

ORIGINAL ARTICLE

Therapeutic vaccination based on side population cells transduced by the granulocyte–macrophage colony-stimulating factor gene elicits potent antitumor immunity

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Among cancer immunotherapies, granulocyte–macrophage colony-stimulating factor (GM-CSF) gene-transduced tumor cell vaccine (GVAX) therapies appear promising and have been shown to be safe and effective in multiple clinical trials. However, the antitumor efficacies of GVAX therapy alone are in some cases limited. Here we showed that GVAX therapy targeting cancer stem cells (CSCs) substantially suppressed tumor development in syngeneic immunocompetent mice recapitulating normal immune systems. CSCs were isolated as side population (SP) cells from 4T1 murine breast carcinoma cell line and transduced with GM-CSF gene delivered by non-transmissible Sendai virus (4T1-SP/GM). Impaired tumorigenicity of subcutaneously injected 4T1-SP/GM depended on CD8⁺ T cells in concert with CD4⁺ T cells and natural killer cells. Mice therapeutically vaccinated with irradiated 4T1-SP/GM cells had markedly suppressed tumor development of subcutaneously transplanted 4T1-SP cells compared with those treated with irradiated cells of non-transduced 4T1-SP cells or non-SP (4T1-NSP/GM) cells. Tumor suppression was accompanied by the robust accumulation of mature dendritic cells at vaccination sites and T-helper type 1-skewed systemic cellular immunity. Our results suggested that CSC cell-based GVAX immunotherapy might be clinically useful for inducing potent tumor-specific antitumor immunity.

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INTRODUCTION

Cancer immunotherapy comprises diverse treatment modalities, which incorporate the specificities of both adaptive and innate immunity. Such therapies include the use of cancer cell vaccines, adoptive T-cell transfer of *ex vivo*-activated T cells and natural killer (NK) cells, and use with an adjuvant to costimulate immune cells.¹ Among them, granulocyte–macrophage colony-stimulating factor (GM-CSF)-secreting cancer cell vaccines (GVAXs), which are genetically modified to produce GM-CSF protein and then irradiated (*ir*) to prevent further cell division, have been shown to induce successful antitumor immune responses in preclinical animal models.^{2–5} Using whole cancer cells, GVAX induces immune responses against both unidentified and known tumor-associated antigens. Multiple clinical trial therapy in patients with solid and hematological cancers have demonstrated the feasibility, safety and objective immunological antitumor effects of GVAXs.^{6–9} However, the antitumor efficacies of GVAX therapy alone are limited, probably because of several reasons, including the induction of myeloid-derived suppressor cell-mediated immune evasion by GM-CSF, which results in the reduced activity of cytotoxic T lymphocytes.^{10,11}

Cancer stem cells (CSCs), which constitute a small fraction of malignant tumors, are considered to establish an immunosuppressive tumor microenvironment that enables them to escape from host immune surveillance.¹² We hypothesized that the introduction of a new cancer treatment targeting CSCs might provide a new opportunity to achieve a clinically beneficial

outcome of GM-CSF. This urged us to investigate to what extent and how CSCs in tumors are susceptible to the GM-CSF-driven antitumor immunological responses remain ambiguous.

Most CSC-related studies have used human CSCs, which could only be xenogenetically transplanted into immunosuppressed mice; this is inadequate to assess the true immunogenicity of CSCs. In this study, we used syngeneic immunocompetent mice recapitulating normal immune systems and side population (SP) cells isolated from 4T1 murine breast carcinoma (4T1-SP) cells as putative CSCs. To develop CSC-based GVAX therapy toward more efficacious immunotherapeutic modalities for patients with advanced cancer, we explored whether mouse CSCs are susceptible to *in vivo* GM-CSF-driven antitumor immunity. The present animal models using enriched mouse SP cells and syngeneic immunocompetent mice will allow a deeper understanding of the interplay between CSC-like cells and host immune responses. Our study underscores the significance of using GM-CSF as a potent immunostimulatory adjuvant in SP cell-based immunotherapies that might improve antitumor effects in the clinical setting.

MATERIALS AND METHODS

Mice and cell culture

Female BALB/c mice aged 6–8 weeks (Charles River Laboratories Japan, Yokohama, Japan) were used for the *in vivo* experiments. All animal experiments were carried out under the Guidelines for Animal Experiments

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of Kyushu University and Law 105 Notification 6 of the Japanese Government. The mouse breast cancer cell line 4T1 was obtained from the American Type Culture Collection and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA) and 1% antibiotic-antimycotic mixed stock solution (Nacalai Tesque, Kyoto, Japan).

Fluorescence-activated cell sorting and SP analysis

Cell suspensions were labeled with Hoechst 33342 dye (Sigma) using the methods described by Goodell *et al.*¹³ with some modifications. Briefly, the cells were resuspended at 1×10^6 cells per ml in prewarmed RPMI-1640 supplemented with 2% fetal bovine serum and 10 mmol l^{-1} HEPES buffer. Hoechst 33342 dye was added to the cell suspensions at a final concentration of $5 \mu\text{g ml}^{-1}$ in the presence or absence of verapamil hydrochloride ($150 \mu\text{M}$; Sigma) and incubated at 37°C for 1 h with intermittent shaking. To detect viable cells, propidium iodide (Nacalai Tesque) was added to the cells at a final concentration of $1 \mu\text{g ml}^{-1}$. Analyses and sorting were performed on either an Epics ALTRA (Beckman Coulter, Fullerton, CA, USA) or FACSARIA II (Becton Dickinson, San Diego, CA, USA) flow cytometer. Flow cytometric data were analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

Sendai virus-mediated gene transduction

For *in vitro* assays, 4T1-SP and 4T1-non-SP (NSP) cells were plated and treated with $100 \mu\text{l}$ serum-free solutions of non-transmissible Sendai viruses (SeV/dF/green fluorescent protein (GFP) or SeV/dF/GM) at predefined multiplicities of infection (MOIs). For *in vivo* assays, shortly after cell sorting, the cells were infected with $100 \mu\text{l}$ virus solution in suspension. After incubation for 1.5 h at 37°C with intermittent shaking, the cells were washed two times with phosphate-buffered saline.

In vivo depletion assay

CD4^+ or CD8^+ T cells were depleted in BALB/c mice by intraperitoneally injecting each mouse with $50 \mu\text{g}$ anti-CD4 or anti-CD8 antibodies, or rat IgG 5, 4 and 3 days before and 3 days after tumor cell injection. Fluorescence-activated cell sorting analysis demonstrated that the respective treatments resulted in $\sim 95\%$ depletion of CD4^+ or CD8^+ T-cell populations in lymph nodes (data not shown). For NK cell depletion, mice were intraperitoneally injected with rabbit anti-asialo GM1 anti-serum (freeze-dried anti-serum diluted at 1:20 in phosphate-buffered saline; Wako, Osaka, Japan) 1 and 7 days before and every 7 days after tumor cell injection. 4T1-SP/GM cells were then subcutaneously injected into the right flank of mice. The tumor volume was calculated by the following formula: tumor (mm^3) = (length) \times (width)² $\times 0.4$. Animals were killed when the tumor diameter exceeded 1.5 cm.

