



A novel regulatory mechanism of naringenin through inhibition of T lymphocyte function in contact hypersensitivity suppression

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ABSTRACT

Naringenin, a flavonoid in grapefruits and citrus fruits, has been reported to exhibit anti-inflammatory and anti-oxidative activities. Contact hypersensitivity (CHS) is a T cell-mediated immune reaction, and the factors released from macrophages also contribute to this response. Previous studies showed that naringenin suppressed CHS by inhibiting activation and migration of macrophages. However, little is known about naringenin's effects on T lymphocytes. Our study indicated that naringenin potently suppressed picryl chloride (PCI)-induced contact hypersensitivity by inhibiting the proliferation and activation of T lymphocytes. In vitro, both of the activated hapten-specific T cells and the T cells stimulated with anti-CD3/anti-CD28 showed growth arrest after naringenin treatment. Furthermore, naringenin reduced CD69 (the protein level) and cytokines such as IL-2, TNF- α , and IFN- γ (the mRNA level) expressions which highly expressed by activated T cells. Meanwhile, naringenin also induced T cell apoptosis by upregulation of Bax, Bad, PARP, cleaved-caspase 3 and downregulation of phosphorylated Akt, Bcl-2. These findings suggest that, besides its anti-inflammatory activities in macrophages, naringenin also showed inhibitory effects on the activation and proliferation of T cells to alleviate symptoms of contact hypersensitivity.

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1. Introduction

Contact hypersensitivity (CHS) is recognized as a classical example of T cell-mediated delayed type immune response which has been studied extensively [1,2]. It is an acute cutaneous inflammatory reaction elicited by applications of a chemical in animals previously sensitized with the same hapten. During this immune response, the activation and proliferation of T lymphocytes plays a crucial role in the process. T cells become activated and expanded after first sensitization. When the skin is challenged by the relevant haptens, the hapten-specific T cells are activated. The cytokines and chemokines produced by these effector T cells recruit and activate a variety of bystander cells including macrophages, subsequently initiating the inflammatory reactions [3,4].

Clinically, corticosteroids such as dexamethasone are usually used to counteract this dermatitis. However, side effects including stomach upset, headache, and weight loss may occur if dexamethasone is given for a few days. More and more studies have showed that several natural products also exhibit anti-anaphylaxis and anti-inflammatory effects on CHS [5]. Naringenin, one of the most abundant flavonoids in grapefruits and citrus fruits, is known as a safe natural product with various bioactive effects [6,7]. Previous studies have reported that naringenin has anti-oxidative,

anti-inflammatory, and anti-cancer activities [8–10]. Naringenin induces apoptosis in various human cancer cells [11–14]. In the anti-carcinogenesis assay in vitro, naringenin caused apoptosis via inhibiting activity of PI3K and expression of bcl-2 [12,14]. In addition, naringenin has been reported to suppress anaphylaxis through inhibiting activation and migration of macrophages. Previous studies have indicated that naringenin significantly inhibited proinflammatory cytokines such as MCP-1, NO, TNF- α in LPS-stimulated RAW 264.7 macrophages [15,16]. Naringenin also showed inhibitory effects on LPS-induced prostaglandin E2 production and COX-2 activity in rat peritoneal macrophages [17]. However, its effect on T lymphocytes is still unclear. Since CHS is known as T cell-mediated immune response, we suppose the inhibitory effect of naringenin on the allergic reaction may also relate to T lymphocytes.

In this study, we investigated the effect of naringenin on PCI-induced CHS and the function of T lymphocytes. The underlying mechanisms which related to activation and proliferation of T cells were investigated in detail. Furthermore, we also examined the intracellular signal transduction pathways involved in regulating apoptosis.

2. Materials and methods

2.1. Animals

Female BALB/c mice (6–8 weeks old, 18–22 g) were obtained from the Experimental Animal Center of Yangzhou University

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(Yangzhou, China). They were maintained with free access to pellet food and water in plastic cages at $21 \pm 2^\circ\text{C}$ and kept on a 12-h light–dark cycle. Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

2.2. Reagents and antibodies

Naringenin (purity > 98%) was obtained from TCM Institute of Chinese Materia Medica (Nanjing, China). Cyclosporin A (CsA) was obtained from Sandoz Ltd. (Basel, Switzerland). Picryl chloride (PCI) was obtained from Nacalai Tesque Inc. (Kyoto, Japan). 2,4,6-Trinitrobenzenesulfonic acid hydrate (TNBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylteraz-olium bromide (MTT), and mitomycin C were obtained from Sigma (St Louis, MO). Dexamethasone was from Xianju Pharmaceutical Co., Ltd. (Zhejiang, China). Glycylglycin was obtained from Nanjing Sunshine Biotechnology Co., Ltd. (Nanjing, China). Annexin V-FITC/propidium iodide (AV/PI) assay kit for flow cytometry was from Jingmei Biotech Co., Ltd. (Shenzhen, China). Fetal bovine serum (FBS) and RPMI 1640 were purchased from Gibco-BRL (Gaithersburg, MD).

