Doctor's Thesis

Multiple antigen-targeted cancer immunotherapy with genetically engineered dendritic cells derived from embryonic stem cells.

複数抗原を標的とした遺伝子改変 ES 細胞由来樹状細胞を用いた癌免疫療法

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学位論文

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1. Contents

2. Summary

Numerous tumor-associated antigens (TAA) have been identified and their use in immunotherapy is considered to be promising. For TAA-based immunotherapy to be broadly applied as standard anti-cancer medicine, methods for active immunization should be improved. In the present study, we demonstrated the efficacy of multiple TAA-targeted dendritic cell (DC) vaccines and also the additive effects of loading α-galactosylceramide (α-GalCer) to DC, using mouse melanoma models. Based on previously established methods to generate DC from mouse embryonic stem cells (ES-DC), four kinds of genetically modified ES-DC which expressed the melanoma-associated antigens, glypican-3 (GPC3), secreted protein acidic and rich in cysteine (SPARC), tyrosinase-related protein-2 (TRP2) or gp100 were generated. Anti-cancer effects elicited by immunization with the ES-DC were assessed in preventive and also therapeutic settings in the models of peritoneal dissemination and spontaneous metastasis to lymph node and lung. The *in vivo* transfer of a mixture of three kinds of TAA-expressing ES-DC protected the recipient mice from melanoma cells more effectively than the transfer of ES-DC expressing single TAA, thus demonstraiting the advantage of multiple as compared to single TAA-targeted immunotherapy. Loading ES-DC with α-GalCer further enhanced the anti-cancer effects, suggesting that excellent synergic effects of TAA-specific cytotoxic T lymphocytes and NKT cells against metastatic melanoma can be achieved by using genetically modified ES-DC. With the aid of advancing technologies related to pluripotent stem cells, iPS cells and ES cells, clinical application of DC highly potent in eliciting anti-cancer immunity will be realized in the near future.

3. Publication list

Fukushima S, Hirata S, Motomura Y, Fukuma D, Matsunaga Y, Ikuta Y, Ikeda T, Kageshita T, Ihn H, Nishimura Y, and Senju S.
Multiple antigen-targeted immunotherapy with □-galactosylceramide-loaded and genetically engineered dendritic cells derived from embryonic stem cells. ***J.Immunotherapy*** in presss

Matsunaga Y, Fukuma D, Hirata S, Fukushima S, Haruta M, Ikeda T, Negishi I, Nishimura Y, and Senju S.
Activation of antigen-specific cytotoxic T lymphocytes by β2-microglobulin or TAP1 gene disruption and the introduction of recipient-matched MHC class I gene in allogeneic ES cell-derived dendritic cells. ***J.Immunol***. 181: 6635-6643, 2008

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4. Acknowledgments

These series of investigations were performed from 2005 to 2009, in the Department of Immunogenetics and the Department of Dermatology, Graduate School of Medical Sciences, Kumamoto University.

I wish to extend my warmest thanks to Professor Yasuharu Nishimura and Associate Professor Satoru Senju, the department of Immunogenetics, and Professor Hironobu Ihn, the department of Dermatology, Graduate School of Medical Sciences, Kumamoto University. They generously gave me advices and suggestions. I would also like to express my gratitude for their spending valuable time to correct my paper.

I am grateful to all staffs and students in the department of Immunogenetics and the department of Dermatology, Graduate School of Medical Sciences, Kumamoto University. They provided me with their valuable time and gave me much advice.

We thank Drs. H. Suemori and N. Nakatsuji for B6, Drs. N. Takakura and T. Suda for OP9, Dr. M. Nishikawa for pCMV-Luc, and Kirin Brewery for α-GalCer.

This work was supported in part by Grants-in-Aid Nos. 16590988, 17390292, 17015035, 18014023, 19591172 and 19059012 from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases launched as a project commissioned by MEXT, Japan, Research Grant for Intractable Diseases from Ministry of Health and Welfare, Japan and grants from Japan Science and Technology Agency (JST), the Uehara Memorial Foundation and the Takeda Science Foundation.

Finally, I would like to thank my family for their support, encouragement, and unshakable faith in my abilities during the course of my studies. Without their support, I may not have persevered in my studies.

5. Abbreviations

αGalCer,	α-galactosylceramide
APC,	Antigen presenting cell;
BCC,	Basal cell carcinoma
β2m,	β2-microglobulin;
BM-DC,	Bone marrow-derived dendritic cell;
CTL,	Cytotoxic T lymphocyte;
ELISA,	Enzyme-linked immunosorbent assay;
ELISPOT,	Enzyme-linked immunospot;
ER,	Endoplasmic reticulum;
ES cell,	Embryonic stem cell;
ES-DC,	Embryonic stem cell-derived dendritic cells;
FITC,	Fluorescein isothipcynate;
GM-CSF,	Granulocyte macrophage colony stimulating factor
HA,	Hemaggulutinin;
HLA,	Human leukocyte antigen;
IFN,	Interferon;
IL,	Interleukin
IRES,	Internal ribosomal entry site;
i.p.,	Intraperitoneally
iPS cell,	Induced pluripotent stem cell;
Luc,	Luciferase;
MHC,	Major histocompatibility complex;
MM,	Malignant melanoma
MF,	Mycosis fungoides
NK,	Natural killer
NKT,	Natural killer T
PBS,	Phosphate-buffered saline;
PCR,	Polymerase chain reaction;
SCC,	Squamous cell carcinoma

s.c., Subcutaneously

TAA, Tumor-associated antigens

TAP, Transporter associated with antigen processing;

TNF, Tumor necrosis factor

6. Introduction

6.1 The prognosis of patients with malignant melanoma

In Japan, the number of patients with malignant skin tumors has increased year by year[1]. Figure 1 displays the annual registered numbers of Japanese patients with basal cell carcinoma (BCC), squamous cell carcinoma (SCC), malignant melanoma (MM) and mycosis fungoides (MF). The patients with malignant melanoma accounted for approximately 20% of these four main skin malignancies.

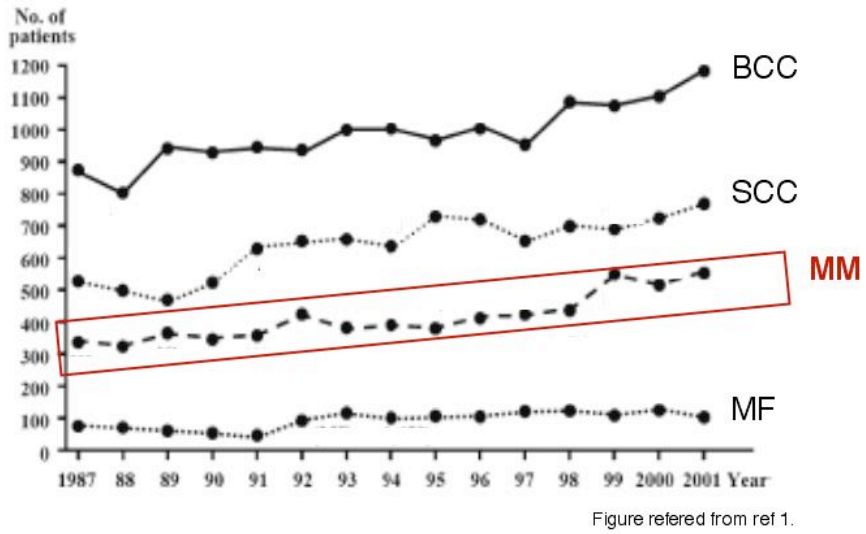

Figure 1. Numbers of patients with skin malignancies

The prognosis of patients with advanced malignant melanoma remains extremely poor.[1] Figure 2A shows survival rate stratified by the stages of melanoma (UICC stage classification). The survival rate of stage IV patients was very low, emphasizing the difficulty of treating malignant melanoma with distant metastases.

Figure 2B demonstrates the survival rate of 150 stage IV patients (M1b; pulmonary metastases and M1c; other metastases or elevated serum levels of LDH) who received chemotherapy, chemotherapy + IFN-beta therapy, or no treatment[1]. The chemotherapy regimen used was DAV (DTIC+ACNU+VCR), CDV (CDDP+DTIC+vindesine), or DACTam (DTIC+ACNU+CDDP+tamoxifen). In Japan, chemotherapy was often combined with intracutanous injection of IFN-beta around the

excision scars of primary melanoma. Survival was significantly higher in the treated groups than in the untreated group ($p<0.0001$). However, there was no significant difference between the chemotherapy-alone group and the chemotherapy + IFN-beta group. These results were obtained by nonrandomized studies, so they should be interpreted with caution. In worldwide, there are no randomized trials comparing the effects of systemic therapies for metastatic melanoma with best supportive care or placebo.[2] Malignant melanoma is chemoresistant and radioresitant. However, malignant melanoma is an immunogenic tumor. Therefore immunotherapy has been considered to be a promising therapy as a treatment for advanced melanoma.

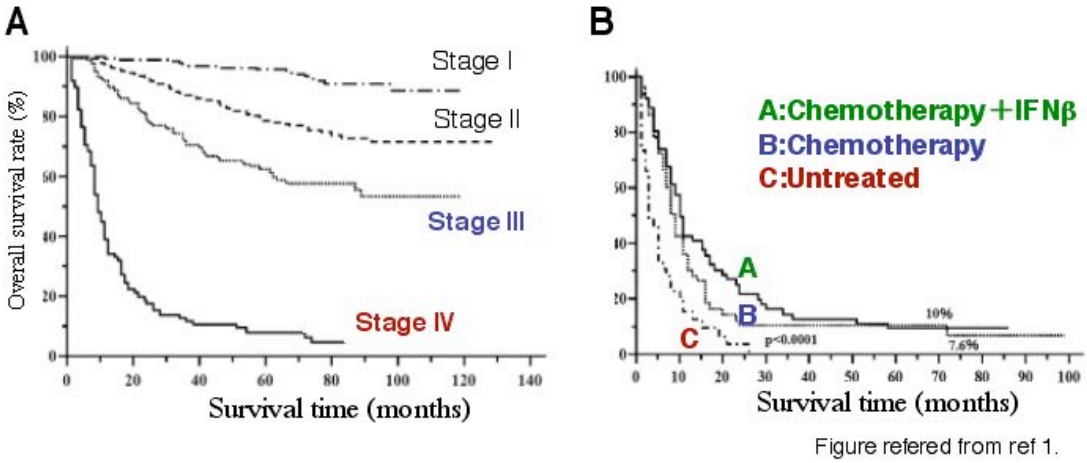

Figure 2. Survival rate of patients with malignant melanoma

6.2. DC vaccine against cancer

Dendritic cells (DC) are potent stimulators of B and T lymphocytes.[3] B cells, which are important in humoral immunity, can directly recognize native antigen through their B cell receptors. However, T lymphocytes, which are important in cellular immunity, require the tumor-associated antigens (TAA) to be processed and presented to them by antigen presenting cells (APC), including DC. The T cell receptors (TCR) recognize antigenic peptides in the context of the major histocompatibility complex (MHC)-encoded molecules expressed on the cell surface of APC. The mature DC express peptide-MHC complex, together with appropriate co-stimulate molecules on the cell surface. This allows the priming of naive $CD4^+$ T helper and $CD8^+$ cytotoxic T lymphocytes (CTL). DC also can activate natural killer (NK) cells [4] and natural killer T

(NKT) cells.[5] So, DC can conduct all of the elements of the immune orchestra, and they are therefore an attractive target and tool for cancer immunotherapy (Fig.3).