In vivo therapeutic studies

On the day of tumor challenge, 1×10^4 sorted 4T1-SP cells were subcutaneously injected into the lower right flank of mice. On days 2 and 9 after tumor challenge, 2×10^4 tumor cells resuspended in $100 \mu\text{l}$ phosphate-buffered saline were subcutaneously injected into the upper right flank of mice. The tumor volume was measured once every other day.

Fluorescence-activated cell sorting analysis of the tumor vaccination site

Eighteen hours after the first vaccination, mice subcutaneously injected with ir.4T1-SP/GFP or ir.4T1-SP/GM cells were killed, and samples from the tumor vaccination sites (TVSs) were collected from each mouse. Mechanical homogenization was applied to prepare a single-cell suspension of the harvested TVS. To analyze the maturation level of dendritic cells (DCs) at the TVS, after blocking with anti-CD16/32 antibody, the cell suspensions were stained with anti-mouse CD80-phycoerythrin, CD11c-peridinin chlorophyll protein/cyanine 5.5 (PerCP/cy5.5) and CD86-phycoerythrin/cyanine 7 (PE/cy7) antibodies. Analysis was carried out using FACSVerse.

Enzyme-linked immunospot assay

Antigen-specific responses were evaluated by a mouse immunospot kit (IFN- γ ELISPOT Kit; BD Pharmingen, Franklin Lakes, NJ, USA). Red blood cell-depleted splenocytes (1×10^5 cells) obtained from the spleens of mice

vaccinated with ir.4T1-SP/GFP or ir.4T1-SP/GM cells were collected and incubated for 48 h at 37°C with 5% CO_2 in the presence or absence of ir.4T1-SP, ir.4T1-NSP or ir.CT26 cells at splenocyte:irradiated tumor cell ratios of 20:1 and 5:1 in a total volume of $200 \mu\text{l}$. Phorbol 12-myristate 13-acetate (50 ng ml^{-1} ; Sigma) and ionomycin ($1 \mu\text{g ml}^{-1}$) were added to cells as a positive control. ELISPOT plates were developed according to the manufacturer's instructions. Color spots were counted using an automated ELISPOT analyzer (Cellular Technology, Cleveland, OH, USA).

Microarray analysis

cRNA was amplified, labeled using an Agilent Low-Input QuickAmp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA) and then hybridized to a SurePrint G3 Mouse GE $8 \times 60 \text{ K}$ Microarray (Agilent Technologies) according to the manufacturer's instructions. The hybridized microarray slides were scanned by an Agilent scanner, and the signals of all probes were calculated using the Feature Extraction Software (Agilent Technologies). Our data have been uploaded to the Gene Expression Omnibus database (accession number GSE53444). Transcripts with a fold change of > 1.5 (upregulated in 4T1-SP cells) and a Z-score of > 2 were considered to be significantly differentially expressed in each cell population. Microarray data analysis was supported by Cell Innovator (Fukuoka, Japan).

Real-time PCR analysis

RNA samples were isolated by RNeasy Kit (Qiagen, Hilden, Germany) and converted to cDNA by using RiverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) under the manufacturer's guidance. Quantitative PCR was performed with Power SYBR PCR Master Mix (Thermo Fisher) in StepOne Plus System (Thermo Fisher). We especially focused on several important oncogenesis-related genes. PCR primers used were as follows: GAPDH F, 5'-AGGTCGGTGTGAACGGATTG-3' and GAPDH R, 5'-GGGGTCGTGATGGCAACA-3'; Ccbp2 F, 5'-CCGGTCGTCTACAGCCTGA-3' and Ccbp2 R, 5'-CATCGTCCGCTTCGAGGT-3'; Ror1 F, 5'-TCCAAGAACCCTGGAAATCT-3' and Ror1 R, 5'-GAAGTAACCAGTGTCTGTGGTG-3'; Abcg2 F, 5'-GAACTCCAGAGCCGTAGGAC-3' and Abcg2 R, 5'-CAGAATAGCATTAAAGCCAGTT-3'.

Statistical analysis

Statistical analyses were performed with the GraphPad Prism 5.0d software package (GraphPad Software, La Jolla, CA, USA). Statistical analysis among groups was performed using the two-tailed unpaired Student's *t*-test, one-way analysis of variance, followed by Tukey's multiple comparison test, or nonparametric Mann-Whitney *U*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Mouse breast cancer 4T1-SP cell sub-population displays diverse CSC-related phenotypes

Because SP cells exhibit properties of both normal stem cells and CSCs,^{14,15} we used the SP fraction as putative CSCs and the NSP fraction as non-CSCs, and isolated these distinct cell sub-populations from 4T1 cells. A characteristic SP fraction prestained with Hoechst 33342 dye was identified, which was defined by low fluorescence staining with both Hoechst 33342 blue and red filters. The SP fraction was abolished by adding verapamil, an inhibitor of ATP-binding cassette transporters including ABCG2. The SP fraction of 4T1 cells, 1–2% sub-population of the entire cell population (Figure 1a), was successfully isolated with $> 90\%$ purity and $> 95\%$ viability. 4T1-SP cells and the NSP fraction (4T1-NSP) were cultured for 2 days and then cell viability was evaluated. The number of propidium iodide-positive dead cells was not significantly different in the two distinctive cellular fractions (Figure 1b). To examine whether 4T1-SP cells possess the properties of CSCs, we firstly compared the clonogenic capacities of 4T1-SP and 4T1-NSP cells. 4T1-SP cells formed four times more colonies than 4T1-NSP cells did, indicating the higher clonogenic capacity of 4T1-SP cells (Figures 1c and d). The stem cell maker expression of stem cell antigen-1 (Sca-1) has been shown to enhance tumorigenicity, implicating its role as CSCs marker;¹⁶ we comparatively analyzed Sca-1 expression between 4T1-SP and