The antibodies used in this study were as follows: mouse anti-CD3 (NA/LE), mouse anti-CD28 (NA/LE), anti-Bcl-2 (BD Pharmingen, San Diego, CA), anti- β -actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Akt, anti-phospho-Akt (ser 473), anti-Bad, anti-Bax, anti-PARP, and anti-cleaved-caspase 3 (Cell Signaling Technology, Boston, MA), peroxidase-labeled anti-mouse and anti-rabbit antibody (KPL, Gaithersburg, ML).

2.3. Picryl chloride-induced contact hypersensitivity

The picryl chloride-induced contact hypersensitivity was measured according to the method reported by Fei et al. [18]. BALB/c mice were sensitized by applying 0.1 ml of 1% picryl chloride (PCI) in ethanol on the abdomen. Five days later, they were challenged with 30 μl of 1% PCI in olive oil on the right ear. After 24 h, the left and right ears thickness were measured with an engineer's micrometer (Mitutoyo Co., Tokyo, Japan), respectively. Ear swelling was evaluated by the difference in thickness between the right and left ears. Naringenin (5 or 10 mg/kg) was orally administered for 6 days after sensitization. Control animals with contact hypersensitivity were given water instead of drugs.

2.4. Cell culture and T cell purification

Mouse lymphocytes were isolated from lymph nodes of BALB/c mice. T cells were purified according to standard procedures using Dynal® mouse T cell Negative Isolation Kit (Invitrogen Dynal AS, Oslo, Norway) via magnetic cell separation. The purity was higher than 97%. All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in a 5% (v/v) humidified CO_2 incubator.

2.5. Hapten-specific T cell proliferation

The detection was according to the report [18]. Splenocytes, isolated from BALB/c mice 5 days after sensitization, were treated with 1.0 mM TNBS (water-soluble analog of picryl chloride) and 25 mg/ml mitomycin C, then incubated at 37°C for 30 min. After being washed 3 times in HBSS (containing 0.6% glycylglycin), the cells were used as stimulator cells (trinitrophenylated splenocytes). Meanwhile, lymph node cells from the above described sen-

sitized mice, which the adherent cells had been removed, were used as responder cells. The 4×10^5 stimulator cells and 2×10^5 responder cells were co-cultured for 72 h. Then cell proliferation was examined by means of MTT assay. The stimulation index (SI) was calculated as follows:

$$\text{Stimulation index} = \text{OD}_{\text{stimulated cells}} / \text{OD}_{\text{nonstimulated cells}}$$

2.6. MTT proliferation assay

Purified mouse T cells were cultured in 96-well plates at a density of 3×10^5 cells/well in RPMI 1640 medium (0.2 ml). After stimulated with anti-CD3 (10 $\mu\text{g}/\text{ml}$) plus anti-CD28 (1 $\mu\text{g}/\text{ml}$), the cells were treated with or without naringenin for 24 h at 37°C in 5% CO_2 , then the cell viability was assessed by MTT assay. MTT (4 mg/ml in PBS, 20 μl per well) was added to each well. After 4 h of additional incubation, remove the culture media, 200 μl DMSO was added to dissolve the crystals. The absorption values at 570 nm were determined.

2.7. Flow cytometric analysis

The mouse lymphocytes were from lymph nodes of BALB/c mice 4 days after sensitization. After cultured at 37°C with or without 1.0 mM TNBS in the presence of drugs for 12 h, the cells were harvested and washed twice with cold phosphate-buffered saline (PBS). Then, the cells were incubated with 2 μl anti-CD69 antibody (FITC conjugated) for 30 min on the ice before flow cytometric analysis.

The purified mouse T cells were harvested and washed twice with cold phosphate-buffered saline (PBS), suspended in 0.5 ml of $1 \times$ binding buffer. After incubation with 2 μl Annexin V-FITC and 2 μl PI for 20 min at room temperature in the dark, flow cytometric analysis was carried out.

Data were analyzed by Cell Quest software.

2.8. Semi-quantification of mRNA expression by means of RT-PCR

The lymph node cells from BALB/c mice 4 days after sensitization were cultured at 37°C with or without 1.0 mM TNBS in the presence of naringenin for 6 h before harvest. The purified mouse T cells were stimulated with anti-CD3 (10 $\mu\text{g}/\text{ml}$) plus anti-CD28 (1 $\mu\text{g}/\text{ml}$), and then they were incubated at 37°C in the presence of naringenin for 12 h. After naringenin treatment, the cells were harvested and washed twice with cold PBS. Total RNA was extracted with TRIzol reagent (Invitrogen). The cDNA synthesis reaction was performed according to standard procedures using ReverTra Ace® kit (Toyobo, Japan). PCR primer sequences (Invitrogen) were as follows: β -actin, forward 5'-ACATCTGCTGGAAGGTGGAC and reverse 5'-GGTACCACCATGTACCCAGG; IFN- γ , forward 5'-CTTCTTCAGCAACGCAAGGC GAAAA and reverse 5'-CCCCAGATACAACCCCGCAATCA; TNF- α , forward 5'-CATCTTCTCAAAATTCGAGTGACAA and reverse 5'-TGGGAGTAGACAAGGTACAACCC; IL-2, forward 5'-CTACAGCGGAAGCA CAGC and 5'-TCCTCAGAAAGTCCACCA. PCR was performed at 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s. The cycling number was 28 for β -actin. Others were 30 cycles. PCR products were electrophoresed in 1.5% agarose gel and visualized by means of ethidium bromide staining.