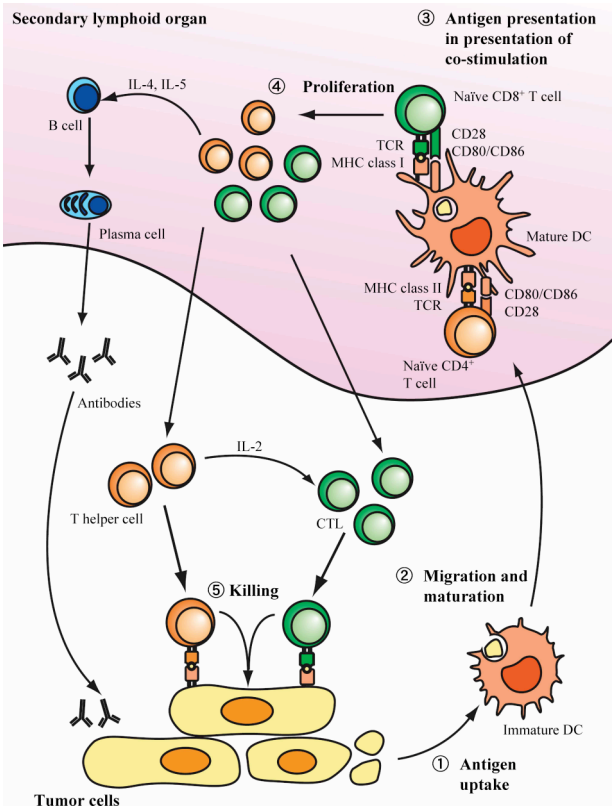

Figure 3. Tumor immunity

Ex vivo-generated, antigen-loaded DC have now been used as vaccines to induce strong T cell immunity.[6] Mice studies have shown that DC loaded with tumor antigen can induce therapeutic and protective antitumor immunity.[7] Numerous reports have shown that DC loaded with dead tumor cells, tumor cell lysates, tumor antigenic proteins or peptides can induce immunity and clinical responses.[8-11] Banchereau *et al*. compared clinical outcomes in several Phase I and Phase II vaccine trials in which DC and other types of vaccine were used for the treatment of patients with melanoma (Table 1).[6, 12-21] Although the rate of objective tumor regression is still limited, these outcomes warrant further exploration to establish the therapeutic value of vaccination with DC.

Vaccine	Total Patients	Responding Patients	Response Rate (%)	References
Peptide vaccine	410	11	2.7	10, 11, 12
Viral vector	160	3	1.9	10
Tumor cells	43	2	4.6	13, 14
Dendritic cells	116	11	9.6	10, 15, 16, 17,18, 19

Table referred from REF.10

Table 1. Clinical outcomes of cancer vaccines in patients with melanoma.

The manipulation of DC, specialized antigen-presenting cells, is a promising strategy to improve the efficacy of cancer immunotherapy.[22,23] Genetic modification to express antigenic proteins has several advantages in comparison to using a peptide, protein, or tumor cell lysate as a means for loading TAA to DC.[24] The expression of TAA by DC circumvents the need for identifying specific CTL epitopes within the protein, and the antigens are continuously supplied for presentation as opposed to a single pulse of peptides or tumor cell lysates.[25] Furthermore, *in vivo* transfer of DC transfected with TAA gene are able to prime CTL reactive to multiple TAA-derived epitopes.[26]

6.2. Embryonic stem cell-derived DC

Others and we have established to generate DC *in vitro* from mouse embryonic stem (ES) cells (ES-DC).[27,28] There are several advantages of using ES cells as a source of DC. (Table 2) ES-DC have the capacity to stimulate T cells, present antigen and migrate to lymphoid tissues upon *in vivo* administration, and their capacity is comparable to that of bone marrow derived DC.[26, 28-30] The genetic modification of ES-DC can be carried out without the use of viral vectors by introducing exogenous genes into undifferentiated ES cells by electroporation and the subsequent induction of their differentiation into ES-DC. In a previous study, ES-DC expressing GPC3 showed protective immunity against mouse melanoma, however, the therapeutic effects were insufficient.[26] Genetically modified DC generated in this laboratory are summarized in Table 3.

1. Infinite proliferative potential
Clinical trials using monocyte-derived dendritic cells (MC-DC) need large amounts of blood from the patients each time.
2. Transfection of ES cells can be done with electroporation
MC-DC introduced with adenovirus vectors encoding TAA have the potential risk accompanying the use of viral vectors and legal restrictions.
3. High gene transferring rate and stable expression
Once a properly transfected ES cell clone is established, it then serves as an infinite source for genetically modified DC.
4. Multiple genes can be introduced to ES cells
ES-DC expressing TAA plus immunostimulating molecules more potently stimulate anti-cancer immunity.

Table 2. Advantages of using ES cells as a source of DC

Genes introduced into ES-DC	Expected effects	References
Activation of immune response		
OVA along with CCL2	Antigen presentation and Attraction of T cells	
OVA along with CXCL9	Antigen presentation and Attraction of T cells	
OVA along with XCL1	Antigen presentation and Attraction of T cells	
Glypican-3	Natural tumor antigen presentation	
TRP2, hgp100 or SPARC	Natural tumor antigen presentation	
Suppression of immune response		
MOG peptide along with TRAIL	Antigen specific immunosuppresion	
MOG peptide along with PD-L1	Antigen specific immunosuppresion	
Others		
SPI-6	Avoidance of DC elimination	

Table 3. Genetically modified ES-DC generated in our laboratory.

6.3. Multiple antigen-targeted immunotherapy

Important issue to be considered is whether it is appropriate to design clinical strategies that combine multiple agents to modulate the immune response.[31] Clinically manifested cancers are often associated with multiple mechanisms to evade immune attacks, such as antigen-loss, active tolerance induction, deficiency in antigen presentation machineries,[32] etc., which are difficult to be addressed successfully with a single agent. To overcome this defense mechanism and to address the low frequency of

cytotoxic T lymphocytes (CTL) that recognize single endogenous TAA, the efficacy of multiple TAA-targeted immunotherapies were examined in the present study. DC were generated expressing endogenous TAA, glypican-3 (GPC3), secreted protein acidic and rich in cysteine (SPARC), tyrosinase-related protein-2 (TRP2) and gp100. The oncofetal protein GPC3, glycosylphosphatidylinositol anchored membrane protein, is specifically overexpressed in human melanoma and hepatocellular carcinoma, and GPC3 can be a candidate target for cancer immunotherapy.[33-35] Clinical trials with GPC3 peptide against hepatocellular carcinoma are now ongoing. SPARC, also called osteonectin, is a matricellular glycoprotein that modulates cellular interactions with the extracellular matrix during tissue remodeling.[36] SPARC or its combination with GPC3 is a useful tumor maker for melanoma.[37, 38]

6.4. Induction of innate immunity

To counter the cancers with deficiencies in antigen presentation machineries it is necessary to induce the innate immunity. α-galactosylceramide (α-GalCer) presented by DC efficiently stimulates NKT cells.[5] Therefore, it is presumed that the *in vivo* transfer of DC simultaneously loaded with TAA and α-GalCer may stimulate both tumor-reactive T cells and NKT cells, resulting in a potent anti-cancer immunity.(Fig. 4)

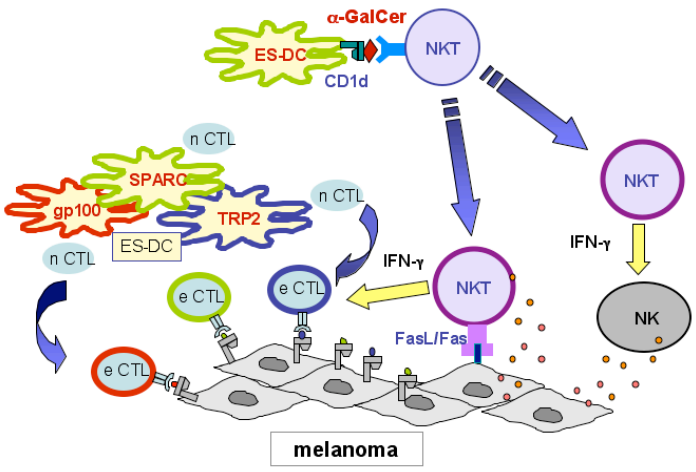

Figure 4. α-GalCer-loaded ES-DC stimulate NKT cells, CTL and NK cells

(nCTL; naïve CTL, eCTL; effector CTL)

6.5. Pre-clinical studies for cancer vaccines

A number of pre-clinical studies have demonstrated the efficacy of cancer vaccines using mouse models. However, cancer vaccine trials in humans have yet to demonstrate a sufficient clinical response,[12, 31] and at present tumor-associated antigens (TAA)-specific cancer immunotherapies are not regarded as the standard medical technology, except for several antibody therapies. Therefore, there is discrepancy between the promising results obtained in mouse studies and those of clinical trials. Although the reasons for this discrepancy vary, one is the improper selection of mouse models.[39] Most mouse studies use models where the tumor cells are inoculated subcutaneously or intravenously. These are convenient to observe the efficacy of immunotherapy. However, to evaluate the efficacy in clinical medicine, experimental systems should be used that reflect the clinical situations where immunotherapy is actually needed, such as cancers accompanying with multiple metastases or with peritoneally disseminated lesions. The present study evaluated the capacity of genetically engineered DC to inhibit peritoneal dissemination and spontaneous metastasis of mouse melanoma to lymph node and lungs.

6.7. Aims of this work

The present study investigated the anti-cancer effects of multiple TAA-targeted immunotherapies with α-GalCer-loaded and genetically engineered ES-DC against mouse melanoma. Anti-cancer effects elicited by immunization with the ES-DC were assessed in preventive and also therapeutic settings in the models of peritoneal dissemination and spontaneous metastasis to lymph node and lung.

7. Materials and methods

7.1. Mice

C57BL/6 mice were obtained from SLC (Hamamatsu, Japan) and maintained under specific pathogen-free conditions. All studies were done with C57BL/6 mice syngeneic to the mouse ES cell line B6 at 6-8 weeks of age. The mouse experiments were approved by the Animal Research Committee of Kumamoto University.

7.2. Cell lines

The ES cell line B6, derived from C57BL/6 blastocysts, was kindly provided by Drs. H. Suemori and N. Nakatsuji (Kyoto University). The method for *in vitro* induction of differentiation of ES cells into DC was described previously,[28] and ES-DC prepared from a 7-day culture in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) were used for all assays. The C57BL/6-derived tumor cell lines, F10 and BL6 sublines of B16 melanoma, a fibrosarcoma cell line MCA205 (MCA), and a thymoma cell line EL4 were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). The cells were cultured in RPMI 1640 supplemented with 10% horse serum. To produce GPC3-expressing MCA (MCA-GPC3), MCA cells were transfected with pCAGGS-GPC3-internal ribosomal entry site (IRES)-puromycin-resistant (puro-R) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), selected with puromycin, and then subjected to cloning by limiting dilution as described previously.[26, 40] To produce EL-4-expressing SPARC (EL4-SPARC), EL-4 cells were transfected with pCAGGS-SPARC-IRES-puro-R same as above. Plasmid DNA encoding firefly luciferase was kindly provided by Dr. M. Nishikawa (Kyoto University), and B16-BL6 cells were transfected with the construct as described above, then the single colonies of G418-resistant cells were picked up, and a clone was selected on the basis of the luciferase activity (B16-BL6/Luc).

