

# A dichloromethane fraction of *Triticum aestivum* sprouts reduces allergic immune response through inhibiting Th2 differentiation in ovalbumin-immunized mice

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**Abstract.** *Triticum aestivum* sprouts are small shoots that germinate from seeds and are consumed as a dietary supplement. The present study aimed to determine whether a dichloromethane fraction isolated from *Triticum aestivum* sprouts (TDF) suppressed the allergic immune response in ovalbumin (OVA)-sensitized mice. *In vivo* experiments were performed by administering TDF or vehicle to mice during the sensitization and this was immediately followed by an intradermal injection of OVA into the ears. Splenocytes isolated from OVA-sensitized mice were pre-treated with TDF and re-challenged with OVA for *ex vivo* evaluation. Results demonstrated that TDF suppressed the inflammatory response in ear tissues and levels of total immunoglobulin (Ig)E and OVA-specific IgE in serum. TDF inhibited the production of interleukin (IL)-4 and expression of GATA-binding protein-3 (GATA-3) transcription factor which regulates the differentiation of naïve T helper (Th) cells into Th2 cells in OVA-stimulated splenocytes. TDF inhibited Th1-associated cytokine interferon- $\gamma$  and IL-12 production and downregulated the expression of Th1 specific transcription factor T-box 21 in OVA-stimulated splenocytes. Overall, these results indicated that TDF attenuates OVA-induced allergic immune response

by suppressing the production of Th2 specific cytokine IL-4, through inhibiting transcription factor GATA-3, and suggests that TDF may exhibit the potential to regulate the immune response in allergic diseases.

## Introduction

Allergies are hypersensitivity reactions initiated in response to various environmental allergens. During sensitization to allergens through skin, and the respiratory and digestive tracts, B cells differentiate into immunoglobulin-producing cells and produce allergen-specific IgE, which binds to high-affinity receptor (Fc $\epsilon$ RI) on surfaces of mast cells in the connective or mucosal tissues (1). Subsequent exposure to the allergen activates mast cells through the cross-linking of Fc $\epsilon$ RI-IgE complexes, and activated mast cells then release amines with inflammatory vascular effects (e.g. histamine), leukotrienes derived from arachidonic acid, heparin, proteases, cytokines and chemokines. These factors promote allergic immune response by regulating the differentiations and activations of various immune cells and induce lymphocyte infiltration to the inflamed site (2). In addition, allergen-specific CD4<sup>+</sup> Th2 cells are known to play important roles in the initiation and maintenance of allergic response (3). Cytokines (e.g. IL-4, IL-5, and IL-13) secreted by Th2 cells induce B cells to produce IgE and activate immune cells, including mast cells, basophils and eosinophils. On a macro scale, these responses cause mucus hypersecretion, epithelium fibrosis and are even associated with tissues damage (4).

Many medications used to treat allergic conditions, including antihistamines and corticosteroids, can cause side-effects. For example, long-term corticosteroid therapy may cause Cushing's syndrome, osteoporosis and adrenal insufficiency (5-7). Therefore, new therapeutic agents for the allergic diseases are today's need. Numerous authors have suggested that plant-derived drugs are intrinsically safer than synthetic drugs (8,9).

*Triticum aestivum* sprouts germinate from seed and contain large amounts of chlorophyll, minerals, enzymes,

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**Key words:** *Triticum aestivum* sprouts, anti-allergic activity, ovalbumin, immunoglobulin E, T helper cells

and other functional entities (10). Eosinophil accumulation and activation have been shown to play an important role in the pathogenesis of allergic inflammation and asthma (11,12). Studies have reported that in thalassemic patients with eosinophilia, *Triticum aestivum* sprouts reduced in the numbers of eosinophils in blood (13). We previously showed that a dichloromethane fraction isolated from *Triticum aestivum* sprouts (TDF) contains large amounts of sterols (e.g.  $\beta$ -sitosterol) and polyunsaturated fatty acids (e.g.  $\alpha$ -linolenic acid) and glycolipids (14). Other studies have reported that  $\beta$ -sitosterol has potential anti-allergic effects (15,16), and increasing evidence indicates polyunsaturated fatty acids alleviate allergic diseases (17,18).