Data were analyzed by BandScan software.

2.9. Western blot

The purified T cells were stimulated with anti-CD3 (10 $\mu\text{g}/\text{ml}$) and anti-CD28 (1 $\mu\text{g}/\text{ml}$) in vitro. After incubation with or without naringenin for 24 h, the cells were harvested and lysed. The protein concentrations were quantified using a BCA protein assay kit

(Pierce, Rockford, IL) according to the manufacturer's specifications. An equal amount of protein was subjected to electrophoresis on SDS-PAGE and transferred by electroblotting to a polyvinylidene fluoride membrane. The membrane was incubated with primary antibody overnight at 4 °C, and then incubated with secondary antibody for 1 h at room temperature. Detection was performed using a 20× LumiGLO Reagent and 20× Peroxide kit (Cell Signaling Technology, Boston, MA).

2.10. Statistical analysis

All results represent as means $\pm$ SD of three independent experiments. Each experiment includes triplicate sets in vitro and eight animals of each group in vivo. Analysis of variance (ANOVA) was used to test the difference between groups. And Student's *t* test was used to test the difference between two groups. *P* < 0.05 was considered to be significant.

3. Results

3.1. Naringenin suppressed contact hypersensitivity in mice

In order to induce the contact hypersensitivity, the mice were sensitized with PCI on day 0 and challenged with PCI on day 5. Naringenin was orally administered every day until the day of sacrifice. As shown in Fig. 1A, Naringenin at 5 and 10 mg/kg potentially inhibited the ear swelling in a dose-dependent manner without obvious side effect. The body and immune organ weights have no change compared with control (Fig. 1B–D). Dexamethasone as

a positive drug also showed a strong inhibition. However, it made the body weight, spleen weight, and thymus weight significantly decrease (Fig. 1B–D) which indicated its toxicity.

3.2. Naringenin inhibited the proliferation of activated T lymphocytes

As a classic model of T cell-mediated delayed-type hypersensitivity (DTH), contact hypersensitivity (CHS) is considered to associate with activation and proliferation of T cells. Therefore, the inhibitory effect of naringenin on CHS may relate to T lymphocytes. To determine naringenin's effect on T cells in CHS, the proliferation of hapten-specific T cell was examined. The stimulation index (SI) reflecting the antigen-induced T cell proliferation was decreased after naringenin treatment (Fig. 2A). We also investigated the influence of naringenin on the proliferation of purified mouse T cells which were activated with anti-CD3/anti-CD28 in vitro. The results also showed that naringenin obviously inhibited the activated T cell amplification (Fig. 2B).

3.3. Naringenin reduced CD69 and cytokines expressions in activated T lymphocytes

CD69 has served as a marker of T lymphocytes undergoing activation. It can be triggered by several agents interacting with the T cell receptor (TCR)/CD3 complex. The results showed that CD69 expression was increased in the hapten-specific T lymphocytes after 12 h incubation with TNBS (1 mM), and naringenin moderately reduced CD69 expression in activated hapten-specific T lymphocytes (Fig. 3A).

