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RESEARCH ARTICLE

Naringenin reduces lung metastasis in a breast cancer resection model

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ABSTRACT

Metastasis is 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. Studies have shown that flavonoid naringenin, a natural product that is mainly present in grapes and citrus, may contribute to cancer prevention. It has many advantages compared to traditional chemotherapeutic drugs, such as low toxicity. To determine whether naringenin can also inhibit metastases, a breast cancer resection model that mimics clinical situations was established. We found that orally administered naringenin significantly decreased the number of metastatic tumor cells in the lung and extended the life span of tumor resected mice. Flow cytometry analysis revealed that T cells displayed enhanced antitumor activity in naringenin treated mice, with an increased proportion of IFN-γ and IL-2 expressing T cells. *In vitro* studies further demonstrated that relief of immunosuppression caused by regulatory T cells might be the fundamental mechanism of metastasis inhibition by naringenin. These results indicate that orally administered naringenin can inhibit the outgrowth of metastases after surgery via regulating host immunity. Thus, 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); depending on the characteristics of the tumor and the condition of the patients, it is often combined with chemotherapy, and sometimes with radiation therapy, endocrine therapy, or immunotherapy. The main problem of surgery is the likelihood of tumor metastasis and local relapse. Most patients have already developed metastases when their cancer is diagnosed, even if the metastases are not clinically detectable. Although chemotherapy may help reduce the risk of metastasis and relapse, severe side effects on normal cells, especially immune cells, limit its long-term usage. In addition, a recent study 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 that have low toxicity and high efficiency 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 underlie T cell tolerance or anergy include negative regulation of the host immune system (e.g. regulatory T cells [Tregs], myeloid-derived suppressor cells [MDSC]), immunosuppression induced by tumor-derived molecules, and lack of effective recognition of tumors by T cells (e.g. the absence of T-cell antigen receptor [TCR] and/or costimulatory signals). Of the above factors, Tregs play a critical role in suppressing antitumor immune responses. They inhibit T cell activation and proliferation by

producing immunosuppressive cytokines transforming growth factor-β (TGF-β) and IL-10. Tregs are apparently elevated in cancer patients, and high amounts of Treg cells are closely correlated with poor prognosis (Liyanage et al., 2002; Beyer and Schultze, 2006). In addition, surgery itself causes a generalized state of immunosuppression due to tissue damage, anesthetic and analgesic drugs, hypothermia, blood loss, transfusion, pain and perioperative distress, etc. (Ben-Eliyahu, 2003; Boomsma et al., 2010; Hogan et al., 2011). The critical factor for successful treatment of tumor resection patients is the elimination of residual tumor cells. Immune cells are distributed all over the body and have a high likelihood to contact tumor cells. Therefore, relieving immunosuppression and restoring systemic antitumor immunity can improve the outcomes of patients undergoing tumor surgery.

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

Naringenin, a flavonoid that is present in high concentrations 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; however most studies focused on its role in primary tumor prevention (So et al., 1996; Le Marchand et al., 2000; Kanno et al., 2005), and few investigated its anti-metastatic effects. We previously found that naringenin could attenuate bleomycin-induced pulmonary fibrosis by improving the immunosuppressive environment (Du et al., 2009). Furthermore, relief of pulmonary fibrosis by naringenin led to a decrease in lung metastasis when challenged with subcutaneously injected or intravenously injected tumor cells. However, it remains unknown whether naringenin may still inhibit metastases in mice without pulmonary fibrosis, especially in the surgery models that closely resemble clinical situations. We hypothesized that immunosuppression might be the reason for metastatic cancer cell survival and the final surgical failure of 4T1 tumor-bearing mice. As naringenin could modulate the immune system of mice with pulmonary fibrosis, it might help the host to fight against metastatic cancer cells and inhibit metastases.

