# Synergistic anti-tumor effects of dasatinib and dendritic cell vaccine on metastatic breast cancer in a mouse model

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**Abstract.** Immunotherapy is currently considered as one of the major anti-tumor modalities, but its efficacy is limited. Dasatinib could improve the expansion and recruitment of cluster of differentiation (CD) $8^+$T cells and natural killer (NK) cells to the tumor microenvironment. The present study aimed to evaluate the synergistic anti-tumor effects of dasatinib with dendritic cell (DC) vaccine in metastatic breast cancer. Dasatinib with DC vaccine was administered to mice inoculated with 4T1 breast cancer cells. Thereafter, tumor volume was measured every other day. On day 34, lung metastasis was assessed with a stereomicroscope. Tumor proliferation and angiogenesis were determined by immunohistochemistry. Apoptosis in tumor tissues was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling. The results showed that although there were no significant differences in tumor volumes between the untreated control, DC vaccine and dasatinib groups, the tumor volume was significantly decreased in the combined treatment group compared to the other three groups. Mice in the combined treatment group showed the longest survival time, while mice treated with either single treatment had a slightly increased survival time compared to the untreated control mice. Additionally, the number of metastatic lung nodules was significantly decreased in combined treatment group compared with the dasatinib alone, DC vaccine alone and untreated control groups. Furthermore, the combined treatment group showed significantly reduced intratumoral microvessel density compared to the other three groups. In addition, the ratios of $CD8^+$ T and NK cells were significantly increased in the combined treatment group compared with the other three groups. These results suggest that dasatinib combined with the DC vaccine is a possible modality for the treatment of metastatic breast cancer.

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*Abbreviations:* DC, dendritic cell; MDSC, myeloid-derived suppressor cell; Tregs, regulatory T cell; SFK, SRC family kinase

*Key words:* breast cancer, dasatinib, dendritic cell, vaccine, lung metastasis

## Introduction

Breast cancer remains the most common malignant tumor in women worldwide and distant metastases is a notable feature of advanced breast cancer (1,2). Despite recent advances in early diagnosis and multidisciplinary therapeutic management, metastatic breast cancer remains challenging to successfully treat (3,4).

Dasatinib is a potent orally-administered multi-target kinase inhibitor that inhibits several kinases, including breakpoint cluster region protein-Abelson murine leukemia viral oncogene homolog, SRC family kinase (SFK), c-KIT and platelet-derived growth factor receptor-β, and has been approved for use in patients with imatinib-refractory leukemia (5,6). SFKhasa key role in numerous cellular signaling pathways that not only modulate the behavior of tumor cells, but also regulate immune effector cells (7-11). For example, Christiansson *et al* (11) found that dasatinib improved the expansion of cluster of differentiation (CD) $8^+$T cells and natural killer (NK) cells in chronic myelogenous leukemia patients. Additionally, patients with NK/T cell lineage lymphocytosis tend to exhibit preferable clinical effects compared with patients without lymphocytosis, indicating that the anti-tumor effect is due to activated immune responses (12). However, available phase I and phase II clinical trial data have confirmed only limited benefits from the use of single-agent dasatinib in diverse types of breast cancer, and the overall response rate was only 4.7% in triple-negative breast cancer (13-16).

Due to the unique ability to initiate and boost powerful anti-tumor T cell responses, *ex vivo*-generated dendritic cells (DCs) are essential for immunotherapy to effectively kill the malignant cells. Notably, dasatinib enhanced the therapeutic efficacy of the DC vaccine *in vitro* and *in vivo* (8,17-19). Nerreter *et al* (18) reported that dasatinib enhanced the

migration of DCs without changing the ability to prime and boost antigen-specific T cell response by reducing the phosphorylation of inhibitory immune receptor siglec-9 and siglec-3 *in vitro* using human peripheral blood mononuclear cell-derived DCs. Lowe *et al* (8) revealed that dasatinib combined with the DC vaccine significantly reduced tumor volume, with enhanced recruitment of $CD8^+$T cells and DCs to tumor-draining lymph nodes and the tumor microenvironment in M05 melanoma mouse models. However, the combined the effect of dasatinib and DC-based vaccine on advanced breast cancer is unknown.

In the present study, a murine 4T1 orthotopic model was established to mimic human stage IV breast cancer, and the synergistic anti-tumor effects of dasatinib and DC vaccine on metastatic breast cancer were evaluated using this model.