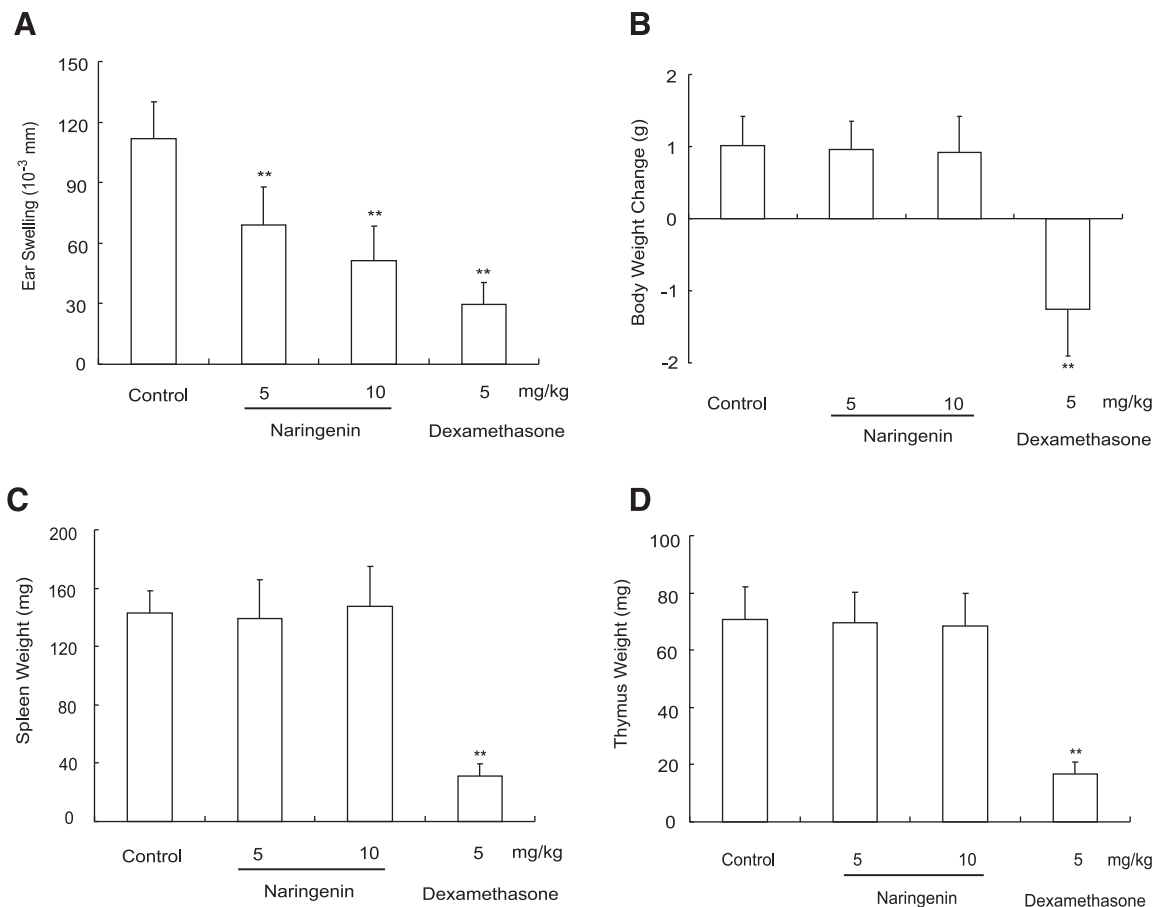


Fig. 1. Effects of naringenin on PCI-induced contact hypersensitivity in mice. The detection of ear swelling (A), body weight (B), spleen weight (C), thymus weight (D). All drugs were given orally for 6 days after sensitization. Each figure indicates the mean $\pm$ SD of eight mice (**P* < 0.05, ***P* < 0.01 vs control).

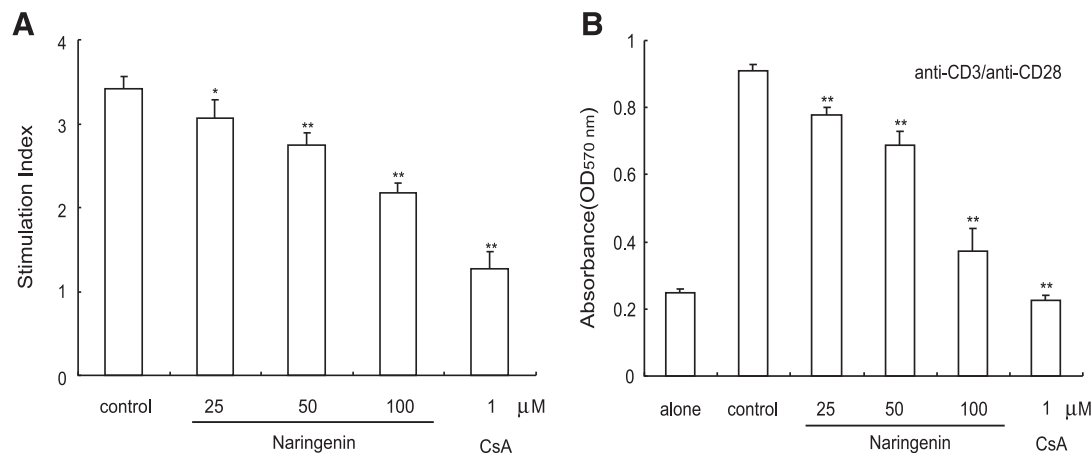


Fig. 2. Effect of naringenin on T lymphocyte proliferation. (A) The lymph node cells from sensitized mice were activated in an antigen-specific manner and proliferated in vitro by trinitrophenylated splenocytes. The stimulated hapten-specific T lymphocytes were treated with naringenin (25, 50, and 100 μ M) or CsA (1 μ M) for 72 h at 37 $^{\circ}$ C, and the cell proliferation were assessed by MTT assay. (B) Purified mouse T cells were stimulated with anti-CD3 (10 μ g/ml) and anti-CD28 (1 μ g/ml). After incubation with naringenin or CsA for 24 h at 37 $^{\circ}$ C, the cell viability was assessed by MTT assay. Data represent means $\pm$ SD of three independent experiments in triplicate (* P < 0.05, ** P < 0.01 vs control).

