1,2,4,5-Tetramethoxybenzene Suppresses House Dust Mite-Induced Allergic Inflammation in BALB/c Mice

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Key Words

1,2,4,5-tetramethoxybenzene · DFE-induced allergic inflammation · Keratinocytes

Abstract

Background: Atopic dermatitis (AD) is the most common allergic inflammatory skin disease. The activation of innate immunity by house dust mite (*Dermatophagoides farinae* extract, DFE) allergen plays an important role in the pathogenesis of AD. We previously showed the inhibitory effect of an extract of *Amomum xanthioides* on allergic diseases, and isolated 1,2,4,5-tetramethoxybenzene (TMB) as a major active component. In this study, we investigated whether TMB relieves DFE-induced allergic inflammation symptoms. **Methods:** We established a DFE-induced allergic inflammation model in BALB/c mice by repeated skin exposure to DFE. To define the underlying mechanisms of action, we used a tu-

mor necrosis factor- α and interferon- γ -activated human keratinocytes (HaCaT cell line) and mouse keratinocytes (3PC cell line) cell line model. **Results:** Oral administration of TMB suppressed allergic inflammation symptoms, such as histopathological analysis and ear thickness, in addition to serum IgE, DFE-specific IgE and IgG2a levels. TMB decreased the serum histamine levels and tissue infiltration of inflammatory cells, including mast cells and eosinophils. TMB also inhibited CD4⁺IFN- γ ⁺, CD4⁺IL-4⁺, and CD4⁺IL-17A⁺ lymphocyte expansion in the draining lymph nodes and expression of the Th2 cytokines in the ear tissue. TMB significantly inhibited the expression of cytokines and chemokines by the downregulation of the mitogen-activated protein kinases and nuclear factor of activated cytoplasmic T cells in HaCaT cells. **Conclusions:** TMB improved DFE-induced allergic inflammation by suppressing the production of proinflammatory cytokines and chemokines. Our results suggest that TMB might be a potential therapeutic agent for AD.

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Introduction

Atopic dermatitis (AD) is a complex and chronic inflammatory skin disease caused by multiple factors, including susceptibility genes, and immunologic and environmental factors [1]. Among the environmental factors, *Dermatophagoides farinae* is important for the pathogenesis of AD through the induction of both acute and chronic AD lesions [2]. Currently, AD is considered a biphasic T cell-mediated disease; Th2 cells predominate the acute phase and the switch to Th1 promotes the chronic phase [3]. Th1/Th2 imbalance is one of the major factors for AD, and Th17 also contributes to its pathogenesis [4, 5]. Th17 cells coordinate local tissue inflammation through the upregulation of proinflammatory cytokines and chemokines [6]. High levels of Th2 cytokines such as interleukin (IL)-4 and IL-13 in AD skin lesions induce immunoglobulin class switching, promoting excessive IgE production [7]. In addition, Th1 cells producing IFN- γ are predominant in the chronic phase of AD, and Th17 cells are increased in the acute phase of AD [8, 9]. The activation of keratinocytes is a hallmark of the pathogenesis of AD [2]. Keratinocytes have an effect on immune responses during the development of AD [10]. In patients with AD, keratinocytes promote inflammation by the secretion of various cytokines and chemokines [11].

Although AD is one of the most common inflammatory skin diseases, therapeutic options for AD patients with moderate-to-severe cases of the disease are limited [5]. Corticosteroids and immunosuppressant drugs are effective treatments for AD. Cyclosporine A (CsA) is a highly effective treatment for AD, which interferes with calcineurin and the nuclear factor of activated T cells (NFAT) [12]. However, the long-term use of corticosteroids and immunosuppressant drugs is associated with a number of potentially serious adverse effects. Recently, several natural extracts have been introduced for the potential treatment of AD. Several studies have demonstrated that the extract of *Amomum xanthioides* has antiallergic, antioxidative and anti-inflammatory effects [13, 14]. In addition, the natural extract β -sitosterol from *A. xanthioides* showed inhibitory effects on AD [15]. We previously showed that 1,2,4,5-tetramethoxybenzene (TMB), extracted from *A. xanthioides*, has antiallergic inflammatory effects [16]. Based on the known antiallergic and anti-inflammatory effects of TMB, we investigated the effects of TMB using DFE-induced allergic inflammation models.

