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Identification of a Highly Immunogenic Mouse Breast Cancer Sub Cell Line,

4T1-S

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List of Abbreviations

4T1-A: 4T1-ATCC

4T1-S: 4T1-Sapporo

DLN: draining lymph node

RLN: remote lymph node

TIL: tumor infiltrating lymphocytes

There is no conflict of interest.

Abstract

Cancer vaccines serve as a promising clinical immunotherapeutic strategy that help to trigger an effective and specific antitumor immune response compared to conventional therapies. However, poor immunogenicity of tumor cells remains a major obstacle for clinical application, and developing new methods to modify the immunogenicity of tumor cells may help to improve the clinical outcome of cancer vaccines. 4T1 mouse breast cancer cell line has been known as poorly immunogenic and highly metastatic cell line. Using this model, we identified a sub cell line of 4T1 – designated as 4T1-Sapporo (4T1-S) – which shows immunogenic properties when used as a vaccine against the same line. In 4T1-S-vaccinated mice, subcutaneous injection of 4T1-S resulted in an anti-tumor inflammatory response represented by significant enlargement of draining lymph nodes, accompanied with increased frequencies of activated CD8 T cells and a subpopulation of myeloid cells. Additionally, 4T1-S vaccine was ineffective to induce tumor rejection in nude mice, which importantly indicate that 4T1-S vaccine rely on T cell response to induce tumor rejection.

Further analysis to identify mechanisms that control tumor immunogenicity in this model may help to develop new methods for improving the efficacies of clinical cancer vaccines.

Introduction

Breast cancer is one of the most frequently diagnosed cancers in young women [1, 2]. The main strategy of management and treatment for breast cancer is early diagnosis, surgical resection and adjuvant systemic therapy. In spite of recent advances in treatment protocols, breast cancer remains the second leading cause of cancer deaths in women in U.S. [1]. Moreover, recent breast cancer statistics showed increased rates of mortality in Japan [2]. Although treatment with conventional chemotherapy has demonstrated considerable efficacies in breast cancer [3], these treatments are frequently accompanied with significant side effects that negatively impact patients' quality of life [4, 5].

Recently, cancer vaccines have emerged as an alternative therapeutic strategy for the treatment of breast cancer that help to provide more specific and less toxic effects than conventional chemotherapies [6-8]. In this regard, several immunotherapeutic approaches have been proposed to generate immunologic response specific for antigens selectively expressed in tumor cells including DNA vaccine, peptide vaccine and whole cell vaccine. In particular, using a whole tumor cell vaccine offer an important advantage of targeting multiple tumor antigens simultaneously [6, 7]. However, several studies have shown that whole tumor cell vaccine were ineffective to induce potent antitumor immune responses in preclinical models and clinical trials

[7, 8]. This may be due to poor immunogenicity of tumor cells used as whole tumor cell vaccine. Immunological heterogeneity of tumor cells has been shown to have significant impact on tumorigenicity and tumor progression [9-11]. During the process of cancer immunoediting, highly immunogenic tumor cell variants are eliminated steadily at the first phase (Elimination), while tumor cell variants with decreased immunogenicity survive immune attacks (Equilibrium), and finally these poorly immunogenic variants dominate and acquire the ability to proliferate and expand (Escape). At this stage, when tumors become clinically diagnosable, tumor cells are rendered poorly immunogenic and characterized by losing major histocompatibility complex class I and II antigens. Thus, improving the efficacies of cancer vaccine requires the modification of tumor immunogenicity in a way that redirects tumor cells from immune-resistant into immune-sensitive state.

In this regard, a recent report has suggested the ability of ionizing radiation to induce immunogenic cell death accompanied with inflammatory response that is capable to control tumor growth, thus changing the potential of tumor cells in radio-immunotherapeutic strategies [12]. Additionally, a mouse breast cancer cell line, 4T1, which is well known as a poorly immunogenic cell line, partially obtains substantially immunogenic properties upon radiation and a potential to respond to immunotherapy [13, 14]. Using the poorly immunogenic 4T1 cell line as a model, we

incidentally identify in this study a sub cell line of 4T1 (designated as 4T1-S), which could induce a protective immune response against challenge of the same line without any manipulation. Vaccination with irradiated 4T1-S induced a potent antitumor inflammatory response represented by striking enlargement of draining lymph nodes and increased frequencies of activated CD8 T cells. Importantly, 4T1-S vaccine was ineffective in nude mice, indicating that the effect of 4T1-S vaccine is T cell-dependent. Identification of the mechanisms that control immunogenicity of 4T1 sub cell line may help to improve the efficacies of cancer cell vaccines in future treatments.

Materials and Methods

Cell lines and animals

Mouse breast cancer cell line (4T1) was purchased from American Type Culture Collection (ATCC). This cell line was designated as 4T1-ATCC (4T1-A). Highly immunogenic sub-clone of 4T1 described in this paper was maintained in our laboratory and named 4T1-Sapporo (4T1-S). 4T1-S underwent no forced gene transduction and was stocked in liquid nitrogen. Both 4T1-A and 4T1-S were cultured in RPMI 1640 medium (WAKO) supplemented with 10% fetal bovine serum (Cell Culture Bioscience), 1% penicillin/streptomycin, 1% L-glutamine, 1mM sodium pyruvate, 1% non-essential amino acids (All from Life technologies) and 50 μ M 2-mercaptoethanol (WAKO). Both 4T1-A and 4T1-S were routinely examined for mycoplasma infection during the period of this study (Lonza). To induce cell death, cells were irradiated at 300 Gy X-rays.

Female BALB/c and BALB/c-nu/nu mice were purchased from Sankyo Labo Service Corporation. All mice used in experiments were at age of 6-10 weeks.