In the present study, we examined the anti-metastatic effects of orally administered naringenin using a highly malignant 4T1 murine breast cancer model and 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 a breast cancer resection model

An orthotopic 4T1 breast cancer resection model was established to mimic clinical situations (Fig. 1A). Primary tumors were resected on day 14, when spontaneous metastasis already occurred (Pulaski and Ostrand-Rosenberg, 2001). Since day 11, a daily dose of 100 mg/kg naringenin was administered orally for 24 days. Efficacy of naringenin was evaluated by the lung clonogenic metastasis assay and the long-term survival experiment. The results showed that the naringenin treatment group (100 mg/kg) exhibited significantly decreased number of lung metastatic colonies compared to the nontreatment surgical control group on both day 3 (13-fold) and day 7 (6-fold) after resection, suggesting that naringenin efficiently inhibits lung metastases ($P < 0.01$; Fig. 1B). Moreover, as shown in Fig. S1, mice that underwent surgical resection showed a ~2.5-fold higher number of metastatic tumor cells than the nonsurgical mice, indicating that surgery promotes tumor metastases.

We further examined the long-term benefit of naringenin. The naringenin treatment group showed a prolonged survival compared to the nontreatment surgical control group (median survival, 74.5 days *vs* 53.5 days, $p < 0.05$; Fig. 1C). At the end of the experiment (on day 160), 3 of 10 (30%) mice in 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 can inhibit tumor metastases to the lung and prolong the life span of tumor resected mice.

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

In order to find whether the antimetastatic effect of naringenin is due to the inhibition of metastatic tumor cell proliferation, we examined the inhibitory effect of naringenin on 4T1 cancer cell proliferation *in vitro* using the MTT assay, and *in vivo* by orthotopic 4T1 tumor growth measurement. Results showed that naringenin did not obviously inhibit tumor proliferation either *in vitro* or *in vivo* (Fig. 2A and 2B).

In the MTT assay, naringenin did not have obvious effect on the cell viability of 4T1 tumor cells, but slightly enhanced cell proliferation at concentrations ranging from 6.25 to 100 μmol/L (Fig. 2A). The *in vivo* assay showed that naringenin did not inhibit tumor growth (Fig. 2B). In addition, in the tumor resection model, there was no significant difference in tumor volume between the naringenin treated mice and the untreated mice at the time of surgery, although naringenin had been given for 3 days (data not shown). These results suggest that naringenin does not kill tumor cells directly.

