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Analysis of Cytotoxic T Lymphocytes Response Activated Dendritic Cells Vaccination Using Breast Cancer Stem-like Cells

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ABSTRACT

In this study, we isolated and evaluated BSCs derived from breast cancer patients, and explored the suitability of BSCs as sources of antigens for DC vaccination against human BSCs, with the aim of achieving BSC targeting and enhanced antitumor immunity. We found that BSCs express high levels of stem cell-associated molecules, CD44+/CD24- and CD133+, but adherent cells express CD44+/CD24+ and low CD133+. The enriched mammospheric cells have a stronger tumorigenic capacity than adherent cells in vivo tumorigenesis. DC vaccination using BSC lysates elicited specific T-cell responses against BSCs. DC vaccination stimulated Th1 response and induced significant IFN- γ production which is positively correlated with the number of cytotoxic T lymphocytes stimulated. Strikingly, using BSC breast cancer model, we demonstrate that vaccination with CTL stimulated DCs pulsed with enriched mammospheric cells lysates, but not pulsed with adherent cells lysates, prolonged survival in animals bearing BSC breast cancer tumors. Therefore, these proof-of-concept results confirmed CD44+/CD24-/CD133+ mammospheric cells have stem cells property and DC immunization with BSCs generates superior antitumor immunity which may accelerate development of BSC-specific immunotherapies and cancer vaccines.

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Introduction

In recent years, there are numerous evidences that have been validated that breast cancer contains a hierarchy of heterogeneous cell populations with different biological properties and that the capability to sustain tumor formation and growth exclusively resides, termed cancer stem cells (CSCs) ^[1-3]. These studies have shown that CSCs are responsible for tumor formation and progression and, interestingly, that they are endowed with stem/progenitor cell properties; in particular, tumor stem cells share the key feature of self-renewal ^[4-5]. Al-Hajjet al. ^[6] identified putative breast cancer tumorigenic cells as CD44+/CD24-/low cells capable to drive tumor formation when a few hundred cells were injected into the mammary fat pad of NOD/SCID mice. The subpopulation of breast cancer stem cells (BSCs) are not effectively targeted by chemotherapeutic agents and radiation in breast cancer ^[4, 7].

The identification of human BSCs presents a novel legend for the development of breast cancer treatments. The stem cell phenotype of breast cancer cells and their limited number within tumor tissues may account for their capability to escape conventional therapies, thus leading to disease relapse although the primary lesion is eradicated ^[4, 7] and are postulated to be the cells responsible for the relapse and progression of cancers after such therapies.

In an analogous fashion, the BSC phenomenon may adversely affect the development of effective immune-therapies for breast cancer. These therapies have involved targeting breast cancer cells that express differentiated tumor antigens which may be selectively expressed on differentiated tumor cells ^[8, 9]. However, BSCs that do not express these antigens may thus escape these immune surveillance.

Clinical trials to treat breast cancer using dendritic cells (DCs) vaccine ^[10, 11] have shown therapeutic efficacy for ER/PR double-negative or triple-negative II/IIIA breast cancer patients. However, the clinical responses to such immune-therapeutic approaches have been confined to a limited amount of breast cancer patients. Nowadays, tumor tissues with heterogeneous populations of cancer cells have been used as antigens either to generate effector T cells or to prime DC vaccines ^[12]. Human tumors are composed of heterogeneous tumor cell clones that differ with respect to proliferation, differentiation, and ability to initiate daughter tumors ^[13]. The apability to target BSCs with current immune methods may be a critical cause for therapeutic failures.

On this basis, we isolated and assessed stem cell phenotype and tumorigenicity of BSCs which derived from patients of

breast infiltrative ductal carcinoma by mammosphere culture. Further, we analyzed the immunogenicity induced by purified BSCs used as special antigens to prime DCs. We demonstrate that BSCs lysates pulsing DC vaccination activated cytotoxic T lymphocytes (CTL) and induced interferon γ (IFN- γ) production against BSCs. In BSC breast cancer models, we also found that DC vaccination with BSCs prolongs survival of tumor-bearing animals. BSC-based vaccines endowed effective protective antitumor immunity which was associated with the induction of cellular responses that directly targeted BSCs via CTLs.

Materials and Methods

1. Isolation and in vitro expansion of progenitor cells from breast tumor specimens.

Tumor specimens were obtained from consenting patients according to the Internal Review and the Medical Ethics Committee of Yanbian University. Sixteen breast lesions, from the histologic diagnostic assessment and sampled by pathologists, were received in the Laboratory within 2 hours of surgery and immediately mechanically disaggregated. Primary Breast carcinoma spheres were cultured as previously described^[14]. Briefly, enzymatic digestion was also required and tissue fragments were incubated at 37 °C for 4 hours in a 1:1 solution of collagenase/hyaluronidase (Sigma-Aldrich

Co. LLC, St. Louis, MO). After filtration through a 40 μ m cell filter, single cells were plated at 1,000 cells/mL in serum-free DMEM-F12 (1:1) (Gibco, Life Technologies Grand Island, NY), supplemented with 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ), 5 μ g/mL insulin (Sigma), and B27 (Gibco). Cells grown in these conditions as non-adherent spherical clusters of cells (usually named “spheres” or “mammospheres”) were enzymatically dissociated every 3-5 days by incubation in an accutase solution (Sigma) for 15 minutes at 37 °C.

Additionally, when breast cancer single cells didn't appear mammospheric growth, and generate cell number decrease for 7 days culture. Culturing single cells carried out adherent culture and adherent cells (BAC) were obtained by in DMEM-F12 (1:1) medium supplemented with 10% fetal bovine serum (Gibco) with growth factors.

2. Flow-cytometric analysis of breast carcinoma-derived cancer stem-like cells

By using a FACS Calibur (Becton Dickinson, San Jose, CA), the expression of a panel of stem cells markers was distinctly evaluated on cells obtained from mammospheres or from adherent cells cultured in serum conditions. The antibodies used were anti-CD44 (conjugated with phycoerythrin, PE),

anti-CD24 (conjugated with fluorescein, FITC), and CD133 (conjugated with phycoerythrin, PE) (BioLegend, Inc., San Diego, CA). The mouse IgG1 antibodies were used as isotype control from Bio-Legend. Staining was done according to the instructions of the manufacturer. Breast carcinoma-derived spheres were processed into single-cell suspension before fluorescence activated cell sorting analysis. Before staining of membrane antigens, unfixed cells were allowed to recover in fresh medium for 1 hour at 37°C in gentle agitation.