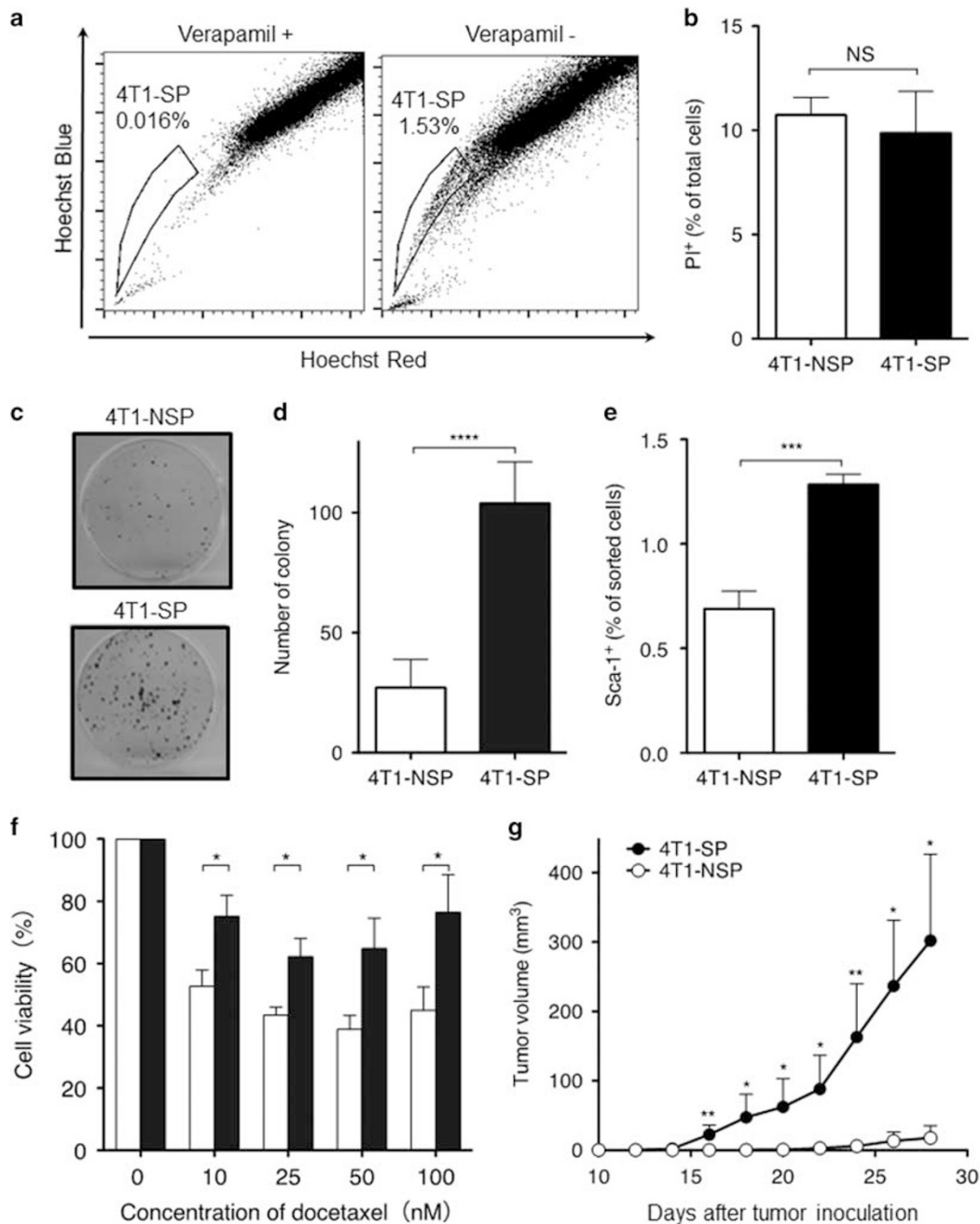


Figure 1. Identification and characterization of SP cells using the Hoechst dye efflux assay. **(a)** 4T1 cells were stained with Hoechst 33342 dye in the presence (left) or absence (right) of verapamil. The SP fraction, which disappeared in the presence of verapamil, was gated and is shown as a percentage of the total viable cell population. **(b)** The frequency of dead cells was quantified by flow cytometry after culturing 4T1-SP and 4T1-NSP cells. Error bars represent the s.d. of the combined data from two independent experiments with similar results. **(c and d)** Comparison of the colony-forming abilities of 4T1-SP and 4T1-NSP cells. The colonies were fixed and stained with crystal violet and then photographed **(c)**. The number of colonies derived from 4T1-SP and 4T1-NSP cells was counted in triplicate **(d)**. Error bars represent the s.d. of two independent experiments (**** $P < 0.0001$). **(e)** Comparison of the expression levels of Sca-1 in 4T1-SP and 4T1-NSP cells by flow cytometry. Error bars represent the s.d. of the combined data from two independent experiments with similar results (*** $P < 0.001$). **(f)** 4T1-SP and 4T1-NSP cells were treated with 10–100 nM DTX. MTS assays were then performed to measure cell viability in response to DTX treatment (* $P < 0.05$). **(g)** Tumorigenic potential of 4T1-SP cells. Subcutaneous tumor growth of 4T1-SP and 4T1-NSP cells (5×10^3 cells per mouse) in the right flank of mice was measured. The mean values and s.e.m. of tumor growth are depicted (* $P < 0.05$ and ** $P < 0.01$). Representative data from two independent experiments with similar results are shown. DTX, docetaxel; MTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NSP, non-SP; SP, side population.

4T1-NSP cells. The Sca-1 expression level in 4T1-SP cells was significantly higher compared with that in 4T1-NSP cells (Figure 1e). Because CSCs exhibit higher chemoresistance,^{17,18} we evaluated the chemoresistances of 4T1-SP and 4T1-NSP cells by anticancer agent docetaxel (DTX) exposure. 4T1-SP cells

displayed significantly higher dose-dependent chemoresistance than 4T1-NSP cells did against DTX (Figure 1f). To evaluate tumorigenicity of 4T1-SP and 4T1-NSP cells, these cells ranging from 5×10^3 to 2×10^4 cells were subcutaneously injected into mice. As few as 5×10^3 of injected 4T1-SP cells developed

Table 1. Tumor formation by subcutaneous administration of 4T1-SP and 4T1-NSP cells in syngeneic BALB/c mice

	Cells transplanted		
	5×10^3 ^a	1×10^4	2×10^4
4T1-NSP	1/6 (16.7%)	4/6 (66.7%)	4/6 (66.6%)
4T1-SP	6/6 (100%)	5/6 (83.3%)	6/6 (100%)

Abbreviations: NSP, non-SP; SP, side population. Assessed on day 30 after the subcutaneous administration of 4T1-SP or 4T1-NSP cells. ^a χ^2 test ($P < 0.05$).

significantly larger tumors than those of 4T1-NSP cells ($P < 0.05$) (Figure 1g). All mice injected with 5×10^3 4T1-SP cells formed tumors, whereas only 16.7% of mice subcutaneously injected with 5×10^3 4T1-NSP cells formed tumors (Table 1). Collectively, these data demonstrated that 4T1-SP cells possessed several characteristic properties of CSCs both *in vitro* and *in vivo*, as reported earlier.¹⁵

Gene transduction mediated by SeV/dF infection does not affect 4T1 cell properties

We used SeV/dF as the gene transduction vector because the SeV receptors, sialic acid residues on cell surface glycoproteins (asialoglycoproteins), are expressed ubiquitously on most mammary cells and are safe and highly effective in gene transduction in panels of mouse and human cancer cells.¹⁹ To evaluate the gene transduction efficiency, 4T1-SP cells were infected with SeV/dF/GFP *in vitro* (4T1-SP/GFP cells). Substantial numbers of GFP⁺ 4T1-SP cells were detected at MOIs of 1–200, with peak expression at an MOI of 50 (Supplementary Figures S1a and b). Sufficient and comparable amounts of mouse GM-CSF and GFP proteins were produced from 4T1-SP and 4T1-NSP cells 72 h after infection with SeV/dF/GM (4T1-SP/GM and 4T1-NSP/GM, respectively) in a virus dose-dependent manner (Figure 2a and Supplementary Figures S1c and d). To exclude the possibility that the exogenous expression of GFP or GM-CSF gene by SeV affected 4T1-SP proliferation, growth curves of 4T1-SP, 4T1-SP/GFP or 4T1-SP/GM cells were evaluated. SeV/dF/GFP and SeV/dF/GM infection, even at the highest MOI of 100, showed negligible effects on the proliferation of 4T1-SP cells (Figure 2b).