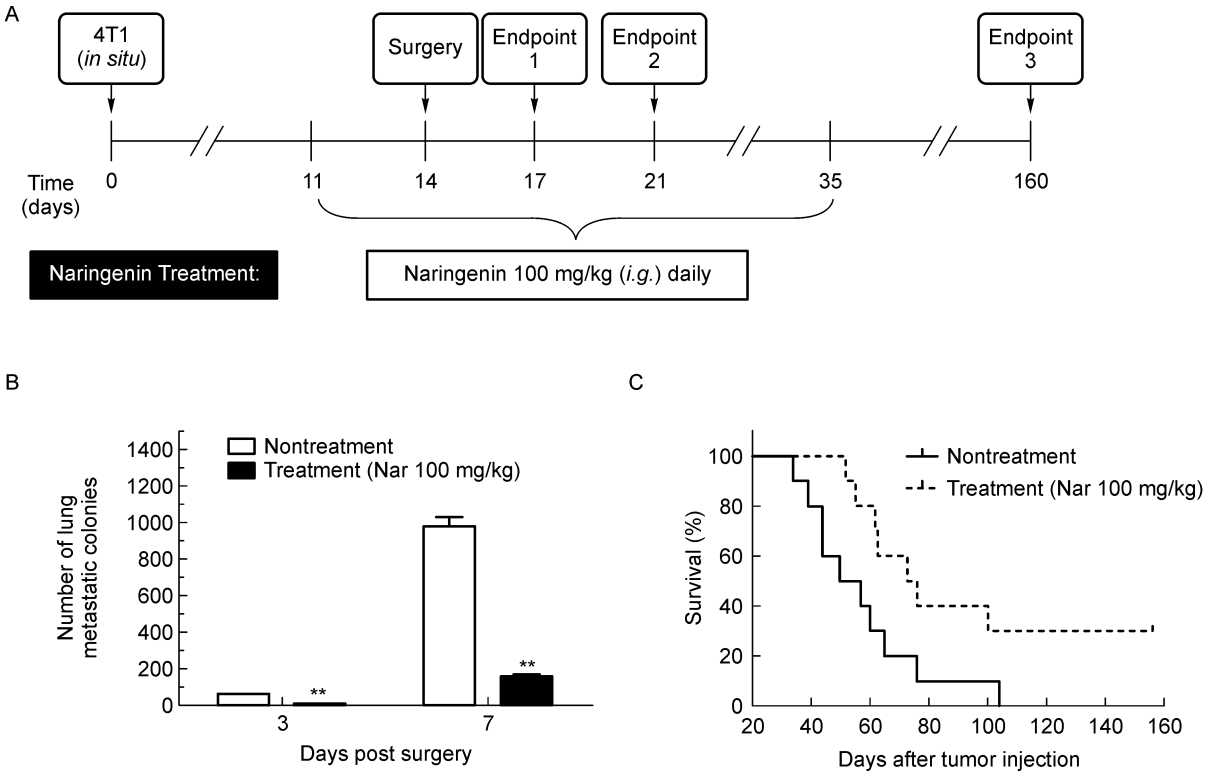

Figure 1. Efficacy of naringenin in a tumor resection model. (A) The schematic diagram of the animal experiment. Briefly, 2.0×10^5 4T1 cancer cells were injected into the fourth mammary fat-pad of female BALB/c mice on day 0. The primary tumor was resected on day 14. Since day 11, mice were given a daily dose of 100 mg/kg naringenin (treatment group) or vehicle (nontreatment group), and the treatment was continued for 24 days. (B) Lung metastases were determined by clonogenic metastasis assay 3 and 7 days after surgery (on days 17 and 21). Data are presented as mean ± SD; $n = 3$ mice/group. $^{**}p < 0.01$ for treatment group *versus* the nontreatment group at corresponding time points (Student's *t*-test). (C) The life span of tumor resected mice that were treated with either naringenin or vehicle was evaluated within 160 days. $n = 10$ mice/group. $p < 0.05$ for treatment group *versus* nontreatment group (log-rank test).

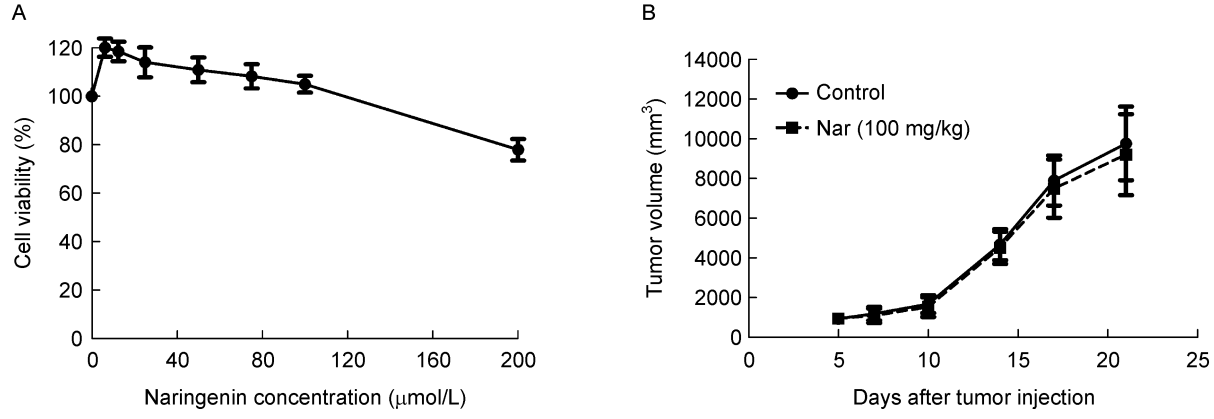

Figure 2. Inhibitory effect of naringenin on 4T1 cell growth. (A) The viability of naringenin treated 4T1 cells was determined by MTT assay. Cells were treated for 24 h and the cell viability was calculated by absorbance of treated cells relative to that of untreated cells. Data are presented as mean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

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

The antitumor immune function is mainly mediated through T cells, especially $CD8^+$ cytotoxic T lymphocytes (CTL) (Rosenberg, 2001). However, in cancer patients, the antitumor function of immune system is often impaired, which leads to uncontrolled tumor growth (Brandacher et al., 2006). Furthermore, surgery itself causes severe immunosuppression, and impaired T cell function after surgery has been frequently observed in several studies (Brune et al., 1999; Ogawa et al., 2000). Thus, we hypothesized that the antimetastatic effect of naringenin was probably due to the recovery of T cell function. To prove this hypothesis, we examined several T cell surface receptors and activation markers, and found that naringenin 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). These results suggest that naringenin can promote T cell activation.