However, the effects of TDF on allergic immune response have not been elucidated. In the present study, we examined the anti-allergic activity and mechanism of action of TDF in an OVA-sensitized mouse model.

## Materials and methods

**Extraction and isolation of plant material.** *Triticum aestivum* were supplied by the Korean National Institute of Crop Science and were germinated on sterile organic feat moss (at  $20\pm 2^\circ\text{C}$ ). The dichloromethane fraction was obtained from *Triticum aestivum* sprouts (TDF) as previously described (14). Briefly, *Triticum aestivum* sprouts were cultivated for two weeks after germination, harvested, lyophilized and crushed to obtain a powder with a predetermined particle size. Frozen powder (30 g) was then extracted with methanol. And the extract was dissolved in 800 ml of water and partitioned sequentially with hexane, dichloromethane ( $\text{CH}_2\text{Cl}_2$ ), ethyl acetate (EtOAc) and n-butanol. TDF was filtered through Whatman filter paper (grade no. 1, diameter: 15 cm) and concentrated under reduced pressure using a rotary evaporator (N-1000; EYELA, Tokyo, Japan).

**In vivo experiments.** Female BALB/c mice (6 weeks old) were purchased from Samtako (Osan, Korea), and acclimated in a pathogen-free facility for 2 weeks prior to experiments. Animals were kept in an air-conditioned room ( $22\pm 2^\circ\text{C}$ ,  $55\pm 10\%$  RH) and fed 5L79 rodent diet (Orient Bio, Seongnam, Korea) throughout the experimental period. The ovalbumin (OVA)-sensitized mouse model of this study was previously described by Park *et al.* (19). After acclimation to the facility's environment, mice were divided into five groups ( $n=5$  each): Group 1; 1% carboxymethyl cellulose (vehicle), group 2; OVA sensitization + vehicle, group 3; OVA sensitization + TDF 100 mg/kg, group 4; OVA sensitization + TDF 200 mg/kg, group 5; OVA sensitization + dexamethasone 0.5 mg/kg. On the first day, all mice were intraperitoneally (i.p.) sensitized with 20  $\mu\text{g}$  OVA (grade V; Sigma-Aldrich, St. Louis, MO, USA) and 1 mg of aluminum hydroxide (Imject® A lum; Thermo Scientific, Cramlington, UK) dissolved in 100  $\mu\text{l}$  of phosphate-buffered saline (PBS, pH 7.4); except mice in the group 1. Two weeks later, mice were administered a second i.p. injection of OVA and alum. To investigate *in vivo* effect of TDF on allergic immune response, OVA-sensitized mice were orally administered TDF or dexamethasone (p.o.) once daily for 13 days following the second OVA injection. All animal experiments were conducted after obtaining approval from

the Institutional Animal Care and Use Committee (IACUC) at Chonbuk National University Laboratory Animal Center.

**Ear swelling test.** The ear swelling test was performed to analyze the allergic response with reference to the previous studies (20,21). Mice were injected subcutaneously with 20  $\mu\text{l}$  of 0.1% (m/v) OVA solution dissolved in PBS into ear skin after the 13 days treatment period. Ear thicknesses of mice were measured using a thickness gauge (Mitutoyo, Tokyo, Japan) at 6 and 24 h after OVA injection.

**Histological examination.** Animals were sacrificed by cervical dislocation method under diethyl ether anesthesia. Ear tissues of animals were harvested at 24 h after final OVA challenge for histological examination. Tissues were fixed with 10% formalin and then embedded in paraffin. Tissue sections (4  $\mu\text{m}$ ) were obtained using a microtome for hematoxylin and eosin (H&E) staining, according to the previous report (22), and stained sections were examined under an optical microscope (CX21; Olympus, Tokyo, Japan).

**Serum analysis of immunoglobulins.** After sacrifice, blood was collected from abdominal inferior vena cava and centrifuged at 3,000 rpm for 10 min. Serum levels of total immunoglobulin (Ig)E and IgG1 were evaluated using commercial ELISA kits (BD Biosciences, San Jose, CA, USA), and the serum levels of OVA-specific IgE and OVA-specific IgG1 were measured using a detection kit obtained from Shibayagi (Gunma, Japan).