7.3. Peptides and cytokines

Known $CD8^+$ T cell epitope peptides were purchased from AnyGen (Gwangju,

Korea) and their amino acid sequences are as follows: mouse TRP2 $_{180\text{-}188}$ （SVYDFFVWL, H-2K^{b} restricted）, mouse gp100 $_{25\text{-}33}$ (EGSRNQDWL, H-2D^{b} restricted) and human gp100 $_{25\text{-}33}$ (KVPRNQDWL, H-2D^{b} restricted). A control peptide derived from OVA $_{257\text{-}264}$ (SIINFEKL, H-2K^{b} restricted) was synthesized by the automatic peptide synthesizer (PSSM8, SHIMADZU, Kyoto, Japan) and subsequently purified by high-performance liquid chromatography. Recombinant mouse GM-CSF, recombinant human IL-2, recombinant murine IFN-γ (PeproTech, London, UK) and IL-4 (ProSpecTechnoGene, Rehovot, Israel) were purchased. α-GalCer was kindly provided by Kirin Brewery Co. (Tokyo, Japan).

7.4. Generation of ES-DC expressing GPC3, SPARC, TRP2 or hgp100

cDNA fragments encoding for total mouse SPARC and TRP-2 were obtained by RT-PCR from B16-F10. Full-length mouse GPC3 and human gp100 (hgp100) cDNA clones were purchased from Invitrogen. cDNA fragments encoding the whole GPC3 protein and a fragment of hgp100 (hgp100 $_{1\text{-}300}$) including the H-2D^{b}-restricted epitope (hgp100 $_{25\text{-}33}$) were isolated from those clones. cDNA for GPC3, SPARC, TRP2 or hgp100 was transferred to a mammalian expression vector pCAGGS-IRES-puro-R, containing the CAG promoter and an IRES-puro-R gene cassette,[41, 42] to generate an expression vector for GPC3, SPARC, TRP-2 and hgp100, pCAGGS-GPC3-IRES-puro-R, pCAGGS-SPARC-FLAG-HA-IRES-puro-R, pCAGGS-TRP2-HA-IRES-puro-R and pCAGGS-hgp100-HA-IRES-puro-R. All constructs contain HA- or FLAG-tag except for that of GPC3 (Fig. 5). To generate transfectant ES cell clones, B6 ES cells were transfected with the expression vectors by electroporation and selected with puromycin as described previously (Fig. 6).[28]

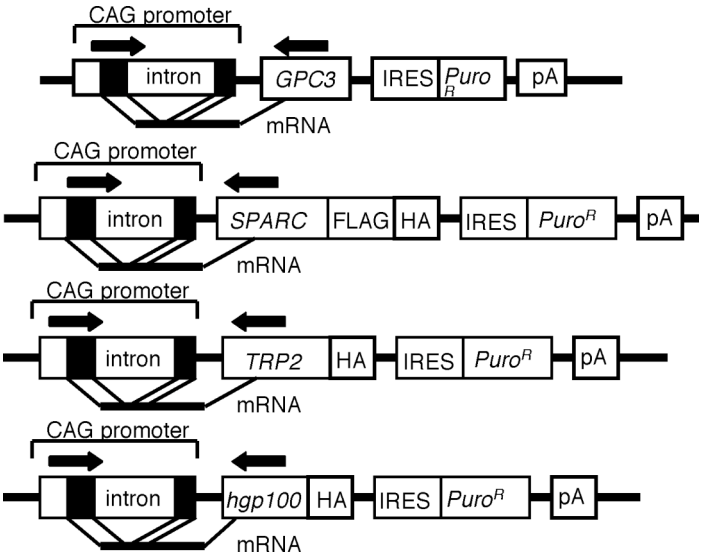

Figure 5. Structure of vectors; pCAGGS-GPC3-IRES-puro-R, pCAGGS-SPARC-FLAG-HA-IRES-puro-R, pCAGGS-TRP2-HA-IRES-puro-R and pCAGGS-hgp100-HA-IRES-puro-R. To obtain the vectors, each cDNA fragment encoding for a full-length of mouse *GPC3, SPARC, TRP2* or *hgp100* $_{1\text{-}300}$ was inserted into a mammalian expression vector, pCAGGS-IRES-puro-R containing the CAG promoter and an IRES-puromycin resistant gene cassette. All constructs contain HA- or FLAG-tag except for that of GPC3.

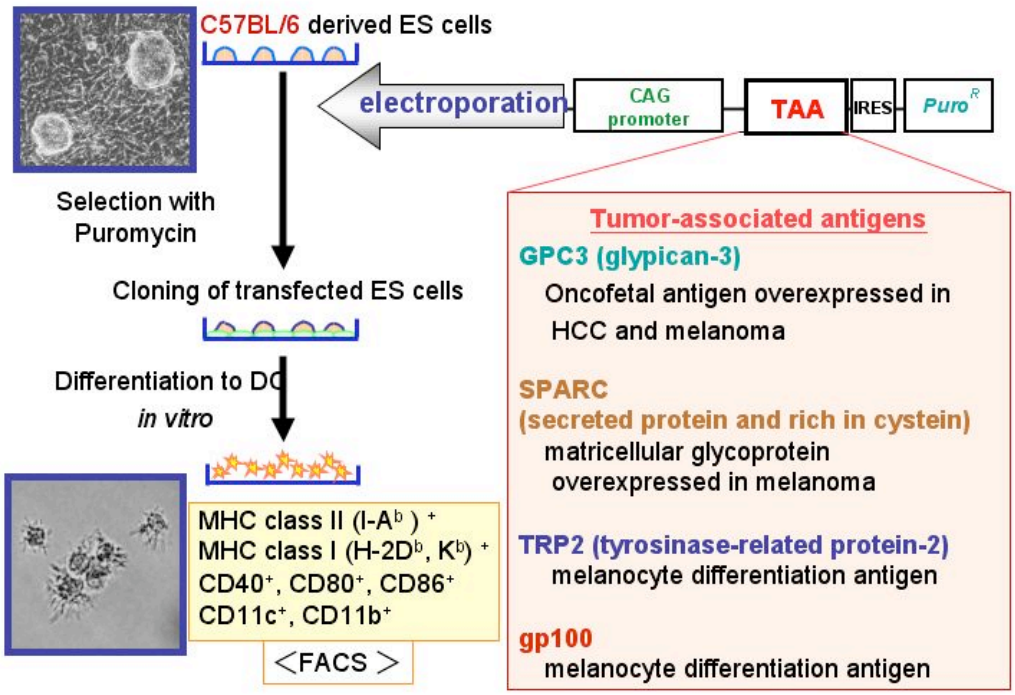

Figure 6. Method to generate ES-DC expressing TAA

Transfectant ES cell clones were subjected to a differentiation culture to generate ES-DC (Fig. 7) [43, 44]. ES cells were suspended in α-MEM supplemented with 20% FCS and seeded (1 x 10^5 cells/ 8 mL medium/well) onto OP9 cell layers in 100-mm dish culture plates. On day 3, half of the medium was removed and 4 mL fresh medium was added to each well. On day 5, cells were harvested using phosphate-buffered saline (PBS)/0.25% trypsin/1 mM EDTA (ethylenediaminetetraacetic acid), reseeded onto fresh OP9 cell layers, and cultured in α-MEM supplemented with 20% FCS and GM-CSF (1000 U/mL). At this step, cells recovered from a 100-mm dish culture plate were suspended in 32 mL medium and seeded into four 100-mm dish. On day 10 (5 days after the transfer), floating cells were recovered by pipetting. On average, 2.5 to 5 x 10^6 cells were recovered from one 100-mm dish, thus indicating 100 to 200 times increase in cell number from undifferentiated ES cells. The recovered cells were transferred to bacteriologic Petri dishes (5 x 10^5 cells/90-mm dish) without feeder cells, and cultured in RPMI-1640 medium supplemented with 10% FCS, GM-CSF (500 U/mL), and 2-mercaptoethanol. On day 17 of cultures, the floating or loosely adherent cells were recovered from culture dishes by pipeting and then were used for the experiments. ES-DC differentiated from GPC3-, SPARC-, TRP2- or hgp100-transfectant ES cells were designated as ES-DC-GPC3, ES-DC-SPARC, ES-DC-TRP2 or ES-DC-hgp100, respectively. Recombinant mouse IL-4 was given to ES-DC at 20 hr before *in vivo* transfer.

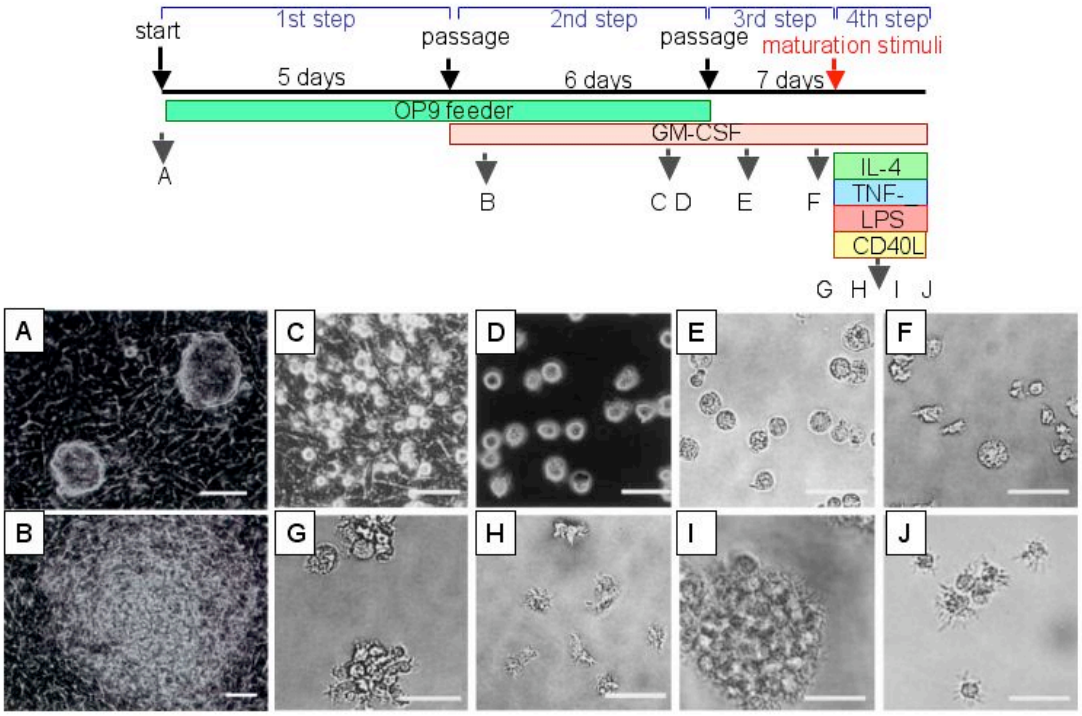

Figure 7. Differentiation and morphology of DC from ES cells.

(A-G) ES cell colonies on OP9 feeder cell layer on day3 (A), day5 (B), day12 (C, D), day17 (E, F) and day 27 (G) of differentiation culture are shown. Cells on day 24 were recovered and stimulated for 2 days with IL-4, TNF-α plus agonistic anti-CD40 mAb (H) or with IL-4, TNF-α plus LPS (I), as described in the materials and methods. Panel (B) and (C) are phase contrast micrographs. Scale bars represent 20 μm.

