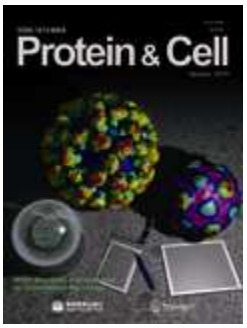




# Naringenin Reduces Lung Metastasis in a Breast Cancer Resection Model

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# Naringenin Reduces Lung Metastasis in a Breast Cancer Resection Model

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**Abstract**

**Metastases are the main cause of death in cancer patients. To improve the outcomes of patients undergoing a surgery, new adjuvant therapies that can effectively inhibit metastases have to be developed. Previous studies have shown that flavonoid naringenin, which is a natural product mainly present in grapes and citrus and has many advantages compared to the traditional chemotherapy drugs, contribute to the cancer prevention. To determine whether naringenin can also inhibit metastases, a breast cancer resection model that mimics clinical situations was applied. We found that orally administered naringenin significantly decreased the number of tumor cells which metastasized to the lung and extended the life span of the tumor resected mice. Flow cytometry analysis showed that T cells displayed enhanced antitumor activity in naringenin treated mice, with increased proportion of IFN-γ and IL-2 expressing T cells. *In vitro* studies further demonstrated that relieving immunosuppression caused by regulatory T cells might be the fundamental mechanism for metastasis inhibition of naringenin. These results indicate that orally administered natural product naringenin can inhibit the outgrowth of metastases after surgery via regulating host immunity. Thus, the low toxic naringenin can be an ideal surgical adjuvant therapy for breast cancer patients.**

**Keywords** Naringenin, breast cancer, surgery, metastasis, immunosuppression, Tregs

## Introduction

Breast cancer is the most common cancer in women and the second leading cause of cancer death among women worldwide (Coughlin and Ekwueme, 2009; Jemal et al., 2010). For most patients, the best treatment is surgery (McCahill et al., 2009), often with chemotherapy, and sometimes with radiation therapy, endocrine therapy, or immunotherapy, depending on the characteristics of the tumor and the patient. The main problem of surgery is the likelihood of tumor metastases and local relapses. Most patients have already developed metastases when the cancer is diagnosed even if the metastases are not clinically detectable. Although chemotherapy may help lower the risk of metastases and relapses, the severe side effects on normal cells, especially immune cells, limit the long term usage of the drugs. In addition, recent research has shown that surgery promotes tumor cell dissemination and metastasis (van der Bij et al., 2008). For these reasons, there is an urgent need to develop new adjuvant therapies with low toxicity that are highly efficient in inhibiting the outgrowth of metastases.

The immune system has been shown to be extremely important in struggling against cancers and maintaining host immune surveillance (Bindea et al., 2010). Immune cells can kill tumor cells and thus inhibit tumor growth and metastasis, or even cause a primary tumor rejection in immunogenic tumor models (Townsend and Allison, 1993). However, in patients and poorly immunogenic tumor models, most tumors are not rejected. Mechanisms that may cause T cell tolerance or anergy include lack of effective recognition of tumors by T cells (e.g. the absence of T-cell antigen receptor [TCR] and/or costimulatory signals), immunosuppression induced by tumor-derived molecules, and negative regulation of the host immune system (e.g. regulatory T

cells [Tregs], myeloid-derived suppressor cells [MDSC]). Of the factors mentioned above, Tregs are the most important in suppressing antitumor immune responses. They inhibit T cell activation and proliferation by producing the immunosuppressive cytokines transforming growth factor-β (TGF-β) and IL-10. Tregs are apparently elevated in cancer patients, and higher levels of Treg cells are closely correlated with poor prognosis (Beyer and Schultze, 2006; Liyanage et al., 2002). In addition, surgery itself may cause immunosuppression, since factors such as tissue damage, anesthetic and analgesic drugs, hypothermia, blood loss, transfusion, pain and perioperative distress contribute to surgery-induced immunosuppression (Ben-Eliyahu, 2003; Boomsma et al., 2010). As we know, for tumor resection patients, the critical factor for successful treatment is the elimination of residual tumor cells. Immune cells are distributed all over the body and have a high likelihood of being in contact with tumor cells. Thus relieving immunosuppression and restoring the antitumor immunity of immune cells would improve their ability to clear remaining tumor cells.

4T1 is a mouse mammary carcinoma cell line with many similarities to human breast cancer, making it a suitable animal model for human breast cancer investigation. 4T1 cells are highly malignant and quickly develop spontaneous metastases to various organs, with pulmonary metastasis being the most common and the predominant cause of death (Chen et al., 2007). 4T1 is lethal even after the primary tumor has been removed (duPre et al., 2008). Furthermore, 4T1 tumor is poorly immunogenic, and resists many immune-based therapies, making it a very challenging model for immunotherapy evaluation (Chen et al., 2007).