3. In vivo tumorigenesis analysis by injecting mammospheric cells into animals

Spheres were collected, enzymatically dissociated, washed in PBS, and kept at 4°C until injection into subcutaneous breast fat pad of right for axillary cavity of 5-week-old SCID mice. Adherent cells were digested with trypsin-EDTA and similarly were injected into the mammary fat pad. Mice were maintained in laminar flow rooms under constant temperature and humidity and received an estradiol supplementation (0.4 mg/kg s.c.) every 10 days for 40 days after cell injection. Mice were inspected for tumor appearance, by observation and palpation, for 15 weeks following cell injection; after this time interval, all mice were sacrificed by cervical dislocation and the presence of each tumor nodule was confirmed by necropsy, including lung, liver, kidney, spleen and lymph node et al.

Experimental protocols were approved by the experimental animal care and use association of Yanbian University.

4. Priming of human DCs with breast cancer stem-like cells lysates

Human immature DCs were prepared from peripheral mononuclear blood cells (PBMCs). PBMCs obtained from a healthy donor were prepared by Ficoll/Paque (Invitrogen, Life Technologies, Grand Island, NY) density gradient centrifugation. PBMCs were seeded (9×10^6 cells/3ml/well) into 6-well plates (Corning Costar Corp., Cambridge, MA) in RPMI 1640. After 1.5 hours of incubation at 37°C, adherent cells were used for DC generation as described. At least 3 to 6 million cells (including BSC and BAC) in suspension were smashed to prepare cells as antigens by Ultrasonic Cell Disrupter (Safer, Nanjing, China). Tumor antigen protein concentrations were determined using Bio-Rad Protein Assay reagents (Hercules, CA).

5. T-cell stimulation and cytotoxic T-cell assays against BSCs

The non-adherent lymphocytes were stimulated with autologous DCs loaded with BSC or adherent cells (BAC) lysates at the ratio of 5:1 (the BSCs lysates and DCs were co-cultured overnight before they were seeded with lymphocytes). Interleukin-2 (IL-2; 300 IU/ml) was added to the cultures the next day and every 3 days thereafter. The lymphocytes were

restimulated with DCs every 5days for up to 3 stimulations, which consistently results in more than 90% of CD3+ T cells (data not shown). CTL-mediated BSC cytotoxicity was tested using the lactate dehydrogenase (LDH) Release Assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay,

Promega) according to the manufacturer's protocol. The target cells were mammospheric cells and adherent cells of breast cancer. The ratio of effector and target cells was 2.5:1, 5:1, 10:1, 20:1 and 40:1. The following formula was used to calculate cytotoxicity:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental-Effector spontaneous-Target spontaneous}}{\text{Target maximum-Target spontaneous}} \times 100\%$$

6. IFN- γ ELISPOT assay cytotoxic T-cell assays against BSCs

The stimulated cytotoxic T cells were incubated with 1×10^4 target cells at the ratio 2.5:1, 5:1, 10:1, 20:1 and 40:1 for 24 hours, and the release of IFN- γ was measured by commercial enzyme-linked immunospot assay (ELISPOT) kit (R&D Systems, Inc., Minneapolis, MN). The spot count and well areas covered of BSC-specific cytotoxic T lymphocytes (CTLs) in the stimulated cells was analyzed by CTL analyzers (Cellular technology Ltd., Shaker Heights, OH).

7. Survival and Statistical Analysis

Data were analyzed with a SPSS16.0 statistical software package (IBM Inc, Armonk, New York). Means of at least 3 independent experiments were reported with standard deviations. The estimated probability of survival was demonstrated

using the Kaplan-Meier method. Any P values less than 0.05 were considered statistically significant.

Results

1. Mammosphere growth of breast carcinoma-Derived Breast Cancer Stem-Like Cells and its expression of stem cells markers

Clinical, pathological information of the cases in this study are summarized in Table 1 and Figure 1. The breast cancer cells were obtained from 16 cases specimens and were seeded in DMEM/F12 (1:1) culture medium added growth factors. Some cases gradually formed smaller initial mammospheric cells after 48 hours culture. But the breast cancer cells of some cases cell gradually occurred apoptosis in the culturing process, some cells were adherent state of

differentiation. These mammospheric cells formed a larger breast balls (Figure 1A and 1B). These adherent cells were cultured in DMEM/F12 (1:1) culture medium added growth factors and fetal calf serum (FCS).

After suspended culture of breast cancer cells of 16 cases specimens, the mammospheric cells express CD44 and CD24 markers which were determined by flow cytometry. Only in part (6 cases, 37.5%) specimens detected a higher proportion of CD44+/CD24-/low cells. The 6 cases of specimens, four cases were Her-2(-), ER (-), PR (-), 1 cases were Her2 (+/++), ER (-), PR(-), 1 cases were HER-2(+), ER(+), PR(-). The figure 1 shown that the CD44+/CD24-/low percent of patient 7 was 97.69% in gated cells (Figure 1E), and patient 11 was 99.45% in gated cells (Figure 1H).

2.Comparison of stem cells markers between the mammospheric cells and the adherent cells

The CD44+/CD24- percent of adherent cells from adherent culture method and mammospheric cells from suspension culture method were 0.08% and 91.87% in gated cells (Figure 2C and 2F). However, the adherent cells highly expressed CD44+/CD24+ double positive cells (61.59%), the mammospheric cells didn't expressed CD44+/CD24+ double positive cells (0.77%).

By comparing CD133 expression level, we found that the mammospheric cells highly express CD133, but the adherent cells lowly express CD133. The adherent cells from patient 9 express 43.78% CD133 in gated cell (Fig 3C), but the mammospheric cells from patient express 96.67% CD133 in gated cell(Fig 3F). To study whether CSCs express certain TAAs, we measured the expression levels of several GBM-associated tumor antigens in both adherent cells and mammospheric cells by cell immune-fluorescence. We also found that both adherent cells express ER, PR and Her2, but mammospheric cells don't express these molecules (Data not shown).

