



(E)-phenethyl 3-(3,5-dihydroxy-4-isopropylphenyl) acrylate gel improves DNFB-induced allergic contact hypersensitivity via regulating the balance of Th1/Th2/Th17/Treg cell subsets

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ABSTRACT

(*E*)-phenethyl 3-(3,5-dihydroxy-4-isopropylphenyl) acrylate gels (THCA354) is a novel polyphenols acrylic acid derivative. To investigate the immunoregulatory mechanisms of THCA354, we established a mouse model of 2,4-dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis (ACD). Responses of Th1, Th2, Th17 and regulatory T cells (Tregs) were determined by flow cytometry, reverse-transcriptase polymerase chain reaction (RT-PCR) and enzyme linked immunosorbent assay (ELISA). Our study found that topical application of THCA354 gel could inhibit ear swelling, reduce inflammatory cell infiltration, down-regulate Th1/Th17 responses and enhance Th2/Treg responses. These findings indicated that THCA354 gel exerted its immunotherapeutic effects by modulating the balance of Th1/Th2/Th17/Treg cell subsets, suggesting that THCA354 gel could be used as a promising drug candidate for intervention of ACD.

1. Introduction

As a common skin inflammatory disease, allergic contact dermatitis (ACD) corresponds to a delayed-type hypersensitivity (DTH) response with a skin inflammation mediated by T lymphocytes [1]. It is an inflammatory reaction characterized by the activation of sensitized T lymphocytes in combination with the corresponding antigens and the release of various cytokines, leading to monocyte infiltration as well as tissue degeneration and necrosis. Many studies have shown that DTH responses are mainly mediated by CD4⁺ T cells [2–4]. CD4⁺ T cells are subdivided into T-helper Th1 and Th2 cells. The balance between the Th1- and Th2-dominant immunities (Th1/Th2 balance) plays a crucial role in the development and maintenance of various immune disorders [5]. Moreover, the abnormal secretion of cytokines, which is caused by the imbalance of T cell subsets, plays an important role in development of ACD. Recently, Th17 cells, as a novel subset of CD4⁺ T cells different from Th1 and Th2 cells, have been shown to induce severe autoimmune responses by secreting inflammatory mediator IL-17 [6,7]. IL-17 plays a fundamental role in activating T cells in allergen-specific T-cell-mediated immune responses, and IL-17 efficiently amplifies the allergic reaction by rendering T cells accessible to recruitment in skin inflammation site in ACD [8]. Besides, CD4⁺ CD25⁺

regulatory T cells (Tregs) are identified to have a pivotal role in the resolution of DTH responses [9,10]. Treg activity is regulated by a specific transcription factor, the forkhead/winged helix (Foxp3) transcription factor. Since Th17 has pro-inflammatory properties and Tregs have anti-inflammatory effects, regulating the balance between Th17/Tregs may provide a new way to effectively treat ACD.

Up to date, the main drugs for ACD treatment, such as glucocorticoid, H1 receptor antagonist and NSAIDs, have severe adverse reactions and undesirable side effects [11]. Therefore, a new drug, which is effective in dermatitis and has fewer side effects, is badly needed. In an early drug screening study of our team, we have identified the best active pharmaceutical ingredients by anti-inflammatory experiments, and a new type of polyphenol acrylic acid derivatives, (*E*)-phenethyl-3-(3,5-dihydroxy-4-isopropyl phenyl) acrylate (THCA354) (Fig. 1), has been synthesized.

Existing studies have shown that the phenolic hydroxyl and double bond in polyphenol compound structure have an effect on anti-inflammatory immune adjustment [12,13]. Early pharmacodynamic studies have confirmed that THCA354 gel can effectively inhibit the mouse auricular swelling induced by terephthalic acid (TPA) and xylene, and it can also effectively reduce the expressions of inflammatory factors IL-1 β , TNF- α and IL-6 in swelling tissue, suggesting that THCA354 gel has

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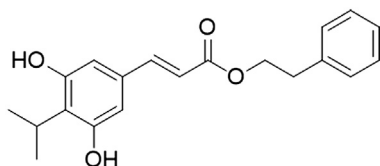


Fig. 1. The structure of THCA354, $C_{20}H_{22}O_4$, MW = 326.14.

significant anti-inflammatory activity [14].