We then confirmed that immunosuppressive molecules expressed on 4T1 cells was not influenced by gene transduction by SeV/dF/GM. Signal transducer and activator of transcription 3 (STAT3) activation has been shown to result in the resistance of tumor cells to chemotherapeutic drugs and the expression level of vascular endothelial growth factor (VEGF) resulting in tumor angiogenesis.^{20–22} We measured the level of phosphorylated (p)-STAT3 in whole-tumor lysates derived from 4T1-SP and 4T1-NSP cells, as well as the production levels of VEGF in culture supernatants. Although 4T1-SP cells expressed higher levels of p-STAT3 and VEGF than 4T1-NSP cells did ($P < 0.01$), no significant difference was found in p-STAT3 and VEGF levels between SeV/dF/GM-transduced group and -non-transduced group (Figures 2c and d). We then evaluated the expression level of the coinhibitory molecule PD-L1, which is well known to relate to aggressive characteristics of human malignancies with poor clinical prognosis.²³ There was no significant difference in PD-L1 expression between SeV/dF/GM-transduced group and -non-transduced group, although isolated 4T1-SP cells expressed a significantly higher level of PD-L1 than 4T1-NSP cells ($P < 0.001$) (Figure 2e).

GM-CSF gene-transduced 4T1-SP cell-based therapeutic vaccination significantly suppresses the tumor growth of subcutaneously transplanted 4T1-SP cells

We next investigated the tumorigenicity of 4T1-SP/GM cells after the subcutaneous injection into immunocompetent mice. Remarkably, mice inoculated with 4T1-SP/GM cells voided the tumor development compared with those inoculated with 4T1-SP and 4T1-SP/GFP cells (Figure 3a), illustrating that genetic modification with GM-CSF on 4T1-SP cells conferred a significant impairment of their tumorigenicity. Furthermore, mice injected with 4T1-SP/GM cells survived longer than control mice did (Figure 3b). These data encouraged us to examine which immune cell sub-populations were directly involved in the GM-CSF-triggered loss of tumorigenicity, thus we depleted CD4⁺ T, CD8⁺ T or NK cells from mice. The tumor rejection observed in mice injected with 4T1-SP/GM cells was markedly abrogated in mice lacking CD8⁺ T, CD4⁺ T and NK cells (Figure 3c), indicating that both innate and adaptive immune cells contributed to the induction of SP cell-specific antitumor immunity.

We next hypothesized that therapeutic ir.4T1-SP/GM cell vaccination might induce superior antitumor immunity against pre-established 4T1-SP cell-derived tumors. We confirmed that ~10 ng of GM-CSF was produced by 1×10^6 4T1-SP/GM cells 8 h after SeV/dF/GM infection (Supplementary Figure S2), indicating that ir.4T1-SP/GM cells produced a sufficient amount of GM-CSF at the TVS. At days 2 and 9 after tumor challenge, ir.4T1-SP, ir.4T1-SP/GFP or ir.4T1-SP/GM vaccine cells were subcutaneously injected into mice. At 18 days after the tumor challenge, growth of 4T1-SP cell-derived tumors was significantly more suppressed in mice treated with ir.4T1-SP/GM cells than with ir.4T1-SP or ir.4T1-SP/GFP cells ($P < 0.05$) (Figure 3d). We also confirmed that mice treated with ir.4T1-SP/GM cells showed prolonged survival compared with control mice without any severe adverse events (data not shown). In the same therapeutic vaccination, mice treated with ir.4T1-SP/GM cells showed a remarkably decreased number of metastatic lung nodules compared with control mice (Supplementary Figures S3a and b). We also demonstrated that ir.4T1-SP/GM cells were more effective than ir.4T1-NSP/GM cells to suppress tumor growth (Figure 3e).

To investigate the possible application of this method to primary tumors, we prepared putative primary 4T1-SP cells by freshly isolating cells from established tumors in 4T1-SP cell-transplanted immunocompetent mice (Supplementary Figure S4a). We genetically modified primary 4T1-SP cells by SeV/dF/GFP or SeV/dF/GM infection and irradiated them as described above (designated as ir.p4T1-SP/GFP and ir.p4T1-SP/GM cells). Mice injected with ir.p4T1-SP/GM cells markedly suppressed the tumor formation by challenge with 4T1-SP cells compared with control mice (Supplementary Figure S4b).

Mice treated with ir.4T1-SP/GM cells exhibit abundant mature DCs at the TVS and systemic antigen-specific T-helper type 1 responses DCs harvested from mice vaccinated with ir.4T1-SP/GM cells expressed significantly higher levels of the costimulatory molecules CD80 and CD86 compared with control mice ($P < 0.01$) (Figures 4a and b). Furthermore, to analyze whether antigen-specific T-helper type 1 cellular immune responses were induced in the therapeutic vaccination model, we quantified the number of IFN- γ -secreting splenocytes from mice vaccinated with ir.4T1-SP/GFP or ir.4T1-SP/GM cells after restimulation with ir.4T1-SP, ir.4T1-NSP or ir.CT26 cells as an irrelevant control. When cocultured in the presence of ir.4T1-SP and ir.4T1-NSP cells, but not ir.CT26 cells, the number of IFN- γ -producing splenocytes from mice vaccinated with ir.4T1-SP/GM cells was significantly higher compared with that of the controls ($P < 0.01$) (Figure 4e and Supplementary Figure S5).