Naringenin, a flavonoid that is present in high concentration in grapefruits and citrus fruits, is considered to be a safe natural product with a wide spectrum of pharmacological activities. Previous studies have indicated that naringenin has anti-cancer effects, but most of them have

focused on the primary tumor prevention (Kanno et al., 2005; Le Marchand et al., 2000; So et al., 1996). Very few studies have investigated the anti-metastatic effects of naringenin, especially on a surgery model that closely reflects clinical conditions. We hypothesize that the immunosuppression in 4T1 bearing mice and patients might be the reason for metastatic cancer cell survival and the final treatment failure. We previously found that naringenin could modulate the immune system of mice with pulmonary fibrosis (Du et al., 2009). Thus naringenin might help the host to fight against metastatic cancer cells and inhibit metastases.

In this study, we used the highly malignant 4T1 murine breast cancer model to examine the effects of orally administered naringenin. We investigated whether naringenin could inhibit metastases in a surgical resection model, and discuss possible mechanisms.

## Results

### Naringenin inhibits lung metastasis and extends survival of mice in the resection model.

An orthotopic 4T1 breast cancer resection model was developed to mimic clinical situations (Fig. 1A). Primary tumors were resected on day 14, at which time spontaneous metastasis has already occurred (Pulaski and Ostrand-Rosenberg, 2001). Naringenin was given orally 3 days before surgery and was persistent for 24 days. The treatment efficacy of naringenin was evaluated by the lung clonogenic metastasis assay and the long-term survival experiment.

Results showed that the naringenin treatment group (100 mg/kg) had significantly decreased lung metastatic colonies compared to the nontreatment surgical control group both on day 3 (13-fold) and day 7 (6-fold) after resection, suggesting that naringenin is efficient in inhibiting lung

metastases ($P < 0.01$)( Fig. 1B). Besides, surgery itself promoted the metastases to lungs, as shown in Supplementary Figure 1. The mice that underwent surgical procedures showed a ~2.5-fold higher number of metastatic tumor cells than the nonsurgical controls.

We further examined the long-term benefit of naringenin. Naringenin was given for three weeks after resection and survival was measured within 160 days. Naringenin treatment group showed a prolonged survival (median survival 74.5 days) compared to the nontreatment surgical control group (median survival 53.5 days, $P < 0.05$) (Fig. 1C). At the end of the experiment (on day 160), 3 of 10 (30%) mice of the treatment group were still alive and tumor-free, while all mice in the nontreatment group died by day 104. Taken together, these results indicate that orally administered naringenin could inhibit the high level of metastases to lungs, and prolong the life span of tumor resection mice.

**Naringenin does not inhibit tumor cell proliferation either *in vivo* or *in vitro***

Two mechanisms may account for the effect of naringenin: the direct inhibitory effect of tumor cell proliferation, and the indirect effect that regulates immune response to tumor cells. We first examined the proliferation inhibitory effect of naringenin on 4T1 cancer cells *in vitro* by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and *in vivo* by orthotopic 4T1 tumor growth. Results showed that naringenin did not obviously inhibit tumor proliferation either *in vitro* or *in vivo* (Fig. 2A and 2B).

For the *in vitro* MTT assay, naringenin did not obviously affect the cell viability of 4T1 tumor cells, with a slightly enhanced cell proliferation at concentration ranging from 6.25 to 100 μM (Fig. 2A). For the *in vivo* assay, the tumor growth curves of the control mice and the naringenin treated mice were quite close, indicating that naringenin has no direct tumor growth inhibitory

effect *in vivo* (Fig. 2B). In addition, for the tumor resection model, there was no significant difference in tumor volume between the naringenin treated group and the nontreatment group at the time of surgery, although naringenin had been given for 3 days (data not shown). Based on these results, we concluded that the effect of naringenin was not due to the direct inhibitory effect of tumor cell proliferation.

**Naringenin promotes T cell activation and restores T cell function in mice**

We next investigated whether naringenin could regulate the immune system and enhance the antitumor immunity. As we know, the antitumor function of immune system is often impaired in cancer patients and this leads to the uncontrolled tumor growth (Brandacher et al., 2006). Here, we examined a series of antitumor related immune cells and molecules to assess our hypothesis.

We examined several cell surface receptors and activation markers, and found that naringenin treatment increased the proportion of activated T cells ($CD44^{high}CD62L^{-}$ subpopulation) in lung $CD4^{+}$ (from 28.4% to 37.3%, $P < 0.01$) and $CD8^{+}$ T cells (from 30.8% to 39.4%, $P < 0.05$) (Fig. 3A). The result suggested that naringenin could promote T cell activation.

We also evaluated the antitumor function of $CD8^{+}$ cytotoxic T lymphocytes (CTL) through their ability to express Interferon-γ (IFN-γ) and IL-2. IFN-γ is regarded to be critical for tumor eradication (Ko et al., 2005)**.** We found that IFN-γ expressing splenic $CD8^{+}$ T cells were sharply decreased in the nontreatment group compared to the normal naïve ones (from 6.8% to 2.2%), and naringenin treatment significantly restored the IFN-γ expressing cells (from 2.2% to 7.8%, *P*

< 0.05) (Fig. 3B and 3C). IL-2 expressing $CD8^+$ T cells were also raised to normal level in the treated group (from 5.6% to 12.1%, $P < 0.01$) (Fig. 3B and 3D).

Taken together, these results indicate that naringenin can promote T cell activation and restore the antitumor function of T cells in tumor resected mice.