In the present study, we established a mouse model of ACD using 2,4-dinitrofluorobenzene (DNFB), and topical THCA354 gel was used to intervene. By investigating the changes of tissue thickness and the degree of lymphocyte infiltration in mouse ear tissue, as well as by detecting Th1, Th2, Th17, Treg-associated cytokines and specific nuclear transcription factors and the frequency of each cell subsets, it was found that THCA354 gel could alter the immune balance of Th1/Th2/Th17/Treg subsets. These findings indicated that THCA354 gel might be a useful drug candidate for the treatment of ACD.

2. Materials and methods

2.1. Reagents and animals

THCA354 ($\geq 99\%$ purity) was synthesized by our group. THCA354 gels (5%) were produced in our laboratory. 1-Fluoro-2,4-dinitrobenzene (DNFB) was purchased from Sigma. Tissue total protein lysis buffer was obtained from Thermo Fisher. The total protein extraction kit was provided by Beyotime. ELISA kits were supplied from DAKWE. Trizol kit was purchased from Introgen. RNA extraction kit was obtained from Tiangen Biotech, Beijing. RNA reverse transcription kit was provided by TaKaRa. Primers for quantitative real-time RT-PCR were synthesized by TaKaRa. Semi-quantitative PCR dye (SYBR Green I) was purchased from TAKARA. Anti-mouse CD3e-FITC, CD4-PE, IFN- γ -APC, IL-4-APC, Foxp3-FITC, CD25-APC and IL-17A-APC were purchased from eBioscience. Ionomycin calcium salt was supplied by from *Streptomyces globatus* was purchased from J&K Scientific. Protein transport inhibitor cocktail was obtained from BD Biosciences.

Female BALB/c mice (weighing 16–18 g) were purchased from Beijing HFK Bioscience. Mice were housed under standard conditions (25 °C) with a 12/12-h light/dark cycle and given free access to rodent food and water. All experimental protocols were approved by the institutional animal care and use committee of Third Military Medical University.

2.2. Induction and assessment of contact dermatitis

To induce ACD, mice were sensitized on the shaved ventral abdomen on day 0 by applying 75 μ L of 0.5% DNFB (dissolved in a mixture of acetone and olive oil, 4:1) [15]. Four days later (day 4), the inner and outer surfaces of the right ears were challenged with 20 μ L 0.5% DNFB, and the left ears were painted with mixture of acetone and olive oil (4:1, v/v) in the same way. On day 5, mice were re-challenged with 20 μ L 0.4% DNFB in the right ears, and only mixture of acetone and olive oil (4:1, v/v) was applied in the left ears. Subsequently, ear thickness was determined by a dial thickness gauge (Mitutoyo) before challenge and 24 h after re-challenge. The ear swelling was calculated as the increase in ear thickness.

2.3. Drug treatment

A total of 50 BALB/c mice were randomly and evenly divided into five groups ($n = 10$) as follows: control group, vehicle group and three THCA354 gel groups. THCA354 (5.0%) was directly added to gel base consisting of carbopol (2.0%), propylene glycol (5.0%), glycerol (5.0%) and water. Topical treatment was performed once daily for

2 days (days 5 and 6) with 12, 24 and 48 mg THCA354 gel (the amount of THCA354 in the gel was 0.6, 1.2 and 2.4 mg respectively) or vehicle (gel matrix, 48 mg).

2.4. Histopathological examination

Mouse ears were collected and subjected to histological examination. These ears were fixed in 10% formalin solution for 24 h, followed by successive dehydration, embedding, sectioning and dewaxing. Subsequently, paraffin-embedded sections were stained with hematoxylin and eosin (H&E), and examined by Motic digital microscope (Olympus). Images were selected from the appropriate representative areas.

2.5. Flow cytometric analysis

T cells from mouse mandibular lymph nodes (LNs) were collected as previously described [16]. Briefly, cells were surface stained with anti-CD4 and anti-CD25 antibodies in the dark at 4 °C for 30 min. For intracellular staining, cells were permeabilized using a fixed/permeabilized solution and incubated with antibodies against Foxp3. Cells were washed for three times and resuspended in phosphate buffered saline (PBS). The results were expressed as the percentage of each cytokine-producing cell population in a total population of CD25+ cells.