## Materials and methods

*Cell culture*. The breast cancer 4T1 cell line was obtained from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 Medium (Hy Clone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal calf serum (FCS; Tianjin Haoyang Biological Products Technology Co., Ltd., Tianjin, China) at 37˚C.

*Mice*. A total of 45 Balb/cmice (32 female and 13 male; 6 weeks old; weight 20±2 g) were provided by Animal Center, Chinese Academy of Medical Sciences (Beijing, China) and fed with *ad libitum* access to purified water and a commercial stock diet which was changed regularly (renewed and added every 12 h). All mice were kept in a pathogen-free environment at a temperature of 21±1˚C with a 12:12 h light: dark cycle and maintained under a relative humidity of 50±10%. All procedures involving animals were approved by the Ethics Committee of Tianjin Medical University (Tianjin, China).

*4T1 cell lysate*. For the preparation of cell lysate, 4T1 cells were washed with PBS three times and the cell concentration was adjusted as $5x10^7$ cells/ml. 4T1 cells were lysed by five freeze-thaw cycles accomplished by 15 min liquid nitrogen submersions followed by 5 min water bath incubations at 55˚C. The lysates were centrifuged at 1,800 x g for 15 min to remove the cell debris at 4˚C. The lysate was aliquoted and stored at -80˚C until use.

*Tumor lysate-pulsed DCs*. Mouse bone marrow-derived cells (BMDCs) were generated from bone marrow precursors. In brief, bone marrow cells were flushed from the femurs and tibias of male BALB/c mice, and subsequently washed and counted. Cells were plated at a concentration of $2x10^6$ cells/100-mm Petri dish in 10 ml RPMI-1640 medium supplemented with 10% FCS, 20 ng/ml murine recombinant granulocyte-macrophage colony-stimulating factor and 10 ng/ml murine recombinant interleukin (IL)-4 (PeproTech, Rocky Hill, NJ, USA) in a humidified incubator (5% $CO_2$ and 37˚C). On days 3 and 6, the media was refreshed. On day 7, 100 $\mu$l cell lysate of 4T1 cells (DC: 4T1, 1:3) was added to the medium and incubated for 18 h. To obtain mature DCs, 1 $\mu$l/ml lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to the medium and cultured for 1-2 days at 37˚C.

*Tumor model and treatment protocol*. In total, $1x10^6$ 4T1 cells were injected subcutaneously into the right mammary gland of 32 BALB/cfe male mice on day 0. On day 10, the animals were randomized into 4 groups (n=8). To find the optimal dose of dasatinib in improving $CD8^+$ T cell recruitment to the tumor microenvironment, a pre-experiment was performed according to Lowe *et al* (8) (data not shown). Dasatinib (15 mg/kg; Selleck Chemicals, Houston, TX, USA) was solubilized in 50 $\mu$l Labrasol [Gattefosse (Shanghai) Trading Co., Ltd., Shanghai, China]. Mice in the dasatinib group were administered with 15 mg/kg dasatinib by daily oral gavage for 7 consecutive days, beginning on day 10. Mice in the DC vaccine group were injected with $1x10^6$ 4T1 lysate-pulsed dendritic cells in 50 $\mu$l PBS in the right mammary gland surrounding the tumor on days 10 and 17. Mice in the control group were left untreated. The combined treatment group received both dasatinib and DC vaccine, as aforementioned. Tumor size was measured every other day in two dimensions using Vernier calipers, and tumor volume were calculated using the formula, $ab^2/2$, where b is the smaller dimension. On day 34, Mice were sacrificed by cervical dislocation. The primary tumors, lungs, livers and spleen were excised, weighed and breast pulmonary nodules were counted using a stereomicroscope (Nikon, Tokyo, Japan).

