Therapeutic effect of α-galactosylceramide-loaded dendritic cells genetically engineered to express SLC/CCL21 along with tumor antigen against peritoneally disseminated tumor cells

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The close cooperation of both innate and acquired immunity is essential for the induction of truly effective antitumor immunity. We tested a strategy to enhance the cross-talk between NKT cells and conventional antigen-specific T cells with the use of αGalCer-loaded dendritic cells genetically engineered to express antigen plus chemokine, attracting both conventional T cells and NKT cells. DC genetically engineered to express a model antigen, OVA, along with SLC/CCL21 or monokine induced by IFN-γ/CXCL9, had been generated using a method based on *in vitro* differentiation of DC from mouse ES cells. The ES-DC were loaded with α-GalCer and transferred to mice bearing MO4, an OVA-expressing melanoma, and their capacity to evoke antitumor immunity was evaluated. *In vivo* transfer of either OVA-expressing ES-DC, stimulating OVA-reactive T cells, or α-GalCer-loaded non-transfectant ES-DC, stimulating NKT cells, elicited a significant but limited degree of protection against the i.p. disseminated MO4. A more potent antitumor effect was observed when α-GalCer was loaded to ES-DC expressing OVA before *in vivo* transfer, and the effect was abrogated by the administration of anti-CD8, anti-NK1.1 or anti-asialo GM1 antibody. α-GalCer-loaded double transfectant ES-DC expressing SLC along with OVA induced the most potent antitumor immunity. Thus, α-GalCer-loaded ES-DC expressing tumor-associated antigen along with SLC can stimulate multiple subsets of effector cells to induce a potent therapeutic effect against peritoneally disseminated tumor cells. The present study suggests a novel way to use α-GalCer in immunotherapy for peritoneally disseminated cancer. (*Cancer Sci* 2005; 96: 889–896)

A means to induce the close cooperation of both innate and acquired immunity would be necessary for the induction of efficient antitumor therapy. Recent studies have shown DC to be potent stimulators of both innate and acquired immunity. The *in vivo* transfer of DC presenting tumor-associated antigens has proven to be efficient in the priming of CTL specific to the antigens. α-GalCer presented by DC efficiently stimulates NKT cells,[1–4] a subset of T cells implicated in the innate immunity against infection and cancer.[5–7] In addition, NKT cells stimulated by the *in vivo* administration of α-GalCer secondarily stimulate conventional T cells.[8,9] It is thus presumed that the *in vivo* transfer of DC simultaneously loaded with tumor-associated antigens and α-GalCer may stimulate both tumor-reactive T cells and NKT cells, thus resulting in a potent antitumor immunity.

Chemokines mediate leukocyte adhesion and homing, and the concordant migration of specific leukocyte subsets induced by chemokines is pivotal for the development of proper immune responses. SLC/CCL21 attracts both T cells and DC to lymphoid tissues through its receptor CCR7, and the effect of SLC is essential for the priming of naive T cells in the initiation phase of the immune response. CXCR3 and its ligands, Mig/CXCL9 and IP-10/CXCL10, mediate the migration of effector/memory T cells and NK cells to the site of inflammation. In addition, a recent study revealed that these chemokines and their receptors also mediate the migration of some subpopulations of NKT cells.[10–12]

As a means for loading the tumor-associated antigens to DC, genetic modification to express antigenic proteins has several advantages. The expression of tumor antigens by DC circumvents the need for identifying specific CTL epitopes within the protein, and by that the antigens are continuously supplied for presentation as opposed to a single pulse of peptides or tumor cell lysates.[13] For the efficient gene transfer to DC, the use of virus-based vectors is required because DC are not easy to genetically modify. Considering the clinical application, however, there are several problems related to the use of virus vectors. These include the inefficiency of gene transfer, the instability of gene expression, and the potential risk accompanying the use of virus vectors. In addition, in many countries, legal restrictions have been placed on the use of virus vectors outside of carefully isolated laboratories.

We and others have established methods to generate dendritic cells *in vitro* from mouse ES cells.[14,15] ES-DC have the

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Abbreviations: Ab, antibody; BM-DC, bone marrow cell-derived dendritic cell; CBF1, (CBA × C57BL/6) F1; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; ES cell, embryonic stem cell; ES-DC, ES cell-derived dendritic cell; α-GalCer, α-galactosylceramide; GM-CSF, granulocyte macrophage colony-stimulating factor; HLA, human histocompatibility leukocyte antigen; IFN-γ, interferon-gamma; IL, interleukin; i.p., intraperitoneally; IP-10, IFN-γ-inducible 10 kDa protein; mAb, monoclonal antibody; Mig, monokine induced by IFN-γ; NK, natural killer; OVA, ovalbumin; s.c., subcutaneously; SLC, secondary lymphoid tissue chemokine.

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capacity to stimulate T cells, present antigen and migrate to lymphoid tissues upon *in vivo* administration, and these capacities of ES-DC are comparable to those of BM-DC. The genetic modification of ES-DC can be carried out without the use of virus vectors by introducing exogenous genes by electroporation into undifferentiated ES cells and the subsequent induction of their differentiation into ES-DC. We can generate multiple gene-transfectant ES-DC by the sequential transfection of ES cells with vectors bearing different selection markers.[16,17] In a previous study, we generated double-transfectant ES-DC expressing SLC or Mig along with a model tumor antigen.[16] Using these double-transfectant ES-DC, we demonstrated that the coexpression of SLC or Mig enhanced the capacity of *in vivo*-transferred ES-DC to activate antigen-specific CTL and to protect the recipient mice from a tumor cell challenge.