**Naringenin treatment down regulates the expression of immunosuppression cytokines in T cells *in vitro*.**

We further investigated the possible mechanisms for the recovery of T cell function *in vitro*. Immune suppression is often observed in animal tumor models and cancer patients, and contribute to the failure of immune therapies (Whiteside, 2006). We hypothesized that the recovery of the antitumor function by naringenin was due to relief of immune suppression. To assess this possibility, we examined the production of immune suppressive cytokines TGF-β1 and IL-10 by naïve splenic T cells, using ELISA and flow cytometry methods. We found that naringenin significantly decreased the $CD4^+$ and $CD8^+$ expressed TGF-β1 to less than 50% of control values ($P < 0.01$)(Fig. 4A). Similar to TGF-β1, the flow cytometric assay revealed a singinficant reduction in IL-10 expressing T cells ($P < 0.01$)(Fig. 4B). These observations indicate that naringenin can inhibite the cytokine mediated suppression.

**Naringenin inhibits the production of Tregs *in vitro***

TGF-β1 and IL-10 are immunosuppressive cytokines that mainly produced by a subpopulation of T cells called regulatory T cells (Tregs), an important kind of immuosuppressive cells. Tumor-derived factors can mediate the conversion of naïve T cells into Tregs. To determine whether naringenin inhibits the conversion of naïve T cells into Tregs, we used naringenin to treat naïve T cells. Results showed that TGF-β1 significantly induced the conversion of naïve T cells to $CD4^+CD25^+Foxp3^+$ Tregs (from 4.5% to 23.7%), and naringenin significantly reverse this process (from 23.7% to 10.7%) (Fig. 5A and 5B). These data indicate that naringenin could inhibit the Treg production, which might be the reason for the recovery of T cell function in mice.

## DISCUSSION

Chemotherapy drugs are commonly used to inhibit metastases in breast cancer patients that undergo surgery (Markiewicz et al., 1996; Shenkier et al., 2004). However, the severe side effects (e.g. immunosuppression, myelosuppression) limit the long term usage of the drugs. Naringenin is a commonly used natural dietary supplement and has a clear safety record. The medium lethal dose (LD50) of naringenin is > 5000 mg/kg in mice and rats (Ortiz-Andrade et al., 2008). In this study, we examined the metastasis inhibition effect of naringenin in a breast cancer resection model for the first time. Results showed that orally administered naringenin significantly decreased the number of tumor cells that metastasized to the lung (Fig 1B) and extended the life span of the tumor resected mice (Fig. 1C), making it a promising adjuvant drug for tumor resection patients.

The *in vivo* experiments were carried out using a most challenging 4T1 model, which is highly malignant and poorly immunogenic. Long-term survival mice have been seldom observed in this

model (Lohr et al., 2000; Pulaski et al., 2000). We found a prolonged survival in naringenin treated mice and 30% mice showed a long-term survival (Fig. 1C). This is probably due to inhibiting metastasis from primary tumors to lungs by naringenin, as tumor cells that metastasized to lungs were significantly decreased (Fig. 1B). The inference was further demonstrated by the *in vivo* and *in vitro* experiments, which showed naringenin did not directly inhibit tumor proliferation (Fig. 2A and 2B). Besides, we found surgery itself could promote metastases to lungs (Supplementary Figure 1). Such a phenomenon is also observed by other researchers (Page and Ben-Eliyahu, 1997; van der Bij et al., 2008). However, the mechanism under the phenomenon remains unclear. It would be interesting to further investigate the mechanism under this surgery induced metastasis.

To determine the possible mechanisms for the antimetastatic effect of naringenin, we examined the T cell function in mice. Flow cytometry analysis showed that the naringenin treated mice had an increased proportion of IFN-γ and IL-2 expressing $CD8^+$ T cells in spleens, and an elevated level of T cell activation marker $CD44^{high}CD62L^-$ in lungs, suggesting that the T cell function had recoverd to some extent (Fig. 3A–D). Since $CD8^+$ T cell is critical in antitumor immunity, the enhanced T cell function of the host could account for the antimetastatic effect of naringenin. This result is in agreement with the inefficacy of naringenin on 4T1 proliferation *in vitro* (Fig. 2A), as no T cell existed in the culture system. However, naringenin treatment was also inefficient in primary tumor inhibition (Fig. 2B). It might be due to the extremely large number of tumor cells and the high proliferation speed. The tumor cells can be eliminated by the increased activated T cells only when tumor cells are present in very small amounts. Once the primary tumor is removed, the restored antitumor function of T cells will present, suggesting that naringenin is suitable for eliminating small portion of tumor cells.

Interestingly, a recent study reported that naringenin could suppress picrylchloride-induced contact hypersensitivity by inhibiting the proliferation and activation of T cells, while our previous study showed that naringenin could enhance T cell function in a bleomycin induced pulmonary fibrosis which is similar to the present result (Du et al., 2009; Fang et al., 2010). The different effects of naringenin on T cells may be the result of using different disease models and different experiment systems. Our results further confirm that naringenin is an immunomodulator—regulating the suppressed or over activated T cells to normal levels.