*Histology and immunohistochemistry*. For histological analysis, primary tumor, lung and liver tissue sections were collected and fixed in 10% formalin for 24 h at room temperature. Subsequent to being embedded in paraffin, specimens were cut into 5 $\mu$m sections and were stained with hematoxylin and eosin (H&E) for 20 min at room temperature. For immunohistochemistry, the tumor sections were prepared in the same manner as the tissue used for H&E staining. Following antigen retrieval (10 mmol/lsodium citrate buffer, pH 6.0; microwave 600 W, 10 min) all tumor slides were blocked with 5% bovine serum albumin (Boster Biological Technology, Pleasanton, CA, USA) for 1 h at room temperature and stained with rabbit anti-mouse natural-killer group 2, member D (NKG2D), CD8, KI67 or CD31 antibodies (cat nos. bs-0938R, bs-10699R, bs-23105R and bs-20322R, respectively; Bioss, Beijing, China) at a dilution of 1:200 at 4˚C overnight, in accordance with the standard avidin-biotin-peroxidase complex staining procedure. Then, avidin-biotin-peroxidase conjugated goat anti-rabbit IgG (cat no. SA1022; Boster Biological Technology; dilution, ready-to-use) was added for 20 min at 25˚C. Finally, diaminobenzidine (Boster Biological Technology) was used for staining for 10-30 min at room temperature. The uniform fields from each section were selected and analyzed by two independent pathologists in a blind manner. Ki67, CD8 and NKG2D-positivecells were counted in five randomly-selected fields from three separate sections at a magnification of x400 under a light microscope (TE200-U; Nikon), with data presented as the percentage of the total number of tumor cells. Microvessel density was counted using five fields per tumor following the criteria described by Weidner *et al* (20).

*Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)*. To assess apoptosis, tumor tissues in different