We also evaluated the antitumor activity of $CD8^+$ T lymphocytes 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 the number of IFN-γ expressing splenic $CD8^+$ T cells sharply decreased in the tumor-bearing mice undergoing surgery compared to the normal control mice (from 6.8% to 2.2%). However, naringenin treatment significantly restored the IFN-γ expressing T cells to normal level (from 2.2% to 7.8%, $p < 0.05$) (Fig. 3B and 3C). In addition, IL-2 expressing $CD8^+$ T cells also elevated to normal levels in the naringenin treated mice (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 downregulates the expression of immunosuppressive 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 it contributes to the failure of immune therapies (Whiteside, 2006). Furthermore, surgery causes a Treg predominant environment, which further intensifies immunosuppression in patients (Hogan et al., 2011). We hypothesized that the recovery of antitumor function by naringenin was due to the relief of immunosuppression. To assess this possibility, we examined the production of immune suppressive cytokines TGF-β1 and IL-10 by naive splenic T cells using ELISA and flow cytometry methods. We found that naringenin significantly decreased the level of secreted TGF-β1 and the numbers of $CD4^+$ and $CD8^+$ cells to less than 50% of control values ($p < 0.01$; Fig. 4A). Flow cytometric assay also revealed a significant reduction in IL-10 expressing T cells ($P < 0.01$; Fig. 4B). These results indicate that naringenin can improve TGF-β1 and IL-10 mediated immunosuppression.

Naringenin inhibits the production of Tregs *in vitro*

TGF-β1 and IL-10 are immunosuppressive cytokines that are mainly produced by a subpopulation of T cells called Tregs, an important type of immunosuppressive cells. Tumor-derived factors can mediate the conversion of naive T cells into Tregs. To determine whether naringenin inhibits the conversion of naive T cells into Tregs, we used naringenin to treat naive T cells. While TGF-β1 significantly induced the conversion of naive T cells to $CD4^+CD25^+Foxp3^+$ Tregs (from 4.5% to 23.7%), naringenin significantly reversed this process (from 23.7% to 10.7%) (Fig. 5A and 5B). These data suggest that naringenin might inhibit Treg production, which, in turn, recovers T cell function.

DISCUSSION

Chemotherapy drugs are commonly used to inhibit metastases in breast cancer patients who undergo surgery (Markiewicz et al., 1996; Shenkier et al., 2004). However, the severe side effects (such as immunosuppression and myelosuppression) limit the long-term usage of chemotherapy. Naringenin is a 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 the present study, we examined the antimetastasis effect of naringenin in a breast cancer resection model. Results showed that orally administered naringenin significantly decreased the number of metastatic tumor cells in the lung (Fig. 1B) and extended the life span of tumor resected mice (Fig. 1C), which makes it a promising adjuvant drug for tumor resection patients.

The *in vivo* experiments were performed using a most challenging 4T1 model, which is highly malignant and poorly immunogenic. Long-term survival is seldom observed in this model (Lohr et al., 2000; Pulaski et al., 2000). However, we found a prolonged survival in naringenin treated mice, among which 30% showed long-term survival (Fig. 1C). This is attributed to the inhibition of lung metastasis by naringenin (Fig. 1B). Because naringenin does not directly inhibit tumor proliferation (Fig. 2A and 2B), its antimetastatic effect may be due to other mechanisms. In addition, we found that surgery itself can promote metastases to lungs (Fig. S1), which is consistent with other reports (Page and Ben-Eliyahu, 1997; van der Bij et al., 2008).

The tumor resection model has its unique characteristics. Surgery may cause tumor cells to detach from primary tumors and disseminate throughout the body (van der Bij et al., 2008). Meanwhile, cell-mediated immunity is suppressed after surgery (Faist et al., 1986; Salo, 1992; Boomsma et al.,