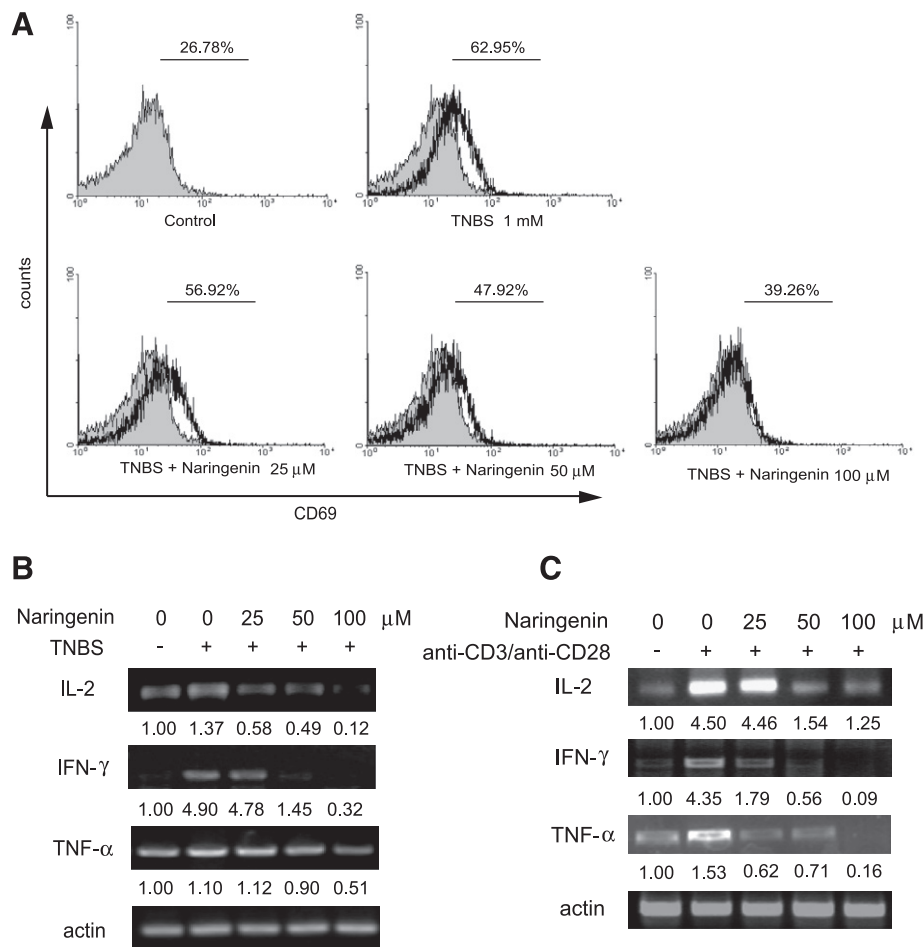


Fig. 3. Effect of naringenin on CD 69 (protein level) and cytokines (mRNA level) expressions. The lymph node cells (5×10^6 cells/well) from 4 days BALB/c mice after sensitization were cultured at 37 $^{\circ}$ C with or without 1.0 mM TNBS in the presence of naringenin. (A) After incubation for 12 h, CD69 expression was determined by flow cytometry. Unstimulated cells served as control. (B) Six hours after culture, the lymph node cells were harvested for mRNA detection. (C) The activated T cells were incubated at 37 $^{\circ}$ C in the presence of naringenin for 12 h. The mRNA expressions of IL-2, IFN- γ , and TNF- α were determined by RT-PCR. The band density was quantified by BandScan software. The relative density of control group (untreated group) is set as 1 unit, and others were compared with control. The data are representative of three independent experiments.

To clarify the mechanism of the anti-proliferation effect of naringenin in activated T lymphocytes, we examined the expression of IL-2 by RT-PCR. It is generally accepted that IL-2 promote

T lymphocytes proliferation. Furthermore, we also detected the production of typical proinflammatory cytokines IFN- γ , TNF- α in the mRNA levels. The results indicated that naringenin inhibited

the mRNA expressions of IL-2, IFN- γ , TNF- α in the activated hapten-specific T cells (Fig. 3B). Additionally, naringenin also showed similar inhibitory effects on purified T lymphocytes which were activated with anti-CD3/anti-CD28 (Fig. 3C).

3.4. Naringenin-induced apoptosis in activated T lymphocytes

Apoptosis plays a pivotal role in the normal development and pathology of a wide variety of tissues [19]. In order to detect the cell apoptosis, flow cytometric analysis with Annexin V and PI labeling was used. Our results demonstrated that naringenin apparently induced apoptosis in activated T lymphocytes (Fig. 4A). Cleaved-caspase 3 and PARP, which serve as markers of cells undergoing apoptosis, were upregulated by treatment of naringenin (Fig. 4B). Moreover, the expressions of phosphorylated Akt, Bcl-2 which perform anti-apoptotic functions obviously decreased in response to

naringenin (Fig. 4C). Naringenin also enhanced the expressions of pro-apoptotic Bax and Bad (Fig. 4C).