As immunosuppression widely exists in cancer patients and contributes to the T cell anergy, we examined the effect of naringenin on immunosuppression related molecules and cells *in vitro* (7, 8). Results showed that naringenin significantly inhibited the production of TGF-β1 and IL-10 (Fig. 4A and 4B). Furthermore, naringenin inhibited the TGF-β1 induced Treg production *in vitro* (Fig. 5A and 5B). These data showed that naringenin could reverse the Treg related immunosuppression. This in turn increased the proportion of activated T cells. Immune system regulation is a complicated process that involved many kinds of cells, such as MDSCs and tumor-associated macrophages (TAMs) (Coffelt et al., 2009; Gabrilovich and Nagaraj, 2009; Marigo et al., 2008). Whether the inhibition of Tregs by naringenin played a definitive role in T cell restoring will need to be clarified by further study.

In summary, our results indicate that orally administered naringenin can inhibit the outgrowth of metastases after surgery via regulating host immunity. The antimetastatic property of low toxic naringenin may make it an ideal surgical adjuvant therapy for breast cancer patients. In addition, naringenin might be efficient in autoimmune diseases as it has an immunomodulating property.

## MATERIALS AND METHODS

### Animals and Reagents

Six- to eight-week-old female BALB/c mice and C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. and allowed to accommodate to the new environment for at least one week. The mice were kept in a temperature-controlled room with a 12-hour light and dark cycle under specific pathogen-free conditions. All animal procedures were conducted in accordance with guidelines established by the NIH and the Institute of Biophysics Animal Care and Use Committee.

Naringenin was purchased from Shanxi Huike Botanical Development Co. Ltd. Cell culture reagents and collagenase type Ⅳ were from Invitrogen. DNase Ⅰ and 6-thioguanine were obtained from Sigma. Recombinant TGF-beta1 was from PeproTech. TGF-β1 ELISA kit was from Promega. The T cell isolation kit was purchased from Miltenyi Biotec. FITC-conjugated anti-CD4 ( GK1.5), Percp-cy5.5-conjugated anti-CD8 (53-6.7), APC-conjugated anti-CD25 (PC-61.5), PE-conjugated anti-CD44 (IM7), APC-conjugated anti-CD62L(MEL-14), purified anti-mouse CD16/32 (93), PE-conjugated anti-IFN-γ (XMG1.2), APC-conjugated anti-IL-2 (JES6-5H4), PE-conjugated anti-IL-10 (JES5-16E3), PE-conjugated anti-Foxp3 (FJK-16s), purified anti-CD3e (145-2C11) and anti-CD28 (37.51) were all purchased from eBioscience.

### Cell Culture

4T1 murine mammary cancer cells (6-thioguanine resistant) were obtained from the American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Animal models**

To examine the effect of naringenin in a tumor resection model, female BALB/c mice were randomly distributed to two groups, the nontreatment surgical control group and the naringenin treatment group. Each group received a mammary fat-pad injection of $2.0\times10^5$ 4T1 cancer cells on day 0 and a tumor resection on day 14. The naringenin treatment group was given a daily dose of 100 mg/kg (i.g.) naringenin suspended in 1% CMC-Na since day 11, and the nontreatment group received only 1% CMC-Na, the treatment was sustained for 24 days. For survival experiment, the life span of the tumor resected mice was observed within 160 days. For lung metastasis determination and other assays, mice were autopsied 3 and 7 days after surgery (on day 17 and day 21), lungs and spleens were carefully harvested for the following assessment. .

For tumor resection procedures, mice were anesthetized with pentobarbital sodium. An incision was then made around the tumor and the tumor was dissected away from the chest wall. The tumor was then excised with a small margin and the incision was closed with stitches.

To examine the effect of naringenin on primary tumor growth, female BALB/c mice were randomly divided into two groups. Each group received a mammary fat-pad injection of $2.0\times10^5$ 4T1 cancer cells. On the following day, one group received a daily dose of 100 mg/kg (i.g.) naringenin suspended in 1% CMC-Na, and the other group received only 1% CMC-Na. The

treatment was sustained for two weeks. And the primary tumor growth was evaluated by measurement with calipers twice a week [volume = 0.5 × (width)$^2$ × (length)].

**Lung clonogenic metastasis assays**

The harvested lungs were rinsed in HBSS to remove the blood. Lungs were minced with scissors and digested at 37°C for 90 min in sterilized HBSS containing 1 mg/ml collagenase type Ⅳ (Invitrogen) and 0.02 mg/ml DNase Ⅰ (Sigma) on a platform rocker. The fragments were then filtered through a 70-μm cell strainer (BD Biosciences) and washed twice in HBSS. Cells were then resuspended in medium containing 60 μM 6-thioguanine (Sigma) and seeded in 6-well tissue culture plates. After 10-14 days, plates were fixed with methanol and stained with 0.03% methylene blue. The 6-thioguanine resistant colonies were stained and counted. A colony represents a metastatic cancer cell (duPre et al., 2008).

**Cell viability assay**

4T1 cells were plated at a density of 7×10$^3$ cells per well in complete RPMI 1640 medium in 96-well plates and were allowed to grow for 24 h. Cells were then exposed to a series of concentrations of naringenin for 24 h, and the viability of cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, the culture medium was discarded and 100 μl MTT solution (0.5 mg/ml in PBS) was added to each well. The plates were incubated for 4 h at 37°C. After incubation, the MTT solution was removed and 100 μl of DMSO was added to each well for 10 min at room temperature. Absorbance was

recorded at 570 nm by a plate reader (Thermo, Germany). The percentage of cell viability was calculated by absorbance of treated cells relative to that of untreated cells (James et al., 2009).