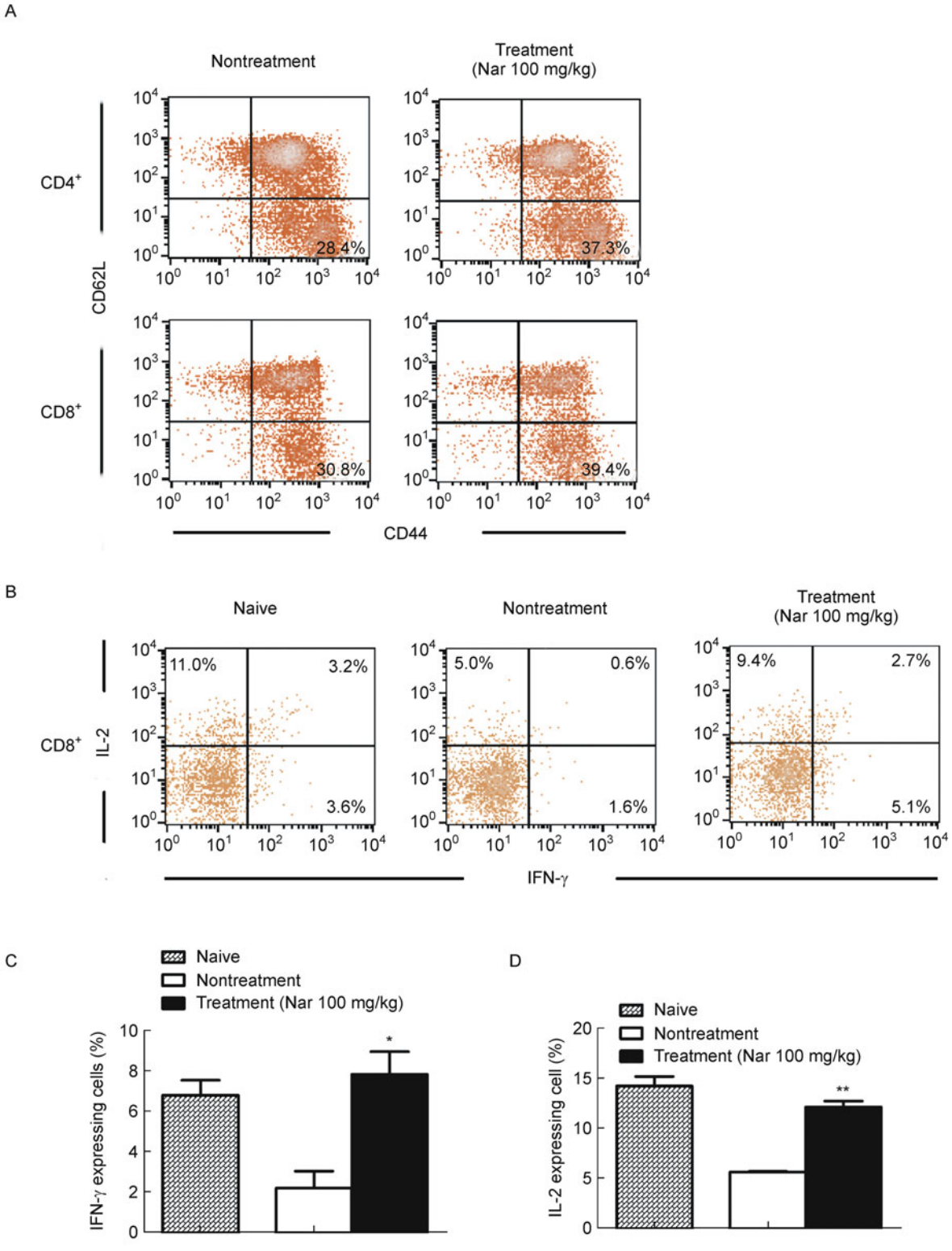

Figure 3. Naringenin promotes T cell activation and restores T cell function in a tumor resection model. Naringenin treated mice, untreated mice or normal naive mice were killed 7 days after surgery. T cells from lungs and spleens were analyzed for expression of cell surface markers and intracellular cytokines using flow cytometry. (A) Naringenin treated mice displayed elevated 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 IFN-γ producing $CD8^{+}$ T cells and IL-2 producing $CD8^{+}$ T cells. The representative bar graph summarizing the flow cytometry results of (B) is shown in (C) and (D), respectively. Data are given as mean ± SD; n = 3 mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$ for treatment group *versus* the nontreatment control (Student's *t*-test).