3.Evaluation of tumorigenesis by in situ transplantation of breast cancer cells in vivo

The mammospheric cells and adherent cells were inoculated into NOD/SCID mice at 1×10^4 , 1×10^5 and 1×10^6 cell number, so we observed tumorigenicity, liver and lung metastases in vivo. The mice produced tumor by inoculating 1×10^4 mammospheric cells after 5 days, while mice inoculated adherent cells need to reach 1×10^6 and formed tumor after 7 days, and its tumorigenic capacity is much lower than the mammospheric group (2/10) (Table 2 and Figure 5A, 5B).

Table1: Summary of clinical, pathological information

Patients Number	Age at diagnosis	Location	Clinical classification	SBR scale	Pathological diagnosis
P1	34	Left breast	Breast infiltrative ductal carcinoma	III	HER-2(+++), ER(-), PR(-)
P2	56	Left breast	Breast infiltrative ductal carcinoma	II	HER-2(-), ER(-), PR(-)
P3	23	Right breast	Breast upper quadrant infiltrative ductal carcinoma	II	HER-2(+), ER(Low, 50-75%), PR(+ >90%)
P4	37	Right breast	Breast infiltrative ductal carcinoma	III	HER-2(+), ER(-), PR(-)
P5	46	Left breast	Breast infiltrative ductal carcinoma	II	HER-2(+), ER(+ 50%), PR(+)
P6	28	Right breast	Breast infiltrative ductal carcinoma	II	HER-2(+), ER(+ 50-75%), PR(+ >90%)
P7	34	Right breast	Breast infiltrative ductal carcinoma	III	HER-2(-), ER(-), PR(-)
P8	27	Right breast	Breast infiltrative ductal carcinoma	III	Her2(+/+), ER(-), PR(-)
P9	54	Left breast	Breast infiltrative ductal carcinoma	II	HER-2(-), ER(++), PR(+)
P10	43	Right breast	Breast infiltrative ductal carcinoma	II	HER-2(+), ER(+ 50%), PR(-)
P11	36	Left breast	Breast infiltrative ductal carcinoma	III	HER-2(-), ER(-), PR(-)
P12	68	Left breast	Breast infiltrative ductal carcinoma	II	HER-2(-), ER(+), PR(-)
P13	53	Right breast	Breast infiltrative ductal carcinoma	III	HER-2(+), ER(+), PR(-)
P14	31	Left breast	Breast infiltrative ductal carcinoma and infiltrating lobular carcinoma	II	HER-2(+), ER(+++), PR(++)
P15	23	Right breast	Breast infiltrative ductal carcinoma	III	HER-2(-), ER(-), PR(-)
P16	26	Left breast	Breast infiltrative ductal carcinoma	II	HER-2(++), ER(++ 50%), PR(++)

It showed that the enriched mammospheric cells have a stronger tumorigenic capacity by suspended culture, which is one of the characteristics of cancer stem cells. Furthermore, the mice grown tumor by inoculating mere 1×10^3 mammospheric cells after 7 days.

We also found that only one mouse appear lung metastasis in transplanting 1×10^6 adherent cells groups, but the mammospheric cells group have occurred liver and lung metastases in all three subgroups after 10 days (Table 2 and Figure 5C, 5D). It indicated that these enriched mammospheric cells have a strong invasive and metastatic capacity.

4. Vaccination with Dendritic Cells Loaded with Breast Cancer Stem-Like Cell Elicits Specific cytotoxic T lymphocytes in vitro

To determine the effect of DC vaccination using CSCs enriched in mammospheric cells, DCs isolated from PBMCs of a healthy donor were primed in vitro using autologous dendritic cells pulsed with mammospheric cells and adherent cells lysates respectively. The mammospheric cells were derived from patient 11, i.e. BSC-11 which express 70.54% CD44+/CD24- cells, and its adherent cells (BAC) were differentiated and obtained by adherent culture of BSC-11 mammospheric cells in DMEM/F12 (1:1) adding 10% FCS without growth factors. Both human mature DCs highly express costimulatory molecules

CD80, CD86, CD83 and CD40. The lymphocytes were restimulated with DCs every 5 days for up to 3 stimulations. The stimulated cells were then tested for their cytotoxic ability against the mammospheric cells (BSC) and adherent cells (BAC) by CTL cytotoxicity assay.

The CTL activities stimulated dendritic cells which were pulsed with the mammospheric cells lysates (BSC-CTL), the adherent cells lysates (BAC-CTL) and no antigens (Blank-CTL) were analyzed by killing the mammospheric cells (BSC) and adherent cells (BAC) respectively. With the increase of Effector:Target (E:T), the cytolysis against these tumor cell lines also continuously increased. It showed that the cytotoxicity of BSC-CTL was significantly higher than of Blank-CTL against the mammospheric cells (** $P < 0.01$) in Figure 4A. However, the cytotoxicity of BSC-CTL against the mammospheric cells hadn't significantly difference with against the adherent cells. Meanwhile Figure 4B also showed that the cytotoxicity of BSC-CTL was significantly higher than of BAC-CTL against the adherent cells was significantly higher than of against the mammospheric cells (** $P < 0.01$) in Figure 4C, and the cytotoxicity of BAC-CTL was significantly higher than of BSC-CTL against the adherent cells (* $P < 0.05$) in Figure 4D.