In vivo model

For the tumor vaccine experiments, irradiated 4T1 cells (2×10^6) were re-suspended in 200 μ l PBS and subcutaneously injected into the right back of mice. 2 weeks after

vaccination, 5×10^4 of live 4T1 cells were re-suspended in 200 μ l PBS and inoculated into the left mammary gland. In the experiments examining lymph nodes, live 4T1 cells (2×10^6) were subcutaneously injected. Animal procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee using an approved protocol (No.11-0160). Tumor size was estimated by ellipsoid volume formulas ($1/2 \times L \times W \times H$).

Flow cytometry

Single cell suspensions were prepared from tumors or lymph nodes and used for flow cytometry analysis after staining with appropriate fluorescent antibodies according to manufacturer's instruction. Fluorescent antibodies used for the staining of mouse cell surface markers were purchased from eBioscience (anti-CD3 and anti-CD8) or Biolegend (anti-CD69 and antibodies for Figure 5). Samples were run on FC500 (BECKMAN COULTER) and analysed using FlowJo software V7.6.5.

Microarray analysis

Total RNAs of 4T1-A and 4T1-S were labeled with Cy3 and hybridized to a Whole Mouse Genome Microarray (Agilent) according to the manufacturer's protocol. Arrays were scanned using the Agilent Technologies Microarray Scanner. Data

were analyzed using the MultiExperiment viewer (<http://www.tm4.org/>). Microarray data are available from the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) with the accession number GSE71926.

Transfection and real time PCR

cDNA for candidate genes was synthesized from 4T1-S mRNA and subcloned into pPyCAG vectors (kindly provided by Dr. H Niwa, RIKEN). The expression vectors were transfected into 4T1-A by electroporation. The expression of transfected genes was examined by real time PCR (Fig.5D). The quantitative PCR reaction was performed using Power SYBR® Green (Life Technologies).

Statistical analysis

Statistical significance was determined by the Student *t* test with a cut-off $P < 0.05$. Data are presented as $\pm$ SEM. The Kaplan-Meier method was used to estimate overall survival, and survival differences were estimated by the log-rank test. All data were analysed using JMP v11.

Results

4T1-S vaccine induces tumor rejection and prolongs survival of mice challenged with live 4T1-S breast cancer cells

At first, we performed a tumor vaccine experiment using 4T1 cell line which was maintained in our laboratory for several years, and thus designated as 4T1-Sapporo (4T1-S). BALB/c mice were vaccinated with irradiated 4T1-S cells (2×10^6) and challenged 2 weeks later with live 4T1-S cells (5×10^4) injected into the mammary glands (Fig.1A). Surprisingly, we found that the vaccination with irradiated 4T1-S cells induced tumor rejection and prolonged survival rate of mice challenged with live 4T1-S cells (Fig.1B, solid line). To further evaluate the vaccine effects of irradiated 4T1 cells, we utilized a new 4T1 cell line purchased from American Type Culture Collection (ATCC) and designated as 4T1-A. 4T1-A and 4T1-S cell lines were cultured at the same condition and examined routinely for mycoplasma infection during the period of this study (data not shown). Interestingly, we found that the vaccination with irradiated 4T1-S showed no effects when mice were challenged with live 4T1-A cells (Fig.1B, dashed line) in contrast to live 4T1-S cells (Fig.1B, solid line). Furthermore, irradiated 4T1-A cells were ineffective to prolong survival of mice challenged with live 4T1-A (Fig.1C, dashed line) or 4T1-S (Fig.1C, solid line) cells. Thus, we concluded that vaccination with irradiated 4T1-S but not 4T1-A is capable to induce a substantial

protection against challenge with live 4T1-S cells. In this study, both 4T1-A and 4T1-S cells were passaged more than 40 times, but no changes in their protective effects were observed (data not shown), suggesting that the number of passage did not affect the immunogenic capacity of 4T1-S cells.

To further confirm the characteristics of 4T1-S cells, we utilized limiting dilution method to establish several single cell-derived subclones from 4T1-A or 4T1-S cell lines. The cell morphology, cell growth rate and FACS pattern of these subclones were similar with those of original lines (not shown, see Fig.2 and 5), which mostly exclude the possibility of any contamination of 4T1 cells with other immunogenic cells from different resource. Then we examined the effects of these subclones when used as vaccines against 4T1-S challenge. As expected, we found that 3 from 6 subclones derived from 4T1-S induced rejection of inoculated live 4T1-S cells, while all 4T1-A-derived subclones (total of 6 subclones) were ineffective (Fig.1D). Together, these results indicate that 4T1-S, but not 4T1-A, contains immunogenic variants that elicit the immunotherapeutic effects of irradiated 4T1-S cells. These results also demonstrate the existence of heterogeneity even in 4T1-S cells.

4T1-A and 4T1-S cells show the same morphology but different proliferative rates in vitro and in vivo

Based on the above results, it was highly anticipated that 4T1-S was coincidentally transformed and obtained the characteristics of tumor vaccine. Next, to confirm any differences between the two cell lines, we compared cell morphologies and cell proliferation in vitro and in vivo. Both 4T1-A and 4T1-S show plastic adherent properties with identical morphologies (Fig.2A). In vitro cell proliferation rate of 4T1-S was significantly higher than 4T1-A (Fig.2B). However, when subcutaneously inoculated, 4T1-A tumors grow more rapidly than 4T1-S (Fig.2C). Consistent with this, survival rate of 4T1-A tumors-bearing mice was shorter than that of 4T1-S (Fig.2D). Together, these results suggest that 4T1-S cell line has acquired a substantial immunogenicity, which results in suppression of tumor growth in vivo.

Vaccine effects of irradiated 4T1-S cells are dependent on T cell response

Cancer vaccines have the capability to trigger potent anti-tumor T cell responses that result in eradication of tumor cells. To examine whether the vaccine effects of irradiated 4T1-S depend on T cell immunity, we performed vaccine experiment using BALB/c-nu/nu mice, a strain that lacks a thymus and thus unable to produce T cells. In this experiment we found that the vaccination with irradiated 4T1-S cells was ineffective to prolong survival rate of BALB/c-nu/nu mice (Fig.3A). Furthermore, survival of mice vaccinated with irradiated 4T1-S was significantly worse when mice

were challenged with 4T1-S compared to 4T1-A ($p=0.0195$) (Fig.3A). Again, vaccination with irradiated 4T1-A cells did not prolong survival rate of BALB/c-nu/nu mice challenged with 4T1-A or 4T1-S cells (Fig.3B). Thus, these results clearly indicate that the vaccine effects by 4T1-S are mediated by T cell response.