Materials and Methods

Animals

Six-week-old female BALB/c mice were purchased from SLC Inc. (Hamamatsu, Japan). The animals were housed with 5–10 mice per cage in a laminar airflow room maintained at a temperature of $22 \pm 2^\circ\text{C}$ with a relative humidity of $55 \pm 5\%$ throughout the study. The care and treatment of the mice were carried out in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Reagents

D. farinae extract (DFE, Greer Laboratories, Lenoir, N.C., USA) was used as an antigen. All reagents were procured from Sigma (St. Louis, Mo., USA) unless otherwise stated. DFE was dissolved in phosphate-buffered saline (PBS) containing 0.5% Tween 20. Recombinant human tumor necrosis factors (TNF)- α and interferon (IFN)- γ were purchased from R&D systems (Minneapolis, Minn., USA).

Extraction of *A. xanthioides* and Isolation of TMB

Fruits of *A. xanthioides* were purchased from Yak-Ryung-Si Market in Daegu, Korea, and identified by Dr. Seung Ho Lee at the College of Pharmacy, Yeungnam University, Korea, where a voucher specimen (YU00194) was deposited in the Natural Product Laboratory. Dried fruits of *A. xanthioides* (9.5 kg) were extracted with methanol (MeOH; 15 liters $\times$ 3) at room temperature. The extracts were concentrated in vacuo to produce a black gum (440 g), which was dissolved in H_2O , continuously partitioned with solvents, and then respectively concentrated to give extracts of *n*-hexane (120 g), methylene chloride (MC; 32.7 g), ethyl acetate (9.8 g) and H_2O (275.1 g). The MC-soluble extract (32.7 g) eluted with a gradient mixture of 100% MC to 100% acetone was chromatographed on a silica gel liquid chromatography to obtain seven fractions (AF1–AF7). TMB was isolated from fraction AF3 (101.2 mg) using preparative HPLC (C_{18} , 250×21.2 mm, flow rate 6 ml/min) and eluted with MeOH: H_2O (gradient from 30:70 to 60:40). Column chromatography was carried out on Merck silica gel (70–230 mesh) and Merck Lichroprep RP-18 gel (40–63 μm). HPLC separation was performed with the Gilson system, a UV detector, and a Luna C_{18} column (250×21.20 mm, 10 μm). Thin-layer chromatography was performed using aluminum plates precoated with silica gel 60 F_{254} and RP-18 F_{254} (Merck, Darmstadt, Germany). Compounds were detected under UV light and then visualized by spraying the plates with vanillin-sulfuric acid reagent (1% ethanolic vanillin solution, 10% ethanolic sulfuric acid) and 10% sulfuric acid followed by heating at 110°C for 1 min. ^1H (250 MHz) and ^{13}C (63 MHz) NMR spectra were recorded on a Bruker 250 MHz (DMX 250) with tetramethylsilane as an internal standard. NMR spectra were recorded on JEOL ECA-500 (^1H , 500 MHz, ^{13}C , 125 MHz) spectrometers. All NMR experiments were performed at 294 K. TMB was dissolved in dimethyl sulfoxide (DMSO) and then diluted with PBS. The proportion of DMSO in the treated samples was controlled to less than 0.1% to minimize the interference.

Cell Culture and Viability

A human keratinocytes cell line (HaCaT) was incubated in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, N.Y., USA), supplemented with 10% fetal bovine serum (FBS) and

antibiotics (100 U/ml penicillin G, 100 µg/ml streptomycin), in a 5% CO₂ incubator at 37°C. A murine keratinocyte cell line (3PC) was cultured in Eagle's minimal essential medium (Lonza, Basel, Switzerland), supplemented with insulin (2.5 µg/ml), epidermal growth factor (5 ng/ml), transferrin (10 µg/ml), *o*-phosphoethanolamine (10 µM), ethanolamine (10 µM), penicillin (50 U/ml), hydrocortisone (0.5 µg/ml), CaCl₂ (0.03 mM) and 1% fetal bovine serum. Cell viability was determined using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide assay (MTT). HaCaT cells were treated with TMB for 24 h and MTT (5 mg/ml) was added into each well that contained a sample and incubated at 37°C for an additional 2 h. Isopropanol was added to dissolve the formazan crystals. The absorbance on each sample compared to that of the control was calculated and expressed as a percentage.