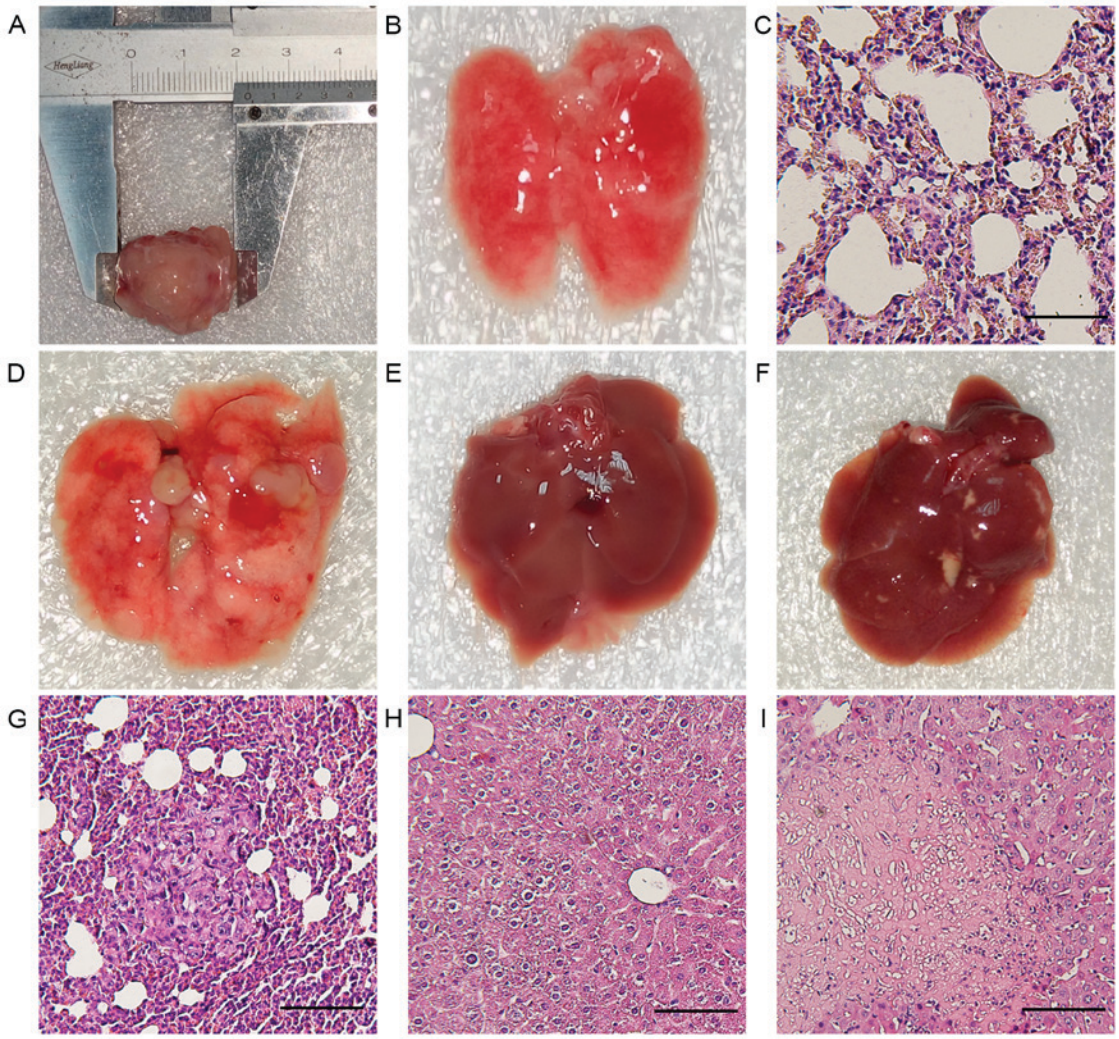


Figure 1. Tumorigenesis and metastasis formation in 4T1 tumor-bearing mice. (A) Primary breast carcinoma extracted from a 4T1 tumor-bearing control mouse. (B and C) Gross and microscopic morphology of lungs extracted from a healthy control mouse. (D) Gross morphology of lungs extracted from a 4T1 tumor-bearing control mouse showing numerous metastatic nodules. (E) Gross morphology of livers extracted from a 4T1 tumor-bearing control mouse. (F) Only one 4T1 tumor-bearing control mouse presented with liver macroscopic yellow necrosis.(G) Microscopic morphology of lungs extracted from a 4T1 tumor-bearing control mouse confirming numerous metastatic nodules. (H) Microscopic morphology of livers extracted from a 4T1 tumor-bearing control mouse. (I) Microscope morphology of livers extracted from a 4T1 tumor-bearing control mouse confirming necrosis. Scale bars, 30 $\mu$m.

groups were subjected to TUNEL assay using the *In Situ* Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. Cells stained brown were considered positive for apoptosis. At least 10 fields at a magnification of x400 were randomly selected under a fluorescent microscope, and the total cells were counted in each field. The percentage of apoptotic positive cells was calculated as the number of apoptotic positive cells/total cells.

*Statistical analysis*. Data were expressed asthemean± standard deviation. GraphPad Prism 6.01 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. Statistical significance of differences between two cohorts was analyzed by Student's t test. Statistical significance of differences between three cohorts was analyzed by one-way analysis of variance followed by Student-Newman-Keuls post hoc test. Log-rank (Mantel-Cox) test was used to compare survival curves. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Dasatinib and 4T1-antigen-loaded DC vaccine reduced tumor growth in mice synergistically*. First tumorigenesis and metastasis formation of 4T1 cells were examined *in vivo*. After 3 weeks, mice developed primary metastatic breast carcinoma (Fig. 1A). The anatomy revealed the presence of numerous macroscopic metastatic foci in all cases. To determine whether the lung nodules were from 4T cells, H&E staining was performed on lung tissues (Fig. 1D and G). In addition, liver tissue was examined by macroscopic and H&E staining analysis. All livers showed no metastases (Fig. 1E and F), but one liver showed macroscopic necrosis (Fig. 1H and I).

To determine whether the combination regime improved the inhibition of tumor growth, tumor-bearing mice were treated with dasatinib and DC-based vaccine. No significant differences were observed in tumor volumes between the untreated control, DC vaccine and dasatinib groups, but tumor volumes were significantly decreased in the combined