In the present study, we evaluated the capacity of α-GalCer-loaded ES-DC to stimulate NKT cells both *in vitro* and *in vivo*, in comparison to that of BM-DC. We next evaluated the antitumor effect of simultaneous stimulation of NKT cells and antigen-specific conventional T cells by the *in vivo* administration of α-GalCer-loaded ES-DC expressing a model tumor antigen, namely OVA. Furthermore, we addressed whether coexpression of SLC or Mig with the antigen by ES-DC could enhance the synergic antitumor effect of NKT cells and conventional T cells.

Materials and Methods

Mice

CBA and C57BL/6 mice were obtained from Clea Animal Co. (Tokyo, Japan) or Charles River (Hamamatsu, Japan) and kept under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to produce CBF1 mice and all *in vivo* experiments were carried out using the CBF1 mice. The animal experiments in this study were approved by the animal experiment committee of Kumamoto University (permission number A16-074).

Reagents

Recombinant mouse GM-CSF was purchased from PeproTech EC (London, UK) and α-GalCer was kindly provided by the Kirin Brewery Co. (Tokyo, Japan). Mouse IL-4 and IFN-γ ELISA kits were purchased from eBioscience (San Diego, CA, USA). Polyclonal rabbit anti-asialo GM1 Ab was purchased from Wako Chemicals (Tokyo, Japan).

Cell lines and preparation of DC

The ES cell line TT2, derived from CBF1 blastocysts,[18] was maintained as described previously.[19] MO4[20] was generated by the transfection of C57BL/6-derived melanoma B16 with the pAc-neo-OVA plasmid. The procedure for inducing the differentiation of ES cells into DC has been reproted previously.[15] ES-DC expressing OVA (ES-DC-OVA) and ES-DC expressing chemokine, SLC or Mig, along with OVA (ES-DC-OVA/SLC or ES-DC-OVA/Mig) were generated as reported previously.[16] ES-DC recovered after a 14-day culture in bacteriological Petri dishes were used for both *in vivo* and *in vitro* assays. To generate BM-DC, bone marrow cells were isolated from CBF1 mice and cultured in RPMI + 10% fetal calf serum + GM-CSF (5 ng/mL) for 7 days, according to the method reported by Lutz *et al*.[21]

Analysis of the activation of NKT cells by DC loaded with α-GalCer

Embryonic stem cell-derived dendritic cells or BM-DC were cultured in the presence of α-GalCer (100 ng/mL) or vehicle (0.00025% Polysorbate-20) alone for 22 h, washed twice, and cocultured with splenic T cells of syngeneic CBF1 mice (5×10^4 DC $+2.5 \times 10^6$ T cells/well in 24-well culture plates). Splenic T cells were isolated using nylon-wool columns, as described previously.[16] After 24 h or 5 days, the cells were recovered and analyzed on their cytotoxic activity by a 4-h ^{51}Cr-release assay using YAC-1 cells (1×10^4 cells/well) as targets in 96-well round-bottomed culture plates at the effector : target ratio indicated. The amount of IL-4 and IFN-γ in the culture supernatant was measured by ELISA. In the analysis of the stimulation of NKT cells *in vivo*, ES-DC or BM-DC loaded with either α-GalCer (100 ng/mL) or vehicle alone, as described above, were injected i.p. into syngeneic CBF1 mice (1×10^6 cells/mouse). After 24 h, the mice were killed and the cytotoxic activity of whole spleen cells against YAC-1 cells was analyzed, as described above.

Tumor challenge experiments

Indicated numbers of MO4 cells were injected s.c. into the shaved left flank region, or i.p. on day 0. On day 3, 1×10^5 genetically modified ES-DC preloaded with either α-GalCer or vehicle alone were transferred i.p. into the mice. The survival rate of the mice was monitored and, in s.c. challenge experiments, the tumor sizes were also determined biweekly in a blinded fashion. The tumor index was calculated as:

Tumor index (mm) = square root of (length × width).

In vivo depletion experiments

The mice were challenged i.p. with 1×10^5 MO4 cells on day 0 and they were injected i.p. with 1×10^5 ES-DC-OVA preloaded with α-GalCer on day 3. To deplete the specific types of cells, the mice were given a total of 14 i.p. injections (days −4, −1, 2, 5, 10, 13, 15, 19, 26, 33, 40, 47, 54 and 61) of mAb, ascites (0.1 mL/mouse/injection) from hybridoma-bearing nude mice, or polyclonal rabbit anti-asialo GM1 Ab (50 μg/mouse/injection). The mAbs used were rat antimouse CD4 (clone GK1.5), rat antimouse CD8 (clone 2.43) and mouse anti-NK1.1 (clone PK-136). Normal rat IgG (Sigma, St Louis, MO, USA) was used as a control (200 μg/mouse/injection).