Induction of DFE-Induced Allergic Inflammation in the Mouse Ear

The induction of DFE-induced allergic inflammation was performed based on our previous research [17, 18]. A schematic experimental procedure is described in online supplementary fig. S2a (see www.karger.com/doi/10.1159/000446510 for all online suppl. material). Mice (total n = 35) were allocated to seven groups (n = 5 per group): vehicle, TMB (25 mg/kg), DFE plus vehicle, DFE plus TMB (1, 5 or 25 mg/kg), or CsA (30 mg/kg). The surfaces of both ear lobes were gently stripped five times with surgical tape (Nichiban, Tokyo, Japan). After stripping, 20 µl of DFE (10 mg/ml) was painted on each ear. The treatment with DFE was repeated twice weekly for 6 weeks. The ear thickness was measured 24 h after DFE application with a dial thickness gauge (Mitutoyo Co., Tokyo, Japan). Two weeks after the first induction, tail bleeding was performed to check the serum IgE level. After confirming an atopic condition by IgE level, TMB (1, 5 or 25 mg/kg) or CsA (30 mg/kg) was orally administered five times per week, until the end of the 6-week induction period (in total 20 times).

On day 42, the animals were euthanized with carbon dioxide. Blood samples were collected from the celiac artery. For serum, the blood was allowed to clot by leaving it undisturbed at room temperature, the clot was removed and centrifuged at 2,000 g (15 min, 4°C) and the supernatant was retained. After blood collection, ears were removed and used for histopathological analysis and RNA extraction. Total serum IgE and IgG2a were measured using an ELISA kit (BD Biosciences, Oxford, UK) according to the manufacturer's instructions. For the detection of DFE-specific IgE, 96-well plates (Nunc, Wiesbaden, Germany) were coated with 10 mg of mite extract. The DFE-specific IgE level was indicated by the OD value.

Histological Observation

The ears (total n = 35) were fixed with 10% formaldehyde and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin (H&E). Infiltrated lymphocytes, thickening of the epidermis and fibrosis in the dermis were observed using a microscope, as described in previous research [19]. For the measurement of mast cell infiltration, skin sections were stained with toluidine blue and the number of mast cells at five randomly selected fields was measured for each sample at a magnification of ×200. Eosinophils in H&E-stained sections were counted in 10 high-power fields at a magnification of ×400. Dermal thickness was analyzed in H&E-stained sections viewed under a magnification of ×200. The thickness was measured in five randomly selected fields from each sample.

Fig. 1. Development of DFE-induced allergic inflammation. TMB (1, 5 or 25 mg/kg) or CsA (30 mg/kg) was orally administered five times per week, beginning on day 16 after the first induction. Ear thickness was measured 24 h after DFE application with a dial thickness gauge. Five mice were placed into each of seven groups: vehicle, TMB (25 mg/kg), DFE plus vehicle, DFE plus TMB (1, 5, or 25 mg/kg), or CsA (30 mg/kg). AD = DFE-induced allergic inflammation. Data are presented as the mean ± SE (n = 5). * p < 0.05, significantly lower than AD.

Histamine Assay

The histamine content was measured using the *o*-phthalaldehyde spectrofluorometric procedure based on a previous study [20]. Blood from the mice (total n = 35) was centrifuged at 400 g for 10 min, and serum was withdrawn to measure the histamine content. The fluorescent intensity was measured using 355-nm excitation and 450-nm filters and the LS-50B fluorescence spectrometer (Perkin-Elmer, Norwalk, Conn., USA).