Injection of 4T1-S increases frequencies of activated CD8 T cells

Next, we aimed to evaluate the frequencies and activation state of CD8 T cells in mice injected with 4T1-S cells. Since vaccination with irradiated 4T1-S cells resulted in rejection of inoculated live 4T1-S cells, it was very difficult to compare tumor infiltrating lymphocytes (TILs) between 4T1-A and 4T1-S tumors after vaccination. Thus, we evaluated the frequencies and activation state of CD8 T cells in draining lymph nodes (DLNs) compared to control remote lymph nodes (RLNs).

By macroscopic observation, a significant enlargement in DLNs was observed when mice were injected with 4T1-S compared to 4T1-A cells, while the size of RLNs was comparable between the two groups (Fig.4A). Furthermore, total cell number was remarkably higher in DLNs isolated from 4T1-S-injected mice compared to 4T1-A, but comparable in RLNs (Fig.4B). Importantly, flowcytometry analysis showed increased frequencies of activated cytotoxic CD8 T cells in DLNs harvested from mice injected with 4T1-S compared with 4T1-A (Fig.4C). We also realized that a certain population

of cells characterized by high forward and side scatter signal values was increased in DLNs isolated from 4T1-S-injected mice (data not shown). Further flowcytometry analysis revealed that a population with expression of CD11b and Ly6G myeloid markers was increased (Fig. 4D). Collectively, these results indicate a unique capability of 4T1-S to induce a potent anti-tumor CD8 T cell response, and the involvement of a certain myeloid subpopulation in the immune response triggered by 4T1-S, which should be clarified in detail in further studies.

4T1-A and 4T1-S cells exhibit different molecular expressions

Finally, we aimed to identify the candidate molecules responsible for vaccine effects of 4T1-S cells. In a microarray analysis, we compared molecular expressions between 4T1-A and 4T1-S, and found that several molecules were differentially expressed between the two subclones (Fig.5A and 5B). Furthermore, we confirmed by flowcytometry that molecules such as CD54 or CD56 (NCAM-1) were highly expressed in 4T1-S than 4T1-A (Fig.5C). Based on these results, we chose several candidate molecules which show high expression in 4T1-S including CD56, Cyp1b1, Thbs3, Oas1a, and Arrb1, and transduced each gene into 4T1-A by transfection. After confirmation of gene expression by real time PCR (Fig.5D), BALB/c mice were vaccinated with irradiated transfectants and challenged after 2 weeks with live 4T1-S

cells as described in Fig.1. However, these vaccines were ineffective to induce rejection of inoculated live 4T1-S (Fig.5E). Therefore, further studies are needed to identify the factors responsible for modifying the immunogenicity of 4T1-S cells.

Discussion

In this study, we identify a sub cell line, named as 4T1-S, which has spontaneously acquired high immunogenic properties, represented by inducing tumor rejection of the same cell line when used as a whole cancer cell vaccine. Since no record of forced gene transduction or mycoplasma infection is available, we assumed that a spontaneous mutation in 4T1-S cells has resulted in the modification of its immunogenicity. In laboratory mice, similar spontaneous mutations were previously reported. For example, Sakaguchi et al have described a mouse substrain showing spontaneously developed rheumatoid arthritis phenotype (SKG mice); subsequently SKG mice were shown to have a point mutation in ZAP-70 gene [15]. Additionally, recent exome sequencing revealed pathogenic mutations in 91 strains of mice with Mendelian disorders [16]. Similarly, 4T1-S line might have a substantial mutation which altered its immunogenicity.

Regarding the mechanism of 4T1-S vaccine, mice challenged with 4T1-S cells showed significant enlargement of draining lymph nodes, accompanied with increased frequencies of activated CD8 T cells. The vaccine effect of 4T1-S was abrogated in nude mice, indicating that this phenomenon is indeed T cell dependent. In addition to CD8 T cells, 4T1-S vaccine also resulted in increased frequency of a certain subpopulation of cells characterized with high expression of CD11b and Ly6G.

CD11b and Ly6G are highly expressed on myeloid cells such as myeloid-derived suppressor cells (MDSC) and neutrophils [17]. CD11b+Ly6G+ neutrophils play important protective roles against viral infections through the secretion of several inflammatory cytokines. On the other hand, MDSCs suppress immune responses and contribute importantly to the maintenance of homeostasis [18]. Following induction by 4T1-S vaccine, CD11b+Ly6G+ cells may play a supportive role through the enhancement of antitumor inflammatory response, or serve as an important suppression mechanism to regulate adaptive immune response following complete eradication of tumor cells. The role of CD11b+Ly6G+ population in our model has not been clarified yet, and further studies are required to understand the significance of this population on the effects of 4T1-S vaccine.

An interesting observation in this study is the specificity of 4T1-S vaccine against 4T1-S but not 4T1-A cells. Based on this observation, we hypothesised that 4T1-S express certain specific factors which might be derived from a mutated gene [19]. Comparison of gene expression has revealed that several molecules were more highly expressed in 4T1-S than 4T1-A cells. However, vaccination with irradiated 4T1-A cells which had been transfected with each of these molecules was ineffective to induce 4T1-S tumor rejection. Therefore, further studies that utilize more comprehensive methods such as next generation sequencing are critically needed to

identify the responsible factors for altering 4T1-S immunogenicity. It is conceivable that the possible responsible factors are not expressed in non-immunogenic 4T1-S variants (Fig. 1D), which would be confirmed in our future study.