7.5. RT-PCR

Total cellular RNA was extracted using the RNeasy Mini Kit (QIAGEN, Maryland, MD) and RT-PCR was done as previously described.[43] Briefly, total RNA was converted into cDNA and PCR was done for 30 cycles for the quantification of *GPC3, SPARC, TRP-2, hgp100* and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) mRNA. The primer sequences for detection of the transgene in ES-DC and endogenous genes expressed in tumor cells are shown in the Table 4. The sense strand primer used for detection of transgene-derived mRNA corresponded to the 5' untranslated region included in the vector DNA. PCR products were visualized by ethidium bromide staining after separation by electrophoresis in a 1.5% agarose gel.

PCR primers used for detection of transgenes expressed in ES-DC

	Sense	Antisense
GPC3	5'-CTGACTGACCGCGTTACTCCCACA-3'*	5'-TAGCAGCATCGCCACCAGCAAGCA-3'
SPARC	5'-CTGACTGACCGCGTTACTCCCACA-3'*	5'-GGCAAAGAAGTGGCAGGAAG-3'
TRP2	5'-CTGACTGACCGCGTTACTCCCACA-3'*	5'-TGGGCAGTCAGGGAATGGAT-3'
hgp100	5'-CTGACTGACCGCGTTACTCCCACA-3'*	5'-GCACCTATCACAGCCAAATG-3'
G3PDH	5'-GGAAAGCTGTGGCGTGATG-3'	5'-CTGTTGCTGTAGCCGTATTC-3'

* The sense strand primer used for detection of transgene-derived mRNA corresponded to the 5' untranslated region included in the vector DNA (Fig. 1A).

PCR primers used for detection of endogenous genes expressed in tumor cells

	Sense	Antisense
SPARC	5'-ATGAGGGCCTGGATCTTCTTTCTC-3'	5'-TTAGATCACCAGATCCTTGTTGATGTCC-3'
TRP2	5'-CCTTTGCGTTGCCCTACT-3'	5'-CTAGGCTTCCTCCGTGTATCTCTT-3'
mgp100	5'-CCCGTGCTTGTGCTGAGTGCTCTG-3'	5'- ATGCTCGACCTGGACACTGGAC-3'

Table 4. PCR primers

7.6. Flow-cytometric analysis and ELISA

The staining of cells and the analysis on a flow cytometer (FACScan, BD Bioscience, San Jose, CA) were performed as previously described.[43, 45] The antibodies and reagents used for staining were: FITC-conjugated anti-HA (clone 3F10, rat IgG1, Fab fragments, Roche, Indianapolis, IN), rabbit anti-gp100 (AnaSpec, San Jose, CA), FITC-conjugated goat anti-rabbit IgG (clone ALI4408, Biosource, Camarillo, CA), anti-mouse CD16/CD32 (clone 2.4G2, rat IgG2b, BD Bioscience), reconbinant soluble dimeric mouse CD1d:Ig (BD Bioscience), PE-conjugated anti-mouse IgG1 (clone A85-1, rat IgG1, BD Bioscience), mouse IgG1 Isotype contral (clone A111-3, mouse IgG1, BD Bioscience), FITC-conjugated anti-mouse TCRβ (clone H57-597, hamster IgG, BD Bioscience) and IntraPrep permiabilization reagent (Beckman Coulter, Fullerton, CA). ELISA was done as described previously.[33, 38] The concentrations of HA and FLAG tagged SPARC proteins in the culture supernatants of ES-DC were measured by ELISA in triplicate wells using anti-FLAG (clone M2; mouse IgG, SIGMA, St Louis, MO) and anti-HA-peroxidase (clone 3F10; rat IgG1; Roche).

7.7. Induction of TAA-specific CTL and cytotoxicity assay

The mice were immunized intraperitoneally (i.p.) with each ES-DC twice

with a 7-day interval. Seven days after the second immunization, spleen cells were isolated from the mice and cultured (2.5 x 10^6 per well) with ES-DC-GPC3, ES-DC-SPARC, ES-DC-TRP2 (7 x 10^4 per well) or 0.1μM hgp100 peptide in 24-well culture plates in RPMI-1640 supplemented with 10% horse serum, rhIL-2 and 2-mercaptoethanol. After culturing for 5 days, the cells were recovered and their cytotoxic activity was analyzed by 4-hr ^{51}Cr release assays using MCA, MCA-GPC3, B16-F10, EL-4, EL4-SPARC and peptide pulsed EL-4 as targets basically by the same method as described previously.[26] B16-F10 cells were pretreated with recombinant murine IFN-γ (1,000 units/mL) before use as target cells as reported previously.[46]

7.8. Subcutaneous tumor model

The mice were immunized i.p. with ES-DC-SPARC or ES-DC-TRP2 twice on days –14 and –7, and B16-F10 cells were inoculated subcutaneously into the shaved back region on day 0. In some experiments, the mice were immunized with a mixture of ES-DC-GPC3, ES-DC-SPARC and ES-DC-TRP2. The tumor sizes were determined biweekly in a blinded fashion and the survival rate was monitored. The tumor volume was calculated as follows: tumor volume (mm^3) = (length x width x height).

7.9. Measurement of luciferase activity

The cells or tissues were homogenized with 2 ml of lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8) and the homogenates were cleared by centrifugation at 10,000 x g for 5 minutes. Twenty-five μl of the supernatant was mixed with 75 μl of dilution buffer (PBS containing $CaCl_2$ 1.8 mM and $MgSO_4$ 0.82 mM) and 100 μl of luciferase assay buffer (Steadyliteplus, PerkinElmer, Norwalk, CT), and at 10 minutes after the mixing the light produced was measured for one second in a luminometer (Tristar LB941, Berthold Technologies, Bad Wildbad, Germany). The luciferase activity was converted to the number of tumor cells using a regression line ($y = 0.1134x^2 - 0.305x + 0.4213$; x=cell number, y=counts per second).

7.10. Experimental peritoneal dissemination

In the preventive experiments, the mice were immunized i.p. with ES-DC-SPARC,

ES-DC-TRP2, ES-DC-hgp100, their mixture (ES-DC-STH), or non-transfectant ES-DC twice on days 0 and 7, and B16-BL6/Luc cells were inoculated i.p. into mice on day 14. On day 28, the mice were euthanized and the greater omentum and pancreas were excised together and then the total luciferase activities were measured. In one experiment, the luciferase activities of the liver, kidney, spleen and peritoneum were also measured. In the therapeutic experiments, B16-BL6/Luc cells were inoculated i.p. into mice on day 0. On days 3 and 10, ES-DC were transferred i.p. into mice. On day 17, mice were euthanized and luciferase activities of the greater omentum and pancreas were measured. In some experiments, ES-DC were cultured in the presence of α-GalCer (100 ng / mL) or vehicle (0.00025% Polysorbate-20) for 20 hr, and washed twice before injection. In one experiment, free α-GalCer (1μg/mouse/transfer) either with or without ES-DC was transferred.

7.11. Analysis of the activation of NKT cells

The activation of NKT cells *in vitro* was analyzed as previously described.[47] In the analysis of the activation of NKT cells *in vivo*, on day 0, ES-DC loaded with either α-GalCer or vehicle were transferred i.p. into mice. On days 1, 7, 17 or 27, the mice were euthanized and the cytotoxic activities of the whole spleen cells against Yac-1 cells were analyzed.

7.12. *In vivo* depletion experiments

The mice were transferred i.p. twice with either α-GalCer or vehicle loaded ES-DC-STH on days 0 and 7, and B16-BL6/Luc cells were inoculated i.p. into mice on day 14. To deplete the specific types of cells, the mice were given a total of 4 i.p. transfers (days –2, 5, 12 and 19) of mAb, ascites (0.1mL/mouse/transfer) from hybridoma-bearing nude mice, or polyclonal rabbit anti-asialo GM1 Ab (Wako, Tokyo, Japan; 20μl/mouse/transfer). The mAbs used were rat anti-mouse CD4 mAb (clone GK1.5), rat anti-mouse CD8 mAb (clone 2.43). Normal rat IgG (Sigma-Aldrich, St. Louis, MO; 200μg/mouse/transfer) was used as a control. The depletion of specific cell subsets by treatment with antibodies was confirmed by a flow cytometric analysis of spleen cells, which showed a >90% specific depletion.

7.13. Spontaneous metastasis experiments

In the preventive experiments, α-GalCer-loaded ES-DC or ES-DC-STH were transferred i.p. into mice twice on days 0 and 7. On day 14, the footpad of mice was inoculated with B16-BL6/Luc cells. On day 35, mice were euthanized, the lungs were excised and total luciferase activities were measured. In the therapeutic experiments, B16-BL6/Luc cells were inoculated into the footpad on day 0. On days 3 and 10, ES-DC were transferred i.p. into mice. On day 17, mice were euthanized and luciferase activities of the inguinal lymph nodes were measured.

7.14. Statistical analysis

Two-tailed Student's *t*-test was used to determine the statistical significance of differences in the tumor growth between the treatment groups. The Kaplan-Meier analysis with the Breslow-Gehan-Wilcoxon test was used to determine that of survival. The Mann-Whitney U test was used to examine the differences of luciferase activities. $P < 0.05$ was considered to be significant. Statistical analyses were performed using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

8. Results

8.1. Generation of ES-DC expressing melanoma antigens

B6 ES cells were transfected with the *GPC3, SPARC, TRP2* and *hgp100* expression vectors; pCAGGS-GPC3-IRES-puro-R, pCAGGS-SPARC-FLAG-HA-IRES-puro-R, pCAGGS-TRP2-HA-IRES-puro-R, pCAGGS-hgp100-HA-IRES-puro-R (Fig. 5) and several transfectant clones were isolated. The transfectant ES cell clones were subjected to differentiation to ES-DC, and the transfectant clone expressing the highest level of each TAA was selected based on the RT-PCR, FACS analysis and ELISA (Fig. 8A-C).

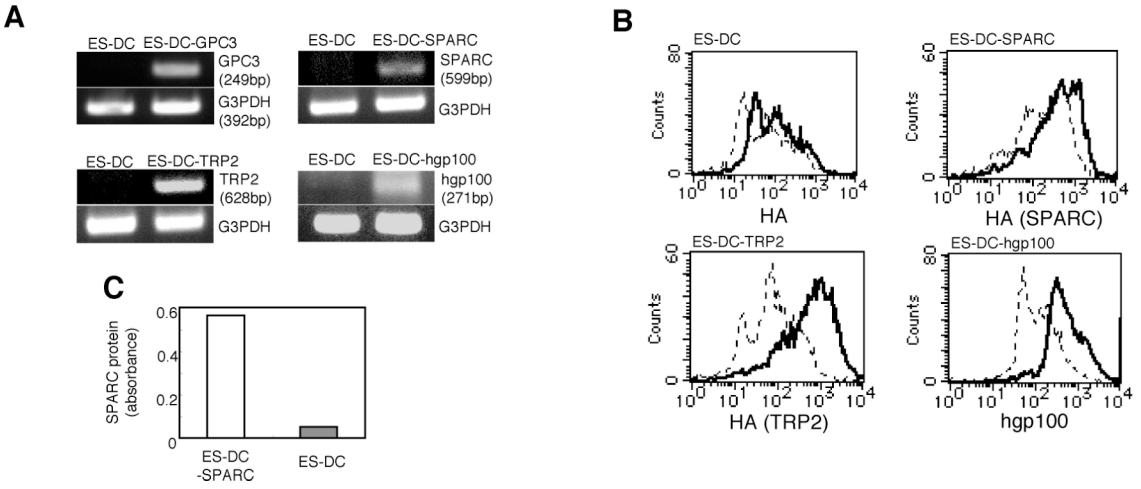

Figure 8. Expression of *GPC3, SPARC, TRP2* and *hgp100* in transfectant ES-DC

A, Expression of *GPC3, SPARC, TRP2* and *hgp100* mRNA detected by RT-PCR in transfectant ES-DC. Primer sets (arrows in Fig.5) were designed to span the intron (917 bp) in the CAG promoter sequence to distinguish the PCR products of mRNA origin from the genome-integrated vector DNA origin. B, The expression of the transfected protein in ES-DC. Transfectant ES-DC were analyzed by flow cytometric analysis using intracellular staining. The staining patterns of specific antibodies (thick line) and negative control (dotted line) are shown. Anti-HA antibodies were used for staining of ES-DC, ESDC-SPARC and ES-DC-TRP2, and anti-hgp100 antibodies were used for staining of ES-DC-hgp100. C, SPARC protein secreted from ES-DC-SPARC or ES-DC (negative control) was measured by ELISA.