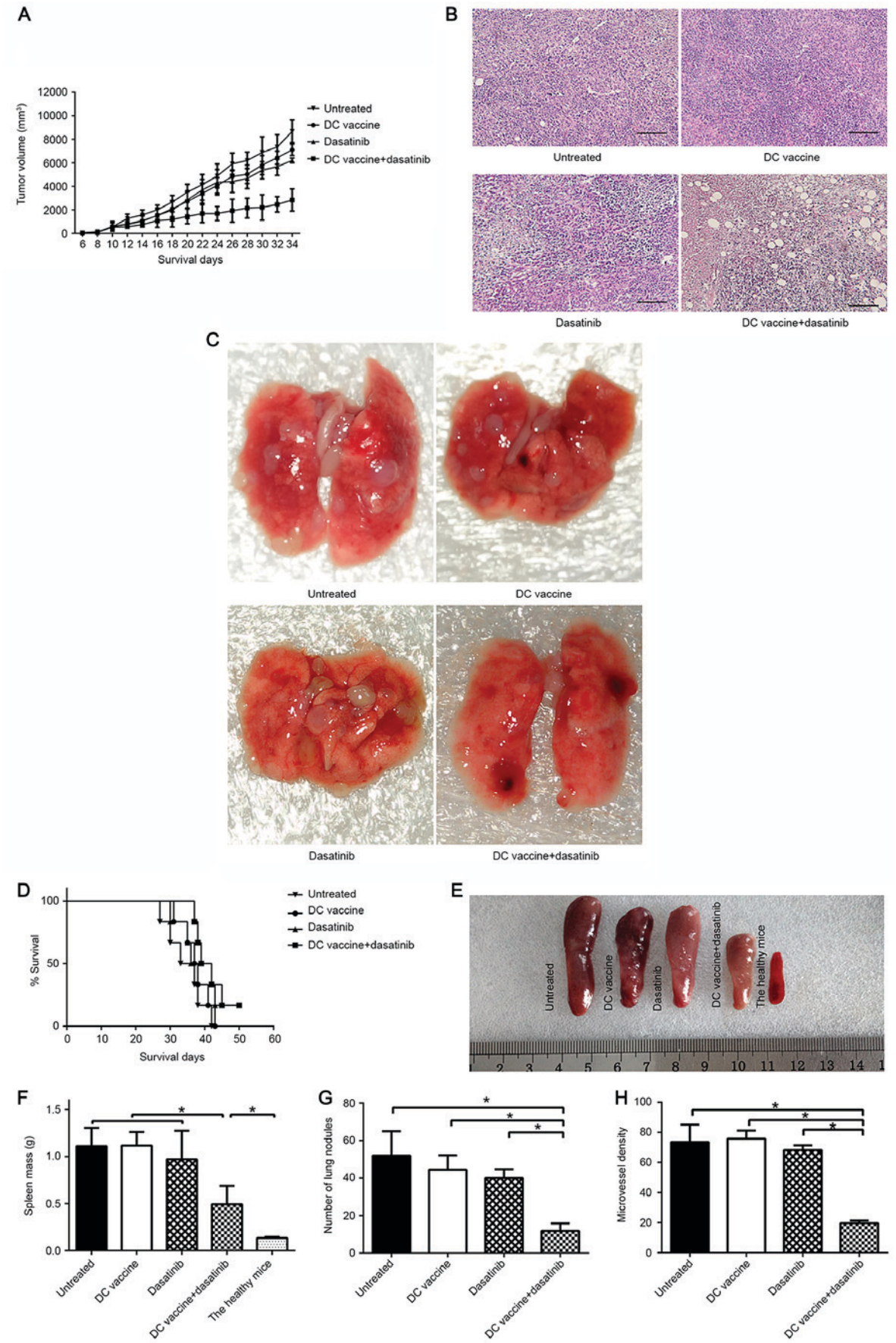


Figure 2. The inhibition of tumor growth, intratumoral angiogenesis and lung metastasis in mice subsequent to different treatments. (A) Tumor volume of the 4T1 breast tumor model was measured every 2 days. Values are presented as the mean ± standard deviation. (B) H&E staining of the tumors of each group. Scale bars, 60 $\mu$m. (C) Representative gross lung image of each group. (D) Kaplan-Meier plot showing the survival rate of the indicated group (n=8 per group). (E) Representative spleen images of each group. (F) All spleen were harvested and weighed. (G) Quantification of lung nodules in each group. (H) Microvessel density was assessed using five fields per tumor. $^{*}$P<0.05. DC, dendritic cell; H&E, hematoxylin and eosin.