The process of cancer immunoediting is classified classically into three phases: elimination, equilibrium and escape [20]. With this in mind, the poorly immunogenic 4T1-A cells locate in the post equilibrium phase, while 4T1-S cells which exhibit immunogenic properties locate in the elimination phase. This is supported by the fact that 4T1-S tumors showed delayed growth in vivo compared to 4T1-A tumors, suggesting that 4T1-S tumors were under immunological pressure in immunocompetent hosts, and the factors that altered 4T1-S immunogenicity have redirected 4T1 cells from the post equilibrium phase back to the elimination phase.

In summary, we introduced in this brief report the discovery of a highly immunogenic breast cancer sub cell line 4T1-S which showed considerable vaccine effects against the same cell line. It would be possible to expand this discovery to a new immunotherapy with an approach of regenerative medicine. For example, dendritic cells from iPS cells could be transduced with the mutated gene which would be isolated from 4T1-S. Thus, identification of the mechanisms that control the immunogenicity of 4T1-S cell may contribute to improve the efficacies of clinical cancer cell vaccines in future treatments.

Acknowledgements

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Figure Legends

Figure.1 Vaccination with irradiated 4T1-S cells prolongs survival of mice challenged with 4T1 breast cancer cells

- (A) A scheme of vaccination experiment. BALB/c mice were vaccinated with irradiated 4T1-A or 4T1-S (2×10^6), and challenged after 2 weeks with 4T1 cells (5×10^4) injected into the mammary glands.
- (B) Survival rate after challenge with live 4T1 cells in mice vaccinated with irradiated 4T1-S cells.
- (C) Survival rate after challenge with live 4T1 cells in mice vaccinated with irradiated 4T1-A cells.
- (D) Survival rate after challenge with live 4T1 cells in mice vaccinated with 6 different subclones of either 4T1-A or 4T1-S.

Figure.2 Similar morphology but different proliferative rates between 4T1-A and 4T1-S

- (A) Photomicrographs of 4T1-A and 4T1-S cells. Scale bars: 100 μm .
- (B) Proliferative rates compared between 4T1-A and 4T1-S cells in in vitro culture.
- (C) Tumor growth in mice injected with 4T1-A or 4T1-S cells.
- (D) Survival rate after injection with 4T1-A or 4T1-S cells.

* $P < 0.05$, ** $P < 0.01$.

Figure.3 Vaccine effects of irradiated 4T1-S cells depend on T cell response

(A) Survival rate after challenge with live 4T1-S cells in BALB/c-nu/nu mice vaccinated with irradiated 4T1-S cells.

(B) Survival rate after challenge with live 4T1-S cells in BALB/c-nu/nu mice vaccinated with irradiated 4T1-A cells.

Figure.4 Vaccination with irradiated 4T1-S induces immunological changes in draining lymph nodes

(A) Macroscopic photographs of draining lymph nodes (DLNs) or remote lymph nodes (RLNs) isolated from mice challenged with subcutaneous injection of 4T1-A or 4T1-S cells (2×10^6).

(B) Comparison of total cell numbers in DLNs or RLNs described in (A). * $P < 0.05$.

(C) Flowcytometry analysis of the frequencies of CD8+CD69+ T cells (gated on CD3+ cells) in DLNs or RLNs described in (A).

(D) Flowcytometry analysis of the frequencies of CD11b+Ly6G+ myeloid cells in DLNs or RLNs described in (A).