8.2. Priming of TAA-specific CTL with genetically modified ES-DC

The capacity of ES-DC-GPC3, ES-DC-SPARC, ES-DC-TRP2 and ES-DC-hgp100 to prime each TAA-specific CTL was analyzed. Other investigators reported that immunization of mice with human gp100 elicited a mouse gp100-specific $CD8^+$ T cell response more efficiently than that with mouse gp100.[48] Therefore, ES-DC expressing human gp100 were generated in the present study. The mice were immunized with ES-DC-GPC3, ES-DC-SPARC, ES-DC-TRP2, ES-DC-hgp100 or non-transfectant ES-DC respectively on days 0 and 7. On day 14, the spleen cells were isolated and cultured with ES-DC-GPC3, ES-DC-SPARC, ES-DC-TRP2 or hgp100 peptide for 5 days. Next, the cells were recovered and their TAA-specific killing activities were analyzed. For the analysis of GPC3- and SPARC-specific CTL, MCA-GPC3 and EL4-SPARC were used as targets. For TRP2- and gp100-specific CTL, EL-4 cells pulsed with previously reported dominant epitopes of TRP2 and gp100 we used. As shown in Fig. 9 (A-D; left), the effector cells primed with ES-DC-GPC3, ES-DC-SPARC, ES-DC-TRP2 or ES-DC-hgp100 exhibited significantly higher killing activities against the target cells that specifically express each TAA. Furthermore, the effector cells activated with each TAA-expressing ES-DC *in vivo* showed significantly higher killing activities against the B16-F10 that naturally express each TAA (Fig. 9A-D; right). On the contrary, spleen cells isolated from mice transferred with non-transfectant ES-DC and co-cultured *in vitro* with each TAA exhibited a basal level killing activity. These results suggest that ES-DC-GPC3, ES-DC-SPARC, ES-DC-TRP2 and ES-DC-hgp100 have the capacity to prime TAA-specific CTL *in vivo*.

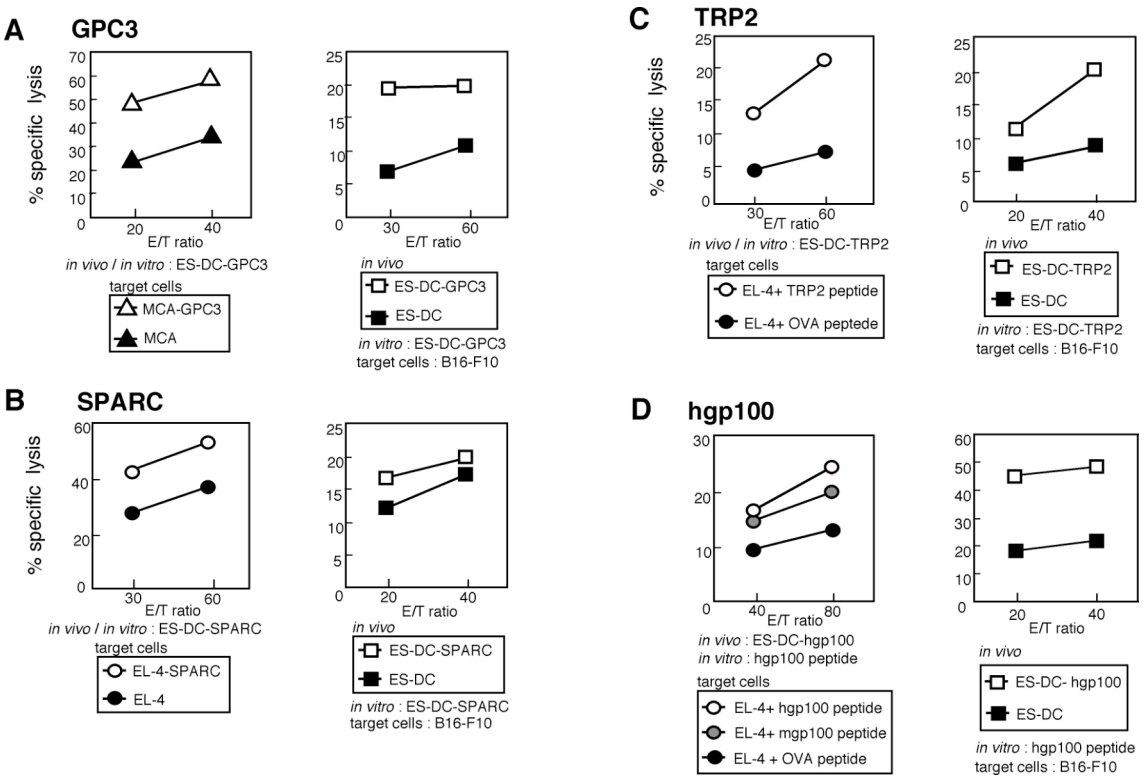

Figure 9. The priming of antigen-specific CTL *in vivo* with ES-DC expressing TAA. The mice were immunized i.p. twice with each 1 x 10^5 ES-DC (as indicated in figure) on days 0 and 7. On day 14, spleen cells were isolated and cultured with 1 x 10^5 ES-DC-GPC3, ES-DC-SPARC, ES-DC-TRP2 or 0.1 μM human gp100 peptide respectively per well in the presence of rhIL-2 (100 units/mL) for 5 days. 4-hr ^{51}Cr release assays were carried out using the obtained resultant cells to evaluate the capacity to kill MCA and MCA-GPC3 (A; left), EL-4 and EL4-SPARC (B; left), EL-4 pulsed with the epitope peptide of TRP2 (C; left) and human or mouse gp100 (D; left), or B16-F10 (A-D; right). The results are expressed as % specific lysis from triplicate assays. The data are each representative of two independent experiments with similar results.

8.3. Protection against subcutaneously inoculated tumor

ES-DC-GPC3 can induce an antigen specific protective effect against 5 x 10^3 subcutaneously inoculated B16-F10 cells naturally expressing GPC3.[26] However, the anti-cancer effect was insufficient when the mice were challenged with a larger number (1 x 10^4 <) of melanoma cells (unpublished observation). The present study examined whether ES-DC-SPARC or ES-DC-TRP2

could protect the recipient mice from a subcutaneous inoculation of 5 x 10^3 B16-F10 cells. Figure 10A shows that immunization with ES-DC-SPARC or ES-DC-TRP2 provided complete protection against 5 x 10^3 B16-F10 inoculations. We observed a certain anti-tumor effect of non-transfectant ES-DC (Fig. 10A). This phenomenon may be due to the fact that even non-transfectant ES-DC produced cytokines with an anti-tumor effect, such as IL-12 and TNF-α. In addition, non-transfectant ES-DC may have presented tumor cell-derived TAA and activated specific CTL, as intrinsic natural DC did. However, the TAA-specific effect elicited by these ES-DC was not significant when the mice were inoculated with 5 x 10^4 B16-F10 cells (unpublished observation). Collectively, ES-DC expressing single TAA could protect the mice from subcutaneous challenge with a relatively small number of tumor cells, however, the effect was insufficient to protect the mice from a challenge with a larger number of tumor cells.

The simultaneous *in vivo* transfer of ES-DC-GPC3, ES-DC-SPARC and ES-DC-TRP2 might protect the recipient mice from a challenge with a relatively large number of B16-F10 cells. Figure 10B demonstrates the transfer of a combination of these TAA-transfectant ES-DC to elicit a significant protection against inoculation with 5 x 10^4 and 1 x 10^5 B16-F10 cells, thus resulting in a significant prolongation of the survival time of the treated mice (Fig. 10C). Therefore, immunotherapy with multiple TAA is more effective than that with a single TAA.

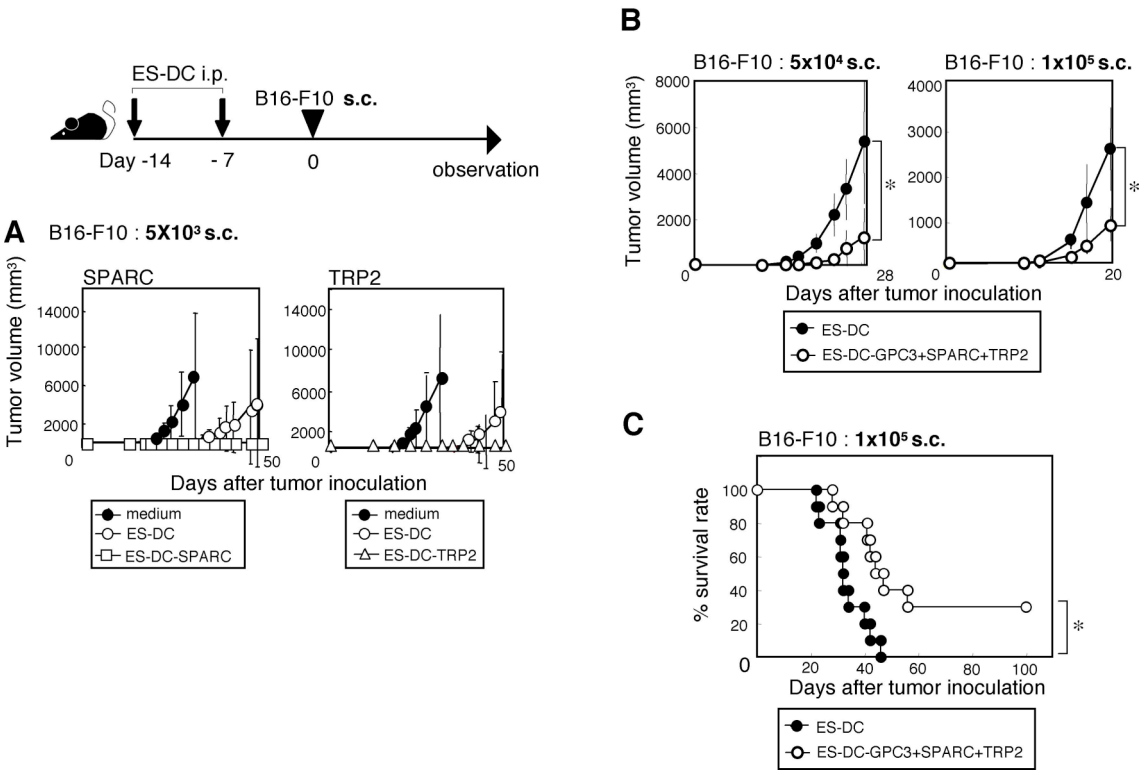

Figure 10. Protection against a subcutaneously inoculated tumor.