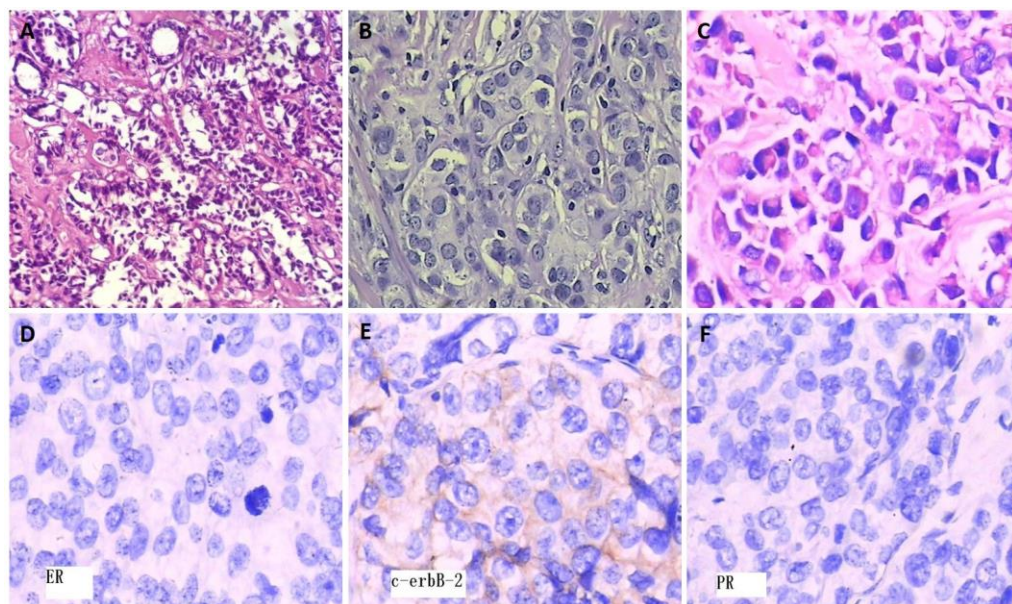


Fig1. The HE and immunohistochemical staining of clinical breast cancer samples. A. The HE staining of SBR III scale of breast infiltrative ductal carcinoma. B. The HE staining of SBR II scale of breast infiltrative ductal carcinoma. C. The HE staining of SBR II scale of Breast infiltrative ductal carcinoma and infiltrating lobular carcinoma. D, E, F. The immunohistochemical staining of SBR II scale of Breast infiltrative ductal carcinoma. D. Estrogen receptor (ER) negative expression in patient 7. E. human epidermal growth factor receptor 2 (HER2, or ErbB-2) negative expression in patient 7. F. Progesterone Receptor (PR) negative expression in patient 7.

Table.2 Tumorigenesis and metastasis of mammospheric and adherent cells in NOD/SCID mice

Tumor cells	Mammospheric cells			Adherent cells		
	1×10 ⁴	1×10 ⁵	1×10 ⁶	1×10 ⁴	1×10 ⁵	1×10 ⁶
In situ tumorigenesis	8/10	10/10	10/10	0/10#	0/10§	2/10#
Liver metastasis	3/10	5/10	6/10	0/10*	0/10#	0/10#
Lung metastasis	5/10	7/10	8/10	0/10*	0/10§	1/10§

Note: By comparison with the mammospheric cells groups, *P=0.05, #P=0.01, §P=0.001

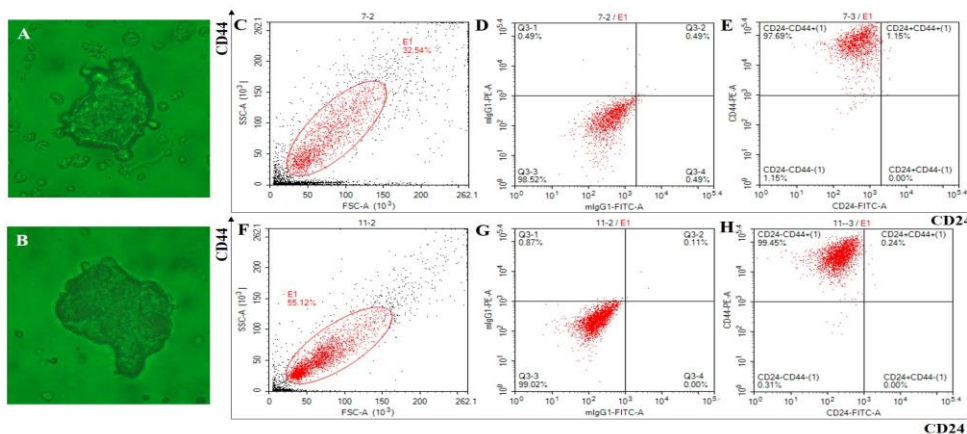


Fig2. Breast carcinoma-derived spheres are enriched in cells expressing cancer stem-like cell markers. (A, B): Breast carcinoma-derived cancer stem-like cells grow as mammospheres (patient 7 and 11). (C, D, E): The mammospheric cells express CD44 but don't express CD24 by Flow cytometry in patient 7. (F, G, H): The mammospheric cells express CD44 but don't express CD24 by flow cytometry in patient 11. Breast carcinoma -derived spheres were processed into single-cell suspension before fluorescence activated cell sorting analysis using antibodies against human CD44 and CD24. (D, G): The results were isotypic control by using mouse IgG1.

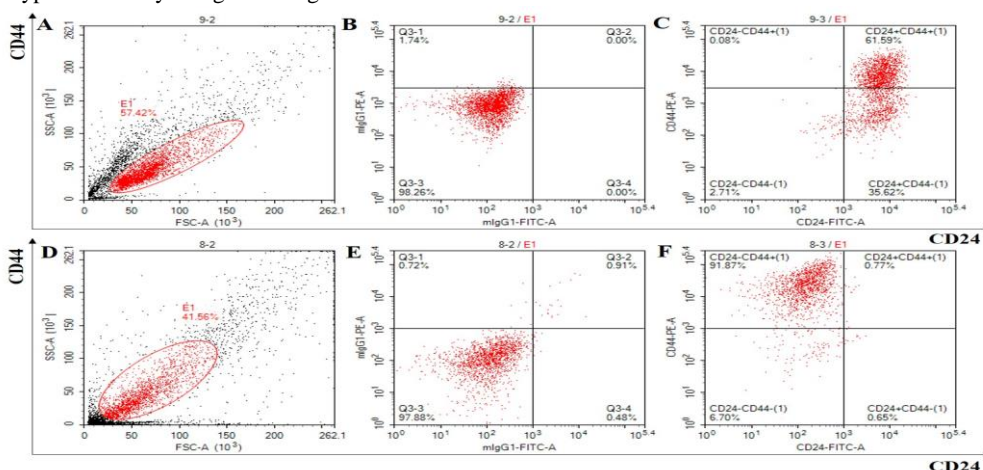


Fig3. The mammospheric cells express cancer stem-like cell markers by comparing with the adherent cells. (A, B, C): Breast carcinoma-derived adherent cells express both CD44 and CD24 by Flow cytometry in patient 9. (D, E, F): The mammospheric cells express CD44 but don't express CD24 by Flow cytometry in patient 8. Breast carcinoma-derived spheres were processed into single-cell suspension before fluorescence activated cell sorting analysis using antibodies against human CD44 and CD24. (B, E): The results were isotypic control by using mouse IgG1.