Statistical analysis

Two-tailed Student's *t*-test was used to determine the statistical significance of differences in cytolytic activity and the tumor growth between the treatment groups. A value of $P < 0.05$ was considered to be significant. The Kaplan–Meier plot for survival was used to determine any significant differences in tumor challenge experiments, using the Breslow–Gehan–Wilcoxon test. In some experiments, the difference in the survival rate between treatment groups was assessed for significance using the χ^2-test. Statistical analyses were made using the StatView 5.0 software (Abacus Concepts, Calabasas, CA, USA).

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Results

Activation of NKT cells by ES-DC pulsed with α-GalCer

Mouse splenic DC and BM-DC loaded with α-GalCer have been reported to efficiently stimulate NKT cells, resulting in the rapid induction of NK cell-like cytolytic activity and the production of cytokines such as IL-4 and IFN-γ.[2,3] We examined whether ES-DC loaded with α-GalCer had the capacity to activate NKT cells, as naturally occurring DC do.

TT2 ES cell-derived non-transfectant ES-DC (ES-DC-TT2) or BM-DC were preincubated with α-GalCer, and then cocultured with splenic T cells isolated from syngeneic CBF1 mice. After 24 h, the cultured cells were recovered and their cytolytic activity against YAC-1 target cells was analyzed by a ^{51}Cr-release assay. The results shown in Fig. 1a indicate that a significant cytotoxity against YAC-1 cells was induced in the splenic T cell preparations by coculture with ES-DC loaded with α-GalCer, in comparison to the coculture with ES-DC loaded with vehicle alone. The cytotoxic activity induced by α-GalCer-loaded ES-DC-TT2 was comparable to that induced by α-GalCer-loaded BM-DC (Fig. 1a). As shown in Fig. 1b, IL-4 and IFN-γ were produced by splenic T cells cocultured with α-GalCer-loaded BM-DC or ES-DC, and a similar amount of the cytokines was produced in the culture with BM-DC and ES-DC preloaded with α-GalCer. If the coculture of T cells with α-GalCer-loaded ES-DC was extended to 5 days, the induced killing activity (Fig. 1c) and the amount of IL-4 and IFN-γ produced was increased in parallel (data not shown).

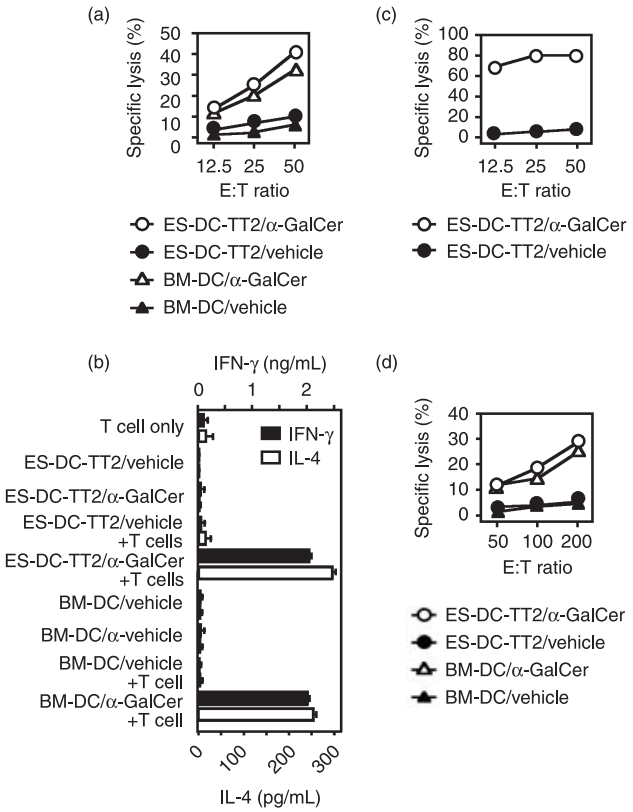

Fig. 1. Activation of NKT cells by the α-GalCer-loaded ES-DC. (a) ES-DC-TT2 or BM-DC were loaded with either α-GalCer (100 ng/mL) or vehicle (Polysorbate-20) alone for 22 h, washed extensively, and cocultured with splenic T cells of syngeneic CBF1 mice (5×10^4 DC + 2.5×10^6 T cells/well in 24-well culture plates). After 24 h of culture, the cells were recovered and the cytotoxic activity of the harvested cells against YAC-1 cells (1×10^4 cells) was analyzed using a 4-h Cr-release assay at the effector : target (E : T) ratios indicated. (b) Amounts of IL-4 and IFN-γ in the supernatant collected at the end of the 24-h coculture were quantified by ELISA. The results are expressed as the mean cytokine production of triplicate assays + SD. (c) The coculture was extended to 5 days and the killing activity of resultant cells was analyzed as in (a). (d) ES-DC or BM-DC were cultured in the presence of either α-GalCer (100 ng/mL) or vehicle alone for 18 h, washed, and injected i.p. into syngeneic CBF1 mice (1×10^6 cells/mouse). After 24 h, spleen cells were isolated from the mice and their cytotoxic activity against YAC-1 cells was analyzed as in (a). The results are expressed as the mean specific lysis of triplicate assays. The SD of triplicates were less than 2%.