FACS

At the end of the experiment, the mice (total n = 35) were euthanized and both auricular lymph nodes were collected from each mouse. The auricular lymph nodes (n = 70) were ground using 70-µm nylon cell strainers (Falcon, Bedford, Mass., USA) to isolate single cells. The cells were stained using a mouse CD4 PerCP-CyTM5.5-FITC-conjugated Th1 (IFN-γ), CD4 PerCP-CyTM5.5-APC-conjugated Th2 (IL-4) and CD4 PerCP-CyTM5.5-PE-conjugated Th17 (IL-17A) phenotyping kit (BD Biosciences) according to the manufacturer's instructions. The fluorescence intensity was detected using a FACSCalibur flow cytometer (BD Biosciences).

Fig. 2. Histological analysis and cell infiltration in DFE-induced allergic inflammation. Representative photomicrographs of ear sections were stained with H&E (original magnification $\times 200$) or toluidine blue (original magnification $\times 200$). Five mice were placed into each of the seven groups indicated. AD = DFE-induced allergic inflammation.

Quantitative Real-Time Polymerase Chain Reaction

To detect the expression of cytokines, a quantitative real-time PCR was performed using a Thermal Cycler Dice TP850 (Takara-bio Inc., Shiga, Japan) according to the manufacturer's protocol. At the end of the in vivo experimental period, the ears were excised and total RNA was isolated. HaCaT cells and 3PC cells were pre-treated with TMB for 1 h, followed by stimulation with TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) for 6 and 24 h, respectively. Total cellular RNA was isolated from cells (2×10^5 cells/24-well plate), using RNAiso Plus (Takara-bio Inc.). The first strand of complementary DNA (cDNA) was synthesized using RT Premix (iNtRON Biotech, Sungnam, Korea). The reverse transcription conditions were at 45°C for 60 min and at 95°C for 5 min. Briefly, 2 μl of cDNA (100 ng), 1 μl of sense and antisense primer solution (0.4 μM), 12.5 μl of SYBR Premix Ex Taq (Takara Bio Inc.) and 8.5 μl of dH_2O were mixed together to obtain a reaction mixture with a final volume of 25 μl in each reaction tube. The conditions for the PCR were similar to our previous report [17] except for the primers of mouse β -actin (F 5'-TAG ACT TCG AGC AGG AGA TG-3', R 5'-TTG ATC TTC ATG GTG CTA GG-3'), mouse TNF- α (F 5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3', R 5'-ACA TTC GAG GCT CCA GTG AAT TCG AAT TCG G-3'), mouse IL-1 β (F 5'-ATA ACC TGC TGG TGT GTG AC-3', R 5'-AGG TGC TGA TGT ACC AGT TG-3'), mouse IL-13 (F 5'-GCA ACA TCA ACA GGA CCA GA-3', R 5'-GTC AGG GAA TCC AGG GCT AC-3'), human TNF- α (F 5'-CCC TCC AGT TCT AGT TCT ATC-3', R 5'-GGG GAA AGA ATC ATT CAA CCA G-3'), human IL-1 β (F 5'-ACG ATG CAC CTG TAC GAT CA-3', R 5'-TCT TTC AAC ACG CAG GAC AG-3'), human IL-6 (F 5'-TTC CCT CAG GAT GGT GTC TC-3', R 5'-AAA TGC CCA ACA GAG GTC AC-3'), human TARC/CCL17 (F 5'-ACT GCT CCA GGG ATG CCA TCG TTT-3', R 5'-ACA AGG GGA TGG GAT CTC CCT CAC-3') and human MDC/CCL22 (F 5'-AGG ACA GAG CAT GGC TCG CCT ACA GA-3', R 5'-TAA TGG CAG GGA GGT AGG GCT

CCT GA-3'). The normalization and quantification of mRNA expression were performed using TP850 software supplied by the manufacturer. The expression levels of β -actin were used for normalization.

Nuclear Protein Extraction

Nuclear protein extraction procedures were based on our previous study [19]. HaCaT cells (1×10^6 cells/6-well plate) were pre-treated with TMB for 1 h, followed by stimulation with TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) for 30 min. After stimulation, cells were washed in 1 ml of ice-cold PBS, centrifuged at 1,200 g for 5 min, resuspended in 400 μl of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM MgCl_2 , 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed and centrifuged at 15,000 g for 30 s. After washing, the pelleted nuclei were resuspended in 50 μl of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, centrifuged at 15,000 g for 5 min at 4°C , and then the supernatant was gathered.