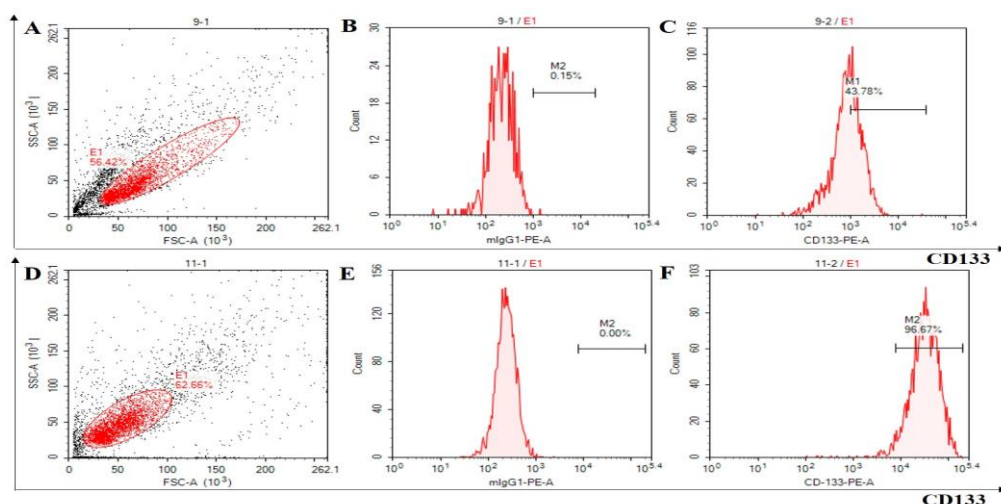


Fig4. The mammospheric cells express cancer stem-like cell markers by comparing with the adherent cells. (A, B, C): Breast carcinoma-derived adherent cells express CD133 lowly by Flow cytometry in patient 9. (D, E, F): The mammospheric cells express CD133 highly by Flow cytometry in patient 8. Breast carcinoma-derived spheres were processed into single-cell suspension before fluorescence activated cell sorting analysis using antibodies against human CD133. (B, E): The results were isotypic control by using mouse IgG1.

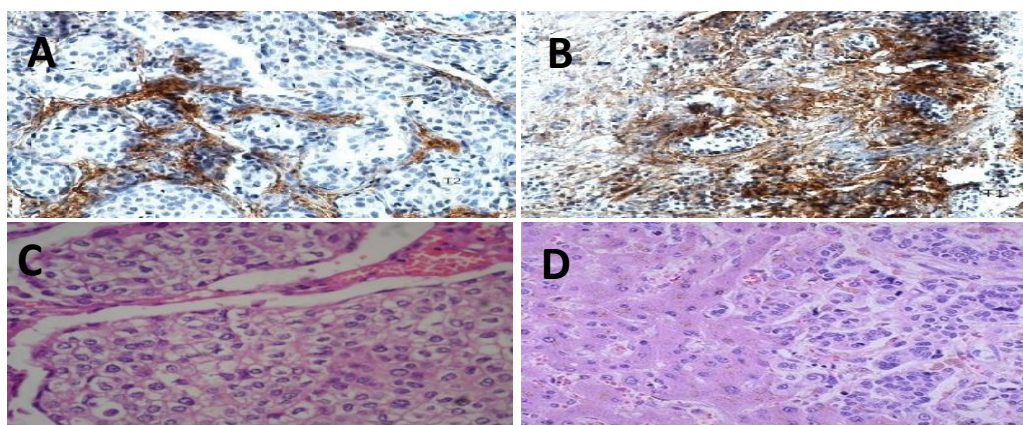


Fig5. The HE staining of tumorigenesis and metastasis of mammospheric and adherent cells in NOD/SCID mice. A. The HE staining of in situ tumorigenesis of mammospheric cells in NOD/SCID mice in situ. B. The HE staining of tumorigenesis of adherent cells in NOD/SCID mice. C. The HE staining of lung metastasis of mammospheric cells in NOD/SCID mice. D. The HE staining of hepatic metastasis of mammospheric cells in NOD/SCID mice.