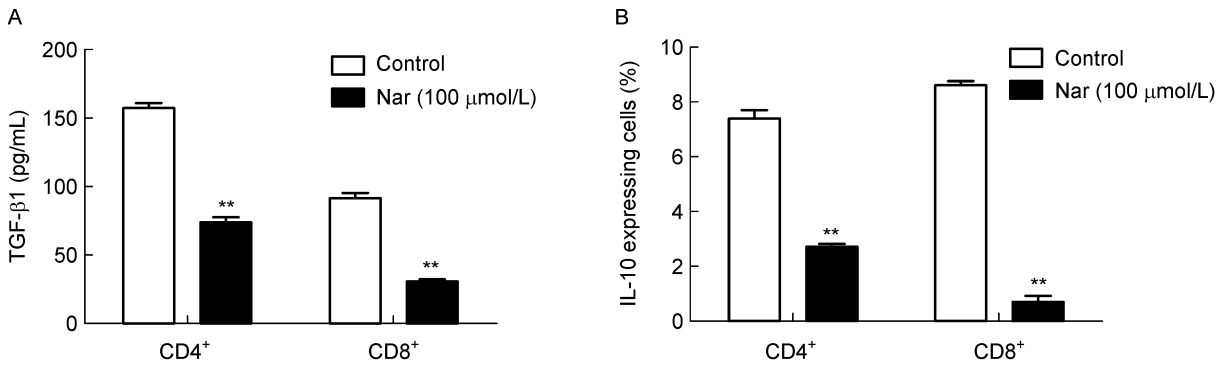

Figure 4. Naringenin treatment downregulates the expression of immunosuppressive cytokines in T cells *in vitro*. The purified splenic T cells or total splenocytes were cultured with or without naringenin (100 μmol/L) for 72 h in the presence of anti-CD3 (3 μg/mL) and anti-CD28 (1 μg/mL) antibodies. (A) The TGF-β1 protein level in the supernatant (X-VIVO 15 serum-free medium) of purified T cells was determined by ELISA. (B) The intracellular expression of IL-10 in splenic T cells was assessed using flow cytometry. Data are presented as mean ± SD; $^{**}p < 0.01$ for naringenin treatment *versus* control (Student's *t*-test).

2010), when impaired T cell function and Treg predominance have been observed in many studies (Shafir et al., 1980; Brune et al., 1999; Ogawa et al., 2000; Hogan et al., 2011). The detached tumor cells and the less efficient immune surveillance may account for the metastasis-promoting effect of surgery. Since the disseminated tumor cells have a great chance of encountering immune cells that are distributed throughout the entire body, restoration of the immune cell function is extremely important for eliminating these tumor cells. Therefore, we examined T cell function of the tumor resected mice, especially in spleen, which could reflect the immune states of the whole body. Flow cytometry analysis showed that naringenin treated mice exhibited an increased proportion of IFN-γ and IL-2 expressing $CD8^+$ T cells in spleen, and an elevated level of T cell activation marker $CD44^{high}CD62L^-$ in the lung, suggesting the recovery of T cell function (Fig. 3A–D). Since T cells are critical for antitumor immunity, enhanced T cell function of the host may contribute to the antimetastatic effect of naringenin. This result can explain the low efficacy of naringenin on inhibiting 4T1 proliferation *in vitro* (Fig. 2A), since no T cell existed in the culture system. However, naringenin treatment also failed to inhibit primary tumor growth (Fig. 2B), which might be due to the extremely large number of tumor cells and a high proliferation rate. Tumor cells could be eliminated by activated T cells only when the tumor cells were present in a very small number. Once the primary tumor was removed, the antitumor function of T cells was restored, suggesting that naringenin is efficient in eliminating a 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 demonstrated that naringenin could enhance T cell function in a bleomycin induced pulmonary fibrosis (similar to the present results) (Du et al., 2009; Fang et al., 2010). The different effects of naringenin on T cells may be caused by different disease models and different experiment systems. The present results confirm that naringenin is an immunomodulator—regulating the suppressed or overactivated T cells to normal levels.