**Preparation of splenocytes.** Spleens were removed from OVA-sensitized mice and filtered using 100  $\mu\text{m}$  cell strainers. Single-cell suspensions and Histopaque® 1119 (Sigma-Aldrich) were mixed and centrifuged at 1,600 rpm for 30 min to remove erythrocytes. The cell pellets so obtained were washed twice with PBS, and cultured in RPMI-1640 (HyClone Laboratories, Logan, UT, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100  $\mu\text{g}/\text{ml}$  of streptomycin at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$  incubator. Splenocytes were used for experiment after stabilization.

**Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR).** Splenocytes were treated with OVA and various concentrations of TDF for 24 h, and total RNA was extracted from splenocytes with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis were performed using the SuperScript™ III First Synthesis system for RT-PCR kit (Invitrogen) and 1  $\mu\text{g}$  of total RNA. Template of cDNA (50 ng) was amplified using 1X reaction buffer, 10 mM dNTP mixture, 5 units of Taq polymerase (GeNet Bio, Seoul, Korea) and RT-PCR primers. Amplified gene products were loaded into 1.5% agarose gel containing ethidium bromide, all gene expressions were normalized vs. GAPDH of each sample. qRT-PCR method was previously described (23). Primer sequences are summarized in Table I.

**Th1/Th2 cytokine assay.** After preparation, splenocytes were incubated with various concentration of TDF in presence or absence of OVA (100  $\mu\text{g}/\text{ml}$ ) for 48 h. Th1 (IFN- $\gamma$ , IL-12) and Th2 (IL-4, IL-5, IL-13) cytokines in cell culture supernatants

Table I. Primer sequences and product size used for RT-PCR and qRT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)
IFN- $\gamma$	CTTCTTCAGCAACAGCAAGGCGAAAA	CCCCCAGATACAACCCCGCAATCA	456
IL-12	AACCTCACCTGTGACACGCC	CAAGTCCATGTTTCTTTGCACC	309
IL-4	GAGATCATCGGCATTTTGAAC	CTTGGAATCATTCATGGTGCA	267
IL-5	GAAAGAGACCTTGACACAGCTG	GAAGTCTTGCAGGTAATCCAGG	277
IL-13	ATGAGTCTGCAGTATCCCG	CCGTGGCAGACAGGAGTGTT	194
GAPDH (RT-PCR)	GCCAAGGTCATCCATGACAAC	GTCCACCACCCTGTTGCTGTA	498
T-bet	ACTTTGAGTCCATGTACGCATCT	AGGATACTGGTTGGATAGAAGAGGT	113
GATA-3	TACCACCTATCCGCCCTATGT	ACACACTCCCTGCCTTCTGT	138
GAPDH (qRT-PCR)	CATGGCCTTCCGTGTTC	CCTGGTCTCAGTGTAGC	152

IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; T-bet, T-box 21; GATA-3, GATA binding protein-3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