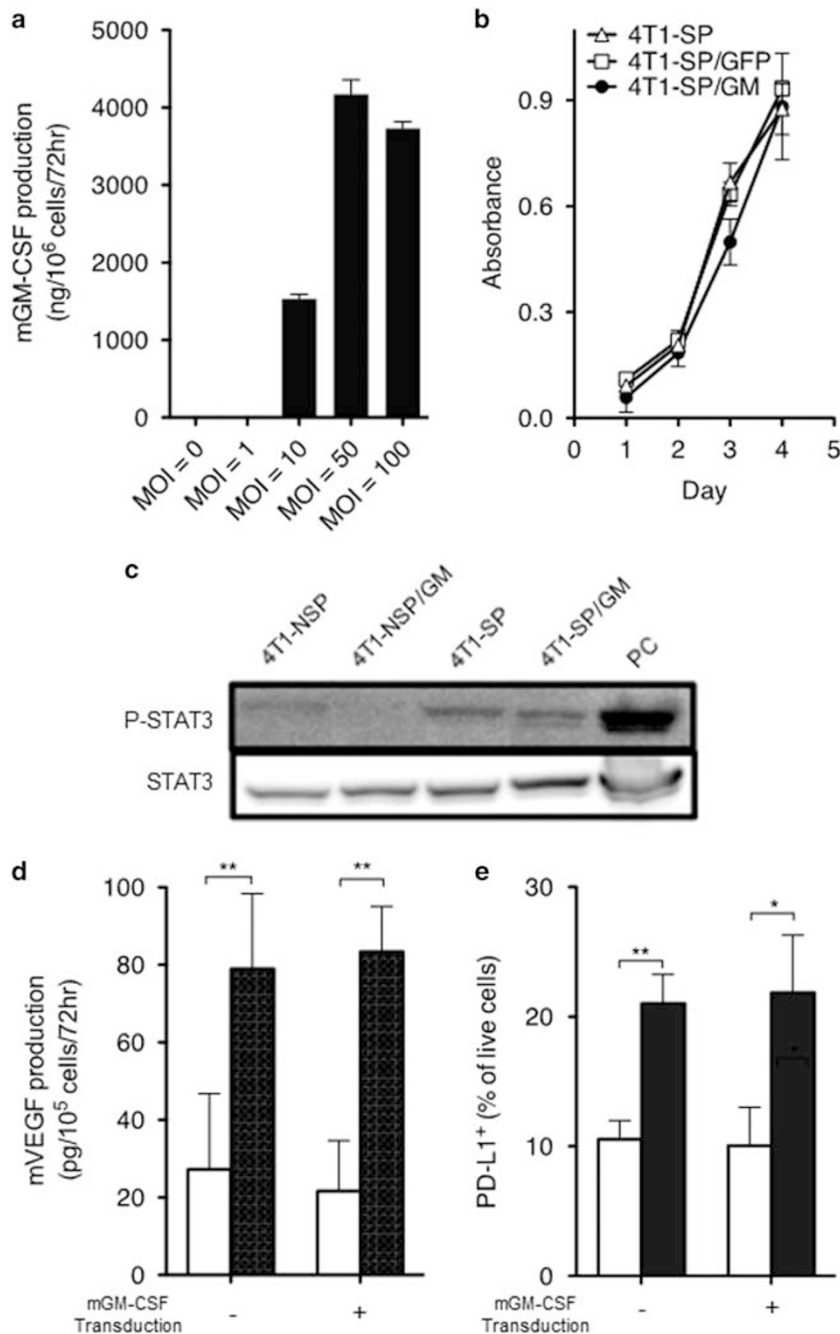


Figure 2. Transduction efficacy of the mGM-CSF gene and functional characterization of 4T1-SP cells as CSCs. **(a)** Cell culture supernatants from 4T1-SP cells infected with SeV/dF/GM at several MOIs were collected to measure the mGM-CSF concentration by ELISA. **(b)** 4T1-SP, 4T1-SP/GFP and 4T1-SP/GM cells were plated in 96-well plates at 1×10^3 cells per well. At each time point (days 1, 2, 3 and 4), MTS assays were performed to analyze the effect of SeV/dF/GFP or SeV/dF/GM infection on cell proliferation rates. Error bars represent the s.d. of triplicate experiments. **(c–e)** Expression levels of p-STAT3, VEGF and PD-L1 were quantified among 4T1-SP, 4T1-SP/GM, 4T1-NSP and 4T1-NSP/GM cells (MOI = 100). Each cell lysate obtained was subjected to western blotting assay for p-STAT3 on Tyr705 detection. The cell lysate from MDA-MB-468, a human breast cancer cell line, was used as a positive control (PC). After 72 h of culture, culture supernatants were harvested, and the expression levels of VEGF were measured **(d)**. Error bars represent the s.d. of triplicate experiments (** $P < 0.01$). 4T1-SP (black bar) and 4T1-NSP (white bar) cells were infected with SeV/dF/GM and cultured for 48 h. The expression level of PD-L1 was then quantified by flow cytometry **(e)**. Error bars represent the s.d. of triplicate experiments (* $P < 0.05$ and ** $P < 0.01$). CSC, cancer stem cell; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; mGM-CSF, mouse granulocyte–macrophage colony-stimulating factor; MOI, multiplicity of infection; MTS, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NSP, non-SP; p-STAT, phosphorylated-signal transducer and activator of transcription 3; SP, side population; VEGF, vascular endothelial growth factor.

Genes related to metastasis, chemoresistance and invasion are more highly upregulated in 4T1-SP cells than in 4T1-NSP cells. Higher antitumor effect of 4T1-SP cells than 4T1-NSP cells promoted us to perform cDNA microarray analysis. Six hundred

ninety-five genes were upregulated in 4T1-SP cells. Among them, we focused on the genes that had been previously reported as cancer-related genes, and their expression was restricted to either the plasma membrane or cytoplasm (Table 2). These genes