We next analyzed the capacity of α-GalCer-loaded ES-DC to activate NKT cells *in vivo*. ES-DC-TT2 or BM-DC were preloaded with α-GalCer or vehicle alone in the same way as described above and i.p. injected into the syngeneic CBF1 mice. After 24 h, the mice were killed and the cytotoxic activity of whole spleen cells against YAC-1 cells was analyzed. As shown in Fig. 1d, a significant degree of cytotoxic activity was induced in the spleen cells by transfer of ES-DC loaded with α-GalCer, but it was not induced by the transfer of those loaded with vehicle alone. The capacity to evoke YAC-1 cell-killing activity of ES-DC and that of BM-DC was similar also *in vivo*. The activated NKT cells are known to activate the cytotoxic activity of NK cells.[22] It is therefore possible that the cytotoxic activity observed in these assays were mostly mediated by NK cells secondarily stimulated by NKT cells. Even so, these data collectively demonstrate that ES-DC had the capacity to present α-GalCer to activate NKT cells, and the capacity was similar to that of BM-DC both *in vitro* and *in vivo*.

Anti-tumor effect of α-GalCer-loaded ES-DC

We assessed whether the activation of NKT cells *in vivo* by α-GalCer-loaded ES-DC had any therapeutic effect against the tumor cells growing *in vivo*. MO4, originating from NK-sensitive B16 melanoma cells, were injected s.c. into the left flank region of mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC-TT2 loaded with α-GalCer or vehicle alone. As shown in Fig. 2a, ES-DC loaded with α-GalCer did not show any therapeutic effect in this s.c. tumor model.

We next investigated the effect of α-GalCer-loaded ES-DC in the peritoneally disseminated tumor model. MO4 cells were injected i.p. into mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC loaded with either α-GalCer or vehicle alone. As shown in Fig. 2b, which indicated the survival rate of the treated mice, the injection of ES-DC-TT2 loaded with α-GalCer elicited a significant ($P < 0.05$) but limited protective effect against the i.p. disseminated tumor cells.

Synergic therapeutic effect of α-GalCer-activated NKT cells and antigen-specific T cells against peritoneally disseminated tumor cells

In a previous study, we demonstrated that the *in vivo* transfer of ES-DC-OVA effectively primed OVA-specific CTL and induced protection against a subsequent challenge with s.c. injected MO4 cells expressing OVA.[16] We investigated

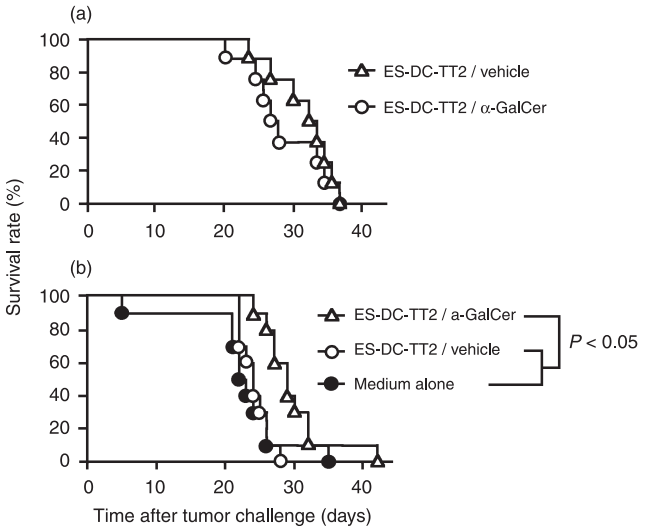

Fig. 2. Anti-tumor effect of *in vivo*-transferred α-GalCer-loaded ES-DC. The mice were (a) inoculated s.c. with MO4 cells (3×10^5 cells/mouse) to the left flank region or (b) inoculated i.p. with MO4 cells (1×10^5 cells/mouse). After 3 days, the mice were treated with i.p. injection of ES-DC-TT2 (1×10^5 cells/mouse) loaded with α-GalCer, vehicle alone or medium alone, and the mouse survival rate was monitored ($n = 10$ per group). In (b), the survival rate of the α-GalCer-loaded ES-DC-TT2-treated group was higher than that of the other two groups and the difference was statistically significant. Data are representative of four independent and reproducible experiments.