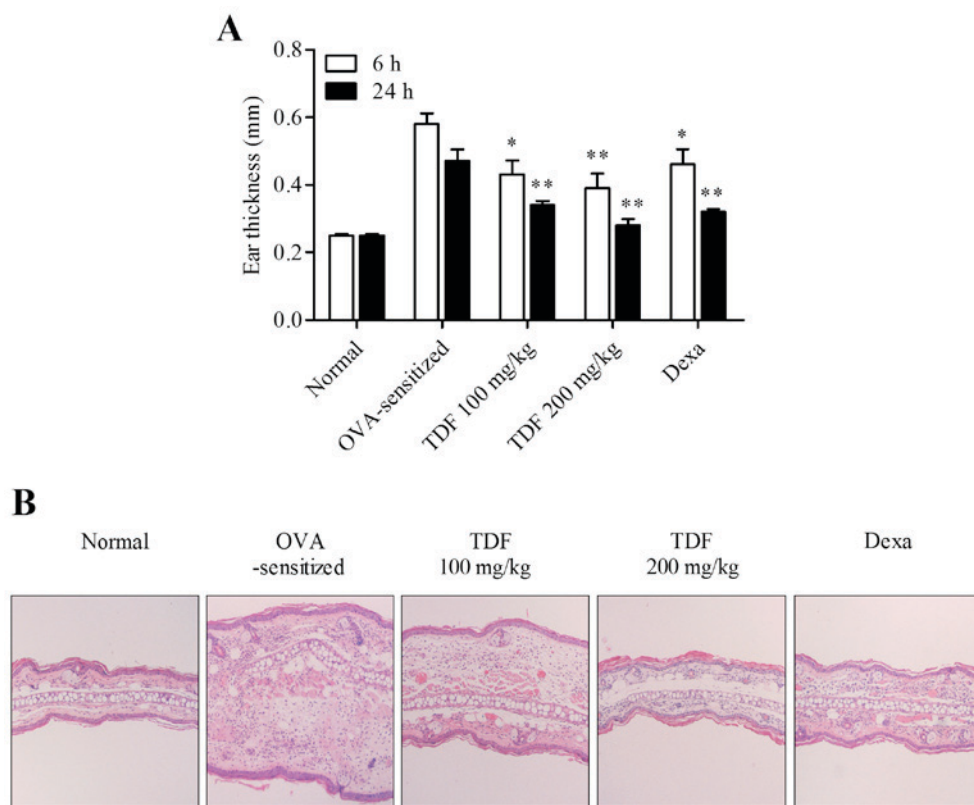


Figure 1. The effect of TDF on allergic skin response in OVA-sensitized mice. (A) Ear swelling response at 6 and 24 h after challenge were measured using a thickness gage. (B) Ear tissues were removed from OVA-sensitized mice at 24 h after 20  $\mu$ l of 0.1% OVA challenge. Samples were fixed with 10% formalin and embedded in paraffin. Histology of ear skin was examined by H&E staining. Original magnification: x400. Results are presented as the means  $\pm$  SEMs of five mice per group. \* $P$ <0.05 and \*\* $P$ <0.01 vs. OVA-sensitized group.

were measured using the following ELISA kits: mouse IFN- $\gamma$ , IL-12 and IL-4 kits were purchased from BioLegend (San Diego, CA, USA) and mouse IL-5 and IL-13 kits from eBioscience (San Diego, CA, USA).

**Statistical analysis.** Results are presented as means  $\pm$  SEMs. The Student's *t*-test was carried out to verify the difference between the control and experimental groups in GraphPad Prism software (version 5.0). Statistical significance was accepted for *p*-values <0.05.

## Results

**Effect of TDF on allergic skin response in OVA-sensitized mice.** Allergic inflammation can be elicited on sensitized subjects when exposure to a specific allergen (24). To investigate the inhibitory effect of TDF on cutaneous allergic response, mice were sensitized with OVA and administered TDF for 13 days, and then challenged with an s.c. injection of OVA into ears. Swelling responses were quantified by measuring ear thicknesses. As shown in Fig. 1A, ear thickness