For analysis of intracellular cytokine production, freshly isolated lymphocytes were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) at a density of 1.2×10^6 cells/mL at 37 °C for 6 h. During 6-h incubation, cells were stimulated with a combination of phorbol 12-myristate 13-acetate (PMA, 10 ng/mL) and ionomycin (1 μ g/ μ L). Then the cells were washed and resuspended in Cell WASH. Cells were directly stained with FITC-conjugated anti-CD4 monoclonal antibody in the dark at 4 °C for 30 min, and fixed with FACS lysing solution for 10 min. Next, cells were washed, pre-incubated with FACS permeabilizing solution for 10 min, and incubated with PE-conjugated monoclonal antibodies against IL-4, IL-17, IFN- γ and their isotype-matched controls in the dark at 4 °C for 30 min. Cells were washed twice and resuspended in phosphate buffered saline (PBS). Flow cytometry was performed using FACS Calibur flow cytometer and Cell Quest software (Beckman Coulter). The results were expressed as the percentage of each cytokine-producing cell population in a total population of CD4+ cells.

2.6. ELISA

Briefly, 1 mL T-PER Tissue protein extraction reagent was added to ear tissue, and then the tissue was homogenized by ultrasound. Tissue homogenates were then centrifuged, and supernatants were collected for cytokine determination. Total protein contents in tissue samples were determined using total protein extraction kit according to the manufacturer's instructions. The contents of inflammatory cytokines were examined using corresponding ELISA kits.

2.7. Semiquantitative RT-PCR

Total RNA was extracted from right ear tissue using tissue RNA extraction kit and reversely transcribed into cDNA using the RNA reverse transcription kit. Expressions of target genes were examined by real-time PCR on a CFX Connect (Bio-Rad) detection system using specific primers (Table 1). Briefly, after an initial denaturation step at 95 °C for 30 s, amplifications were carried out with 42 cycles at a melting temperature of 95 °C for 10 s, an annealing temperature of 60 °C for 20 s and an extension temperature of 72 °C for 20 s.

2.8. Statistical analysis

Data were expressed as means $\pm$ standard deviations. Correlation

Table 1
Primers for quantitative real-time RT-PCR.

Items	Sense primer	Antisense primer
β-Actin	GAGACCTTCAACACCCAGC	ATGTCACGCACGATTTC
IFN-γ	TCAAGTGGCATAGATGTGAAG	CGCTTATGTTGTTGCTGATGG
IL-4	TTGTCATCCTGCTCTCTTCTC	TCTGTGGTGTCTCTCGTTGC
T-bet	GATCGTCTGCAGTCTCTCC	AACTGTGTTCCCGAGGTGTC
GATA3	ACCGGGTTCGGATGTAAGTCG	TACCATCTCGCCGCCACAGT
IL-17	ATCTGTGTCTCTGATGCTGTTG	AACGGTTGAGGTAGTCTGAGG
ROR-γt	CGCACCAACCTCTTTTCACG	TGGCAAACTCCACCACATACTG
IL-10	TGCCAAGCCTTATCGGAAATG	ACTCTTACCTGCTCCACTG
TGF-β	GAGGCGGTGCTCGCTTTGTA	CGTTGTTGCGGTCCACCATT
Foxp3	ACACCCAGGAAAGACAGCAACC	CTCGAAGACCTTCTCAACACG
IL-6	TAGTCTTCTACCCCAATTTCC	TTGGTCTTAGCCACTCTTC

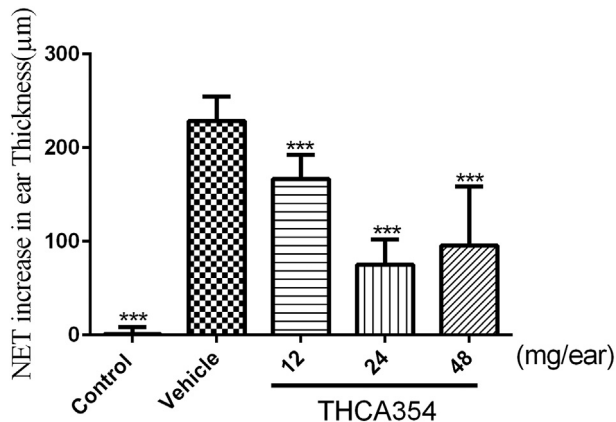


Fig. 2. The effect of THCA354 gel on ear thickness of DNFB-challenged mice. Data were shown as the means $\pm$ S.D. (n = 10) ***P < 0.001, vs. the vehicle group.