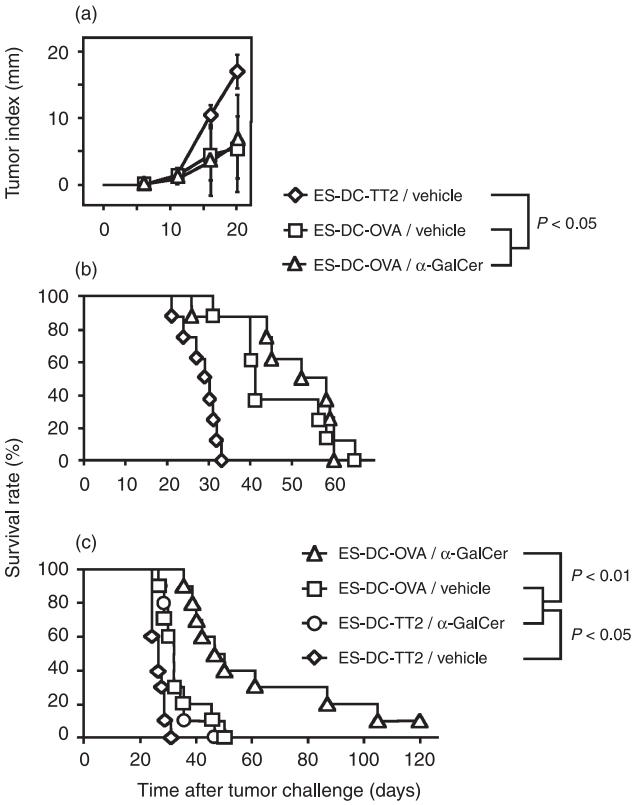

Fig. 3. Synergic effect of α-GalCer loading and the expression of tumor antigen on the protection against tumors induced by ES-DC. MO4 cells (3×10^5 cells/mouse) were injected s.c. into the left flank region of the mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC-TT2 (1×10^5 cells/mouse) loaded with vehicle alone, ES-DC-TT2 loaded with α-GalCer, or ES-DC-OVA loaded with α-GalCer. After that, the tumor sizes were determined (a) and survival rate was monitored (b). Both the differences in tumor index (a) and in mouse survival rate (b) between the vehicle-loaded ES-DC-TT2-treated group and other two groups were statistically significant. (c) MO4 cells (1×10^5 cells/mouse) were injected i.p. into the mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC-OVA or ES-DC-TT2 (1×10^5 cells/mouse) loaded with α-GalCer or vehicle alone. Thereafter, the mouse survival rate was monitored. The survival rate of the α-GalCer-loaded ES-DC-OVA-treated group was higher than that of the other groups and the difference was statistically significant. The survival rates of the vehicle-loaded ES-DC-OVA-treated group and α-GalCer-loaded ES-DC-TT2-treated group were higher than that of vehicle-loaded ES-DC-TT2-treated group and the difference was statistically significant. Each group included 10 mice. Data are representative of three independent and reproducible experiments.

whether the loading of α-GalCer to ES-DC-OVA before *in vivo* transfer would enhance the therapeutic effect against pre-established MO4 tumor. The mice were challenged s.c. with MO4 cells, and then 3 days later they were treated by i.p. injection of ES-DC-OVA preloaded with α-GalCer or vehicle alone. As shown in Fig. 3a,b, compared to the transfer of ES-DC-TT2, the transfer of ES-DC-OVA, loaded with either α-GalCer or vehicle alone, elicited a significant antitumor effect in this therapeutic model, as observed in the previously reported prevention (prophylactic) model.[16] However, the loading of α-GalCer to ES-DC-OVA did not improve the effect, based on either the tumor growth or the mouse survival time (Fig. 3a,b). These results suggest that the activation of NKT cells by α-GalCer loaded to ES-DC does not enhance the therapeutic effect of antigen-specific T cells against s.c. growing tumor cells.

We next investigated the effect of transfer of α-GalCer-loaded ES-DC-OVA against peritoneally disseminated tumor cells. Mice were i.p. inoculated with MO4 cells and 3 days later they were treated by an i.p. injection of ES-DC-OVA loaded with α-GalCer or vehicle alone, or ES-DC-TT2 loaded with vehicle alone. As shown in Fig. 3c, the therapeutic effect of the transfer of ES-DC-OVA loaded with vehicle alone was significant ($P < 0.05$) in comparison to the treatment with ES-DC-TT2 loaded with vehicle alone, but the effect was less marked than the effect observed in the s.c. growing tumor model (Fig. 3b). In contrast, the treatment with ES-DC-OVA loaded with α-GalCer elicited a potent effect to prolong the survival time of the mice. Given that the antitumor effect elicited by α-GalCer-loaded non-transfectant ES-DC was also limited (Figs 2b,3c), these results indicate that the NKT cells activated by α-GalCer presented by ES-DC and OVA-specific CTL primed by OVA antigen presented by the same ES-DC acted synergistically to protect the mice.

Subsets of effector cells contributing to the antitumor effect induced by α-GalCer-loaded ES-DC that expressed a model tumor antigen

To determine the effector cells exhibiting the observed antitumor effect induced by adoptive transfer of α-GalCer-loaded ES-DC expressing OVA, we carried out depletion experiments by injecting the mice with Abs specific to several subsets of effector cells during the tumor cell challenge and treatment with ES-DC. Figure 4a shows the

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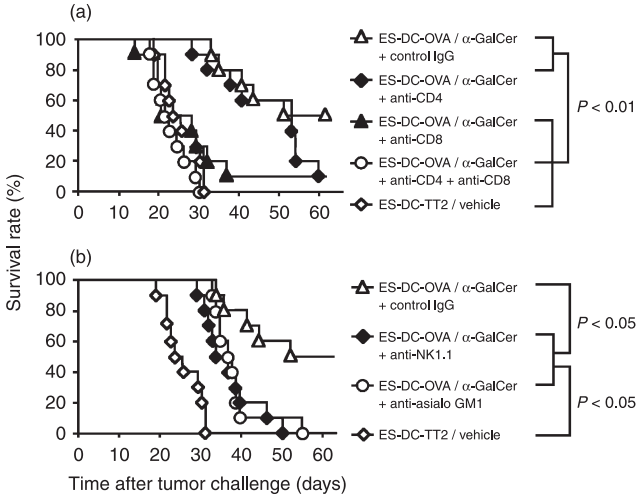