A, 1 x 10^5 ES-DC-SPARC or ES-DC-TRP2 were transferred i.p. into mice twice on days -14 and -7, and 5 x 10^3 B16-F10 cells were inoculated subcutaneously into the shaved back region on day 0. B, 3 x 10^5 of non-transfectant ES-DC (control) or a mixture of ES-DC-GPC3, ES-DC-SPARC and ES-DC-TRP2 that consisted of 1 x 10^5 of each ES-DC was transferred i.p., and 5 x 10^4 or 1 x 10^5 B16-F10 cells were inoculated in the same schedule as above. The tumor sizes were determined biweekly in a blinded fashion and the survival rate was monitored (C). The tumor volume was calculated as follows: tumor volume (mm3)= (length x width x height). The data are the mean ± s.d. (A-C, n = 5; D, n=10; *, $P < 0.05$). The data are each representative of two independent experiments with similar results.

8.4. Prevention of peritoneal dissemination of melanoma by preimmunization with multiple TAA-targeted ES-DC

Experiments on preventing the growth of tumors inoculated subcutaneously are often performed and they are convenient models to observe the effects of cancer

immunotherapy, but they do not accurately reflect clinical situations. In a pre-clinical study of cancer immunotherapy, it is important to examine in the metastatic models that are resistant to cancer immunotherapy. Therefore, the effects of ES-DC were evaluated in the peritoneal dissemination model.[29, 47] Previously, the survival rate was the only endpoint in the peritoneal dissemination model. To evaluate tumor metastasis, labeling of cells by introducing reporter gene is a promising technique because the metastatic cells can be quantified. A firefly luciferase gene was introduced into a mouse melanoma B16-BL6 cell line which is a highly metastatic subline of B16, as previously described [49] and established a transfectant clone B16-BL6/Luc. The expression of *SPARC*, *TRP2* and mouse *gp100* mRNA were detected by RT-PCR analysis in B16-BL6 (Fig. 11A) and *gpc3* mRNA was not detected (unpublished observation). The luciferase activities in the homogenates of B16-BL6/Luc cells indicated that the luciferase activity was correlated with the number of B16-BL6/Luc cells at least in the range from 7 to 60,000 counts per second (Fig. 11B). The luciferase activity of the homogenate of each organ was confirmed on day 17 after i.p. inoculation of 1 x 10^4 B16-BL6/Luc cells **(**Fig. 11C). As others have reported, the greater omentum and pancreas were the most common site of metastasis in the early stage of peritoneal dissemination.[50] Because the luciferase activity of the greater omentum and pancreas were significantly higher than other organs such as the spleen, liver, kidney and peritoneum, the luciferase activities of greater omentum and pancreas were measured to evaluate the metastasis of abdominal organs in the subsequent experiments.

The preventive effects of ES-DC were evaluated in the peritoneal tumor dissemination model (Fig. 11D). Immunization with ES-DC-SPARC, ES-DC-TRP2 or ES-DC-hgp100 did not show a TAA-specific, anti-tumor effect to inhibit the dissemination when 1 x 10^4 B16-BL6/Luc cells were inoculated. However, simultaneous *in vivo* transfer of ES-DC-SPARC, ES-DC-TRP2 and ES-DC-hgp100 completely prevented the tumor dissemination. Therefore, the potency of immunotherapy with multiple TAA was confirmed also in the peritoneal dissemination model.

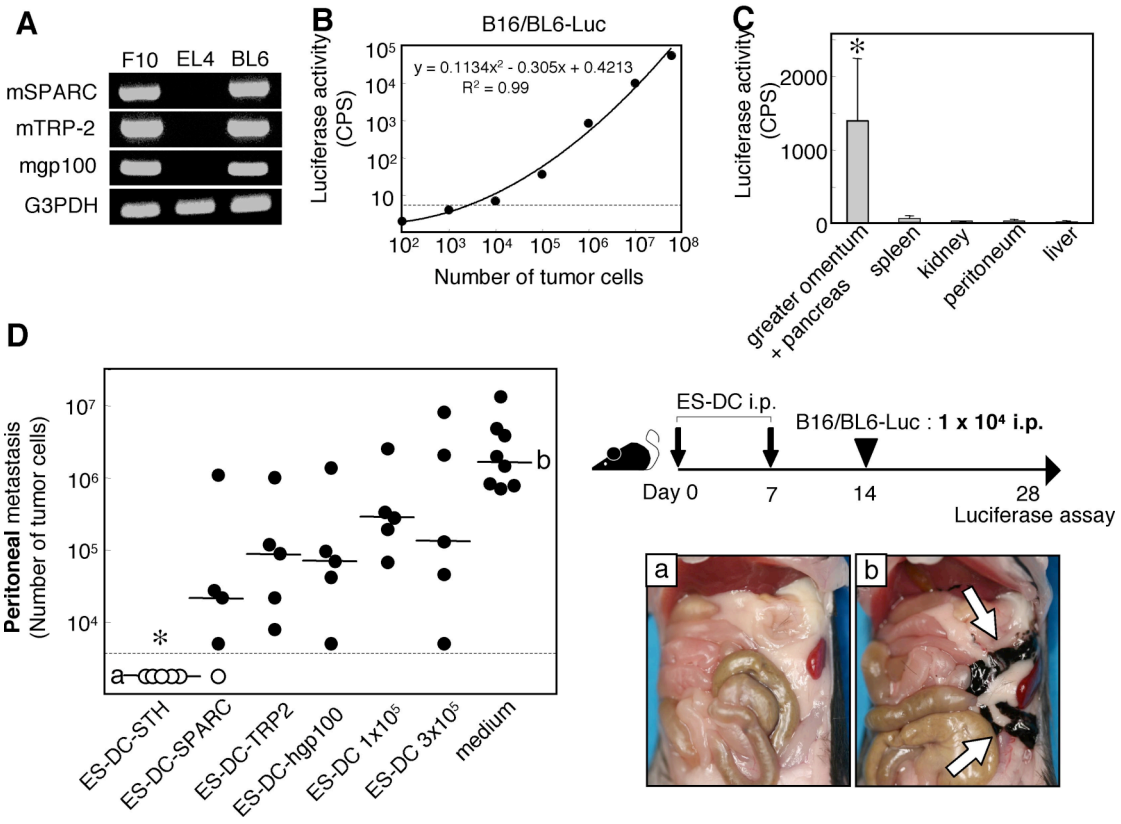

Figure 11. The prevention of peritoneal dissemination of melanoma by multiple TAA-targeted immunization with ES-DC.

A, The expressions of SPARC, TRP2 and mouse gp100 mRNA in B16-BL6 were detected by RT-PCR. B, Luciferase activities in the homogenates of B16-BL6/Luc cells at the indicated numbers are shown. C, Luciferase activity of the homogenate of organs isolated on 17 days after i.p. inoculation of 1 x 10^4 B16-BL6/Luc cells. The data is shown as the counts per second (CPS). D, ES-DC-SPARC (1X10^5 cells), ES-DC-TRP2 (1X10^5 cells), ES-DC-hgp100 (1X10^5 cells), non-transfectant ES-DC (1X10^5 cells), non-transfectant ES-DC (3X10^5 cells) or a equal mixture of ES-DC-SPARC, ES-DC-TRP2 and ES-DC-hgp100 (ES-DC-STH; 3X10^5 cells in total) were inoculated i.p into mice twice on days 0 and 7, and 1X10^4 B16-BL6/luc cells were inoculated i.p into mice on day 14. Mice were euthanised on day 28. The greater omentum and pancreas were excised together and the total luciferase activity was measured. The luciferase activity was converted to the number of tumor cells using a regression line shown in B. Typical examples of peritoneal dissemination of B16-BL6/Luc cells are shown in mice inoculated with (left; a) ES-DC-STH or (right; b) medium. (C, n=5; D, n=5-8; *, $P < 0.05$). Dotted line indicates the detection limit. The data are each representative of two independent experiments with similar results.

8.5. Activation of NKT cells by α-GalCer-loaded ES-DC

Others have reported that α-GalCer loaded on mature DC induced more prolonged interferon-γ production by NKT cells and better protection against B16 melanoma than free α-GalCer.[5] The capacity of ES-DC was first confirmed to activate NKT cells *in vitro*. As shown in Fig. 12A, NKT cells detected by α-GalCer/CD1d dimer represented 1.61% of splenic T cells prior to stimulation with α-GalCer-loaded ES-DC (Day 0). After stimulation, $TCR\beta^+$ $dimer^+$ cells almost disappeared on day 3, probably reflecting activation-induced down-regulation of the surface TCR.[51, 52] This population began to reappear on day 5 and was dramatically increased (31.7%) on day 7, consistent with previous reports that activated NKT cells remain quiescent for a while, but eventually proliferate. The cultured cells recovered on day 5 exhibited strong cytotoxic activities against Yac-1 cells (Fig. 12B). These results indicate that α-GalCer-loaded ES-DC induced significant activation and of NKT cells *in vitro*. Next the duration of activation of NKT cells induced by ES-DC was analyzed *in vivo*. On day 0, ES-DC loaded with either α-GalCer or vehicle were injected i.p. into mice. On days 1, 7, 17 or 27, the mice were euthanized and the cytotoxic activity of whole spleen cells against Yac-1 cells was analyzed. Figure 10C showed the Yac-1 cell-killing activities of spleen cells reflecting activation of NKT cells. The killing activities sustained for one week, and after two weeks the effect decreased to the background level.

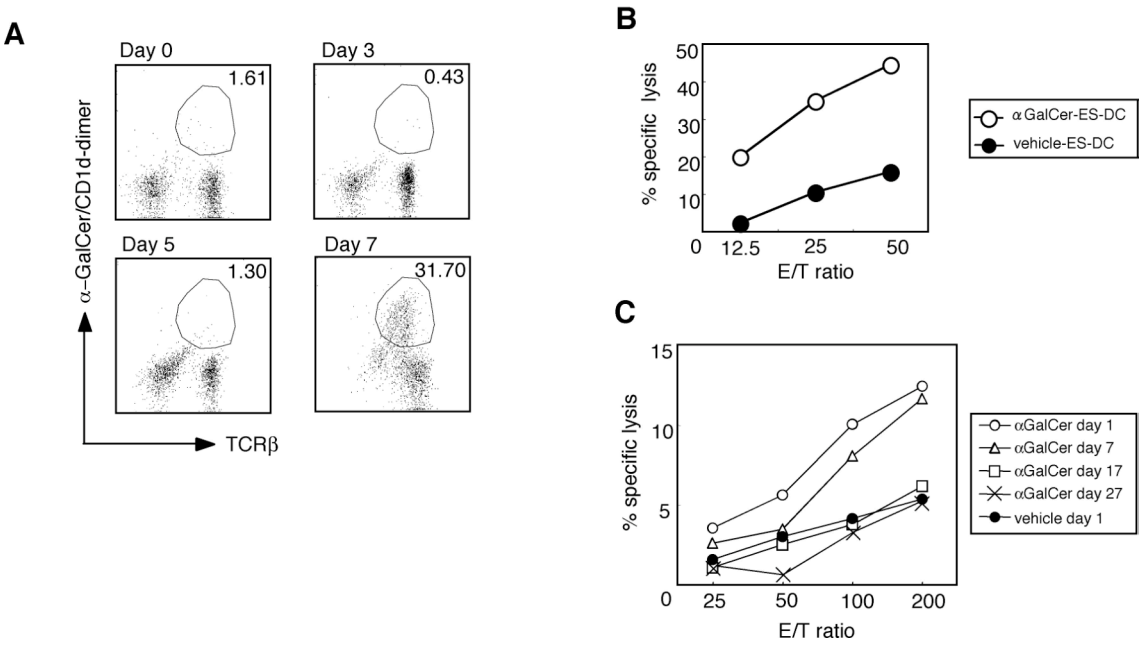

Figure 12. The activation of NKT cells by α-GalCer-loaded ES-DC.