As immunosuppression is severe in cancer patients undergoing surgery and probably contributes to T cell anergy, we examined the effect of naringenin on immunosuppression related molecules and cells *in vitro* (Liyanage et al., 2002; Beyer and Schultze, 2006). The results revealed that naringenin significantly inhibited the production of TGF-β1 and IL-10 (Fig. 4A and 4B). Furthermore, naringenin inhibited TGF-β1 induced Treg production *in vitro* (Fig. 5A and 5B). These data demonstrate that naringenin can reverse Treg related immunosuppression, which in turn increase the proportion of activated T cells. TGF-β signaling is essential for the transcription of Foxp3, a transcription factor necessary for the production of Tregs (Chen and Konkel, 2010). A recent study showed that naringenin could inhibit TGF-β ligand-receptor interaction, thus inhibiting TGF-β signal transduction and the downstream gene expression (Yang et al., 2011). Therefore, a possible mechanism is that naringenin inhibits TGF-β ligand-receptor interaction, and the suppressed TGF-β signaling leads to reduced transcription of Foxp3, making it difficult for naive CD4 T cells to develop into Tregs.

The regulation of the immune system is a complicated process and involves many types of cells, such as MDSCs and tumor-associated macrophages (TAMs) (Marigo et al., 2008; Coffelt et al., 2009; Gabrilovich and Nagaraj, 2009). Whether the inhibition of Tregs by naringenin plays a definitive role in T cell restoration should be clarified in future studies.

In summary, our results indicate that orally administered naringenin can inhibit the outgrowth of metastases after surgery via regulating host immunity. The antimetastatic properties and low toxicity of naringenin make it an ideal surgical adjuvant therapy for breast cancer. In addition,