Figure.5 Different molecular expression is observed between 4T1-A and 4T1-S

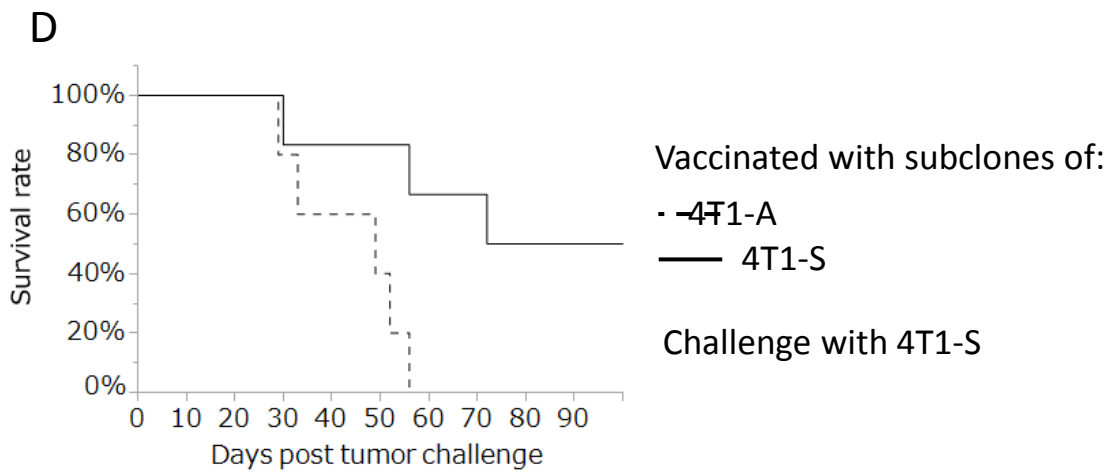
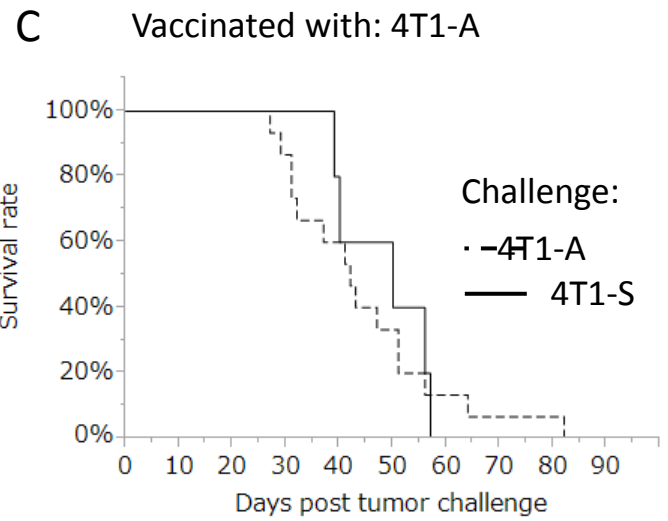
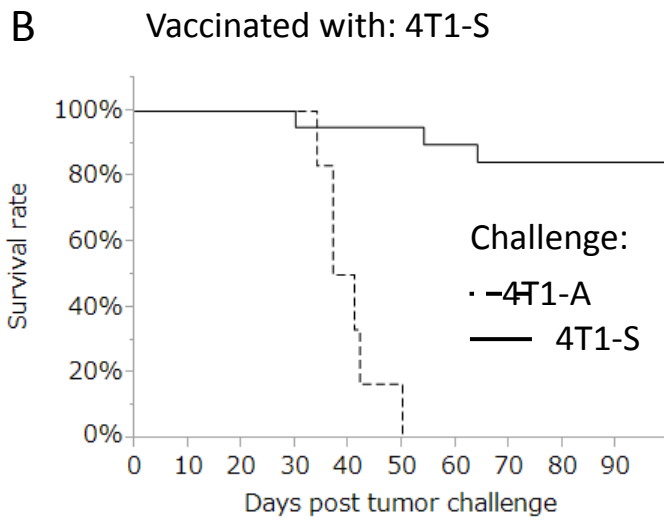
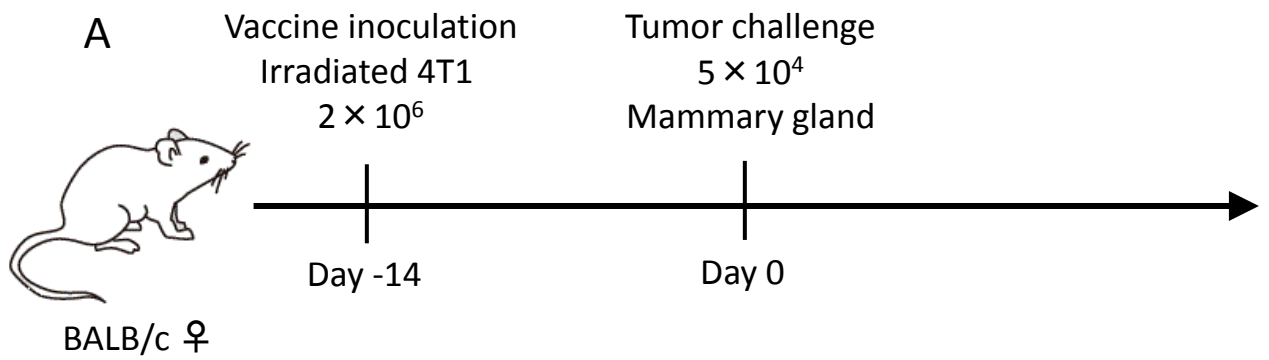
(A) MA plots reveal gene expression in 4T1-A and 4T1-S cells.

(B) Heat maps for gene expression analysis by microarray data between 4T1-A and 4T1-S described in (A). A1-A2 and S1-S2 represent 4T1-A and 4T1-S, respectively.

(C) Expression of representative cell surface molecules in 4T1-A and 4T1-S cells by flowcytometry. Gray histogram: 4T1-A, Solid line: 4T1-S.

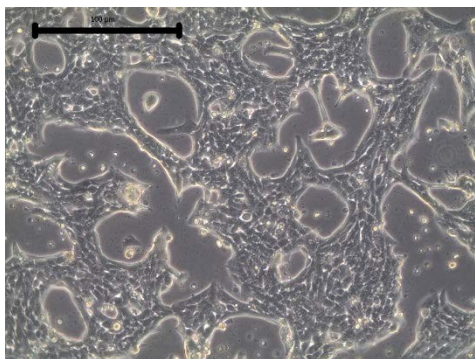
(D) Overexpression of indicated genes in 4T1-A was confirmed by real time PCR.

(E) Tumorigenesis in mice vaccinated with irradiated transfectants and challenged with live 4T1-S. Tumor formation was estimated by macroscopic observation of established tumors at day 30.

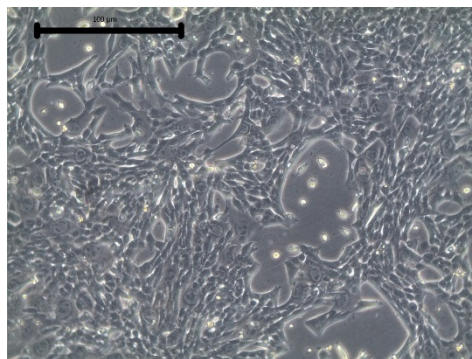


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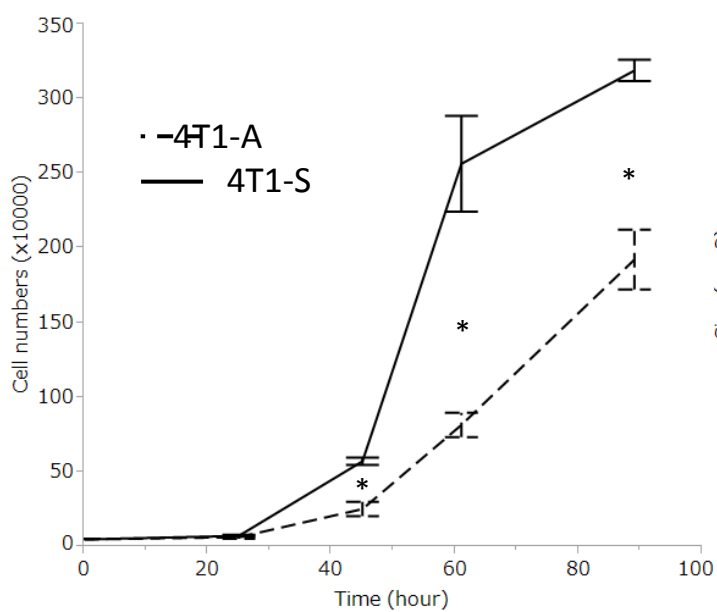
4T1-A