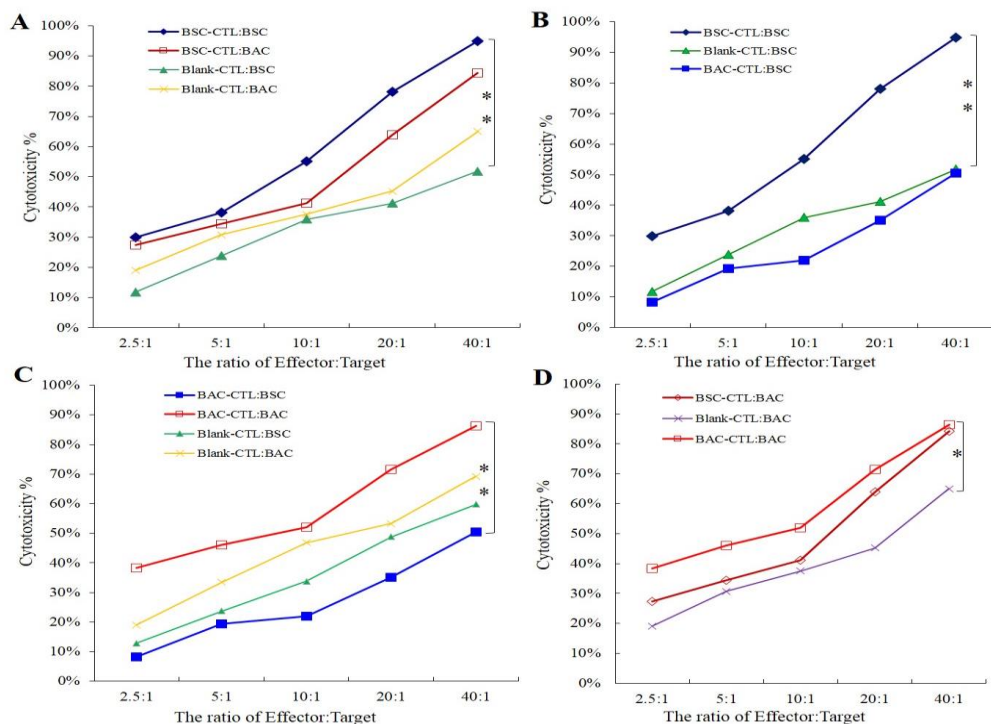


Fig6. The cytotoxic activities of dendritic cells activated cytotoxic T lymphocytes (CTL) against the mammary cells and adherent cells by pulsing with ammospheric and adherent cells lysates respectively. (A): The cytotoxic activities of dendritic cells against the mammary cells (BSC) and adherent cells (BAC) by pulsing with the ammospheric cells lysates (BSC-CTL) and no antigens (Blank-CTL) respectively. The cytotoxicity of BSC-CTL was significantly higher than of Blank-CTL against the mammary cells (** $P < 0.01$). (B): The cytotoxic activities of dendritic cells against the mammary cells (BSC) by pulsing with the ammospheric cells lysates (BSC-CTL) and the adherent cells lysates (BAC-CTL) respectively. The cytotoxicity of BSC-CTL was significantly higher than of BAC-CTL against the mammary cells (** $P < 0.01$). (C): The cytotoxic activities of dendritic cells against the mammary cells (BSC) and adherent cells (BAC) by pulsing with the adherent cells lysates (BAC-CTL) and no antigens (Blank-CTL) respectively. The cytotoxicity of BAC-CTL against the adherent cells was significantly higher than of against the mammary cells (** $P < 0.01$). (D): The cytotoxic activities of dendritic cells against the adherent cells (BSC) by pulsing with the ammospheric cells lysates (BSC-CTL) and the adherent cells lysates (BAC-CTL) respectively. The cytotoxicity of BAC-CTL was significantly higher than of BSC-CTL against the adherent cells (* $P < 0.05$).

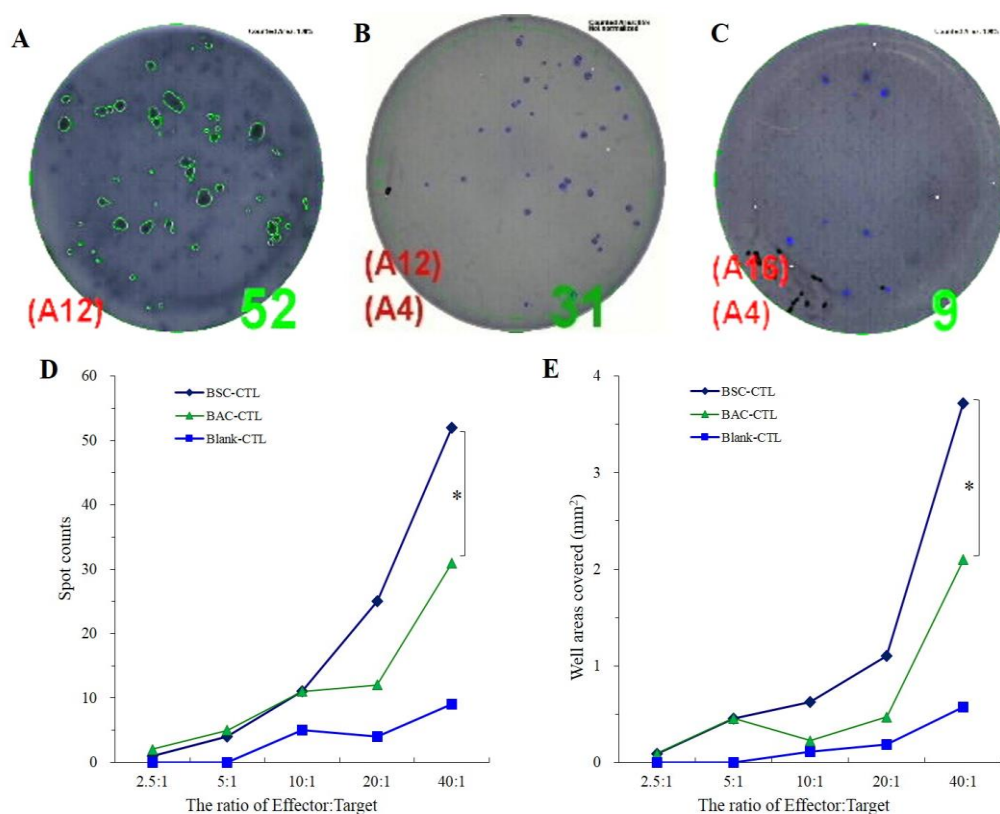


Fig 7. Results of the IFN- γ ELISPOT assay against the mammopheric cells (BSC) following CTL activated DCs pulsed with the ammopheric cells lysates(BSC-CTL) and the adherent cells lysates(BAC-CTL) respectively. (A, B, C): Spots were produced by BSC-CTL, BAC-CTL and blank-CTL against the mammopheric cells at E:T=10:1by the IFN- γ ELISPOT assay respectively. (D): The IFN- γ ELISPOT results of BSC-CTL were avidently higher than BAC-CTL by spot counts (* $P < 0.05$). (E): The IFN- γ ELISPOT results of BSC-CTL were avidently higher than BAC-CTL by well areas covered (* $P < 0.05$).