**Isolation of lymphocytes**

Spleens were harvested from mice and were filtered through 70-μm cell strainers to prepare single cell suspensions. Erythrocytes were subsequently removed by lysis using ammonium chloride. After washing, splenocyte preparations were used for subsequent analysis.

Lungs were harvested, rinsed, and then digested at 37°C for 90 min in sterilized HBSS containing 1 mg/ml collagenase type Ⅳ and 0.02 mg/ml DNase Ⅰ on a platform rocker. The fragments were then filtered through a 70-μm cell strainer and washed twice in HBSS. Cells were subsequently fractioned by centrifugation at 2500 rpm for 20 min on a discontinuous gradient consisting of 70% and 35% Percoll solutions (GE Healthcare). The lymphocytes were recovered from the interface and were used for subsequent flow cytometry analysis.

**Purification of T cell subpopulation**

Splenocytes from normal naïve C57BL/6 mice were pelleted and resuspended in MACS buffer (PBS with 0.5% BSA and 2 mM EDTA, pH7.2) to a final concentration of $1.1 \times 10^8$ cells/ml. The CD4 or CD8 micro beads (Miltenyi Biotec) were utilized as manufacturer's directions. Briefly, 10 μl of CD4 or CD8a microbeads were added to $10^7$ splenocytes and incubated for 15 min at 4℃. Cells were then washed and resuspended to $2\times 10^8$ cells/ml. Splenocytes were seperated using positive-selection MS columns. Finally, the CD4 or CD8 T cells were flushed

out, resuspended in X-vivo medium or complete PRIM 1640 medium for further TGF-β1 production assay or Treg induction assay.

**T cell activation and cytokine production assays**

For *in vivo* T cell activation assay, the isolated lung lymphocytes from naringenin treated mice or untreated surgical control mice were directly surface-stained with fluorochrome-conjugated anti-CD4, anti-CD8, anti-CD44 and anti-CD62L antibodies. For IL-2 and IFN-γ production assay of *in vivo* experiment, the isolated splenocytes from naringenin treated mice, untreated mice or normal naïve mice were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 500 ng/ml Ionomycin (Sigma) for 5 h. After further incubation with Brefeldin A Solution (eBioscience) for 3 h, stimulated cells were surface stained with anti-CD8 antibodies, fixed and permeabilized in fixation/permeabilization buffer (eBioscience), and then stained with anti-IL-2 and anti-IFN-γ antibodies (Chiang et al., 2007).

For T cell cytokine production assay *in vitro*, the isolated splenocytes or purified T cells from normal naïve C57BL/6 mice were cultured with or without naringenin for 72 h in the presence of anti-CD3 (3 μg/ml) and anti-CD28 (1 μg/ml) antibodies. Culture supernatant concentration of TGF-β1 from purified T cells was determined by ELISA (Promega) according to the protocols provided by the manufactures. Cells were further stimulated, surface stained, fixed and permeabilized and finally intracellularly stained for IL-10 production, as mentioned above.

The antibody stained cells were analyzed by flow cytometry (FACSCalibur) and CellQuest software.

### Treg induction assay

Isolated splenocytes of normal naïve C57BL/6J mice were purified using CD4 MACS beads (Miltenyi Biotec, Germeny). The purified $CD4^+$ T cells were cultured with anti-CD3 antibody (3 μg/ml), anti-CD28 antibody (1 μg/ml) and TGF-β1 (5 ng/ml) to induce regulatory T cells (Kong et al., 2009). Naringenin was supplemented to the culture at concentration 0 and 100 μM and maintained for 72 h. Cells that treated with neither TGF-β1 nor naringenin were used as normal controls. Cells were surfaced stained with anti-CD4 and anti-CD25 antibodies. To detect Foxp3 expression, the surface-stained cells further underwent an intracellular staining process with anti-Foxp3 antibodies.

### Statistical analysis

Data were presented as mean ± SD. Statistical tests were performed using the Student's *t* test when comparing two groups. When comparing three or more groups, ANOVA was performed with a post hoc Bonferroni test to determine which two groups showed significant differences. Survival curves were calculated by the Kaplan-Meier method and analyzed by the log rank test (GraphPad software, version 5.0). In all tests, $P < 0.05$ was considered to be statistically significant.