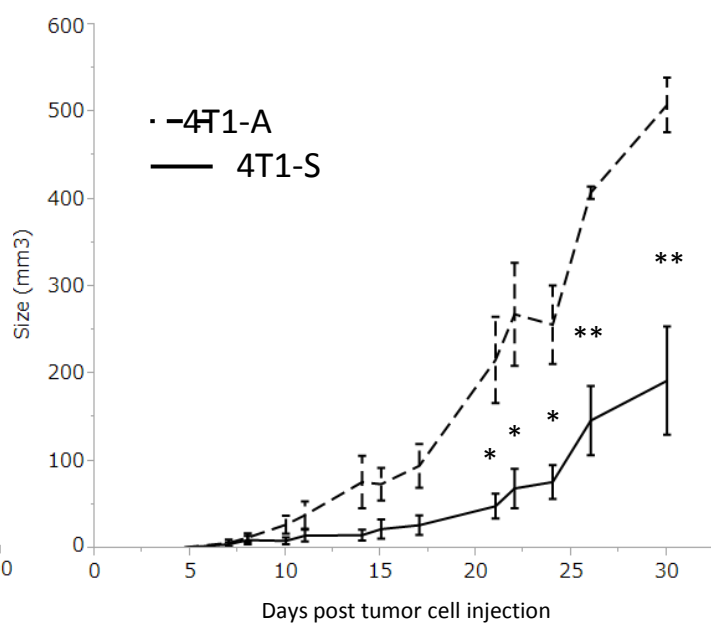
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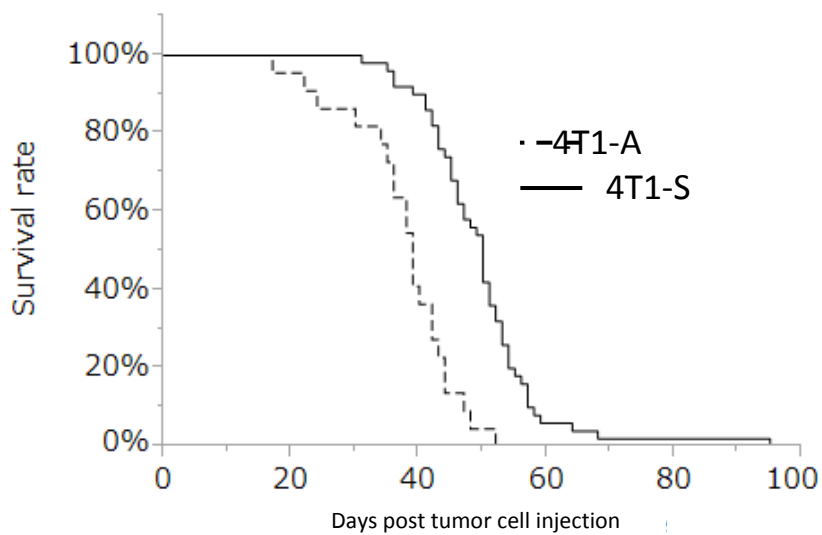
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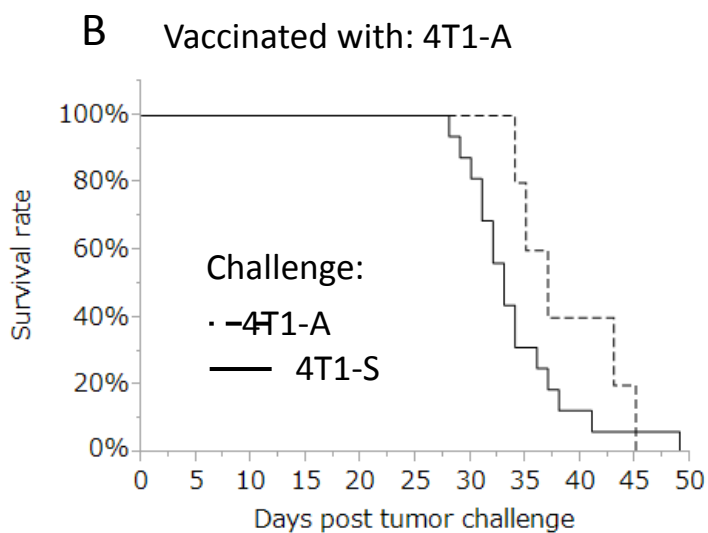
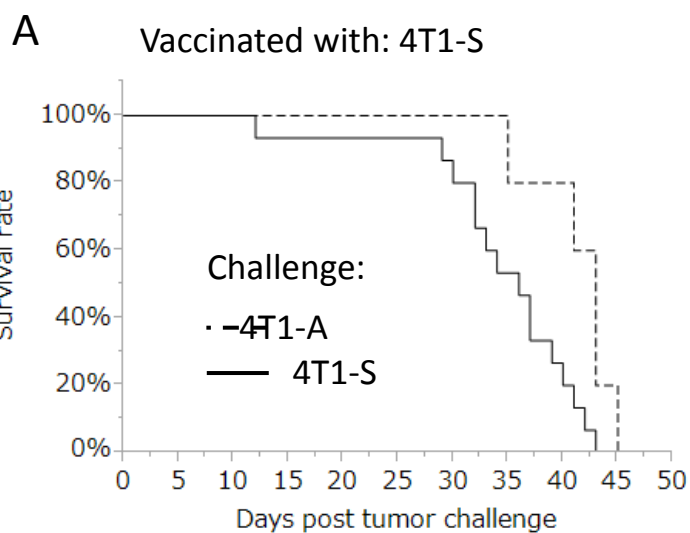