A, ES-DC were cultured in the presence of α-GalCer or vehicle alone for 20 hr, washed twice, and co-cultured with splenic T cells of syngeneic mice (5 x 10^4 DC+ 2.5 x 10^6 T cells / well in 24-well culture plate). On day 0, 3,5 or 7, the cells were analyzed by flow cytometric analysis. The percentage of NKT cells, defined as TCRβ$^+$ and α-GalCer/CD1d dimer reactive cells, is indicated. B, On day 5 the effecter cells recovered from the culture were analyzed for their cytotoxic activities by a 4-hr ^{51}Cr-release assay using Yac-1 cells as a target cells (1 x 10^4 cells/well) at the indicated effecter: target ratio. C, *In vivo* activation of NKT cells by α-GalCer-loaded ES-DC. On day 0, ES-DC loaded with either α-GalCer or vehicle alone were transferred i.p. into mice (1 x 10^6 cells /mouse). On day 1, 7, 17 or 27, the mice were euthanized and the cytotoxic activities of whole spleen cells against Yac-1 cells were analyzed, as described above. The results are expressed as % specific lysis from triplicate assays.

8.6. Potent anti-cancer effects of multiple TAA-targeted ES-DC loaded with α-GalCer

We evaluated the effects of multiple TAA-targeted and α-GalCer-loaded ES-DC in several metastatic models. As shown in Fig. 13A, simultaneous *in vivo* transfer of vehicle-loaded ES-DC-SPARC, ES-DC-TRP2 and ES-DC-hgp100 did not

show a significant inhibition of the dissemination of 5 x 10^4 B16-BL6/Luc cells, when 5 times more cells were injected i.p. in comparison to the experiments shown in Fig. 11D. While α-GalCer-loaded ES-DC without TAA induced a significant but a partial protection, a mixture of three kinds of TAA-transfectant ES-DC loaded with α-GalCer completely protected the mice from tumor dissemination. As shown in the Fig. 13B, α-GalCer-loaded ES-DC expressing TAA induced a significantly more potent protection than either 1μg of free α-GalCer alone (the commonly used dose) or that injected simultaneously with ES-DC expressing TAA. These results clearly indicate the advantage of loading α-GalCer to DC in order to improve the anti-cancer effects. To analyze the effector cell populations induced by α-GalCer-loaded ES-DC with TAA, *in vivo* depletion of $CD4^+$T, $CD8^+$T, or NK cells with specific antibodies was performed as shown in Fig. 13C. The preventive effects of the immunization with α-GalCer-loaded TAA expressing DC (compared to vehicle–loaded TAA expressing DC) were almost totally abrogated when CD4+T cells, CD8+T cells, or NK cells were depleted. Thsese results suggest that all of three effector cell subsets were essential in order to achieve the protective effect.

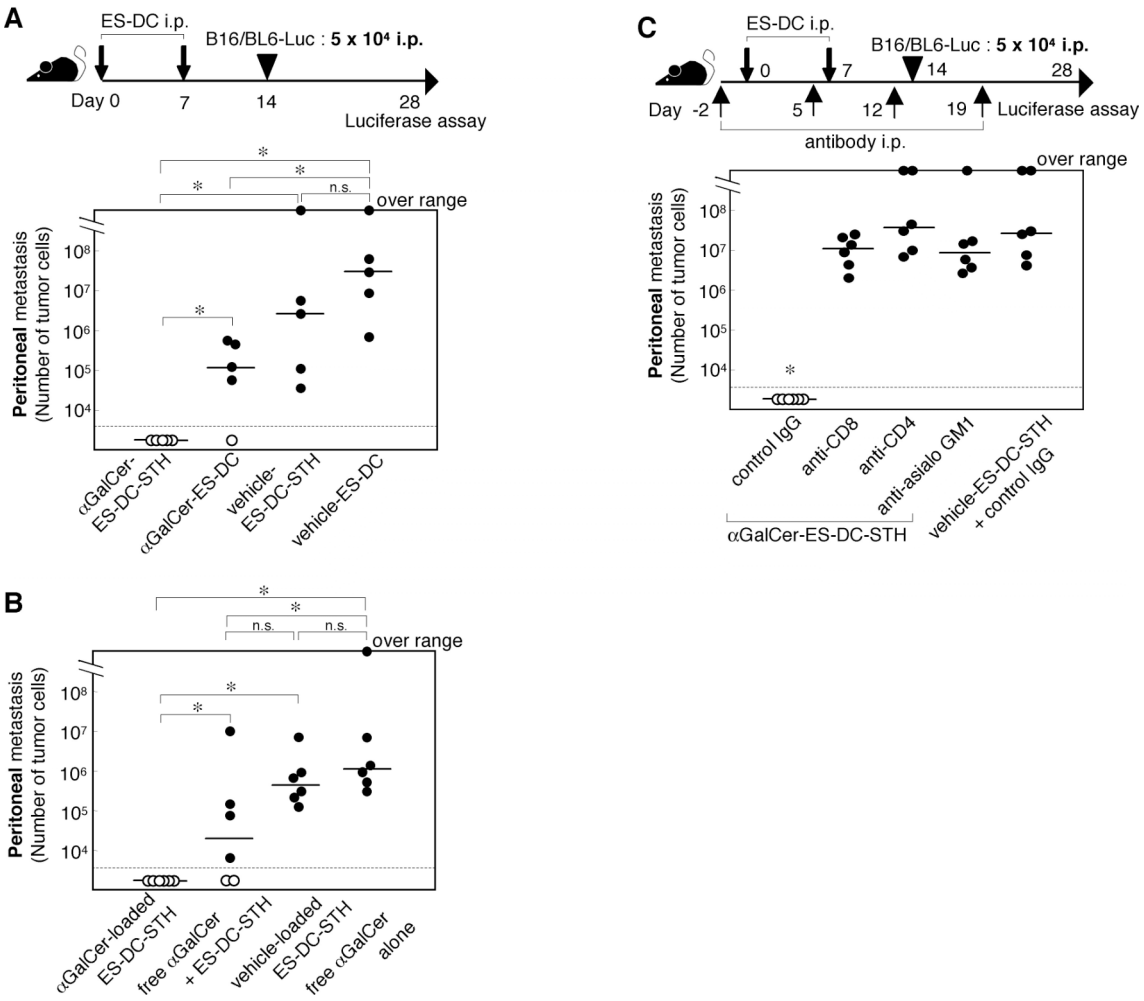

Figure 13. Preventive anti-cancer effects of multiple TAA-targeted vaccinations with α-GalCer loaded ES-DC.

A, The mixture of ES-DC-SPARC, ES-DC-TRP2 and ES-DC-hgp100 (3 x 10^5) loaded with either α-GalCer (αGalCer-ES-DC-STH) or vehicle (vehicle-ES-DC-STH) or non-transfectant ES-DC loaded with either α-GalCer (αGalCer-ES-DC) or vehicle (vehicle-ES-DC) were transferred i.p. into mice twice on days 0 and 7, and 5 x 10^4 B16-BL6/Luc cells were inoculated i.p. into mice on day 14. On day 28, the mice were euthanized and the greater omentum and pancreas were excised together and the total luciferase activities were measured. B, 3 x 10^5 αGalCer-loaded ES-DC-STH, free α-GalCer (1μg/mouse/transfer) combined with ES-DC-STH, vehicle-loaded ES-DC-STH, free α-GalCer alone (1μg/mouse/transfer) were transferred i.p. into mice twice on days 0 and 7. 5 x 10^4 B16-BL6/Luc cells were inoculated and luciferase activities were measured using the same protocol as A. C, $CD4^+$ T, $CD8^+$ T or NK cells were depleted *in vivo* by the i.p. transfer of anti-CD4 mAb, anti-CD8 mAb or polyclonal rabbit anti-asialo GM1 Ab. During this procedure, the mice were immunized with αGalCer- or vehicle-loaded ES-DC-STH and challenged i.p. with B16-BL6/Luc cells in the same protocol as A. Dotted line indicates the detection limit. (A, n=5, B and C, n=6; *, P <0 .05). The data are each representative of two independent experiments with similar results.

Spontaneous pulmonary metastasis was induced by inoculation of B16-BL6/Luc cells into the footpad as previously described.[53] The mixture of α-GalCer-loaded ES-DC-SPARC, ES-DC-TRP2 and ES-DC-hgp100 or α-GalCer-loaded ES-DC without TAA were transferred i.p. into mice twice on days 0 and 7. On day 14, 2 x 10^6 B16-BL6/Luc cells were inoculated into the footpad of mice. On day 35, the mice were euthanized, the lungs were excised and luciferase activities were measured. As shown in Fig. 14, *in vivo* transfer of a mixture of three kinds of TAA-transfectant ES-DC loaded with α-GalCer induced significant protection compared to α-GalCer-loaded ES-DC without TAA also in the spontaneous pulmonary metastasis model. In this model, we have no evidence indicating that the loading of α-GalCer to TAA-expressing ES-DC provided any benefit in regard to inhibiting the local tumor growth in the primary lesion. This result may be due to the tissue distribution of NKT cells. NKT cells are known to mainly localize in the liver, lung, spleen, bone marrow and peritoneal cavity.

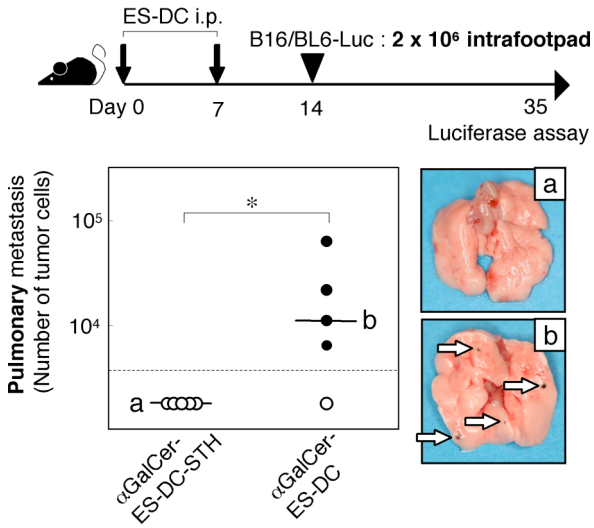

Figure 14. Potent anti-cancer effects of multiple TAA-targeted vaccinations with α-GalCer loaded ES-DC.

3 x 10^5 of αGalCer-ES-DC-STH or αGalCer-ES-DC were transferred i.p. into mice twice on days 0 and 7. On day 14, 2 x 10^6 B16-BL6/Luc cells were inoculated into the footpad of mice. On day 35, the mice were euthanized, the lungs were excised and luciferase activities were measured. Typical examples of pulmonary metastasis of B16-BL6/Luc cells in mice are shown: (above; a) αGalCer-ES-DC-STH (below; b) αGalCer-ES-DC.