4. Discussion

In this study, we first examined the effect of naringenin on PCI-induced contact hypersensitivity (CHS). The results showed that orally administered naringenin remarkably suppresses the PCI-induced ear swelling response in CHS. The inhibition ratio is 54.1% in high dose group (Fig. 1A) without any obvious adverse effect. The body and organ weights have no change compared with the controls (Fig. 1B–D), suggesting that naringenin is effective with no apparent toxicity. Meanwhile, the animals after routine dose of dexamethasone treatment showed obvious side effects such as body weight loss and immune organ atrophy (Fig. 1B–D).

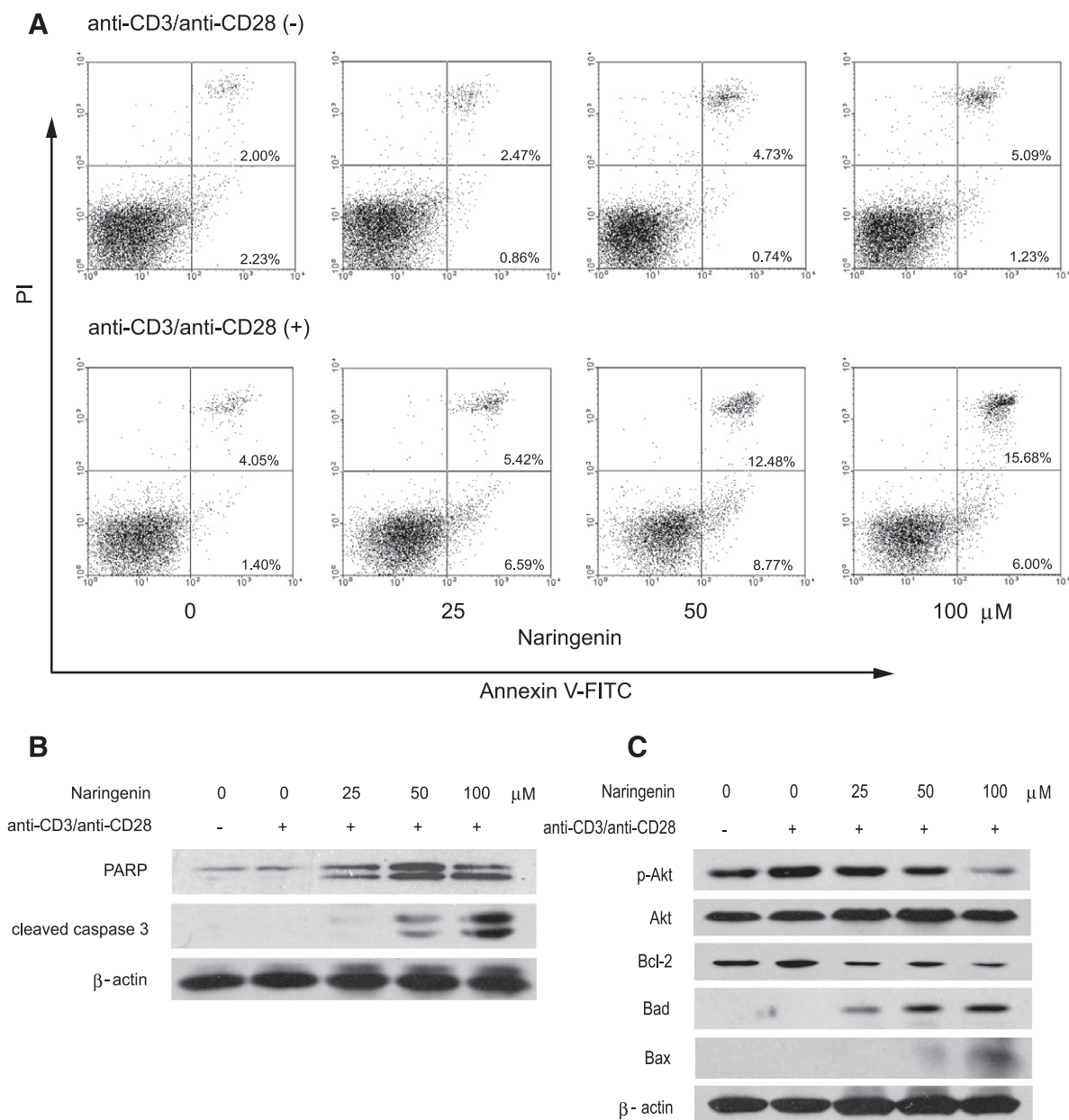


Fig. 4. Naringenin-induced apoptosis in activated T lymphocytes. The purified T cells were stimulated with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) in vitro. After incubation with or without naringenin for 24 h, the cells were harvested. (A) The cells were assayed for the Annexin V-positive populations. (B,C) Total cellular proteins (20 μg) were subjected to SDS/PAGE, and the expressions were determined by Western blotting. The data represent three different experiments.

