3709, 2002.
10 Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, Hutchinson A, Geller M, Liu N, Annable R, Shay J, Kirchhoff K, Nishi N, Ando Y, Hayashi K, Hassoun H, Steinman RM and Dhodapkar MV : Sustained expansion of NKT cells and antigen-specific T-cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. J Exp Med *201*: 1503-1517, 2005.
11 Nieda M, Okai M, Tazbirkova A, Lin H, Yamaura A, Ide K, Abraham R, Juji T, Macfarlane DJ and Nicol AJ: Therapeutic activation of V dendritic cells24+Vbeta11+ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. Blood *103*: 383-389, 2004.
12 Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M, Iizasa T, Nakayama T, Taniguchi M and Fujisawa T: A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. Clin Cancer Res *11*: 1910-1917, 2005.
13 Motohashi S, Nagato K, Kunii N, Yamamoto H, Yamasaki K, Okita K, Hanaoka H, Shimizu N, Suzuki M, Yoshino I, Taniguchi M, Fujisawa T and Nakayama T: A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. J Immunol *182*: 2492-2501, 2009.
14 Della Bella S, Gennaro M, Vaccari M, Ferraris C, Nicola S, Riva A, Clerici M, Greco M and Villa ML: Altered maturation of peripheral blood dendritic cells in patients with breast cancer. Br J Cancer *89*: 1463-1472, 2003.
15 Sakakura K, Chikamatsu K, Takahashi K, Whiteside TL and Furuya N: Maturation of circulating dendritic cells and imbalance of T-cell subsets in patients with squamous cell carcinoma of the head and neck. Cancer Immunol Immunother *55*: 151-159, 2006.

Received March 7, 2014
Revised May 15, 2014
Accepted May 16, 2014