Finally the effects of the *in vivo* transfer of a mixture of three kinds of TAA-transfectant ES-DC loaded with α-GalCer in the therapeutic setting on lymph node metastasis and peritoneal dissemination were evaluated. On day 0, 2 x 10^6 B16-BL6/Luc cells were inoculated into the footpad or 1 x 10^4 tumor cells were inoculated i.p. into mice. On days 3 and 10, ES-DC were transferred i.p., and on day 21 or 17 respectively, the mice were euthanized and luciferase activities were measured. Multiple TAA-targeted ES-DC loaded with α-GalCer induced significant therapeutic effects compared to α-GalCer-loaded ES-DC without TAA in the model of spontaneous metastasis to inguinal lymph node (Fig. 15A). As shown in Fig. 15B, while the *in vivo* transfer of α-GalCer-loaded ES-DC without TAA or vehicle-loaded ES-DC with TAA showed insufficient effects, a mixture of three kinds of TAA-transfectant ES-DC loaded with α-GalCer induced a significant therapeutic effect in the peritoneal dissemination model.

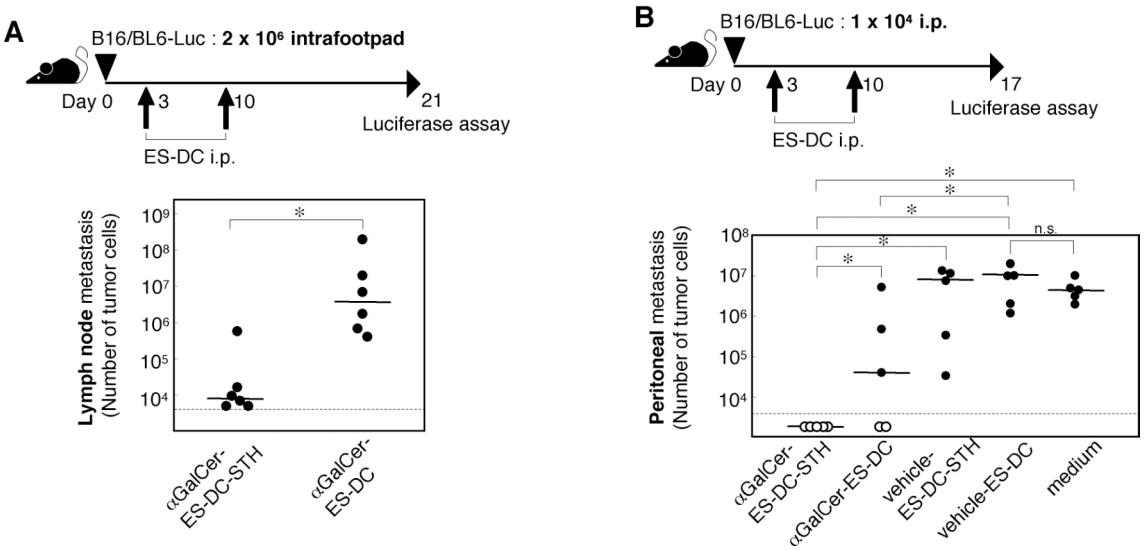

Figure 15. Therapeutic anti-cancer effects of multiple TAA-targeted vaccinations with α-GalCer loaded ES-DC.

A, On day 0, 2 x 10^6 B16-BL6/Luc cells were inoculated into the footpad of mice. On day 3 and 10, each ES-DC was transferred i.p. On day 21, the mice were euthanized and the luciferase activities of the inguinal lymph nodes were measured. B, On day 0, 1 x 10^4 B16-BL6/Luc cells were inoculated i.p. into mice. On day 3 and 10, each ES-DC was transferred i.p. On day 17, the mice were euthanized and the luciferase activities of the greater omentum and pancreas were measured. Dotted line indicates the detection limit. (A and C, n=5, B, n=6; *, P <0 .05). The data are each representative of two independent experiments with similar results.

9. Discussion

The anti-cancer effects of multiple TAA-targeted immunotherapies against mouse melanoma were evaluated by utilizing α-GalCer-loaded and genetically engineered ES-DC. Four TAA that were naturally overexpressed in melanoma were selected, GPC3, SPARC, TRP2 and gp100. SPARC is expressed in various types of cancer tissues [54, 55] and implicated in evasion of cancers from immune attack.[56, 57] The present study is the first to demonstrate that SPARC can be a target antigen for cancer immunotherapy. Figure 9B, 10A and 11D shows that the effect of SPARC as the target antigen was comparable to the previously known melanoma associated antigen, TRP2 or gp100.

CTL specific to each TAA were sensitized by the *in vivo* transfer of DC transfected with *GPC3, SPARC, TRP2* or *hgp100* gene. However, the anti-cancer effects of ES-DC expressing single TAA *in vivo* were insufficient (Fig. 11D*)*. These results are quite similar to the situation of recent T cell-targeted cancer immunotherapy. Using DC expressing multiple TAA for cancer immunotherapy makes sense and several studies have been reported.[19, 58, 59] However, there are very few reports that directly demonstrate the advantage of multiple as compared to single TAA-targeted immunotherapy.[60] As shown in Fig. 10B-C and 11D, preimmunization with the mixture of three independent TAA transfectant ES-DC protected the mice more efficiently than the ES-DC expressing single TAA. The enhancement of anti-tumor immunity by the transfer of a mixture of three kinds of TAA-transfectant DC could be due to an increase of number of CTL attacking tumor cells and a low frequency of immune escape.

In the past decade, α-GalCer has been attracting attention as a novel immunostimulatory reagent for cancer immunotherapy. CD1d is monomorphic and thus CD1d-α-GalCer-complex on DC can stimulate the NKT cells of any recipients. Based on the promising results of preclinical studies demonstrating the anti-cancer effects of α-GalCer-loaded DC,[5] several phase I clinical studies have been conducted. Although the activation and expansion of NKT cells by the administration of α-GalCer-loaded DC has been observed, the results seemed to be unsatisfactory from the viewpoint of the clinical effects.[61-64] The present study evaluated the effects of loading α-GalCer onto

ES-DC expressing endogenous TAA to induce anti-cancer immunity. Upon loading with α-GalCer, ES-DC had a capacity to activate NKT cells (Fig. 13). Figure 13C showed that the killing activity induced by the *in vivo* administration of α-GalCer-loaded ES-DC through the activation of NKT cells was sustained one week, and after two weeks the effect decreased to the background level. Despite this transient NKT cell activating capacity of α-GalCer-loaded ES-DC, anti-cancer effects induced by a mixture of three kinds of TAA-transfectant ES-DC loaded with α-GalCer showed a potent effect on inhibiting the growth of B16-BL6/Luc at two or five weeks after the administration as shown in Fig. 13-15. On the other hand, α-GalCer-loaded ES-DC without TAA showed an insufficient effect under the same conditions. This suggests that α-GalCer-loaded ES-DC have potent, antigen non-specific effect in the early phase after administration, but sustained anti-cancer effect requires activation of TAA specific CTL induced by ES-DC expressing TAA. Interestingly, the others reported that NKT cells activation induced by α-GalCer-loaded mature DC helped to boost adaptive immunity *in vivo*.[63] We performed an IFN-γ ELISPOT assay to investigate whether the loading of α-GalCer to TAA expressing DC would enhance the TAA specific immunoresponse. However, no significant enhancement was observed when the mice were immunized with α-GalCer-loaded TAA expressing DC in comparison to the vehicle-loaded TAA expressing DC (unpublished observation). The preventive effects of the immunization with α-GalCer-loaded TAA expressing DC (compared to vehicle–loaded TAA expressing DC) were almost totally abrogated when the $CD4^+$T cells, $CD8^+$T cells, or NK cells were depleted (Fig. 13C). Collectively, we considered that the enhanced anti-tumor effects induced by α-GalCer-loaded TAA expressing DC came from the cooperative work of CD4+ T cells, CD8+ T cells and NK cells.

Anti-cancer immunotherapy with DC loaded with HLA-binding peptides derived from TAA 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. In addition, the culture to generate DC should be done separately for each patient and 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, has the capacity to propagate infinitely and multiple gene-transfectant ES-DC can be generated by the sequential transfection of ES cells with vectors bearing different selection markers.[29, 43, 44] Generation and genetic modification of ES-DC from human ES cells is achieved by the currently established method.[65] It may therefore be possible to generate multiple gene-transfectant human ES-DC expressing TAA plus immunostimulating molecules, which could thus more potently stimulate anti-cancer immunity than monocyte-derived DC do.

Considering the future clinical application of ES-DC, 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 the patients will be available for most cases. Mouse ES-DC administered into semi-allogenic recipients, sharing one MHC haplotype with the ES-DC, effectively primed antigen-specific CTL, thus suggesting that ES-DC can survive for a sufficient period to stimulate antigen-specific CTL restricted by the shared MHC class I.[66] However, in the same semi-allogenic setting, the five times injection of non-antigen-loaded ES-DC significantly reduced the efficiency of priming of antigen-specific CTL induced by the subsequent injection of antigen-loaded ES-DC (unpublished observation). Therefore, repetitive stimulation with ES-DC expressing allogenic MHC may result in activation and expansion of allogenic MHC class I-reactive CTL, and in such recipients subsequently transferred ES-DC may be rapidly eliminated. Repeated immunization may be required in clinical applications to induce strong anti-cancer immunity. Therefore, the problem of the histoincompatibity between ES cell lines and recipients should be resolved. The methods for targeted the gene-modification of human ES cells and for targeted chromosome elimination of mouse ES cells have been developed. To overcome the problem of histoincompatibility, genetic modification to inhibit expression of endogenous HLA class I in ES-DC may be effective. A disruption of the genes for the molecules necessary for the cell surface expression of HLA class I molecules, such as TAP or β2-microglobulin (β2M), is presumably feasible. Along this line, we recently reported that the efficient activation of antigen-specific CTL were induced by TAP1 or β2M disrupted- and recipient-matched MHC class I introduced-mouse ES-DC.[30] We are now preparing to introduce expression

vector encoding for β2M-linked form of recipient-matched HLA class I heavy chain into TAP1- or β2M-deficient human ES cells.

Previous studies on ES-DC were done using well-established TT2 ES cells.[26, 29, 47] The present study confirmed that ES-DC could be generated from B6 ES cells with the same method previously established using TT2 ES cells.[28] ES-DC generated from B6 ES cells were comparable to TT2 ES cells in differentiation, proliferation, surface phenotype and antigen presentation. In addition, ES-DC could be generated from other ES cell lines (unpublished observation), thus suggesting that the method to generate ES-DC can be applied to various types of mouse ES cells.

On the other hand, other studies have reported the generation of induced pluripotent stem (iPS) cells from adult human dermal fibroblasts with the defined four factors: Oct3/4, Sox2, Klf4, and c-Myc.[67] Human iPS cells are similar to human ES cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity. DC can be generated from mouse iPS cells (unpublished observation) and testing is underway to determine whether DC could be generated from human iPS cells. Tailor-made medicine may therefore someday be possible if 'iPS-DC' can be generated in the future.

10. Conclusions

Anti-cancer effects elicited by immunization with the ES-DC were assessed in preventive and also therapeutic settings in the models of peritoneal dissemination and spontaneous metastasis to lymph node and lung. The *in vivo* transfer of a mixture of three kinds of TAA-expressing ES-DC protected the recipient mice from melanoma cells more effectively than the transfer of ES-DC expressing single TAA, thus demonstraiting the advantage of multiple as compared to single TAA-targeted immunotherapy. Loading ES-DC with α-GalCer further enhanced the anti-cancer effects, suggesting that excellent synergic effects of TAA-specific cytotoxic T lymphocytes and NKT cells against metastatic melanoma can be achieved by using genetically modified ES-DC. With the aid of advancing technologies related to pluripotent stem cells, iPS cells and ES cells, clinical application of DC highly potent in eliciting anti-cancer immunity will be realized in the near future.

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