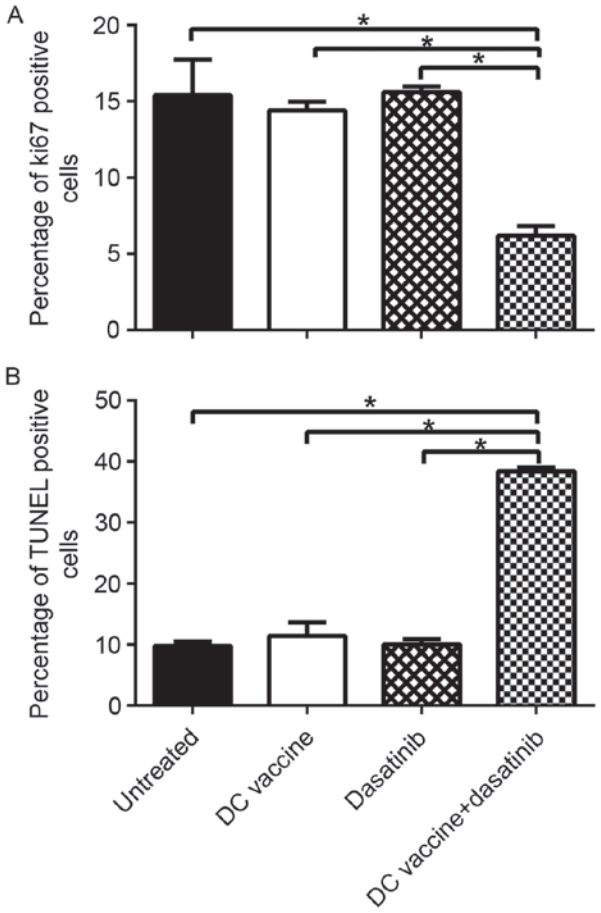


Figure 3. Changes in the proliferation and apoptosis of 4T1 tumor cells after different treatments. Immunohistochemical analysis of Ki67 in each group of tumors. (A) Ki67-positive cells in each group of tumors were measured. Immunohistochemical analysis of apoptosis in each cohort of tumors. (B) TUNEL positive cells in each group of tumors were measured. *P<0.05. DC, dendritic cell; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

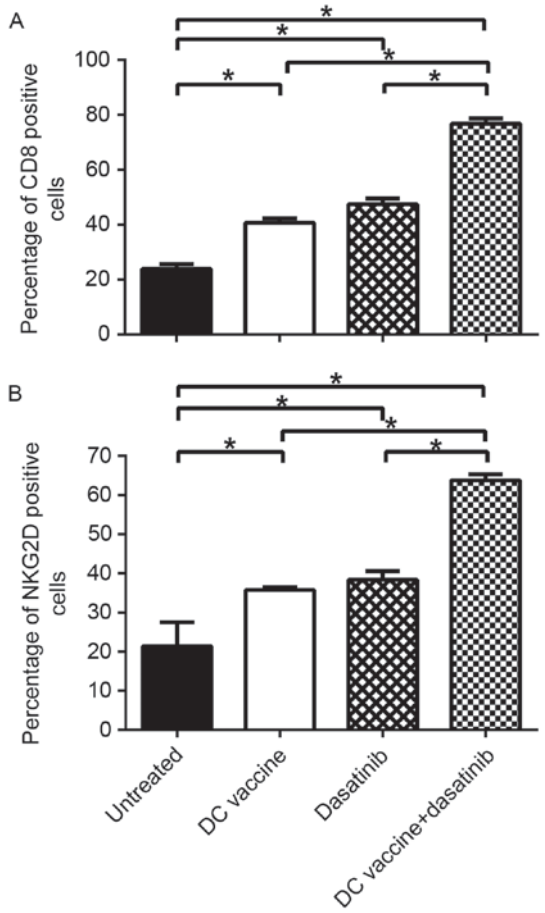


Figure 4. Alterations in the number of $CD8^+$ T cells and NK cells subsequent to different treatments. $CD8^+$ T cells and NK cells in each cohort were identified by standard immunohistochemistry and the ratio of (A) $CD8^+$ T cells and (B) NK cells were examined in all tumors. *P<0.05. CS, cluster of differentiation; NK, natural killer; DC, dendritic cell; NKG2D, natural-killer group 2, member D.

treatment group compared to the other three groups (Fig. 2A; P<0.05 vs. all other groups after 22d tumor-inoculation). H&E staining of tumor tissue revealed irregular, disrupted tumor cells with large, blue-black nuclei (Fig. 2B). The spleen were excised and weighed on day 34 (Fig. 2E). The spleen mass was significantly decreased in the combined group or healthy control group compared to other groups (Fig. 2F; P<0.05). Mice treated with dasatinib combined with DC vaccine showed the longest survival time, and mice treated with either single treatment lived slightly longer compared with untreated control mice (non-significant; P>0.05); however, these differences were not statistically significant (Fig. 2D).