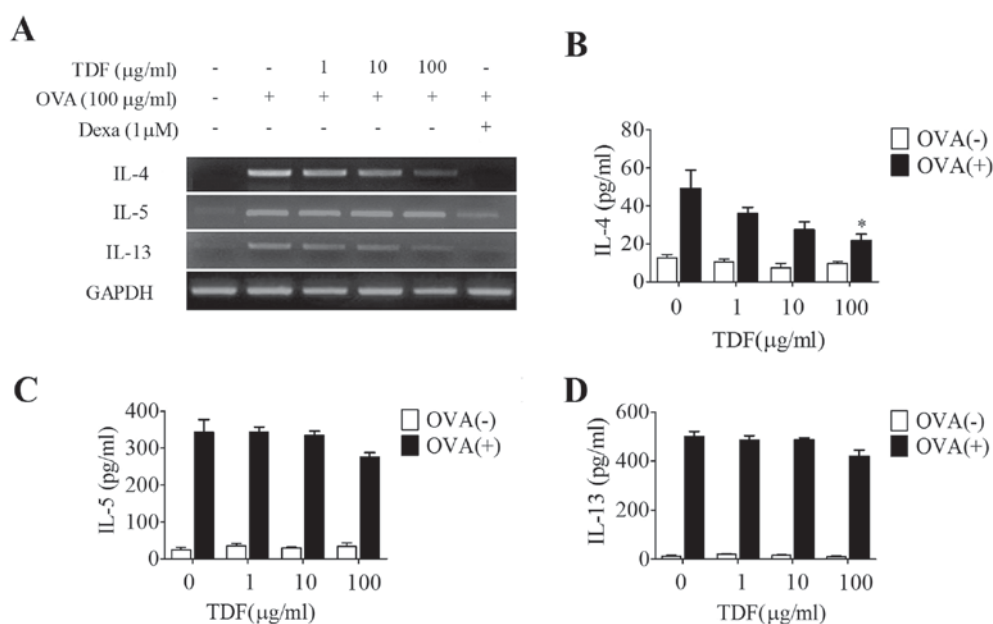


Figure 2. The effect of TDF on Th2 cytokine production in splenocyte culture. The impact of TDF on the Th2 subset was analyzed in splenocytes isolated from OVA-sensitized mice. Splenocytes were incubated with the indicated concentrations of TDF (1, 10, 100 µg/ml) in the absence or presence of 100 µg/ml OVA. (A) The mRNA expressions of Th2 response genes (IL-4, IL-5, IL-13) were assayed by RT-PCR. GAPDH was used as an internal control. Secretions of Th2 response genes (B) IL-4, (C) IL-5 and (D) IL-13 into cell supernatants was measured by ELISA. Results are presented as the means ± SEMs of independent three experiments. \*P<0.05 vs. control.

Table II. The level of total IgE and IgG1 in serum of TDF treated OVA-sensitized mice.

Group	Total IgE (µg/ml)	Total IgG1 (µg/ml)
Normal	0.90±0.44 (15)	847.18±181.42 (35)
OVA-sensitized	6.02±1.03 (100)	2389.45±206.82 (100)
TDF 100 mg/kg	4.30±1.28 (71) <sup>a</sup>	2374.91±60.66 (99)
TDF 200 mg/kg	3.98±0.98 (66) <sup>a</sup>	2316.73±156.32 (97)
Dexa	4.12±1.28 (68) <sup>a</sup>	1594.45±222.29 (67) <sup>b</sup>

Data are shown as mean ± SEMs. <sup>a</sup>P<0.05 and <sup>b</sup>P<0.01 vs. OVA-sensitized group.

Table III. The level of OVA-specific IgE and IgG1 in serum of TDF treated OVA-sensitized mice.

Group	OVA-specific IgE (U/ml)	OVA-specific IgG1 (U/ml)
Normal	ND	ND
OVA-sensitized	105.74±14.68 (100)	4741.24±1185.42 (100)
TDF 100 mg/kg	71.58±10.43 (68) <sup>a</sup>	4721.40±634.74 (100)
TDF 200 mg/kg	57.84±5.35 (55) <sup>b</sup>	4692.22±892.12 (99)
Dexa	63.48±10.22 (60) <sup>a</sup>	3432.45±793.27 (72) <sup>a</sup>

Data are shown as mean ± SEMs. <sup>a</sup>P<0.05 and <sup>b</sup>P<0.01 vs. OVA-sensitized group. ND, Not detectable.

increases were significant compared to normal group at 6 and 24 h post-challenge. And TDF reduced these increases.

Histological analyses of ear tissues revealed marked increases in inflammatory cell infiltration into ear skin tissues after challenge in OVA-sensitized group. But these inflammatory cells infiltrations were inhibited dose-dependently by TDF (Fig. 1B). These results demonstrated that administration of TDF contributes to improving the allergic inflammatory response.