Western Blot

Samples for the Western blot were prepared as described in previous research [21]. Briefly, cells (1×10^6 cells/6-well plate) were stimulated for 30 min for MAPKs and NFAT. Cells were then rinsed twice with ice-cold PBS and the total cell lysates were gathered in 200 μl of lysis buffer. The lysates were spun in a micro-centrifuge for 20 min at 4°C and the supernatant was collected. Proteins were electrophoresed using 8–12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were stained with reversible Ponceau S to ensure equal loading of the samples in the gel. The nucleus NFATc1 was assayed using anti-NFATc1 antibodies (Santa Cruz Biotech., Santa Cruz, Calif, USA). The phosphorylation of ERK, JNK and STAT1 was determined using

Fig. 3. Histological analysis and cell infiltration in DFE-induced allergic inflammation. **a, b** Epidermal and dermal thickness were analyzed in H&E-stained sections, viewed at a magnification of $\times 200$. **c** Eosinophils in H&E-stained sections were counted in 10 high-power fields at a magnification of $\times 400$. **d** Mast cells in toluidine blue-stained sections were counted in five fields at $\times 200$. Five mice were placed into each of seven groups: vehicle, TMB (25 mg/kg), DFE plus vehicle, DFE plus TMB (1, 5 or 25 mg/kg), or CsA (30 mg/kg). AD = DFE-induced allergic inflammation. Data are presented as the mean $\pm$ SE ($n = 5$). * $p < 0.05$, significantly lower than AD.

anti-phospho-ERK, anti-phospho-JNK and anti-phospho-STAT1 antibodies (Cell Signaling, Beverly, Mass., USA). Immunodetection was performed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, Mass., USA).

Statistical Analysis

Statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, Calif., USA). Treatment effects were analyzed using one-way analysis of variance followed by Dunnett's test. A value of $p < 0.05$ was used to indicate statistically significant differences.

Results

Effects of TMB on DFE-Induced Allergic Inflammation in Mice

To determine the effect of TMB on AD, we used a DFE-induced allergic inflammation mouse model. Dur-

ing the induction period, ear swelling was measured 24 h after each induction. There was no difference in the ear swelling between the groups until 2 weeks. After 3 weeks of induction the oral administration of TMB (1, 5 or 25 mg/kg) showed significant improvements in ear thickness (fig. 1; online suppl. fig. S1). TMB and CsA, a positive control, did not alter the body weight of the animals (online suppl. fig. S2b).

Histological analysis showed alterations, such as epidermal and dermal thickness, hyperkeratosis and infiltration of eosinophils in DFE-induced allergic inflammation. The oral administration of TMB (1, 5, or 25 mg/kg) markedly diminished the thickness of epidermal and dermal layers and eosinophil infiltration compared with DFE-induced allergic inflammation (fig. 2, fig. 3a–c; online suppl. fig. S3a). Photographs of the mice applied with DFE and each drug are shown in online supplementary

Fig. 4. Serum levels of immunoglobulin and histamine in DFE-induced allergic inflammation. **a** Histamine levels were detected using a fluorescent plate reader. Serum total IgE levels (**b**), DFE-specific IgE levels (**c**) and serum total IgG2a levels (**d**) were measured by ELISA. Samples of the vehicle, TMB (25 mg/kg), DFE plus vehicle, DFE plus TMB (1, 5 or 25 mg/kg), or CsA (30 mg/kg) groups were collected by orbital puncture on day 42. AD = DFE-induced allergic inflammation. Data are presented as the mean $\pm$ SE (n = 5). * p < 0.05, significantly lower than AD.

figure S3b. Mast cells are tissue immune cells that originate from hematopoietic cells and are a major source of histamine and proinflammatory cytokines [22, 23]. Skin sections were stained with toluidine blue for the measurement of mast cell infiltration. We examined the infiltration of mast cells in the ear tissue and the serum levels of histamine. TMB decreased both the infiltration of mast cells and serum histamine (fig. 3d, 4a). The efficacy of TMB was similar to the CsA.