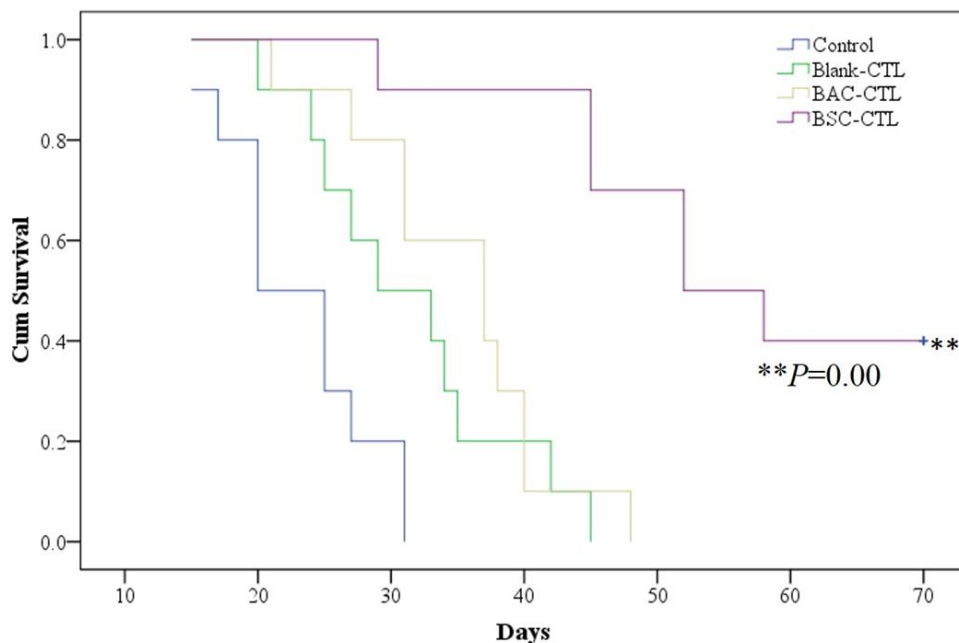


Figure 8. Therapeutic effect of cytotoxic T cells (CTL) activated mammosphere-pulsed dendritic cells (DCs) against tumor in NOD/SCID mice by transplanted the mammospheric cells in situ. Mice were injected subcutaneous breast fat pad with 1.0×10^4 the mammospheric cells on day 0 and were vaccinated s.c. on days 7, 14, and 21 with CTL activated different tumor antigen-pulsed dendritic cell vaccines: control (no antigens), adherent cells, and mammospheric cells ($n=10$ for each group). Kaplan-Meier survival curve showed that mice treated with CTL activated mammospheric cells lysate-pulsed DCs have longer survival than the other groups (** $P=0.00$). The surviving animals with cancer stem-like cell vaccination were sacrificed after 70 days and slowly growing brain tumors were detected.

The cytotoxicity of BAC-CTL against the adherent cells also hadn't significantly difference by comparing with BSC-CTL. The results suggested that superior immune responses after vaccination with DCs pulsed with BSC are likely due to improved target cell recognition by antigen-specific CTLs.

5. IFN- γ ELISPOT assay of Specific cytotoxic T lymphocytes Elicited Dendritic Cells Loaded with Breast Cancer Stem-Like Cells

As shown in Figure 5, BSC lysates-loaded DCs stimulated Th1 response and induced significant IFN- γ production. Next, we tested the effect of DC vaccination using

mammospheric cells and adherent cells lysates as antigens. Stimulation of PBMCs using each of the two breast cancer cells antigens induced significant T-cell responses as indicated by robust IFN- γ production in response to analysis stimulation (Fig. 5A, 5B). The spot counts was 52 and 31 spots which were produced in BSC-CTL and BAC-CTL against the mammospheric cells at E:T=10:1 respectively. The IFN- γ production of BSC-CTL were evidently higher than of BAC-CTL by spot counts (* $P < 0.05$) in Figure 5D. Moreover the IFN- γ ELISPOT results of BSC-CTL were evidently higher than BAC-CTL by well areas covered (* $P < 0.05$) in Figure 5E. The IFN- γ production of both BSC-CTL and BAC-CTL were evidently higher than Blank-CTL at spot counts and well areas covered respectively (* $P < 0.01$ and * $P < 0.05$) (Figure 5D and 5E). Moreover, with the increase of Effector: Target (E: T), spot counts and well areas covered of IFN- γ ELISPOT also enhanced gradually. DC vaccination induced significant IFN- γ production which is positively correlated with the number of cytotoxic T lymphocytes (CTLs) stimulated. Distinctly, IFN- γ ELISPOT suggested that DC-induced IFN- γ production likely resulted from activation of antigen-specific CTLs, so stimulated Th1 response.

6. DC Vaccination Using Breast Cancer Stem-Like Cell Prolongs Survival in a BSC Breast Tumor Model

To investigate whether DC vaccination targeting BSCs may induce antitumor immunity in vivo and improve survival for tumor-bearing animals, we established a BSC breast cancer model for DC vaccination studies. BSCs were isolated and cultured by non-serum DMEM/F12 (1:1) medium from breast cancer tissue of patient 11 and demonstrated that BSC-11 express 70.54% CD44+/CD24- cells and can initiate invasive and metastatic breast carcinoma in vivo tumorigenesis above. Breast cancer adherent cells (BAC) were differentiated and obtained by adherent culture of BSC-11 mammospheric cells in DMEM/F12 (1:1) adding 10% FCS without growth factors. DCs were isolated from a healthy donor, and were pulsed with BSC-11, its adherent cells lysate and didn't pulsed. The cytotoxic T lymphocytes were restimulated with DCs every 5 days for up to 3 stimulations and used as immunotherapy of mice bearing BSC breast carcinoma. Animals bearing BSC breast carcinoma were vaccinated by tail intravenous injection on days 7, 9, and 11 after tumor implantations. Mice bearing BSC breast carcinoma vaccinated with CTL stimulated no antigens-pulsed DC all expired, with a median survival of 31.4 days. Mice vaccinated with CTL stimulated DCs pulsed with BAC cells lysate also expired, with median survival dates of 35 days, respectively. In contrast, Mice vaccinated with CTL stimulated

DCs pulsed with BSC cells lysate had a median survival of 56.1 days when survival was monitored up to 70 days after inoculation, at which point 40% of the mice were still alive (Fig.6). Kaplan-Meier survival curve showed that mice treated with CTL activated mammospheric cells lysate-pulsed DCs have longer survival than the other groups (**P=0.00). The data demonstrate that DC immunotherapy could induce specific immune response targeting cancer stem-like cells and significantly prolong survival in a mice breast carcinoma model, suggesting that immunotherapy selectively targeting cancer stem cells could be a novel effective strategy to treat malignant breast carcinoma patients.