It is believed that the activation and proliferation of T cells against an antigen contributed to the inflammatory process in CHS response. In the experiment of hapten-specific lymphocyte proliferation, we found that naringenin decreased the cell number in a concentration-dependent manner (Fig. 2A), indicating that the activity of naringenin against CHS was due to its limitation of T cell amplification. It is known that T cell activation requires two signals. The interaction of TCR $\alpha\beta$ /CD3 and their coreceptors, CD4 and CD8, with the MHC-peptide complex releases the first signal. The second signal is a costimulatory signal, usually delivered by interactions between B7 molecules on DCs and CD28 molecules on T cells [20]. The anti-CD3/anti-CD28 mAbs are usually used to activate and expand T lymphocytes by imitating signals 1 and 2. To evaluate the effect of naringenin on purified T lymphocytes, we also examined the proliferation of T cells stimulated with anti-CD3/anti-CD28. The results showed that naringenin remarkably reduced the activated T cell amplification (Fig. 2B).

To determine the mechanisms of T cell growth arrest with naringenin treatment, we first investigated the expression of CD69 in the hapten-specific T cells. CD69 is recognized as an early activation marker expressed in T lymphocytes following stimulation. It plays a very important role in regulation of immune functions including the induction of intracellular signaling. Previous studies indicated that the increased CD69 expression in T lymphocytes correlated with T lymphocytes proliferation [21]. Our results demonstrated that CD69 expression decreased following naringenin treatment in activated hapten-specific T lymphocytes (Fig. 3A), indicating naringenin reduced T lymphocytes amplification by inhibiting the activation of T cells. Furthermore, as contact allergens preferentially induce Th1-predominant responses, the cytokines IL-2 and IFN- γ produced by type-1 cells play pivotal roles in PCI-induced CHS. IL-2 is considered to promote the activation and proliferation of T cells, and IFN- γ secreted by activated T lymphocytes contribute to stimulate the macrophages [22]. In addition, another important proinflammatory cytokine TNF- α is also involved in CHS response. Pasparakis et al. demonstrated that TNF- α KO mice have a decreased CHS response [23]. The mRNA expressions of IL-2, IFN- γ , and TNF- α are related to T lymphocyte activation. Our data indicated that naringenin reduced the expression of IL-2 in the mRNA levels (Fig. 3B and C). Apparent reduction of this cytokine was found in high concentration group (100 μ M). The results also demonstrated that IFN- γ and TNF- α were reduced by naringenin, suggesting its suppression on T lymphocyte activation (Fig. 3B and C).

The further investigation about the effect of naringenin on cell cycles is carried out. However, we did not observe cell cycle arrest but apoptosis in activated T cells after naringenin treatment (data not shown). Therefore, to validate the findings, we detected the cell apoptosis by flow cytometric analysis with Annexin V-FITC/PI staining. The results confirmed that naringenin induced the activated T lymphocytes apoptosis (Fig. 4A). Apoptosis plays an important role in counteraction of the excessive immune responses. It is considered as an essential mechanism to eliminate the activated T cells and maintain immune homeostasis [24]. In our study, naringenin triggered apoptosis of activated T cells rather than native T cells. The suppression of the excessive T lymphocytes proliferation may contribute to the therapeutic effect of naringenin on CHS. Additionally, the expression of PARP and cleaved-caspase 3 were examined. The cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis. Caspase 3 is one of the key executioners of apoptosis, and its activated form is cleaved-caspase 3. Our results indicated that naringenin upregulated the cleavage of PARP and caspase 3 in activated T cells, which further confirmed the apoptosis induction by naringenin (Fig. 4B). To study the mechanisms in detail, the Akt pathway and Bcl-2 family which have been demonstrated to regulate apoptosis are con-

sidered. Akt exhibits a variety of anti-apoptotic functions. The activation of Akt signaling promotes cellular growth and survival. In Bcl-2 family, Bax and Bad are activators, while Bcl-2 acts as an inhibitor in apoptosis. Our data showed that naringenin apparently reduce the expressions of phosphorylated Akt and Bcl-2, meanwhile enhance the expressions of Bax and Bad (Fig. 4C).

In conclusion, naringenin potentially suppress the immune responses in CHS by inhibiting proliferation in activated T lymphocytes. The growth arrest in T cells induced by naringenin is due to the inhibition of activation, induction of apoptosis but not cell cycle arrest. These findings extended our understanding of naringenin's effect on CHS, and might provide a new approach to study the bioactivities of naringenin.