Effects of TMB on the Serum Immunoglobulin and Expression of Cytokines

AD is associated with the Th2 phenotype, elevated IL-4 and IL-13 secretion, and increased serum IgE levels [24]. IL-4 mediates IgE isotype switching, and IFN- γ mediates IgG2a isotype switching [8]. To investigate the role of TMB on the Th1 or Th2 response, we examined the

serum levels of total IgE, DFE-specific IgE and IgG2a. Oral administration of TMB reduced total serum IgE, DFE-specific IgE and IgG2a levels compared to the DFE-induced allergic inflammation group (fig. 4b–d).

AD is characterized by a biphasic, T cell polarization, dominated by the infiltration of Th2 cells and cytokines such as IL-4, IL-5 and IL-13 in the acute phase and switch to a Th1 phenotype in chronic AD lesions [9]. In addition, Th17 cells have been described as essential factors in the pathophysiology of AD [9]. We investigated whether TMB regulates the Th1, Th2 and Th17 immune responses by measuring T cell populations and the expression of inflammatory cytokines in DFE-induced allergic inflammation. CD4⁺ T cells were isolated from the auricular lymph nodes and analyzed by FACS analysis. The CD4⁺IFN- γ ⁺, CD4⁺IL-4⁺ and CD4⁺IL-17A⁺ populations were significantly decreased by TMB compared to the DFE-induced

allergic inflammation group (fig. 5; online suppl. fig. S4). The mRNA expression of Th1 (TNF- α and IFN- γ), Th2 (IL-4, IL-13 and IL-31) and Th17 (IL-17A) was markedly increased in DFE-induced allergic inflammation; TMB suppressed the expression of TNF- α , IFN- γ , IL-4, IL-13, IL-31 and IL-17A in a dose-dependent manner (fig. 6).

Effects of TMB on the Activation of Keratinocytes

A keratinocyte model was used to clarify the biological functions and molecular mechanisms of TMB. We first tested the cytotoxicity of TMB in human keratinocyte cell lines (HaCaT). TMB did not show cytotoxicity up to 10 μ M (online suppl. fig. S5). CsA, an immunosuppressant for severe AD, was used as a positive control. Keratinocytes promote chronic, self-amplifying loops of immune activation by producing inflammatory factors [25]. Therefore, human keratinocytes produce thymus and activation-regulated chemokines (TARC/CCL17) and macrophage-derived chemokines (MDC/CCL22) in response to TNF- α /IFN- γ , which are thought to play an important role in the pathogenesis of AD [2, 26]. To investigate the inhibitory effects of TMB on the TNF- α /IFN- γ -induced expression of cytokines and chemokines in HaCaT cells, the cells were pretreated with TMB for 1 h and then stimulated with TNF- α /IFN- γ for 6 h. Quantitative real-time PCR showed that TMB suppressed the TNF- α /IFN- γ -induced gene expression of TNF- α , IL-1 β , IL-6, TARC/CCL17 and MDC/CCL22 in a dose-dependent manner (fig. 7a). In addition, the inhibitory effects of TMB on the TNF- α /IFN- γ -induced expression of cytokines were confirmed using mouse keratinocytes (3PC cells) (fig. 7b).

Previous studies showed that intracellular MAPK signaling pathways were activated by TNF- α /IFN- γ in HaCaT cells [27]. The JAK/STAT signaling pathways contribute to the production of chemokines (TARC/CCL17 and MDC/CCL22) in TNF- α /IFN- γ -induced HaCaT cells [28]. In addition, the NFAT family of transcription factors plays an important role in the immune system by the regulation of proinflammatory cytokines [29]. To investigate the mechanism responsible for the inhibitory effect of TMB on cytokine expression, we examined the effect of TMB on the TNF- α /IFN- γ -induced activation of MAPKs, STAT1 and NFATc1. HaCaT cells were pretreated with TMB for 1 h and then stimulated with TNF- α /IFN- γ for 30 min. TMB reduced the TNF- α /IFN- γ -induced phosphorylation of ERK and NFATc1, but did not alter the activation of JNK and STAT1 (fig. 8). These results suggest that TMB inhibits TNF- α /IFN- γ -induced expression of cytokines and chemokines by reducing activation of ERK and NFATc1 in keratinocytes.