coefficients and statistical significance were determined using SPSS 17.0 package. Unpaired Student's *t*-test and one-way ANOVA were used for comparisons between clinical groups. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of THCA354 gel on DNFB-induced ACD

To explore the effect of THCA354 gel treatment on the allergic progression of DNFB-induced ACD, we first assayed the severity of allergic inflammation by measuring the ear thickness increment after 24 h of DNFB re-challenge. As shown in Fig. 2, the ear thickness of DNFB-challenged mice was significantly increased compared with the control group, while it was significantly decreased in the THCA354 gel groups compared with the vehicle group. Moreover, the 24-mg THCA354 gel group had the best effect with an inhibition rate of 67.19%. The above-mentioned results indicated that THCA354 gel could significantly suppress the inflammatory responses in DNFB-challenged mice.

3.2. Morphological analysis

Tissue sections and H&E staining further confirmed the histological response in the ears of ACD mice. From Fig. 3, we showed that compared with the control group (Fig. 3A), the ear tissue swelling, epidermal hyperplasia and lymphocyte infiltration were significantly increased in the vehicle group (Fig. 3B) that received DNFB stimulation, suggesting that the ACD model was successfully established. However, the ear swelling and inflammatory cell infiltration were significantly decreased in the THCA354 gel groups (Fig. 3C, D and E) compared with the vehicle group (Fig. 3B), which was consistent with the ear swelling test results. Histomorphological analysis showed that treatment with THCA354 gel could inhibit inflammatory infiltration and skin edema in the ears of ACD mice.

3.3. Effects of THCA354 gel on Th1/Th2 balance in DNFB-induced ACD

ACD is generally considered to be a model of Th1 cell-mediated disease and is closely related to the balance between Th1/Th2 cell subsets. To investigate the underlying mechanism of the therapeutic efficacy of THCA354 gel on ACD, protein levels of IFN-γ and IL-4 in mouse ear tissues were first measured by ELISA. The expression of IFN-γ, a Th1 cytokine, was significantly suppressed in THCA354-treated ACD mice (Fig. 4A), while the expression of IL-4, a Th2 cytokine, was significantly increased by THCA354 gel in ACD mice (Fig. 4B). Then we detected the expressions of IFN-γ and IL-4 at the mRNA level by RT-