*Effect of TDF on levels of serum immunoglobulins in OVA-sensitized mice.* IgE production is considered the hallmark of allergic diseases, and thus, we investigated whether TDF can regulate the serum levels of immunoglobulins in OVA-sensitized mice. It has been reported that IL-4 secreted by Th2 cells promotes the productions of IgE and IgG4 in human and of IgE and IgG1 in mice (25). We found significant

increases in the levels of serum total IgE and IgG1 in the OVA-sensitized group (Table II). Serum total IgE and IgG1 levels were about 6.6 and 2.8-fold greater, respectively, in the OVA-sensitized group than in the normal control group. Notably, serum levels of total IgE were significantly lower in the TDF 100 and 200 mg/kg groups than in the OVA-sensitized group, whereas total IgG1 levels were similar.

To add, we obtained similar result after examination of OVA-specific IgE and IgG1 (Table III). OVA-specific IgE and IgG1 levels were obvious in the OVA-sensitized group but barely detectable in the normal control group. TDF treatment concentration-dependently suppressed serum OVA-specific IgE levels, but serum OVA-specific IgG1 levels remained unchanged in the TDF treated group.

*Effect of TDF on Th2-related cytokine level in splenocytes isolated from OVA-sensitized mice.* By cellular interaction



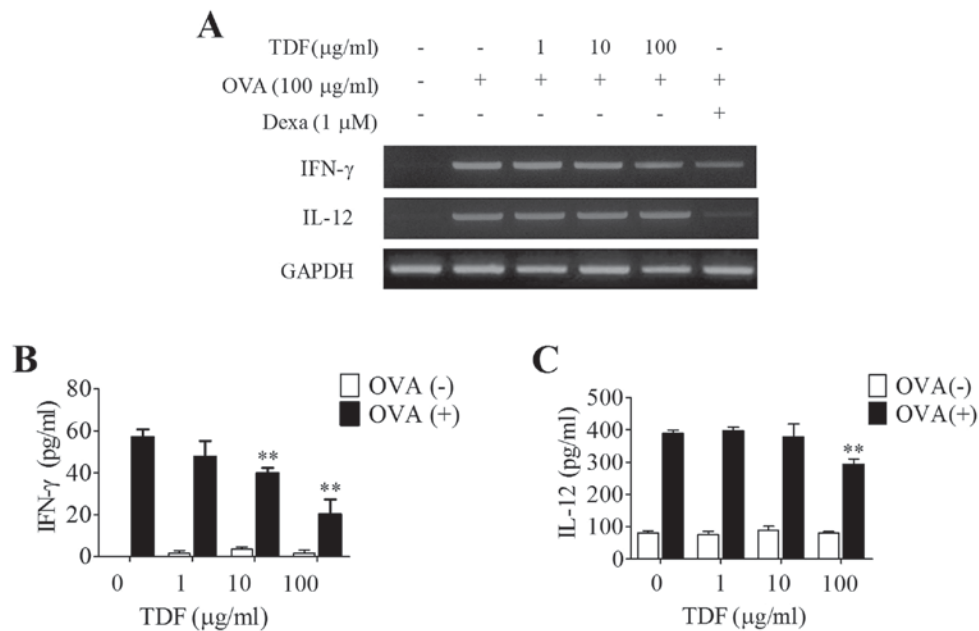


Figure 3. The effect of TDF on Th1 cytokine production in splenocyte culture. The impact of TDF on the Th1 subset was analyzed in splenocytes isolated from OVA-sensitized mice. Splenocytes were incubated with the indicated concentrations of TDF (1, 10, 100 μg/ml) in the absence and presence of 100 μg/ml OVA. (A) The mRNA expressions of Th1 response genes (IFN-γ, IL-12) were assayed by RT-PCR. GAPDH was used as an internal control. Secretions of Th1 response genes (B) IFN-γ and (C) IL-12 in cell supernatant were measured by ELISA. Results are presented as the means ± SEMs of independent three experiments. \*\*P<0.01 vs. control.

and cytokine secretion, Th cells play a key role in the class switching of B cells. Of the functional Th subsets, Th2 cells are important for enhancing allergic immune response. To determine the effect of TDF on Th2 response, we examined the mRNA and protein levels of Th2-related genes in splenocytes obtained from OVA-sensitized mice.