Fig. 5. Phenotypes of T lymphocytes in the draining lymph node of DFE-induced allergic inflammation. Both auricular lymph nodes were collected from each mouse. From the isolated single cells, the numbers of CD4⁺IFN- γ ⁺ (a), CD4⁺IL-4⁺ (b) and CD4⁺IL-17A⁺ (c) were detected by FACSCalibur. Five mice were placed into each of the seven groups: vehicle, TMB (25 mg/kg), DFE plus vehicle, DFE plus TMB (1, 5 or 25 mg/kg), or CsA (30 mg/kg). AD = DFE-induced allergic inflammation. Data are presented as the mean $\pm$ SE (n = 10). * p < 0.05, significantly lower than AD.

Fig. 6. Expression of cytokines in the ears of mice with DFE-induced allergic inflammation. Ears were excised and total RNA was isolated. The quantitative real-time PCR was performed as described in Materials and Methods. Five mice were placed into each of the seven groups indicated. AD = DFE-induced allergic inflammation. Data are presented as the mean $\pm$ SE (n = 5). * p < 0.05, significantly lower than AD.

Fig. 7. Expression of cytokines and chemokines in TNF-α/IFN-γ-stimulated keratinocytes. **a** HaCaT cells were pretreated with TMB (0.1, 1.0 or 10 μM) or CsA (10 μM) 1 h before stimulation with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for 6 h. **b** 3PC cells were pretreated with TMB (10 μM) or CsA (10 μM) 1 h before stimulation with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for 24 h. Expression levels of cytokines were determined by quantitative real-time PCR. Data are presented as the mean $\pm$ SE (n = 5). * p < 0.05, significantly lower than the TNF-α/IFN-γ-stimulated group.

Discussion

Several studies reported that natural compounds occupy a place of choice in the current pharmacopoeia [1]. Recently, many natural extracts have been introduced for the potential therapeutic agents for treating skin inflammatory disorders such as AD [15, 30]. *A. xanthioides* is a medicinal herb which has various medicinal effects, such as antimicrobial, antioxidative, anticancer and anti-in-

flammation properties [31]. Previously, we reported the inhibitory effect of methanol extracts of *A. xanthioides* in mast cell-mediated allergic inflammations [13]. To clarifying the role of *A. xanthioides*, we isolated TMB from *A. xanthioides* by the activity-guided fractionation. In our previous study, the novel compound TMB isolated from *A. xanthioides* was shown to have antiallergic inflammatory effects in mast cells and animal models [16]. Based on the known antiallergic and anti-inflammatory effects

Fig. 8. Activation of MAPKs, STAT1 and NFATc1 in TNF- α /IFN- γ -stimulated HaCaT cells. Cells were pretreated with TMB (10 μ M) or CsA (10 μ M) 1 h before stimulation with TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) for 30 min. The activation of MAPKs, NFATc1 and STAT1 was analyzed by Western blot. The data shown represent three independent experiments. The ratios of bands were measured using ImageJ software. Actin was used as a loading control. N-NFATc1 = Nucleus NFATc1.

of TMB, we demonstrated the effect of TMB on AD using *in vivo* and *in vitro* models.

DFE is known to contribute to the development of AD via the activation of inflammasomes in the epidermal keratinocytes [1, 32]. Previous research showed that repeated application of DFE produced AD symptoms, including epidermis thickening, fibrosis in the dermis and the infiltration of inflammatory cells (eosinophils and mast cells) [33]. Mast cell-derived histamine and inflammatory mediators contribute to itching and inflammation in patients with AD, and they secrete multiple mediators affecting eosinophils [10]. In addition, histamine could be a significant mediator in AD patients; infiltration of eosinophils in the skin of AD patients also aggravated the inflammatory response [10, 34]. In our results, oral ad-

ministration of TMB diminished the symptoms of AD, such as ear thickness, dermal and epidermal thickness, eosinophils and mast cell infiltration, and serum histamine levels. Oral administration of CsA is widely used in patients with severe AD [5]. Previous research has shown that CsA is important in the pathogenesis of itch in AD [35]. In the DFE-induced allergic inflammation model, oral administration of TMB at high doses (25 mg/kg) had a similar anti-inflammatory effect as CsA (30 mg/kg). These results imply that TMB relieves itching and inflammatory responses in AD through a reduction of the inflammatory cells.