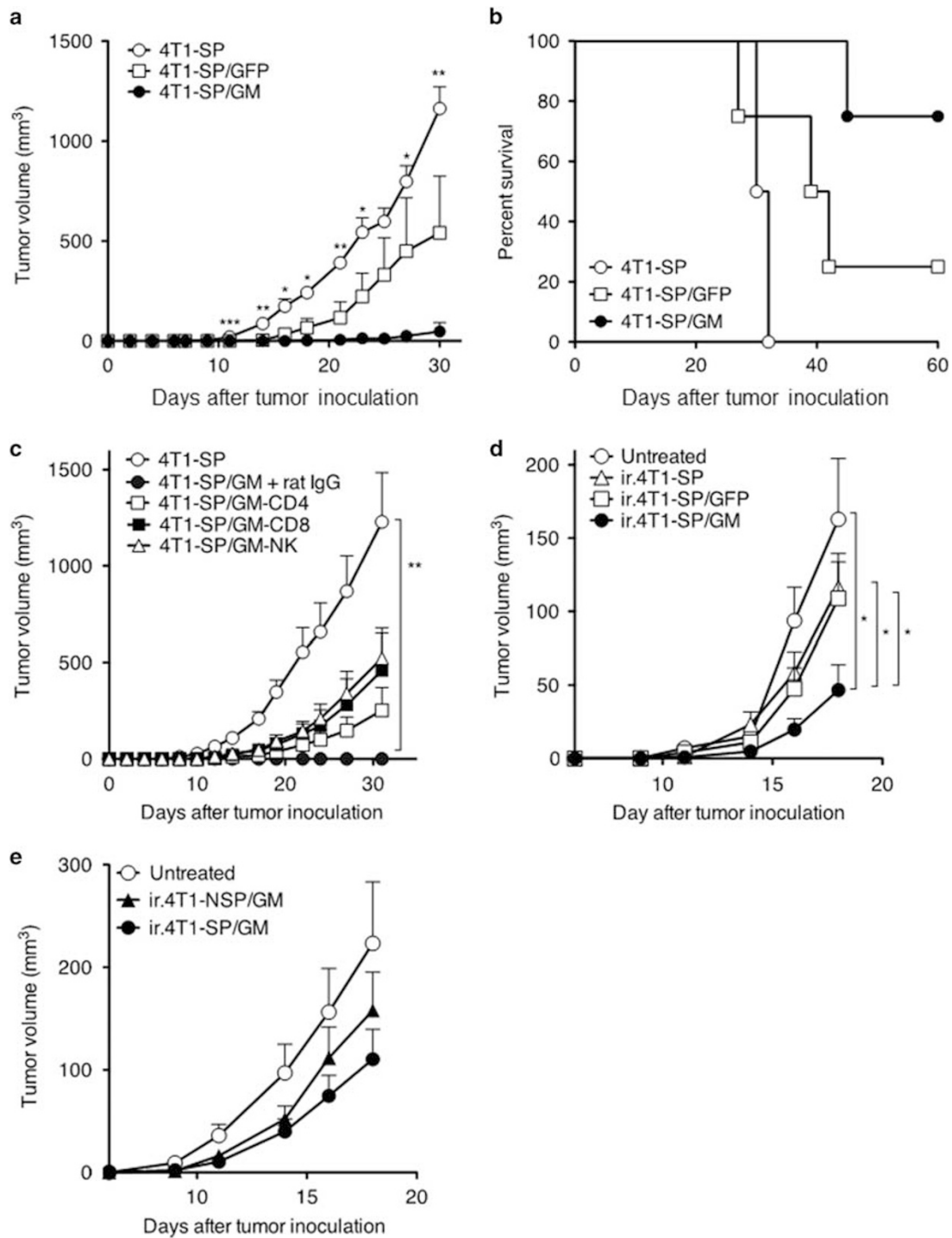


Figure 3. Impaired tumorigenicity of subcutaneously injected 4T1-SP/GM cells and therapeutic antitumor effects of ir.4T1-SP/GM and ir.4T1-SP/GM cell-based vaccines. **(a and b)** A total of 1×10^4 4T1-SP, 4T1-SP/GFP or 4T1-SP/GM cells were subcutaneously inoculated into BALB/c mice. **(a)** Tumor growth curves are shown as the means \pm s.e.m. ($*P < 0.05$, $**P < 0.01$ and $***P < 0.001$). **(b)** Kaplan–Meier survival curves are shown. Representative data from five independent experiments with similar results are shown. **(c)** BALB/c mice received intraperitoneal injections of indicated neutralization antibodies. Error bars represent the s.e.m. of the combined data from two independent experiments ($**P < 0.01$). **(d and e)** Therapeutic tumor vaccination in mice subcutaneously transplanted with 4T1-SP cells. Each group received the indicated cellular vaccinations on days 2 and 9 after the tumor challenge. Tumor volumes of pre-established 4T1-SP cells are shown as the means \pm s.e.m. ($*P < 0.05$) **(d)**. Comparison of the therapeutic effects of ir.4T1-SP/GM and ir.4T1-NSP/GM cells **(e)**. Error bars represent the s.e.m. of the combined data from three independent experiments with similar results. GFP, green fluorescent protein; GM, granulocyte–macrophage; NSP, non-SP; SP, side population.