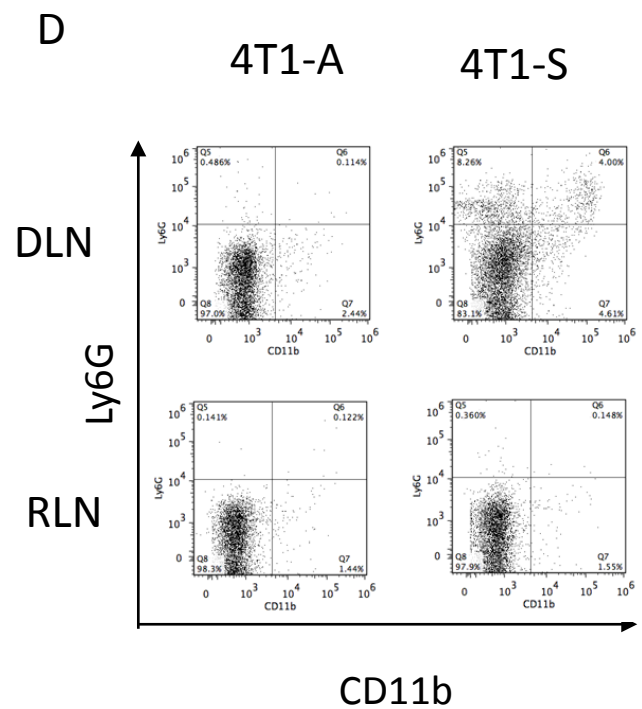
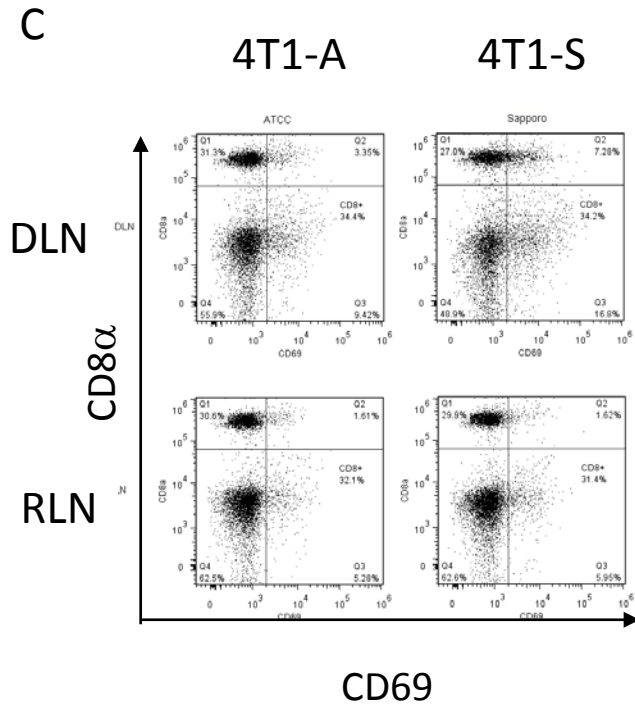
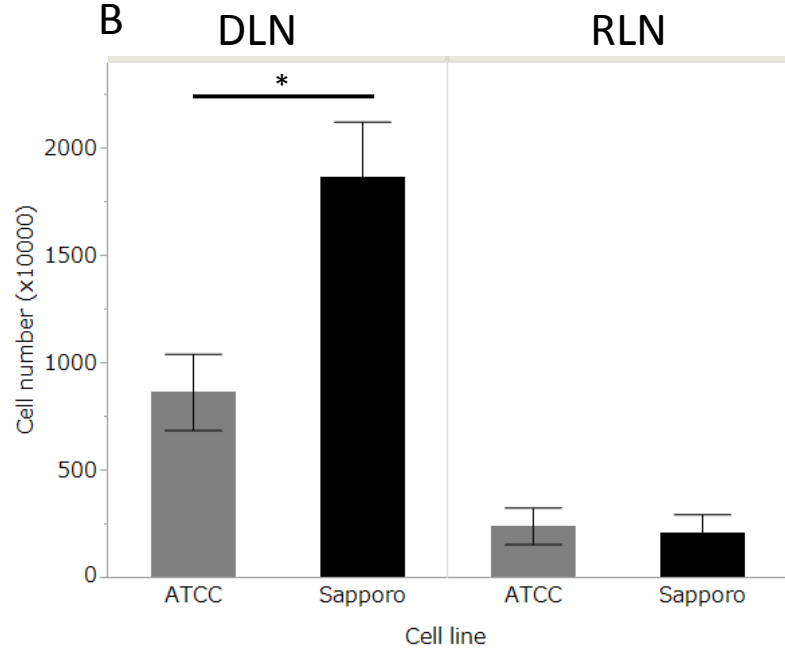
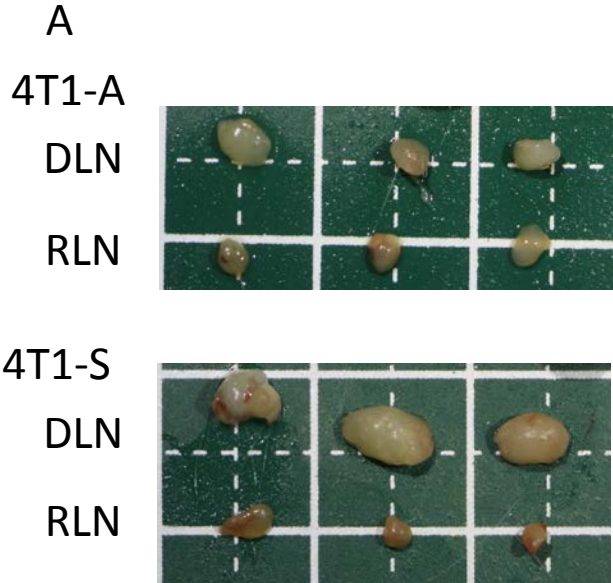
C