Fig. 4. Effector cells involved in the antitumor effect exerted by adoptive transfer of α-GalCer-loaded, antigen-expressing ES-DC. The mice were challenged i.p. with 1×10^5 MO4 cells on day 0 and injected i.p. with 1×10^5 ES-DC-OVA preloaded with α-GalCer on day 3. To deplete the specific types of cells, the mice were given i.p. injections of mAb or polyclonal rabbit anti-asialo GM1 Ab. The effect of the injection of anti-CD4, anti-CD8, or a combination of these two Abs is shown in (a). The effect of anti-NK1.1 or rabbit anti-asialo GM1 Ab is shown in (b). As a control, the survival of the mice treated with normal rat IgG is shown in both (a) and (b). Each group included 10 mice. In (a), the survival rates of α-GalCer-loaded ES-DC-OVA plus control IgG-treated group and α-GalCer-loaded ES-DC-OVA plus anti-CD4-treated group were higher than those of the other three groups and the difference was statistically significant. In (b), the survival rates of α-GalCer-loaded ES-DC-OVA plus anti-NK1.1 or anti-asialo GM1-treated groups and those of the other two groups were statistically different. The experiment was carried out once.

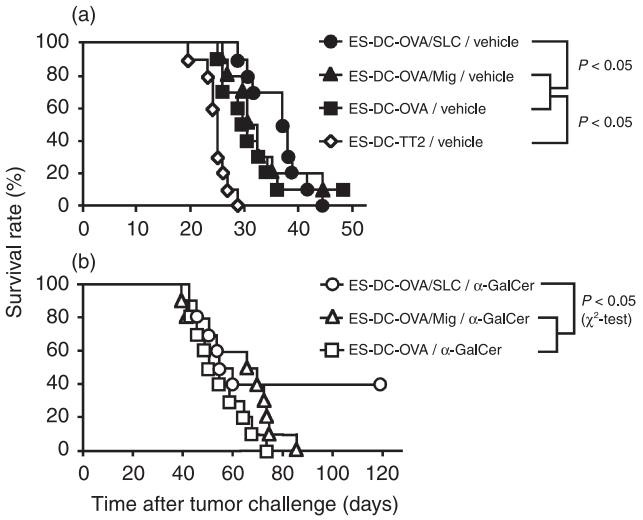

Fig. 5. Enhanced antitumor effect elicited by α-GalCer-loaded ES-DC expressing SLC along with OVA. (a) MO4 cells (1×10^5 cells/mouse) were injected i.p. into the mice and, 3 days later, the mice were treated with i.p. injection of ES-DC-TT2, ES-DC-OVA, ES-DC-OVA/Mig, or ES-DC-OVA/SLC (1×10^5 cells/mouse), all loaded with vehicle only. Thereafter, the survival rate of the mice was monitored. The differences in the survival rate between ES-DC-OVA/Mig-treated or ES-DC-OVA-treated group and the other two groups were statistically significant. (b) The mice were challenged with MO4 cells as in (a) and treated with either ES-DC-OVA, ES-DC-OVA/SLC or ES-DC-OVA/Mig, all loaded with α-GalCer. The frequency of mice from the ES-DC-OVA/SLC-treated group surviving for more than 100 days (four out of 10 mice) was significantly higher than that of the other two groups (0 out of 10 mice in each group), according to the χ^2-test. The experiment was carried out once.

effect of the injection of anti-CD4 or anti-CD8 mAbs or a combination of these two mAbs. The injection of anti-CD8 mAb almost totally abrogated the effect of the treatment with the ES-DC, thus suggesting that $CD8^+$ OVA-specific CTL played an important role in protecting the mice from the tumor. Compared to the effect of anti-CD8 mAb, the injection of anti-CD4 mAb had far less influence on the protective effect against the tumor, thus indicating the function of $CD4^+$ helper T cells to be not essential.

Figure 4b shows the effect of the injection of rabbit anti-asialo GM1 Ab, depleting NK cells, and also that of anti-NK1.1 mAb, depleting both NK and NKT cells. Treatment with either of these two kinds of Ab decreased the effect of α-GalCer-loaded ES-DC-OVA to a level similar to that elicited by vehicle-loaded ES-DC-OVA. These results indicate that NK cells played an essential role in the enhanced antitumor effect caused by the activation of NKT cells by α-GalCer.

Enhanced antitumor effect elicited by α-GalCer-loaded ES-DC expressing SLC along with OVA

We previously found that the coexpression of SLC or Mig, T cell-attracting chemokines that natural DC do not produce, along with OVA by ES-DC significantly enhanced their capacity to prime OVA-specific CTL and also to induce a protective immunity against s.c. injected MO4 cells.[16] A recent study revealed that these two chemokines induced chemotaxis not only of conventional T cells but also of some subpopulations of the NK cells and NKT cells.[10–12,23] We therefore examined whether the coexpression of such chemokine by ES-DC expressing OVA would also have an enhancing effect in protection against the i.p. growing MO4 cells.