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## ABBREVIATIONS

CTL, cytotoxic T lymphocytes; IFN-γ, interferon-gamma; IL-2, interleukin 2; IL-10, interleukin 10; LD50, medium lethal dose; MDSCs, myeloid-derived suppressor cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nar, naringenin; Tregs, regulatory T cells; TAMs, tumor-associated macrophages; TCR, T-cell antigen receptor; TGF-β, transforming growth factor-β

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**Figure legends**

**Figure 1. Efficacy of naringenin in tumor resection model.** The schematic diagram of the animal experiment was shown in (A). Briefly, $2.0\times10^5$ 4T1 cancer cells were injected into the fourth mammary fat-pad of female BALB/c mice on day 0. Primary tumor was removed on day

14. Since day 11, mice were given a daily dose of 100 mg/kg naringenin (treatment group) or vehilcle (nontreatment group), and the treatment was persistent for 24 days. (B) Lung metastases were determined by clonogenic metastasis assay 3 and 7 days after surgery (on day 17 and 21). Data are given as mean ± SD; $n = 3$ mice/group. $^{**}P < 0.01$ when compared with nontreatment group at corresponding time points (Student's *t* test). (C) The life span of the tumor resected mice that treated with either naringenin or vehicle was evaluated within 160 days. $n = 10$ mice/group. $P < 0.05$ for treatment group relative to the nontreatment group (log-rank test).

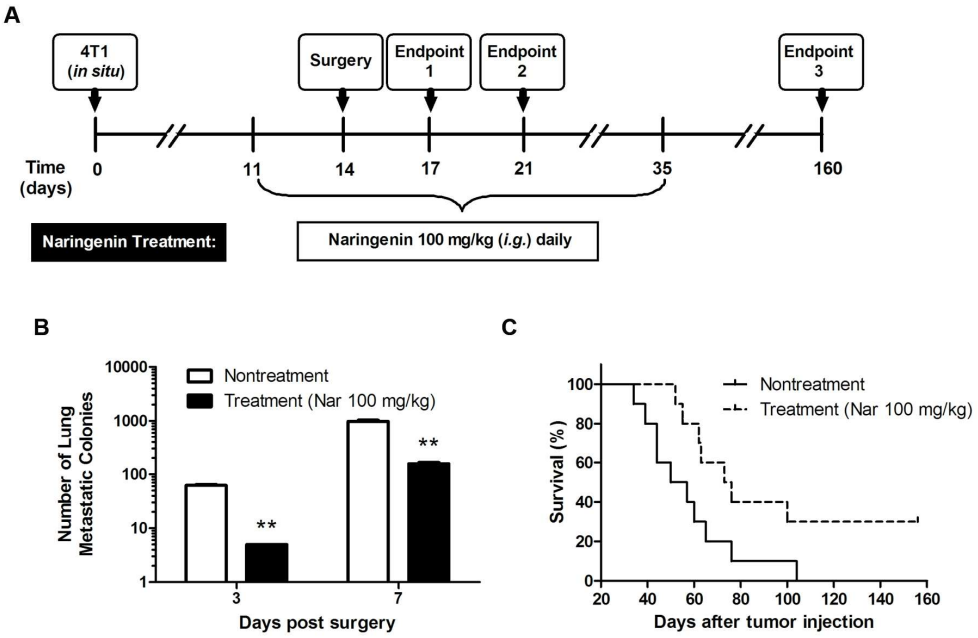
**Figure 2. Growth inhibitory effect of naringenin on 4T1 cells.** (A) The viability of naringenin treated 4T1 cells was determined by MTT. Cells were treated for 24 h and the cell viability was calculated by absorbance of treated cells relative to that of untreated cells. Data are given as mean ± SD. (B) The *in vivo* growth inhibitory effect of naringenin was examined by primary tumor growth. Mice received a mammary fat-pad injection of $2.0\times10^5$ 4T1 cancer cells on day 0. Naringenin was given for two weeks since the following day at 100 mg/kg (i.g.). Data are given as mean ± SD; $n = 10$ mice/group.

**Figure 3. Naringenin promotes T cell activation and restores T cell function in tumor resection model.** Naringenin treated mice, untreated mice or normal naïve mice were killed 7 days after surgery. T cells from lungs and spleens were analyzed for expression of cell surface markers and intracellar cytokines using flow cytometry. (A) Naringenin treated mice showed an increased proportion of activated T cells ($CD44^{high}CD62L^-$) in $CD4^+$ (*Upper*) and $CD8^+$ (*Lower*) T cells from lungs. $n = 3$ mice/group. (B) Naringenin increased the proportion of $CD8^+$ T cells producing IFN-γ and $CD8^+$ T cells producing IL-2. The representative bar graph summarizing

the flow cytometry results are shown in (C) and (D), respectively. Data are given as mean ± SD; $n = 3$ mice/group. $^{*}P < 0.05$, $^{**}P < 0.01$ when compared to the nontreatment controls (Student's $t$ test).

**Figure 4. Naringenin treatment down regulates the expression of immunosuppression cytokines in T cells *in vitro*.** The purified splenic T cells or total splenocytes were cultured with or without naringenin (100 μM) for 72 hours in the presence of anti-CD3 (3 μg/ml) and anti-CD28 (1 μg/ml) antibodies. (A) The TGF-β1 protein level in the supernatant (X-VIVO 15 serum-free medium) of the purified T cells was determined by ELISA. (B) The intracellular expression of IL-10 in splenic T cells was accessed using flow cytometry. Data are given as mean ± SD; $^{**}P < 0.01$ when compared with controls (Student's $t$ test).