*Combination of dasatinib and DC vaccine elicited superior inhibition on metastatic lung nodules and intratumoral angiogenesis*. The mice in all treatment groups were sacrificed prior to lung nodule quantification. The incidence of lung metastases in all four groups was the same, with 100% of mice developing lung metastases. However, the number of metastatic lung nodules was significantly decreased in the combined treatment group compared with the dasatinib alone, DC vaccine alone and untreated control groups (Fig. 2C and G; P<0.05). Furthermore, immunohistochemistry of tumor sections was performed using CD31 as an indicator of angiogenesis. It was found that microvessel density was slightly decreased in the dasatinib alone or DC vaccine alone groups compared to the untreated control group. By contrast, the combined treatment group showed significantly reduced inhibition of intratumoral microvessel density compared to the other three groups (Fig. 2H; P<0.05).

*Dasatinib and DC vaccine inhibited the proliferation and enhanced the apoptosis of 4T1 tumor cells synergistically*. To assess the inhibition of proliferation and the induction of apoptosis after different treatments, immunohistochemistry of Ki-67, as a maker of proliferation, and TUNEL assay, as an indicator of apoptosis, were performed. DC vaccine group, dasatinib group and combination group exhibited decreased Ki-67 expression level related to untreated control group (Fig. 3A) (P<0.05). However, only the combined treatment exhibited a significantly increased inhibitory effect on proliferation compared to the untreated control. Furthermore, TUNEL assay showed that apoptotic cells were significantly increased in mice of combined treatment group compared to untreated control group or either single treatment group (Fig. 3B; P<0.05).

*Combination of dasatinib and DC vaccine enhanced the expansion of CD8$^+$ T cells and NK cells in the tumor microenvironment*. Previous studies have indicated that the antitumor effect of dasatinib is largely dependent on the expansion of CD8$^+$T cells and NK cells. Subsequently, the presence of immune cells in the tumor microenvironment was measured by immunohistochemistry. Compared to untreated control mice, the ratio of CD8$^+$ T cells in tumor tissues from the DC vaccine, dasatinib or combined treatment groups was significantly increased ($P<0.05$). Notably, in mice treated with dasatinib plus DC vaccine, the expression level of CD8$^+$ T cells was significantly increased compared with the mice treated with dasatinib alone or DC vaccine alone (Fig. 4A; $P<0.05$). Similarly, the ratio of NK cells was significantly increased in the combined treatment group compared with the other three groups (Fig. 4B; $P<0.05$). Overall, these data indicate that the expansion of CD8$^+$T cell and NK cell at tumor site contributes to high anti-tumor efficacy of combination of dasatinib and DC vaccine.

## Discussion

The rationale for combining dasatinib with DC vaccine in this study relied on the immunostimulatory off-target effects of dasatinib on several immune cells (7-11). In order to improve its potent immunostimulatory ability, dasatinib has been studied in combination with cytotoxic therapies, including chemotherapy, which may cause the release of tumor cell antigens, a crucial trigger for immune responses (21). However, cytotoxic therapies showed limited clinical efficacy, with potential increased frequency of adverse events (22). Immunotherapy is an ideal therapeutic adjuvant, as it is more effective in enhancing immune response and has low toxicity (23). Additionally, dasatinib combined with DC vaccine has resulted in marked effects in certain tumor models (8,19). In the present study, 4T1 cell-induced breast cancer was used to demonstrate that the combination of dasatinib and DC vaccine significantly inhibits tumor growth and metastasis.

Recruitment of regulatory T cell (Tregs) and myeloid-derived suppressor cells (MDSCs) to the tumor microenvironment is one of the important mechanisms of immune tolerance (24,25) and contributes to tumor initiation and progression (26,27). Increased levels of MDSC and Tregs are associated with poor prognosis in multiple tumor types (28,29). Previous studies *in vitro* showed dasatinib inhibits all subsets of T cells, without impairing its viability (30-33). However, previous studies have reported opposite immunostimulatory effects *in vivo* (5,6,10,11,34). It is hypothesized that the inhibitory effect of dasatinib on suppressor cells, including Tregs and MDSCs, far outweigh the inhibition on other effector T cells, resulting in Treg and MDSC-mediated effector T cells activation and expansion (35,36). Another possible explanation for this discrepancy is that *in vitro* culture may not completely reproduce *in vivo* conditions; the plasma half-life of dasatinib was reported to be only 3-4 h (37), and the transient inhibition on T cells could be reversed by drug removal (31,37) *in vivo*. In addition, multiple preclinical and clinical studies showed that other TKIs, such as sunitinib (38-42) and axitinib (43,44), can decrease immunosuppressing MDSCs and Tregs in solid tumors via mechanisms involving c-kit, Stat3 and possibly VEGF inhibition. Similar mechanisms on dasatinib are thought to be helpful to suppress Tregs and MDSCs (8,19), but this possibility requires additional investigation.