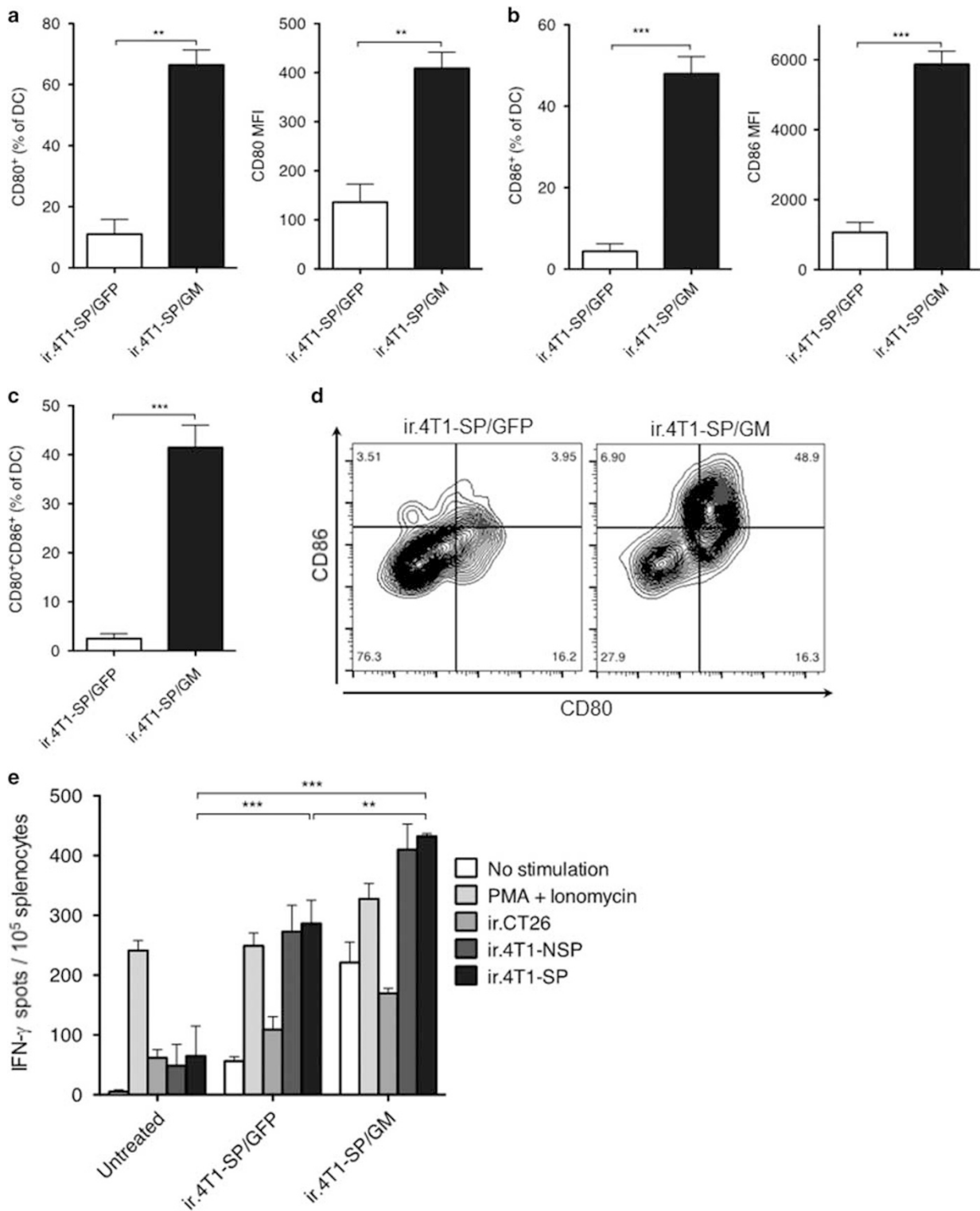


Figure 4. *In vivo* immunological assays during 4T1-SP cell-based therapeutic vaccinations. **(a and b)** Assessment of DC maturation at the TVS of mice treated with ir.4T1-SP/GFP or ir.4T1-SP/GM cells. Cell suspensions prepared from the TVS of indicated mouse groups were subjected to FACS analysis. Values represent the mean \pm s.e.m. of the frequencies (left panel) and mean fluorescence intensities (right panel) of CD80⁺ **(a)** and CD86⁺ **(b)** cells relative to the total CD11c⁺ cell population at the TVS. **(c)** Bar graphs representing the means \pm s.e.m. of the frequencies and CD80⁺CD86⁺ cells relative to the total CD11c⁺ cells. **(d)** Representative contour plots depicting CD80 and CD86 expression gated on CD11c⁺ cells. Representative data from two independent experiments with similar results are shown (** $P < 0.01$ and *** $P < 0.001$). **(e)** ELISPOT assay to quantify the number of IFN- γ -secreting splenocytes. At 10 days after the second 4T1-SP cell-based vaccination, splenocytes harvested from 4T1-SP tumor-bearing untreated mice or mice treated with the ir.4T1-SP/GFP or ir.4T1-SP/GM cells were cocultured with the indicated stimulator cells. Results are expressed as the mean number of spots \pm s.e.m. of triplicate determinations per 10⁵ splenocytes (** $P < 0.01$ and *** $P < 0.001$). DC, dendritic cell; FACS, fluorescence-activated cell sorting; GM, granulocyte-macrophage; IFN, interferon; ir, irradiated; TVS, tumor vaccination site.

Table 2. Upregulated genes in 4T1-SP cells compared with 4T1-NSP cells

Gene symbol	Accession no.	Gene description	Z-score
<i>Dcx</i>	NM_001110222	<i>Mus musculus doublecortin, transcript variant 1</i>	4.24
<i>Galr1</i>	NM_008082	<i>Mus musculus galanin receptor 1</i>	4.2
<i>Tuba 1b</i>	NM_011654	<i>Mus musculus tubulin, alpha 1B</i>	4.16
<i>Abcg2</i>	NM_011920	<i>Mus musculus ATP-binding cassette, sub-family G (WHITE), member 2</i>	3.98
<i>Ccbp2</i>	NM_021609	<i>Mus musculus chemokine-binding protein 2</i>	3.9
<i>Ube2c</i>	NM_026785	<i>Mus musculus ubiquitin-conjugating enzyme E2C</i>	3.66
<i>Ror1</i>	NM_013845	<i>Mus musculus receptor tyrosine kinase-like orphan receptor 1</i>	3.29
<i>Pbk</i>	NM_023209	<i>Mus musculus PDZ-binding kinase</i>	2.91
<i>Tbk1</i>	NM_019786	<i>Mus musculus TANK-binding kinase 1</i>	2.92
<i>Nek2</i>	NM_010892	<i>Mus musculus NIMA (never in mitosis gene a)-related expressed kinase 2</i>	2.85

included the genes categorized into subgroups based on their malignancy-related phenotypes such as prognostic markers in cancer patients, chemo- or radioresistance, metastasis and cellular proliferation (Figures 5a and b).

DISCUSSION

Breast cancer is the leading cause of death in women because of recurrence or metastasis after standard therapies, including surgery, chemotherapy and radiotherapy. The prognosis of patients with metastatic breast cancer remains poor, with a 5-year survival rate of 23.3%.²⁴ Breast CSCs are a relatively rare sub-population of tumor cells that are resistant to conventional therapies and responsible for relapse or metastasis.^{25–27} Therefore, novel treatment approaches to specifically target breast CSCs are expected to induce long-term clinical benefits for patients with advanced breast cancer. To date, most of the studies investigating the CSC compartment isolated from human tumors or tumor cell lines have explored their high tumorigenic potential by transplantation into immune-compromised mice.²⁸ However, in these mouse models, the xenografts may not fully recapitulate the activities of *bona fide* CSCs in human cancers²⁹ as they lack adaptive immune responses, leading to failure of comprehensive immunological assessment.³⁰ Therefore, to evaluate precisely the immunogenicity of CSCs in syngeneic immunocompetent hosts, we used purified mouse breast cancer 4T1-SP cells possessing diverse characteristics of CSCs as tumor antigen sources to fully prime adaptive immunity. We confirmed that 4T1-SP cells exhibited stem cell properties and immunoregulatory characteristics, as evidenced by their increased capacity to form colonies and high expression of Sca-1, p-STAT3, VEGF and PD-L1, which were not affected by genetic manipulation via SeV/dF/GM infection. One possible reason for the negligible influence by SeV/dF/GM transduction might be the fact that 4T1-SP cells did not express the GM-CSF receptor protein, thereby evading the paracrine effects of GM-CSF produced by the gene-transduced cells.

Despite the characteristics of 4T1-SP cells, such as low susceptibility to the cytotoxic effects of DTX, high tumorigenicity in mice and immunosuppressive phenotypes, 4T1-SP/GM cells strikingly lost their tumorigenicity in immunocompetent mice and induced strong immunostimulatory activities. As expected, CD8⁺ T cells (cytotoxic T lymphocytes) in mice injected with 4T1-SP/GM cells had a predominant role in exerting substantial antitumor immunity, supporting our previous findings that cytotoxic T lymphocytes act as the main effectors in GM-CSF-driven *in vivo* antitumor immunity, together with CD4⁺ T cells.^{2,31} Importantly, we demonstrated that therapeutic vaccines comprised an irradiated GM-CSF-producing breast SP cell-enriched sub-population as the antigen source, conferring greater antitumor immunity than control vaccines of ir.4T1-NSP/GM cells did. These results

suggest that CSC-associated antigens derived from SP cells appear to be more effective to induce therapeutic antitumor effects than unsorted cancer cells or surgically removed autologous tumor cells. The ELISPOT assay showed that CSC-associated antigen-specific T-helper type 1 cellular immunity was induced in ir.4T1-SP/GM cell-treated mice whose splenocytes produced a significantly higher IFN- γ . Furthermore, substantial therapeutic antitumor effects of vaccinations using GM-CSF-transduced primary 4T1-SP cells were observed. Notably, vaccination with ir.4T1-SP/GM cells efficiently inhibited lung metastasis. Collectively, SP cell-based GVAX therapy may provoke potent CSC-associated antigen-specific immune responses as an effective modality to improve the overall survival of patients with breast cancer by suppressing relapse and metastasis.