**Figure 5. Naringenin inhibits regulatory T cells production *in vitro*.** The purified splenic $CD4^+$ T cells were cultured with anti-CD3 antibody (3 μg/ml), anti-CD28 antibody (1 μg/ml) and TGF-β1 (5 ng/ml) to induce regulatory T cells. Naringenin was supplemented to the culture at concentration 0 and 100 μM and maintained for 72 h. Cells that treated with neither TGF-β1 nor naringenin were used as normal controls. The proportion of regulatory T cells ($CD4^+CD25^+Foxp3^+$) in total $CD4^+$ T cells was analyzed by flow cytometry. Representative flow cytometry data and bar graph summarizing the results are shown in (A) and (B), respectively. Data are given as mean ± SD; $^{**}P < 0.01$ (Student's $t$ test).

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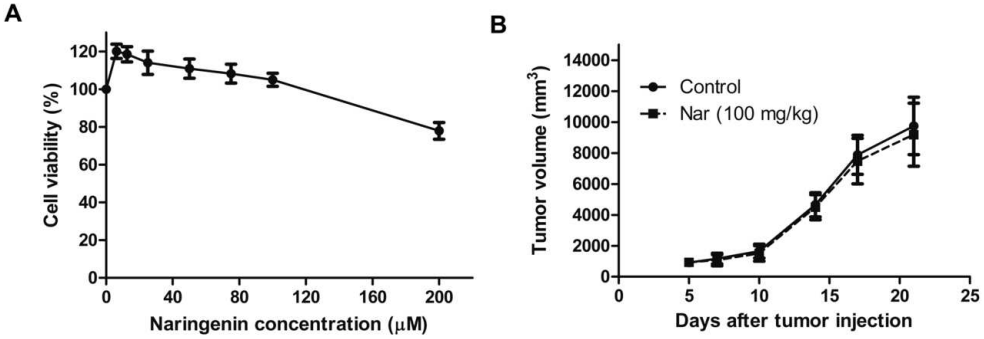


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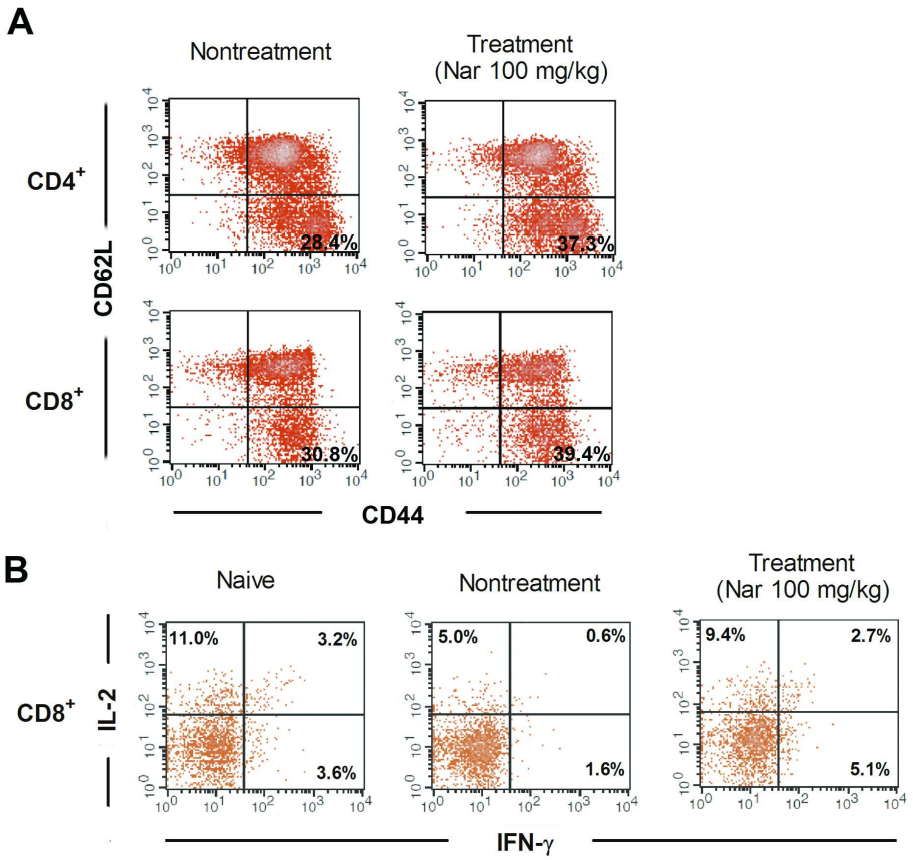


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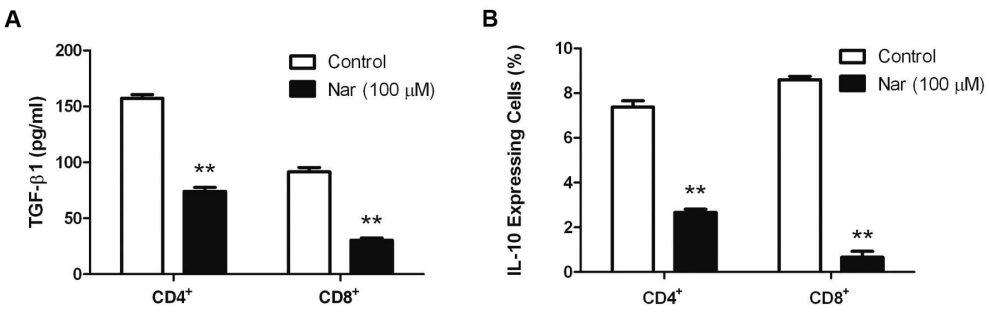