The repertoire of chemokines in the tumor microenvironment is known to regulate the migration and infiltration of leukocytes via binding their various G protein-coupled receptors. Dasatinib combined with the anti-X40 regimen decreases the ratio of Tregs to CD8$^+$ effector T cells partly by upregulating CXCL9, CXCL10 and CXCL11 chemokines at the tumor site, resulting in substantially improved therapeutic efficacy compared to treatment with either single modality (19). Furthermore, the chemokine ligand/receptor binding would cause a positive feedback loop that attracts more effector T cells into the tumor site by producing more interferon (IFN)-γ, which further upregulates CXCL9, CXCL10 and CXCL11 (45,46). Lowe *et al* (8) showed that treatment of mice inoculated with M05 melanoma cells with dasatinib and peptide-pulsed DC improved the recruitment of these chemokines to tumor microenvironment with increased secretion of IFN-γ and activation and recruitment of Type-1, vaccine-induced CXCR3$^+$/CD8$^+$ tumor-infiltrating lymphocytes into the tumor microenvironment.

DC-based immunotherapy is considered to effectively recognize and eradicate the malignant cell population, including intratumoral, peritumoral, distant and widely disseminated cancer cells. However, DC-based immune monotherapy has shown only limited transient response (47-49), which is in line with the present findings using DC vaccine alone. Previous studies suggest that dasatinib pretreatment could improve the tumor microenvironment to be ready for effective DC-based immunotherapy. Wolfl *et al* (17) observed that dasatinib significantly improved the production of IL-12p70 during TLR2/4-triggered DC activation. Nerreter *et al* (18) found that the number of migratory DCs in a dasatinib-pretreated LPS-matured DC cohort was significantly increased compared with the LPSonly-matured DC cohort.

The present study confirmed that the treatment protocol using dasatinib and DC vaccine was more effective in inhibiting 4T1 breast tumor growth and metastasis compared to either single therapy with dasatinib or DC vaccine. The synergistic therapeutic effect appeared to be mainly dependent on the expansion of CD8$^+$T cells and NK cells. Yang *et al* (19) reported that the superior therapeutic effect of dasatinib and anti-OX40 was largely due to T cell-mediated immunity, as CD4$^+$ or CD8$^+$T-cell depletion experiment showed that reduced CD4$^+$ or CD8$^+$ T cells level lead to shorter survival time. Although the NK-cell-mediated innate immune response can mount antigen-independent anti-tumor responses and may be important to drive an effective immune response, the generation of tumor-specific CTL is considered essential for effective anti-tumor immunity.

In conclusion, the protocol using dasatinib with 4T1-antigen-loaded DC vaccine demonstrated synergistic antitumor efficacy on 4T1 breast cancer cell proliferation, apoptosis, metastasis and angiogenesis, with promising immunostimulatory effects for the expansion of CD8$^+$ T cells and NK cells. Due to the unique ability of priming and boosting T cells and NK cells, DC should be mainly responsible for the achieved therapeutic effect. However, the addition of dasatinib leads to significant synergistic immunostimulatory effects.

Therefore, dasatinib combined with DC vaccine is a possible modality for the effective treatment of metastatic breast cancer.

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**Availability of data and materials**

The datasets generated and analyzed in the present study are included in this published article.

**Authors' contributions**

NS and XW conceived and designed the study. NS and HG performed the experiments and wrote the paper. NS, HG, JR and SH collected, analyzed and interpreted the data of the work. JR and SH reviewed and edited the manuscript. All authors read and approved the manuscript.

**Ethics and consent to participate**

All procedures involving animals were approved by the Ethics Committee of Tianjin Medical University. All animal studies also comply with the ARRIVE guidelines and the AVMA euthanasia guidelines 2013.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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