We first assessed the effect of the expression of such chemokines by ES-DC without preloading with α-GalCer. We analyzed the capacity of ES-DC-OVA/SLC or ES-DC-OVA/Mig, ES-DC expressing OVA simultaneously with SLC or Mig, to induce protection against i.p. disseminated MO4 cells, comparing the capacity with that of ES-DC-OVA. The effect elicited by ES-DC-OVA/Mig was not higher than that elicited by ES-DC-OVA. Thus, the expression of Mig did not enhance the antitumor effect (Fig. 5a). However, expression of SLC by ES-DC did enhance the protective effect, although the effect of SLC in this i.p. tumor model was less evident than that observed in the s.c. tumor model reported previously.[16]

We next evaluated the effect of the expression of either SLC or Mig on the antitumor effect elicited by α-GalCer-loaded ES-DC-OVA. α-GalCer ES-DC-OVA/Mig and α-GalCer ES-DC-OVA exhibited a similar degree of protection, thus indicating that the coexpression of Mig by α-GalCer-loaded ES-DC-expressing OVA did not have any additive effect (Fig. 5b). In contrast, α-GalCer-loaded ES-DC-OVA/SLC exhibited a far more potent protective effect than α-GalCer ES-DC-OVA/Mig or α-GalCer ES-DC-OVA did. We observed that 40% of the mice treated with α-GalCer-loaded ES-DC-OVA/SLC completely rejected the tumor cells (Fig. 5b). These results suggest that the SLC produced by

ES-DC augmented the synergic effect of antigen-reactive CTL, α-GalCer-activated NKT cells, and probably NK cells.

As we reported previously,[16] coexpression of SLC along with OVA in ES-DC enhanced the capacity of ES-DC to prime OVA-specific CTL upon *in vivo* transfer. The data shown in the Fig. 5a also indicate that coexpression of SLC enhanced the capacity of ES-DC to induce antitumor immunity mediated by OVA-specific CTL in the absence of α-GalCer. To assess the effect of SLC produced by ES-DC on the activation of NKT or NK cells, we compared the capacity of α-GalCer-loaded ES-DC-OVA and α-GalCer-loaded ES-DC-OVA/SLC to stimulate NKT cells by an experiment similar to that shown in Fig. 1d. As a result, we observed that the capacity of α-GalCer-loaded ES-DC to induce YAC-1 cell-killing activity was not enhanced by expression of SLC (data not shown). Thus, effect of SLC produced by ES-DC to enhance the activation of NKT and NK cells was not detected at least by this short-term (24 h) assay. Based on these observations, it may be considered that the expression of SLC by ES-DC dominantly enhanced the activation of antigen-specific CTL rather than NKT or NK cells.

Discussion

In the present study, we evaluated the effect of loading α-GalCer to ES-DC expressing a model tumor antigen on their capacity to induce antitumor immunity. Upon loading with α-GalCer, ES-DC had a capacity comparable to that of BM-DC to stimulate NKT cells (Fig. 1). The *in vivo* administration α-GalCer-loaded non-transfectant ES-DC had some antitumor effect in an i.p. disseminated tumor model but not in an s.c. growing tumor model (Fig. 2). The difference in the effect of loading of α-GalCer to ES-DC in between the two models may be accounted for by the tissue distribution of NKT cells. NKT cells localize mainly in the liver, lung, spleen, bone marrow and peritoneal cavity.[6,11,24,25] In parallel with these observations, the loading of α-GalCer to ES-DC-OVA enhanced their antitumor effect against i.p. disseminated but not s.c. growing MO4 tumor cells (Fig. 3).

In a previous study, we observed that the protective effect against s.c. growing MO4 cells by transfer of ES-DC-OVA was almost totally abrogated by the depletion of either of CD4 or CD8 T cells.[16] In contrast, in the present study, the depletion of $CD8^+$ T cells but not $CD4^+$ T cells diminished the antitumor effect against i.p. MO4 cells elicited by α-GalCer-loaded ES-DC-OVA (Fig. 4a). These results indicate that CTL play a pivotal role in both conditions, and that $CD4^+$ T helper cells were not essential in the protective immunity against i.p. tumor cells on the occasion of simultaneous activation of NKT cells. The reason for the dispensability of $CD4^+$ T helper cells may be that NKT cells and NK cells, secondarily activated by NKT cells, provide help to OVA-specific CTL.[26] The data shown in Fig. 4b revealed that the depletion of NK cells decreased the effect of α-GalCer-loaded ES-DC-OVA to a degree similar to that elicited by vehicle-loaded ES-DC-OVA, indicating that NK cells played an essential role in the enhancement of the antitumor effect obtained by loading α-GalCer to ES-DC-OVA. Collectively, $CD8^+$ CTL, NKT cells and NK cells played essential roles in the antitumor effect obtained by α-GalCer to ES-DC expressing