We observed increases of IL-4, IL-5 and IL-13 mRNA and protein levels in OVA-treated cells. However, TDF treatment at 100 μg/ml attenuated IL-4 mRNA expression and decreased IL-4 secretion (Fig. 2A and B). IL-5 and IL-13 mRNA (Fig. 2A) and protein levels tended (non-significantly) to be lower in TDF treated cells than in OVA-treated cells (Fig. 2C and D).

**Effect of TDF on Th1 cytokine levels in splenocytes isolated from OVA-sensitized mice.** Th1 and Th2 cells can inhibit each other by secreting cytokines, and naïve T cells differentiate into Th1 cells in the presence of IL-12, and Th1-derived cytokines (e.g. IFN-γ) are able to suppress Th2 differentiation (26). To investigate the effect of TDF on Th1 response, we examined Th1 response genes in splenocytes obtained from OVA-sensitized mice. Pre-treating splenocytes with TDF had no effect on the mRNA expressions of IFN-γ and IL-12 (Fig. 3A). However, IFN-γ and IL-12 secretions were significantly reduced by TDF, suggesting TDF reduced the levels of proteins involved in Th1 response without significantly changing mRNA levels (Fig. 3B and C).

**Effect of TDF on the expressions of Th specific transcription factors in splenocytes isolated from OVA-sensitized mice.** Due to the observed inhibition of Th1/Th2 related cytokine production by TDF in splenocyte culture, we further examined the expressions of transcription factors involved in skewing T cell polarization toward Th1 and Th2 cells (Fig. 4). As

shown in Fig. 4A, TDF markedly inhibited the mRNA expression of T-bet, which plays a key role in Th1 differentiation. Likewise, the mRNA expression of the Th2 specific transcription factor GATA-3 was also inhibited dose-dependently by TDF (Fig. 4B).

## Discussion

The prevalence of allergic diseases has increased in association with the western lifestyle in recent decades. Furthermore, it has been reported more than 25% of the populations of industrialized countries are under the influence of allergic diseases. The clinical manifestations of allergic diseases such as allergic rhinitis, asthma, atopic dermatitis, food allergy, allergic conjunctivitis and anaphylaxis can occur alone or in combination (27-29). OVA-sensitized animal models are commonly used to mimic chronic allergic diseases such as asthma and atopic dermatitis in human (30,31). In the present study, we investigated the potential ameliorative role of TDF in allergic immune response using OVA-sensitized mouse model.

IgE mediated-mast cell degranulation induces an immediate hypersensitivity reaction to causes allergic inflammation. For example, edema is caused by the movement of immune cells on inflamed site, the expansion of blood vessels, and increased vascular permeability (32). In the present study, allergic cutaneous reaction was suppressed in TDF treated groups as compared with to the OVA-sensitized group. And this effect was comparable to that induced by dexamethasone (the positive control).

Allergic reactions are initiated by enhanced IgE production induced by activation of the Th2 pathway. Especially, antigen-specific IgE have a key role on mast cells. Mast cells which have FcεRI-bound IgE produce a diverse array