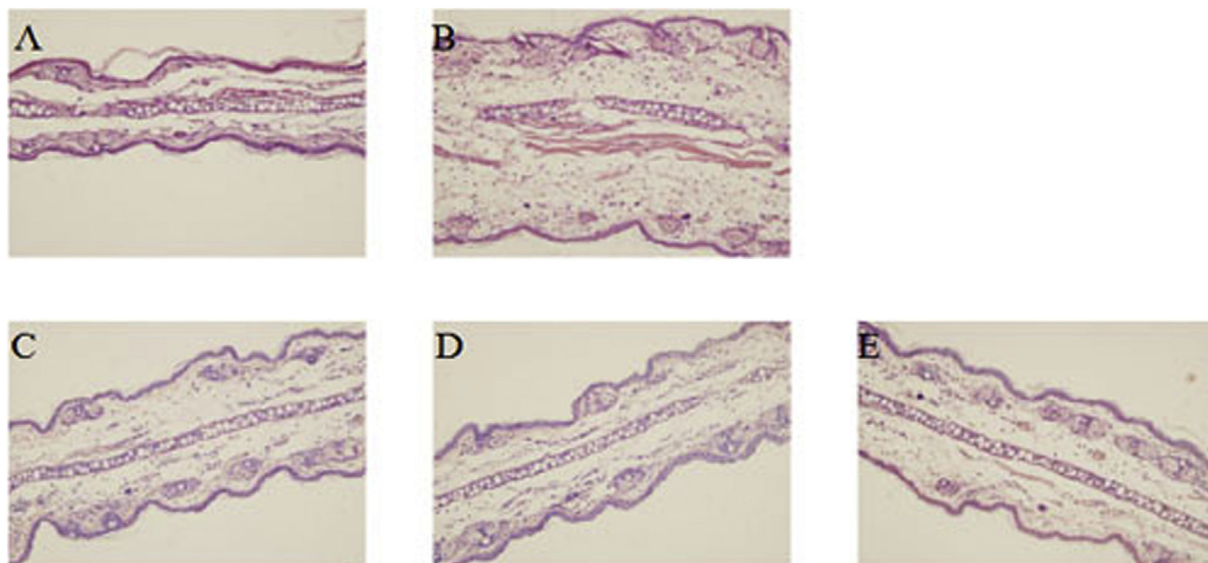
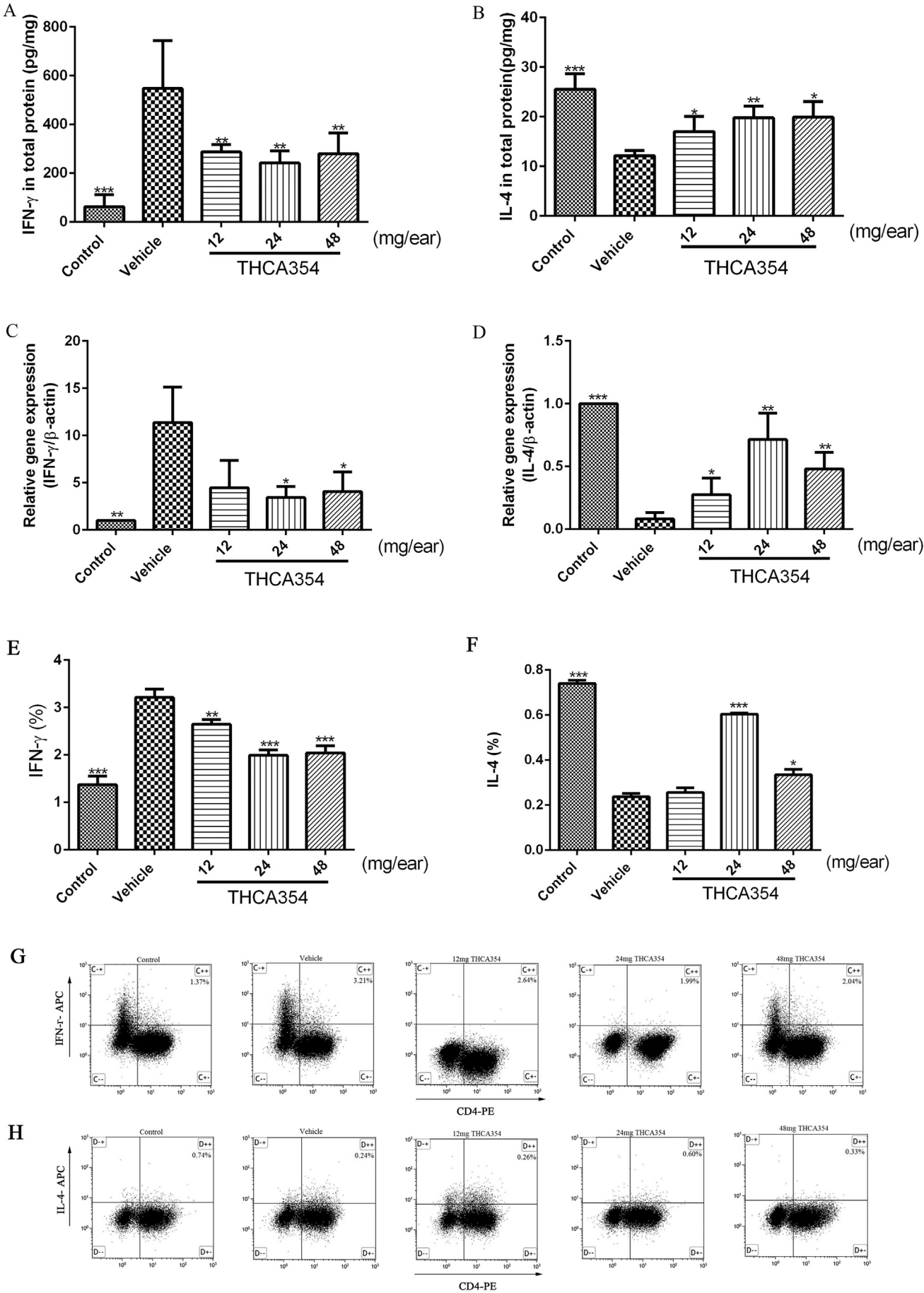


Fig. 3. Representative micrographs of HE-stained sections of mice ears sensitized with DNFB. (A) Control; (B) DNFB + vehicle; (C) DNFB + 12 mg THCA354; (D) DNFB + 24 mg THCA354; (E) DNFB + 48 mg THCA354.