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References

- [1] H.N. Eisen, L. Orris, S. Belman, Elicitation of delayed allergic skin reactions with haptens; the dependence of elicitation on hapten combination with protein, *J. Exp. Med.* 95 (1952) 473–487.
- [2] M.H. Flax, J.B. Caulfield, Cellular and vascular components of allergic contact dermatitis, *Am. J. Pathol.* 43 (1963) 1031–1053.
- [3] A. Cavani, C. Albanesi, C. Traidl, S. Sebastiani, G. Girolomoni, Effector and regulatory T cells in allergic contact dermatitis, *Trends Immunol.* 22 (2001) 118–120.
- [4] S. Grabbe, T. Schwarz, Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity, *Immunol. Today* 19 (1998) 37–44.
- [5] J.L. Rios, E. Bas, M.C. Recio, Effects of natural products on contact dermatitis, *Curr. Med. Chem.* 4 (2005) 65–80.
- [6] D. Chen, M.S. Chen, Q.C. Cui, H. Yang, Q.P. Dou, Structure-proteasome-inhibitory activity relationships of dietary flavonoids in human cancer cells, *Front. Biosci.* 12 (2007) 1935–1945.
- [7] J. Szejtli, L. Szente, Elimination of bitter, disgusting tastes of drugs and foods by cyclodextrins, *Eur. J. Pharm. Biopharm.* 61 (2005) 115–125.
- [8] G. Du, L. Jin, X. Han, Z. Song, H. Zhang, W. Liang, Naringenin, a potential immunomodulator for inhibiting lung fibrosis and metastasis, *Cancer Res.* 69 (2009) 3205–3212.
- [9] G. Galati, M.Y. Moridani, T.S. Chan, P.J. O'Brien, Peroxidative metabolism of apigenin and naringenin versus luteolin and quercetin: glutathione oxidation and conjugation, *Free Radic. Biol. Med.* 30 (2001) 370–382.
- [10] L.M. Knowles, D.A. Ziggrossi, R.A. Tauber, C. Hightower, J.A. Milner, Flavonoids suppress androgen-independent human prostate tumor proliferation, *Nutr. Cancer* 38 (2000) 116–122.
- [11] Y.C. Chen, S.C. Shen, H.Y. Lin, Rutinoid at C7 attenuates the apoptosis-inducing activity of flavonoids, *Biochem. Pharmacol.* 66 (2003) 1139–1150.
- [12] C.Y. Jin, C. Park, J.H. Lee, K.T. Chung, T.K. Kwon, G.Y. Kim, B.T. Choi, Y.H. Choi, Naringenin-induced apoptosis is attenuated by Bcl-2 but restored by the small molecule Bcl-2 inhibitor, HA 14-1, in human leukemia U937 cells, *Toxicol. In Vitro* 23 (2009) 259–265.
- [13] S. Kanno, A. Tomizawa, T. Ohtake, K. Koiwai, M. Ujibe, M. Ishikawa, Naringenin-induced apoptosis via activation of NF-kappaB and necrosis involving the loss of ATP in human promyeloleukemia HL-60 cells, *Toxicol. Lett.* 166 (2006) 131–139.
- [14] J.H. Park, C.Y. Jin, B.K. Lee, G.Y. Kim, Y.H. Choi, Y.K. Jeong, Naringenin induces apoptosis through downregulation of Akt and caspase-3 activation in human leukemia THP-1 cells, *Food Chem. Toxicol.* 46 (2008) 3684–3690.
- [15] S. Hirai, Y.I. Kim, T. Goto, M.S. Kang, M. Yoshimura, A. Obata, R. Yu, T. Kawada, Inhibitory effect of naringenin chalcone on inflammatory changes in the interaction between adipocytes and macrophages, *Life Sci.* 81 (2007) 1272–1279.
- [16] S.Y. Lyu, W.B. Park, Production of cytokine and NO by RAW 264.7 macrophages and PBMC in vitro incubation with flavonoids, *Arch. Pharm. Res.* 28 (2005) 573–581.
- [17] Y. Takano-Ishikawa, M. Goto, K. Yamaki, Structure-activity relations of inhibitory effects of various flavonoids on lipopolysaccharide-induced prostaglandin E2 production in rat peritoneal macrophages: comparison between subclasses of flavonoids, *Phytomedicine* 13 (2006) 310–317.
- [18] M. Fei, X. Wu, Q. Xu, Astilbin inhibits contact hypersensitivity through negative cytokine regulation distinct from cyclosporin A, *J. Allergy Clin. Immunol.* 116 (2005) 1350–1356.
- [19] M.O. Hengartner, The biochemistry of apoptosis, *Nature* 407 (2000) 770–776.

- [20] B. Wang, C. Feliciani, I. Freed, Q. Cai, D.N. Sauder, Insights into molecular mechanisms of contact hypersensitivity gained from gene knockout studies, *J. Leukoc. Biol.* 70 (2001) 185–191.
- [21] C.Y. Leung, L. Liu, R.N. Wong, Y.Y. Zeng, M. Li, H. Zhou, Saikosaponin-d inhibits T cell activation through the modulation of PKC θ , JNK, and NF-kappaB transcription factor, *Biochem. Biophys. Res. Commun.* 338 (2005) 1920–1927.
- [22] S. Pastore, A. Cavani, G. Girolomoni, Epidermal cytokine and neuronal peptide modulation of contact hypersensitivity reactions, *Immunopharmacology* 31 (1996) 117–130.
- [23] M. Pasparakis, L. Alexopoulou, V. Episkopou, G. Kollias, Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response, *J. Exp. Med.* 184 (1996) 1397–1411.
- [24] A. Strasser, M. Pellegrini, T-lymphocyte death during shutdown of an immune response, *Trends Immunol.* 25 (2004) 610–615.