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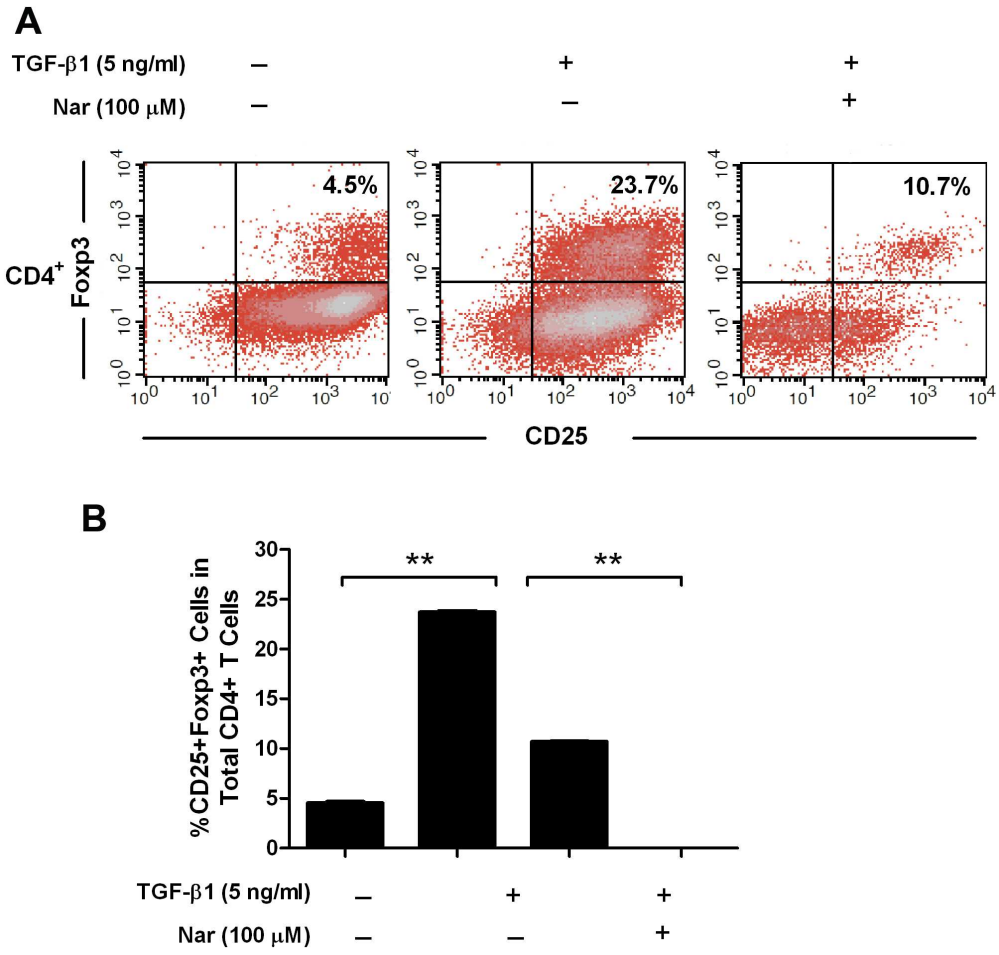


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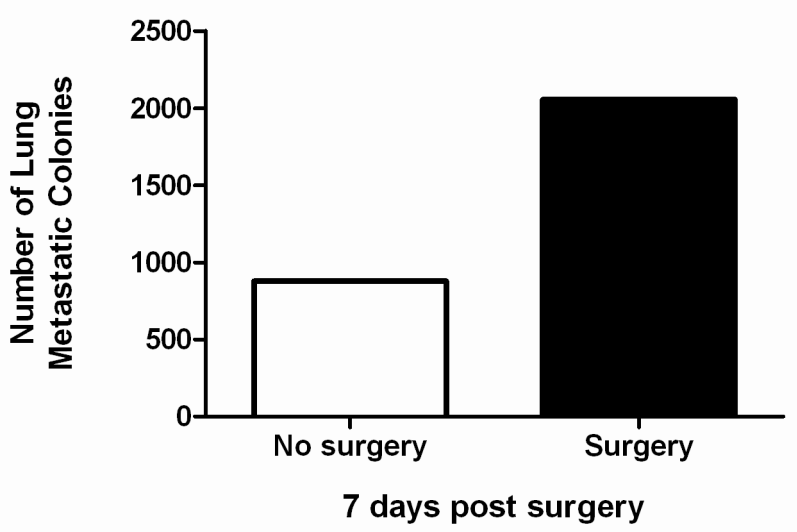
## Supplementary Figure




**Supplementary Figure 1. The impact of surgery on tumor metastases.** Briefly, $2.0\times10^5$ 4T1 cancer cells were injected into the fourth mammary fat-pad of female BALB/c mice on day 0. On day 14, mice were received a primary tumor resection or not. Lung metastases were determined by clonogenic metastasis assay 7 days after surgery (on day 21).