(caption on next page)

Fig. 4. Effect of THCA354 gel on Th1/Th2 cytokine. (A) The expression of IFN- γ at the mRNA level; (B) the expression of IL-4 at the mRNA level; (C) the level of IFN- γ by ELISA; (D) the level of IL-4 by ELISA; (E) the percentages of CD4 + IFN- γ + T cells (Th1) were analyzed by flow cytometry; (F) the percentages of CD4 + IL-4 + T cells (Th2) were analyzed by flow cytometry; (G) representative FACS plots for CD4 + IFN- γ + T cells; (H) representative FACS plots for CD4 + IL-4 + T cells. Data were shown as the means $\pm$ S.D. * P < 0.05, ** P < 0.01, *** P < 0.001, comparison with the vehicle group.

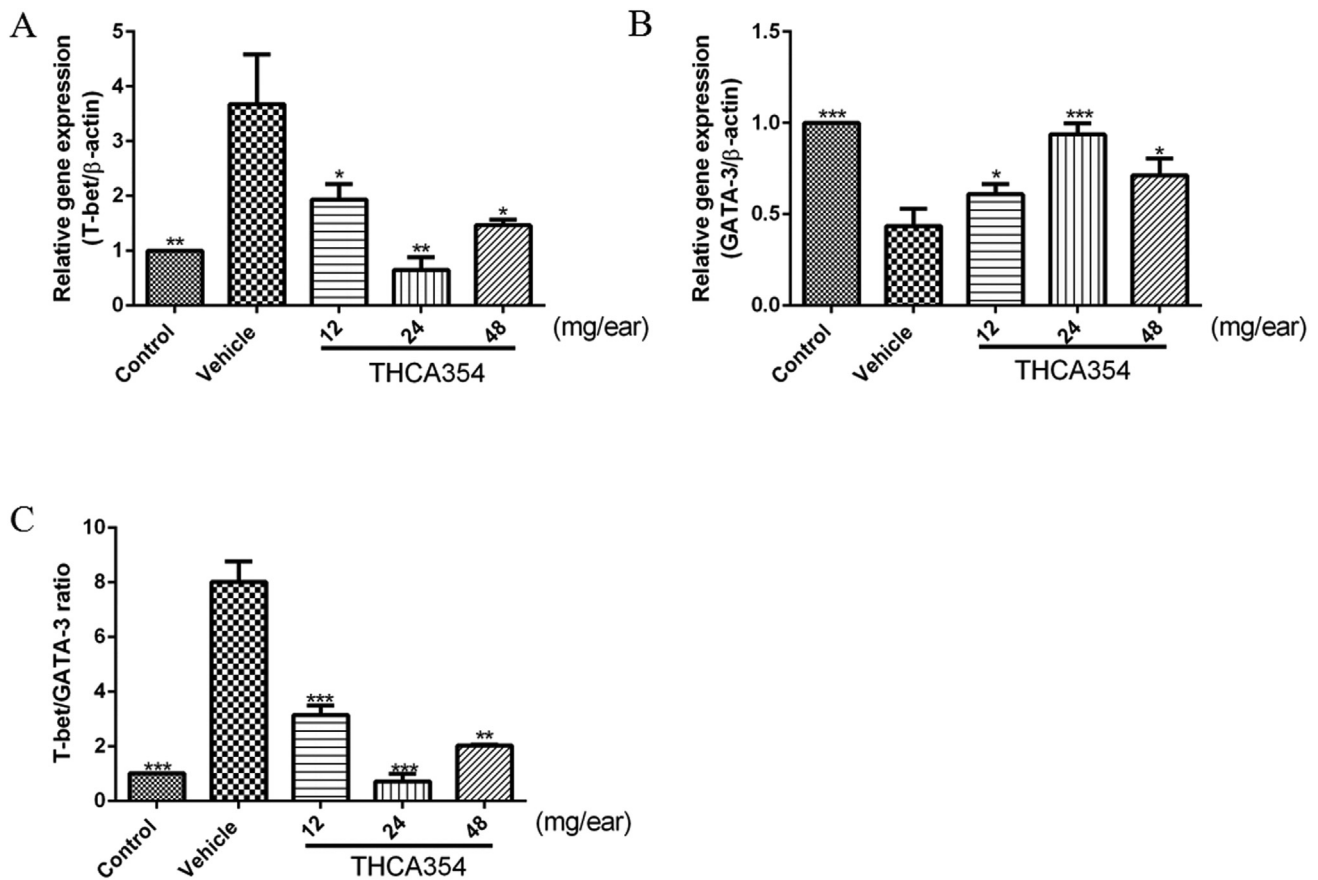


Fig. 5. Effect of THCA354 gel on the mRNA expression of transcription factors involved in Th1 and Th2 differentiation. (A) Quantitative real-time PCR of T-bet; (B) quantitative real-time PCR of GATA-3; (C) the ratio of relative gene expression for T-bet/GATA-3. Data were shown as the means $\pm$ S.D. * P < 0.05, ** P < 0.01, *** P < 0.001, vs. the vehicle group.

PCR. Similar to protein levels, THCA354 gel treatment significantly inhibited the gene expression of IFN- γ but increased the gene expression of IL-4 (Fig. 4C and D). In addition, the percentages of IFN- γ - and IL-4-positive cells were determined by flow cytometry (Fig. 4E and F), showing that THCA354 gel treatment decreased IFN- γ -positive cells and increased IL-4-positive cells (Fig. 4G and H).

To further determine the possible immune mechanisms involved in the protective effect of THCA354 gel on DNFB-induced ACD, we examined the expressions of transcription factors T-bet (for Th1) and GATA-3 (for Th2) at the mRNA level, which regulate Th1 and Th2 differentiation, respectively (Fig. 5A and B). The ratio of T-bet/GATA-3 in the THCA354 gel groups was significantly lower compared with the vehicle group (Fig. 5C). These results suggested that the therapeutic effect of THCA354 gel on ACD was associated with the Th1/Th2 balance.