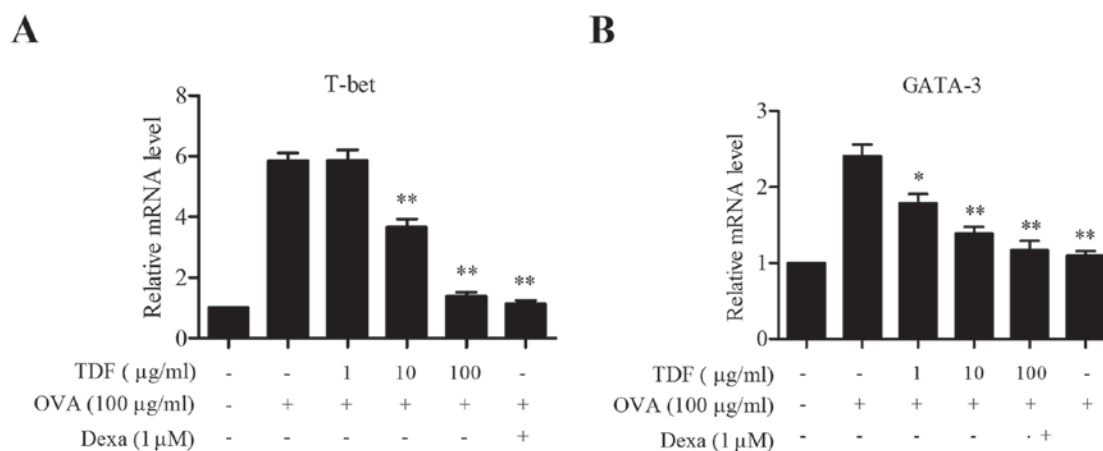


Figure 4. The effect of TDF on the expression levels of Th1/Th2 specific transcription factors in splenocytes. The mRNA expressions of the Th1 specific transcription factor (A) T-bet and Th2 specific transcription factor (B) GATA-3 were assayed by qRT-PCR. Results are presented as the means  $\pm$  SEMs of independent three experiments. \*P<0.05 and \*\*P<0.01 vs. control.

of biologically active mediators when re-exposed to the antigens (33). We observed that TDF treatment significantly inhibited the total and OVA-specific IgE, when compared to the OVA-sensitized group. Our *in vivo* results suggest the observed suppression of allergic inflammation by TDF was due to IgE down-regulation.

We also investigated T cell regulation by TDF to explore further the natures of these IgE reductions. After an antigen encountered and bound by antigen presenting cells (APCs) in the body presents, the antigen to naïve CD4<sup>+</sup> T cells, which then differentiate into functionally distinct subsets as determined by the micro-environmental background.

Th2-biased immune responses commonly results in atopy, and is observed in blood and lung in the presence of asthmatic conditions, and thus, medications targeting Th cells are viewed as promising therapeutic strategies for the treatment of allergic diseases (34). Differentiation of naïve Th precursor cells into Th2 cells is characterized by the productions of Th2 cytokines (e.g. IL-4, IL-5 and IL-13). During Th2-development, Th2 signaling initiates activation of signal transducer and activator of transcription (STAT)-6 in the presence of IL-4, which is followed by the expressional up-regulation of GATA-3 (35). We observed TDF decreased the production of IL-4 and the mRNA expression of transcription factor GATA-3 induced by OVA stimulation in splenocyte, which result shows that TDF suppressed Th2 differentiation by inhibiting IL-4 production.

On the other hand, during the development of Th1 lineage cells, antigen stimulates APCs to secrete Th1-promoting cytokines such as IL-12 and IFN- $\gamma$ . In naïve Th precursor cells during T cell antigen receptor (TCR) engagement, IFN- $\gamma$  activates STAT-1 and then downstream T-bet expressed in T cells. Furthermore, T-bet is known as a specific regulator for Th1 differentiation and to be related to IFN- $\gamma$  production (36). Our results show that TDF inhibited the productions of the Th1 cytokines IFN- $\gamma$  and IL-12 and decreased T-bet expression. Although Th1 and Th2 responses can block each other, this result confirmed that TDF does not participate in the induction of Th1 response and suggested that there are other mechanisms for inhibition of Th2 response. In addition, we examined the effect of TDF on other cell subsets such as

regulatory T cells which have protective roles in the presence of excessive immune response. However, TDF was not found to have a significant effect on this subset (data not shown).

*Triticum aestivum* sprouts are known as contain flavonoids such as isoorientin, isoscaparin and luteolin as well as sterols and polyunsaturated fatty acids (37,38). These constituents have been reported to have the potential to ameliorate allergic diseases. For example, isoorientin was reported to suppress the release of histamine and leukotrienes in guinea pig lung mast cells activated by OVA (39), and luteolin was found to inhibit mast cell-mediated allergic inflammation and to attenuate immediate and late-phase asthmatic responses (40,41). Consistent to these studies, these factors could be evidences for beneficial effect of *Triticum aestivum* sprouts on allergic diseases.

In conclusion, our data reveal that TDF reduces OVA-induced allergic immune response by inhibiting Th2 differentiation mediated by the activation of GATA-3 and IL-4, and suggest that TDF could be useful for the treatment of allergic diseases. Further studies are required to elucidate the molecular targets associated with the TDF mediated inhibition of allergic immune response.

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