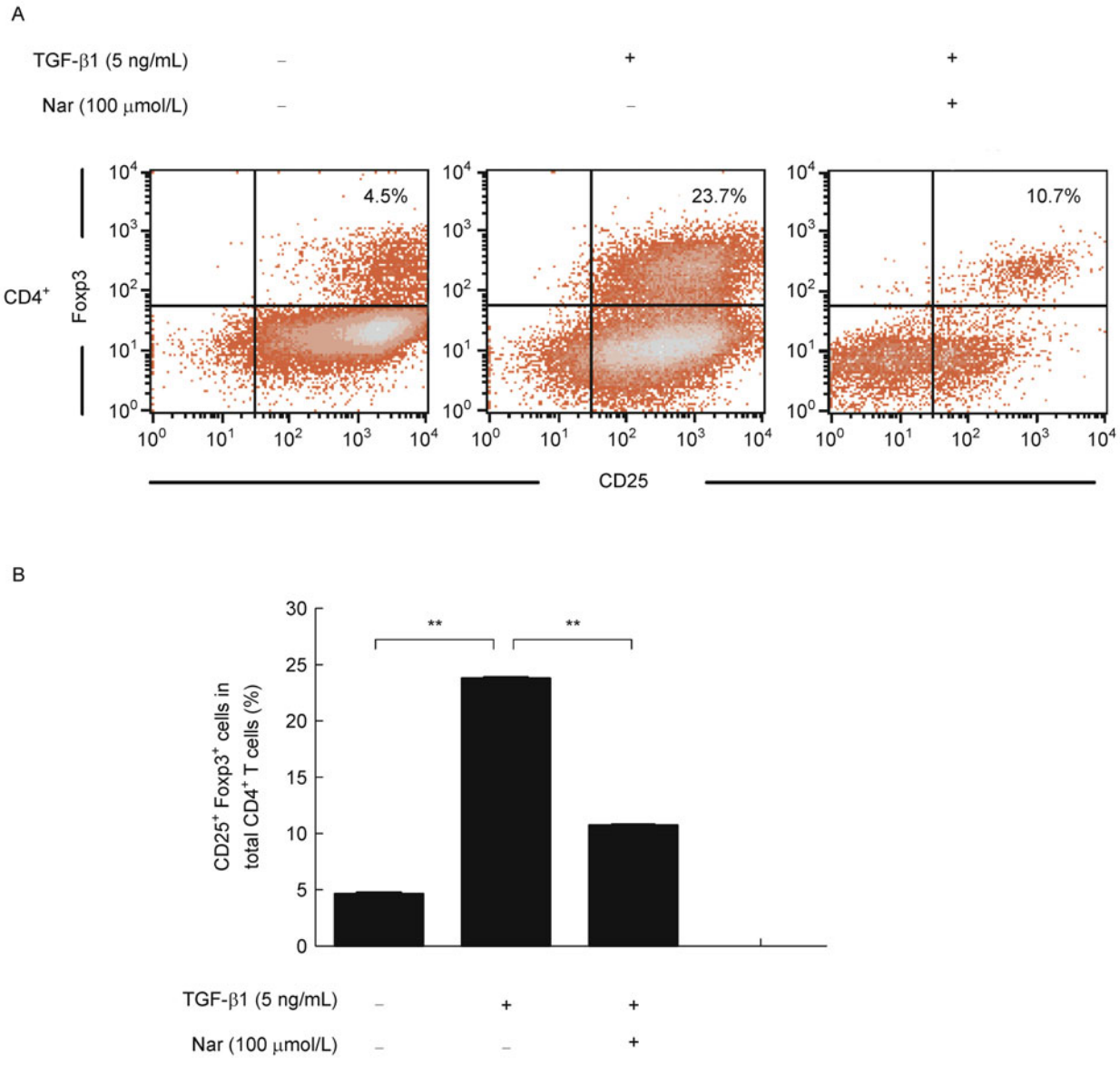

Figure 5. Naringenin inhibits regulatory T cell production *in vitro*. Purified splenic $CD4^+$ T cells were cultured with anti-CD3 antibody (3 μg/mL), anti-CD28 antibody (1 μg/mL) and TGF-β1 (5 ng/mL) to induce regulatory T cells. Naringenin was supplemented to the culture at concentrations of 0 and 100 μmol/L for 72 h. Cells that were 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 presented as mean ± SD; $^{**}p < 0.01$ (Student's *t*-test).

naringenin might be efficient in autoimmune disease treatment for its immunomodulating activities.

MATERIALS AND METHODS

Animals and reagents

Six- to eight-week-old female BALB/c mice and C57BL/6 mice were purchased from the 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 of the USA and the Animal Care and Use Committee of the Institute of Biophysics, CAS, Beijing.

Naringenin was purchased from Shanxi Huike Botanical Development Co. Ltd. Cell culture reagents and type IV collagenase was from Invitrogen. DNase I and 6-thioguanine were obtained from Sigma.

Recombinant TGF-β1 was from PeproTech. A 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 U/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 divided into two groups: the nontreatment surgical control group and naringenin treatment group. Each group received a mammary fat-pad injection of 2.0×10^5 4T1 cancer cells on day 0 and a tumor resection on day 14. The naringenin treatment group was administered a daily dose of 100 mg/kg (i.g.) naringenin (suspended in 1% CMC-Na) since day 11, and the nontreatment group received 1% CMC-Na only; the treatment was sustained for 24 days. For survival experiments, the life span of 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 days 17 and 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 2.0×10^5 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 1% CMC-Na only. The treatment was sustained for two weeks. The primary tumor growth was evaluated by caliper measurement twice a week [volume = 0.5 × $(\text{width})^2$ × (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 type IV collagenase (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 the medium containing 60 μmol/L 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. One colony represents one 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 96-well plates in complete RPMI 1640 medium and were cultured 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 at 37°C for 4 h. 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 type IV collagenase 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 rotation/min 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 naive C57BL/6 mice were pelleted and resuspended in the MACS buffer (PBS with 0.5% BSA and 2 mmol/L EDTA, pH 7.2) to a final concentration of 1.1×10^8 cells/mL. The CD4 or CD8 microbeads (Miltenyi Biotec) were used according to the manufacturer's instructions. Briefly, 10 μL of CD4 or CD8a microbeads were added to 10^7 splenocytes and incubated at 4°C for 15 min. Cells were then washed and resuspended to 2×10^8 cells/mL. Splenocytes were separated using positive-selection MS columns. Finally, the $CD4^+$ or $CD8^+$ T cells were flushed out, and 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* assay of T cell activation, 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. To analyze the *in vivo* production of IL-2 and IFN-γ, the isolated splenocytes from naringenin treated mice, untreated mice or normal naive 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 the *in vitro* assay of T cell cytokine production, the isolated splenocytes or purified T cells from normal naive 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. The concentration of TGF-β1 from purified T cells in the supernatant was determined by ELISA (Promega) according to the manufacturer's protocols. Cells were further stimulated, surface stained, fixed and permeabilized, and finally intracellularly stained for IL-10, as described above.

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

Treg induction assay

Isolated splenocytes of normal naive 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 concentrations of 0 and 100 μmol/L for 72 h. Cells 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 were further intracellularly stained with anti-Foxp3 antibodies.

Statistical analysis

Data were presented as mean ± SD. Statistical analyses were performed using the Student's *t*-test when comparing two groups. To compare 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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