DISCUSSION

Large numbers of proofs had supported the notion that a small subpopulation of breast cancer stem-like cells is responsible for cancer progression [15], therapy resistance [16], and relapse [17]. The identification and classification of stem and progenitor cell lineages in breast cancer remains under development. In the current study, we isolated and evaluated human BSCs derived from breast cancer patients by non-serum culture, so these BSCs can mammospheric growth and be enriched. The enriched mammospheric cells over-express high levels of stem cell-associated molecules, more than 90% CD44+/CD24- and CD133+ at gated cells, but breast cancer

adherent cells(BACs) don't express CD44+/CD24-/Low, are CD44+/CD24+ and low CD133+. The tumorigenic characteristic of enriched mammospheric cells is significant difference with BACs in vivo, and have a stronger tumorigenic capacity than adherent cells in vivo tumorigenesis. The tumorigenic capacity of mammospheric cells merely need mere 1×10^3 in SCID mice. Moreover, We still found that the mammospheric cells group have occurred liver and lung metastases in all three orders of cells magnitude, but only one mouse appear lung metastasis in transplanting 1×10^6 adherent cells groups. It explained that these enriched mammospheric cells have a strong invasive and metastatic capacity. The reason is many research reports that CD44+/CD24-/Low cells with breast stem/progenitor cells properties, which were able to form new tumors when as few as 103 cells were injected into SCID mice [18, 19]. In addition, The BSCs highly express CD133+ phenotype which have the capacity of transdifferentiation and contributed to vasculogenic mimicry (VM) in triple-negative breast cancer [3]. Sunitinib induced hypoxia in TNBCs, and Twist1 expression induced by hypoxia accelerated VM by increasing population of CD133+ cells [20]. VM was pivotal factor and responsible for invasive and metastatic capacity of breast cancer.

To inhibit breast cancer relapse more

effectively, it would require any cancer therapy urgently, including immunotherapy, to target breast cancer stem cells. There have been some research work to develop treatments by targeting BSCs at present. Liu P et al. found that Liposome encapsulated Disulfiram blocked NF- κ B activation and specifically targeted the CSC population in vitro and in vivo and showed very strong anticancer efficacy [21]. At targeting other cancer stem cells, some study has applied DC as a tool to target CSCs. By vaccination with DCs pulsed with the lysate of glioma stem cells, primed T cells were capable of selective targeting CSCs [12, 22]. DCs pulsed with ALDH (high) CSC lysates induced significantly higher protective antitumor immunity than DCs pulsed with the lysates of unsorted whole tumor cell lysates in both melanoma and squamous cancer models [23]. Here we explored the suitability of BSCs antigens for DC vaccination against human BSCs. The results showed that the CTL cytotoxicity stimulated dendritic cells pulsed with the mammospheric cells lysates (BSC-CTL) was significantly higher than of no antigens-pulsing DCs stimulated CTL (Blank-CTL) and adherent cells-pulsing DCs stimulated CTL (BAC-CTL) against the mammospheric cells (**P<0.01 and **P<0.01 respectively). But the cytotoxicity of BSC-CTL against the mammospheric cells hadn't significantly difference with against the adherent cells. The cytotoxicity of

BAC-CTL against the mammospheric cells also hadn't significantly difference with Blank-CTL. Meanwhile we also found that the cytotoxicity of BAC-CTL against the adherent cells was significantly higher than of against the mammospheric cells (**P<0.01), and the cytotoxicity of BAC-CTL was significantly higher than of BSC-CTL against the adherent cells (*P<0.05). But the cytotoxicity of BAC-CTL against the adherent cells hadn't significantly difference by comparing with BSC-CTL. The results suggested that superior immune responses after vaccination with DCs pulsed with BSC stimulate Th1 response and are likely due to improved target cell recognition by antigen-specific CTLs.

Established CTL clones were analyzed using the IFN- γ ELISPOT assay, tumor antigen-loaded DCs induced significant IFN- γ production [24]. The study showed that DC vaccination using BSC lysates can induced significant IFN- γ production which is positively correlated with the number of cytotoxic T lymphocytes (CTLs) stimulated. With the increase of Effector:Target (E:T), spot counts and well areas covered of IFN- γ ELISPOT also enhanced gradually. BSC-CTL and BAC-CTL produced 52 and 31 spots against the mammospheric cells at E:T=10:1 respectively. The IFN- γ production of BSC-CTL were evidently higher than of BAC-CTL by spot counts

and well areas covered (* $P < 0.05$ and * $P < 0.05$ respectively) The IFN- γ production of both BSC-CTL and BAC-CTL were evidently higher than Blank-CTL at spot counts and well areas covered respectively (* $P < 0.01$ and * $P < 0.05$). DC-induced IFN- γ production likely resulted from activation of antigen-specific CTLs, so stimulated Th1 response.

For further exploring the possibility of targeting BSCs in DC vaccination, we build on our extensive experience of DC vaccination for human breast cancer patients by using both human samples and a syngeneic animal breast cancer model. In BSC breast cancer models, mice vaccinated with Blank-CTL and BAC-CTL all expired, with a median survival of 31.4 days and 35 days respectively. In contrast, Mice vaccinated with BSC-CTL had a median survival of 56.1 days when survival was monitored up to 70 days after inoculation at 40% mice. Kaplan-Meier survival curve showed that mice treated with CTL activated mammospheric cells lysate-pulsed DCs have longer survival than the other groups (** $P = 0.00$). It demonstrated that vaccination with CTL stimulated DCs pulsed with enriched mammospheric cells lysates prolonged survival in animals bearing BSC breast cancer tumors. Two recent studies using animal models demonstrated the potential of DC vaccination targeting CSCs in cancer immunotherapy [25, 26]. The MHC-non-restricted immune cells, such as

macrophages and NK cells. So, these cells, the only potentially able to recognize and eliminate MHC-non-expressing stem tumor cells [25]. Prostate stem cell antigen vaccinated TRAMP mice had a 90% survival rate at 12 months of age. In contrast, all control mice had succumbed to prostate cancer or had heavy tumor loads [26]. Several reports have described the killing of CSCs via nonspecific immune effector cells, such as natural killer cells [27] and $\gamma\delta$ T lymphocytes [28].

In summary, we explored the suitability of BSCs as sources of antigens for DC vaccination against breast cancer, with the aims of achieving BSC-targeting and better antitumor immunity. We found that CD44+/CD24-/CD133+ mammospheric cells have stem cells property and strong tumorigenic function. DC vaccination using BSC antigens elicited a potent antigen-specific Th1 response that activated antigen-specific CTLs and induced IFN- γ production. Therefore, DC immunization with BSCs produces superior antitumor immunity may help develop novel and more effective cancer immunotherapies.

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