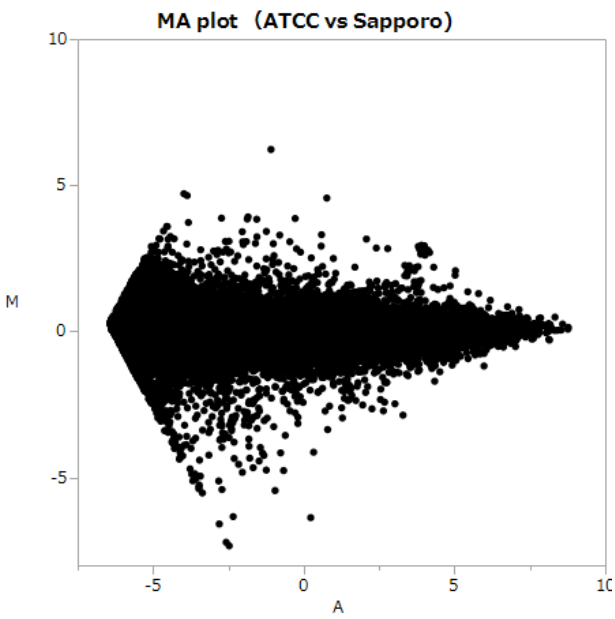
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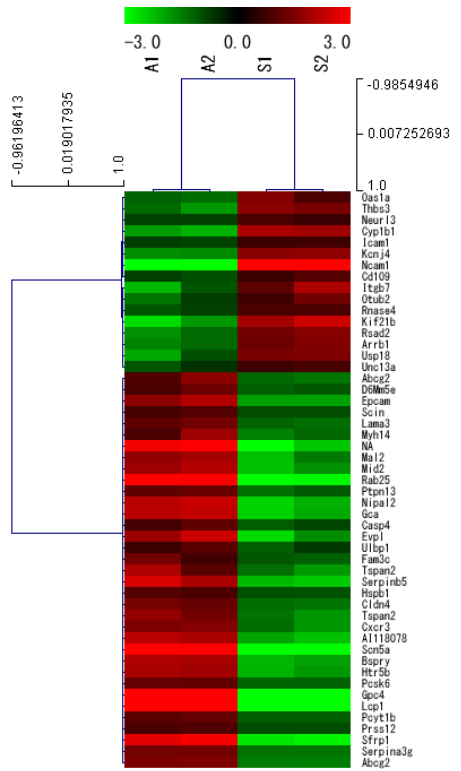




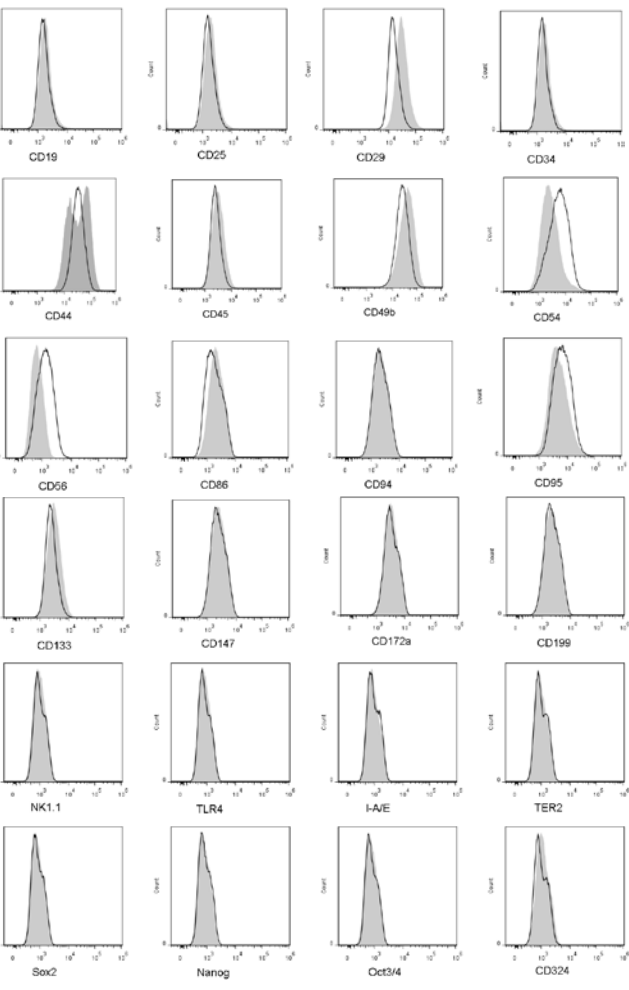
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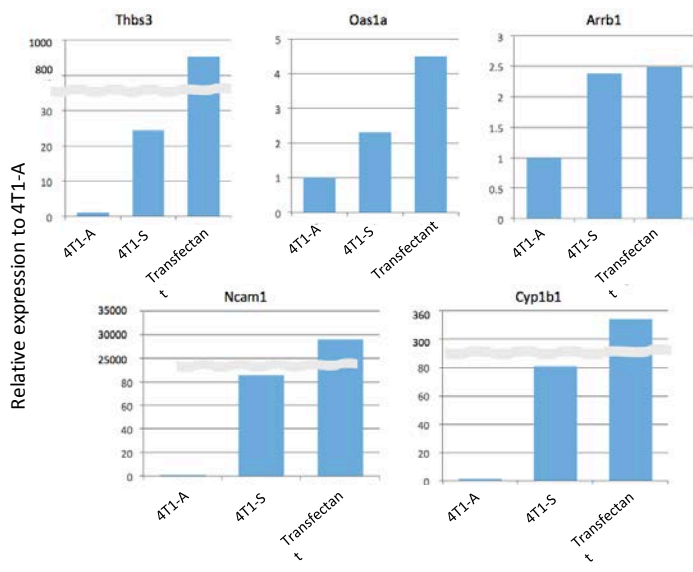
B



C



D



E

	Tumorigenesis	%
Oas1a	5/5	100
Arrb1	4/4	100
Ncam1 (CD56)	5/5	100
Thbs3	5/5	100
Cyp1b1	4/4	100