Only a few studies have reported that DC vaccine therapies using CSCs as antigen sources confer effective protective or therapeutic antitumor immunity in mouse or rat models.^{32,33} Here we showed for the first time that a therapeutic strategy using GM-CSF-driven SP cell-based vaccines markedly suppressed tumor development by the challenge with isolated breast cancer SP cells through both enhanced innate and adaptive immunity. Our immunological findings suggested that DCs, which link the innate and adaptive arms of the immune system, were more mature at the TVS stimulated by vaccinations with ir.4T1-SP/GM cells than the control. However, further investigation is required to assess whether these mature DCs at the TVS effectively phagocytose CSC-associated antigens.

Because GM-CSF production from the 4T1-SP/GM cells was 1531 ng/1 $\times 10^6$ cells per 72 h at an MOI of 10, probably equivalent to over 40 ng/1 $\times 10^6$ cells per 48 h required for GVAX therapy to provoke *in vivo* antitumor immunity,³⁴ it is conceivable that we detected substantial antitumor effects by ir.4T1-SP/GM cells. However, the antitumor effects need to be improved by modifying SP cell-based GVAX therapies with respect to administration route, frequency of administration, amount of mGM-CSF produced from cells and combinatorial use with other cytokines.^{35–37}

Although we demonstrated that SP cell-based cell vaccines were effective in controlling tumor growth, it is very difficult to prepare sufficient number of SP cells as antigen sources from surgically removed tumor tissue because of the small SP cell fraction in primary tumors.³⁸ Accordingly, the development of a new method to expand or amplify SP cells *in vitro* will be required to translate our findings to the clinical setting.

To ascertain potential CSC-associated antigens responsible for the superior antitumor effects of 4T1-SP cell-based vaccines compared with those of 4T1-NSP cell-based vaccines, cDNA microarray analysis was conducted to compare the gene expression profiles between 4T1-SP and 4T1-NSP cells. We selected the top 10 cancer-related genes predominantly expressed and localized in either the plasma membrane or cytoplasm of 4T1-SP cells as candidates for CSC-associated

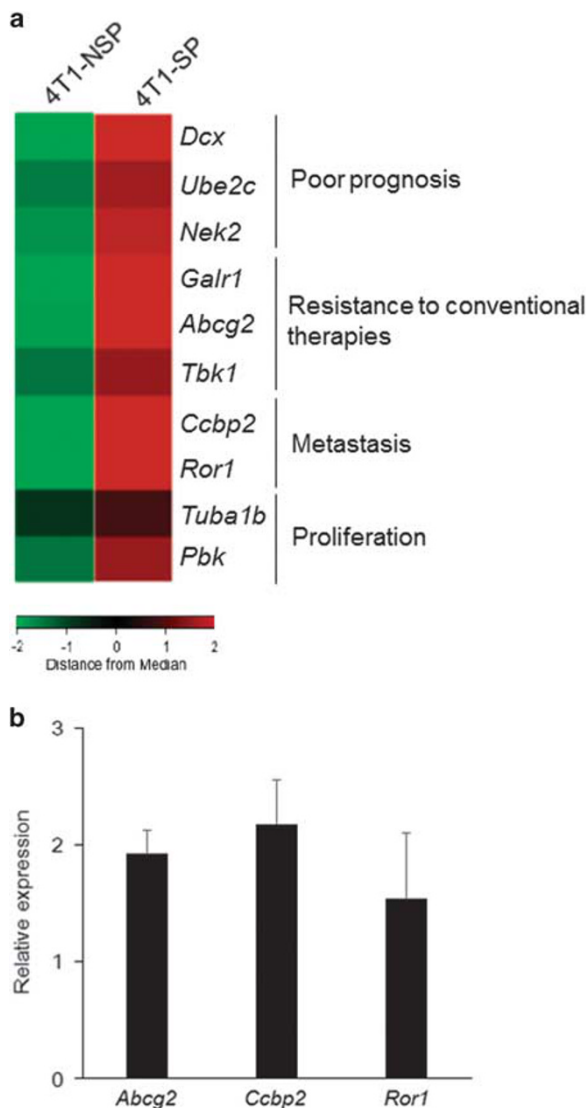


Figure 5. Heat map representing fold change patterns of the most highly differentially expressed genes in 4T1-SP cells. **(a)** A heat map was generated to compare fold-change patterns of the most highly differentially expressed genes in 4T1-SP cells, the likely cancer stem-like fraction and 4T1-NSP cells. The 10 most highly expressed genes were categorized into subgroups based on unfavorable cancerous phenotypes such as poor prognosis, metastasis, resistance to conventional therapies and cellular proliferation. Right panel (red): upregulated genes; left panel (green): downregulated genes. **(b)** Real-time PCR analysis to validate the result of microarray analysis. We especially focused on oncogenesis-related genes, and expression levels of the indicated genes in 4T1-SP cells compared with those in 4T1-NSP cells were calculated by relative quantification using the ddCt method with GAPDH as endogenous control. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NSP, non-SP; SP, side population.

antigens, including *Abcg2*, well known as Hoechst 33342 efflux transporter.³⁹ Among them, *Ror1* (receptor tyrosine kinase-like orphan receptor), *Ube2c* (ubiquitin-conjugating enzyme E2C) and *Galr1* (galanin receptor 1) have been shown to correlate with unfavorable cancerous characteristics such as metastasis,^{40,41} poor prognosis^{42,43} and chemoresistance⁴⁴ in various human solid cancers, including breast cancer. Other candidate genes were *Pbk* (PDZ-binding kinase), which has recently been characterized as a

novel cancer/testis antigen with oncogenic activity in breast cancer,⁴⁵ and *Nek2* (NIMA-related kinase 2), whose expression has been shown to be associated with disease recurrence in patients with breast cancers.^{46,47} These findings underscore the possibility that these candidate genes might be promising antigenic targets for future immunotherapy to eradicate breast CSCs. Sca-1-specific T cells, for example, prominently expressed in 4T1-SP cells could be one of the putative CSC-associated antigen-specific T cells and might partially contribute to the observed antitumor effects by ir.4T1-SP/GM cell-driven vaccinations. However, further studies are warranted to validate the most critical gene responsible for breast CSC-directed antitumor immunity.

In conclusion, 4T1-SP cells with CSC phenotype elicited susceptibility to GM-CSF-driven antitumor responses in both *in vivo* tumorigenicity and therapeutic vaccination assays. Our animal models using enriched mouse SP cells and syngeneic immunocompetent mice will allow a deeper understanding of the mutual interplay between CSC-like cells and host immune responses, and enable us to develop a novel cancer immunotherapy exploiting GM-CSF gene-transduced cancer SP cells for improving the overall survival of cancer patients.

CONFLICT OF INTEREST

KT receives research funding from SBI Pharmaceutical and Shinnihonsei-yaku to develop novel cancer therapies. The terms of this arrangement have been reviewed and approved by the University of Tokyo in accordance with its conflict of interest policies. The other authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cancer Gene Therapy website (<http://www.nature.com/cgt>)