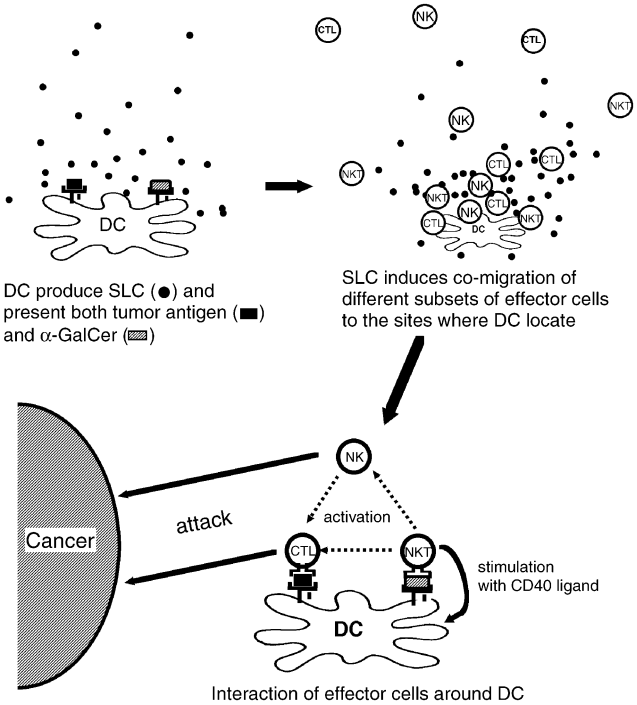

Fig. 6. A schematic depiction of the enhanced cross-talk of different subsets of effector cells induced by α-GalCer-loaded ES-DC expressing OVA plus SLC. SLC secreted by ES-DC induces the comigration of different subsets of effector cells, including NKT cells, NK cells and antigen-specific T cells, to the sites where the injected ES-DC are located. The effector cells of both innate and acquired immunity gathered around ES-DC, which present both α-GalCer and tumor antigen, thus closely interacting to develop a potent antitumor immunity.

the antigen (Fig. 6). Presumably, the sequential stimulation of NKT cells and NK cells augmented the antitumor effect of OVA-specific CTL[9] and probably the interactions of effector cells were mediated by IFN-γ and IL-2.[22,27–31]

The data shown in Fig. 5b indicate that the expression of SLC by ES-DC enhanced the antitumor effect induced by the transfer of α-GalCer-loaded ES-DC expressing OVA. SLC has been reported to attract not only conventional T cells and DC but also NKT cells.[10,23] SLC also induces chemotaxis of $CD56^{bright}$ $CD16^-$ NK cells and has a costimulatory effect on the proliferation of NK cells.[32] Thus, SLC probably induced the comigration of conventional T cells, NKT cells and NK cells to the sites where ES-DC were located, and, as a result, the close interaction of such multiple subsets of effector cells may have occurred (Fig. 6).

In the past decade, α-GalCer has been attracting attention as a novel immunostimulatory reagent for antitumor therapy. Based on the promising results of preclinical studies demonstrating antitumor effects of α-GalCer,[2,25,33] several phase I clinical studies on anticancer immunotherapy by the direct intravenous administration of α-GalCer or the administration of α-GalCer-loaded DC have been carried out.[34–37] Although the activation and expansion of NKT cells by the administration of α-GalCer has been observed, the results seemed to be unsatisfactory from the viewpoint of the clinical effect. The present study demonstrated that α-GalCer is useful for induction

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of immunity against peritoneally disseminated tumor cells, especially when it is loaded to DC genetically engineered to express tumor antigen. Although metastasis of melanoma to visceral organ sites is observed frequently in patients with advanced (stage IV) malignant melanoma, peritoneal dissemination of melanoma is very rare. Thus, we are planning another study with more clinical relevance, using models of cancer with a high tendency to peritoneal dissemination.

In recent years, a number of tumor-associated antigens have been identified. These antigens are potentially good targets for immunotherapy. To establish truly effective anticancer immunotherapy, a method for potently polarizing the immune system toward these antigens is essential. Antitumor immunotherapy with DC loaded with HLA-binding peptides derived from tumor antigens has been tested clinically in many institutions. In most cases, the DC are generated by culture of monocytes obtained from peripheral blood of the patients. To generate a sufficient number of DC for treatment, apheresis, a procedure that is sometimes invasive for patients with advanced stages of cancer, is necessary to obtain a sufficient number of monocytes as a source for DC. In addition, the culture to generate DC should be done separately for each patient and for each treatment, and thus the procedure used at present may be too labor-intensive and expensive to be applied broadly in a practical setting. Alternately, the source of ES-DC, ES cells, have the capacity to propagate infinitely. We would thus be able to use human ES cells as an infinite source of DC. In addition, we will be able to generate genetically engineered DC without the need to use virus vectors, as mentioned above. We may thus be able to generate multiple gene-transfectant ES-DC expressing tumor antigen plus immunostimulating molecules, which could be more potent in stimulating antitumor immunity than monocyte-derived DC are.

Regarding the future clinical application of ES-DC, we recently established a method for generating ES-DC from ES cells of a non-human primate, namely the cynomolgus monkey, and also for their genetic modification (unpublished data). We believe that this method would be applicable to human ES cells, although some modifications may be necessary. Considering the future clinical application of ES-DC technology, allogenicity (i.e. differences in the genetic background) between patients to be treated and ES cells as a source for DC may cause problems. However, it is expected that human ES cells sharing some HLA alleles with patients will be available for most cases. We recently found that antigen-expressing ES-DC potently primed antigen-specific CTL after the transfer to semiallogeneic mice sharing some MHC alleles with the ES-DC, and protected the recipient mice from subsequent challenge with tumor cells bearing the antigen.[38] CD1d is monomorphic and thus a CD1d-α-GalCer-complex on ES-DC can stimulate the NKT cells of any recipients. α-GalCer would thus be an ideal adjuvant to enhance the immune response toward the tumor antigens presented by ES-DC.

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