Both Th1 and Th2 cytokines contribute to AD skin lesions, Th2 cytokines for acute AD and Th1 cytokines for chronic AD [8]. Cytokines are known to affect immunoglobulin isotype switching [8]. IL-4 and IL-13 are critical for Th2 cell differentiation, IgE production and eosinophil recruitment [36]. Total IgE levels are elevated in AD patients compared to healthy individuals [37]. IL-31 is produced by Th2 cells and acts as an itch mediator in AD [38]. In addition, IFN- γ is expressed in chronic AD and drives the production of IgG2a [39]. IL-17A is highly expressed in acute AD, which has been described as an essential factor for AD [9]. Conclusively, the downregulation of Th1, Th2 and Th17 cytokines is necessary to modulate AD symptoms [40]. In our DFE-induced allergic inflammation mouse model, Th1 (IFN- γ), Th2 (IL-4, IL-13 and IL-31) and Th17 (IL-17A) cytokines were upregulated in the skin of mouse ears and the draining lymph node cells. TMB significantly diminished the expression of all the Th1, Th2 and Th17 cytokines in a dose-dependent manner. In addition, TMB markedly inhibited the serum level of total IgE, DFE-specific IgE and IgG2a. These results suggest that TMB controlled AD immune responses by the downregulation of Th1, Th2 and Th17 cytokines. Based on our results, we could assume that TMB suppresses both acute and chronic AD phases.

Keratinocytes secrete cytokines and chemokines after exposure to proinflammatory cytokines and Th2 cytokines from T cells [34]. Keratinocytes from AD patients produce a unique profile of cytokines and chemokines following some stimulation [41]. Several studies have reported that keratinocytes are one of the main sources of TRAC/CCL17 and MDC/CCL22, which are the key mediators for the development of AD [42–44]. Stimulation with TNF- α /IFN- γ in HaCaT cells produces TRAC/CCL17 and MDC/CCL22, which are known to be upregulated in AD-like skin lesions [45]. In the present study, TMB suppressed the TNF- α /IFN- γ -induced gene expression of TNF- α , IL-1 β , IL-6, TRAC/CCL17 and MDC/

CCL22 in HaCaT cells. In addition, TMB also suppressed the TNF- α /IFN- γ -induced gene expression of TNF- α and IL-1 β in 3PC cells. These results complement the inhibitory effect of TMB in DFE-induced allergic inflammation.

CsA is a potent immunosuppressant and often prescribed for AD [46]. CsA is known to inhibit the NFAT-mediated signaling pathway [46]. NFAT, a transcription factor, plays an important role in the immune system through regulation of the expression of proinflammatory cytokines [29, 47]. The MAPK pathways also play an important role in inflammatory diseases and lead to the systemic inflammatory response syndrome [48]. In particular, ERK enhances the phosphorylation of NFAT [49]. In our results, TMB markedly inhibited the activation of TNF- α /IFN- γ -stimulated ERK and NFATc1 in HaCaT cells. We assume that TMB inhibited the TNF- α /IFN- γ -stimulated expression of proinflammatory cytokines and chemokines by the blocking of ERK and NFATc1 in keratinocytes. However, further studies are needed in order to understand the exact mechanisms of TMB.

In the present report, we demonstrated that oral administration of TMB suppressed the development of AD symptoms in in vivo and in vitro models. TMB suppressed AD symptoms in a dose-dependent manner in the DFE-induced allergic inflammation models. TMB also inhibited the proinflammatory cytokines and chemokines through a reduction of ERK and NFATc1 in keratinocytes. Furthermore, the inhibitory effect of TMB on AD was comparable to that of CsA. We have provided conclusive evidence that TMB could be a potential therapeutic candidate for AD.

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