3.4. Effects of THCA354 gel on Th17 in DNFB-induced ACD

Th17 cells are a newly discovered subset of CD4 + T cells with IL-17 as the main effector. They are involved in various inflammatory and autoimmune diseases [17]. Retinoic acid orphan receptor gamma (ROR- γ t) is a specific transcription factor for Th17 cells and plays an important role in the differentiation of Th17 cell subsets. Moreover, IL-6 is essential for inducing Th17 development and activation of ROR- γ t

[18]. To investigate immunomodulatory effect of THCA354 gel on Th17, we subsequently evaluated the role of THCA354 gel on Th17. The protein and mRNA expression levels of IL-17 were determined by ELISA and RT-PCR, respectively, and both analyses showed that THCA354 gel significantly inhibited the expression and production of IL-17 (Fig. 6A and B). Next, we examined the cellular phenotypes of Th17 cell by FACS. Compared with the vehicle group, the frequency of CD4 + IL-17 + T cells in THCA354 gel groups was significantly reduced (Fig. 6C and D). To further understand the underlying mechanism of THCA354 gel on Th17, we examined the mRNA expression of IL-6 and ROR- γ t by RT-PCR. These results showed that the expression of IL-6 and ROR- γ t in the THCA354 gel groups was much lower compared with the vehicle group (Fig. 6E and F). These results demonstrated that THCA354 gel inhibited Th17 differentiation and reduced IL-17 secretion.

3.5. Effects of THCA354 gel on Tregs in DNFB-induced ACD

Tregs is a class of T cell subsets with immunomodulatory effects [19]. Recent studies have found that Tregs play a significant role in the development of ACD [20]. Treg activity is regulated by a specific transcription factor, namely forkhead box P3 (Foxp3) and IL-10, TGF- β are the major secretory cytokines released by Tregs. Therefore, in order to confirm the mechanism of action of THCA354 gel on Tregs, we first detected the expressions of IL-10 and TGF- β by RT-PCR. Fig. 7A and B

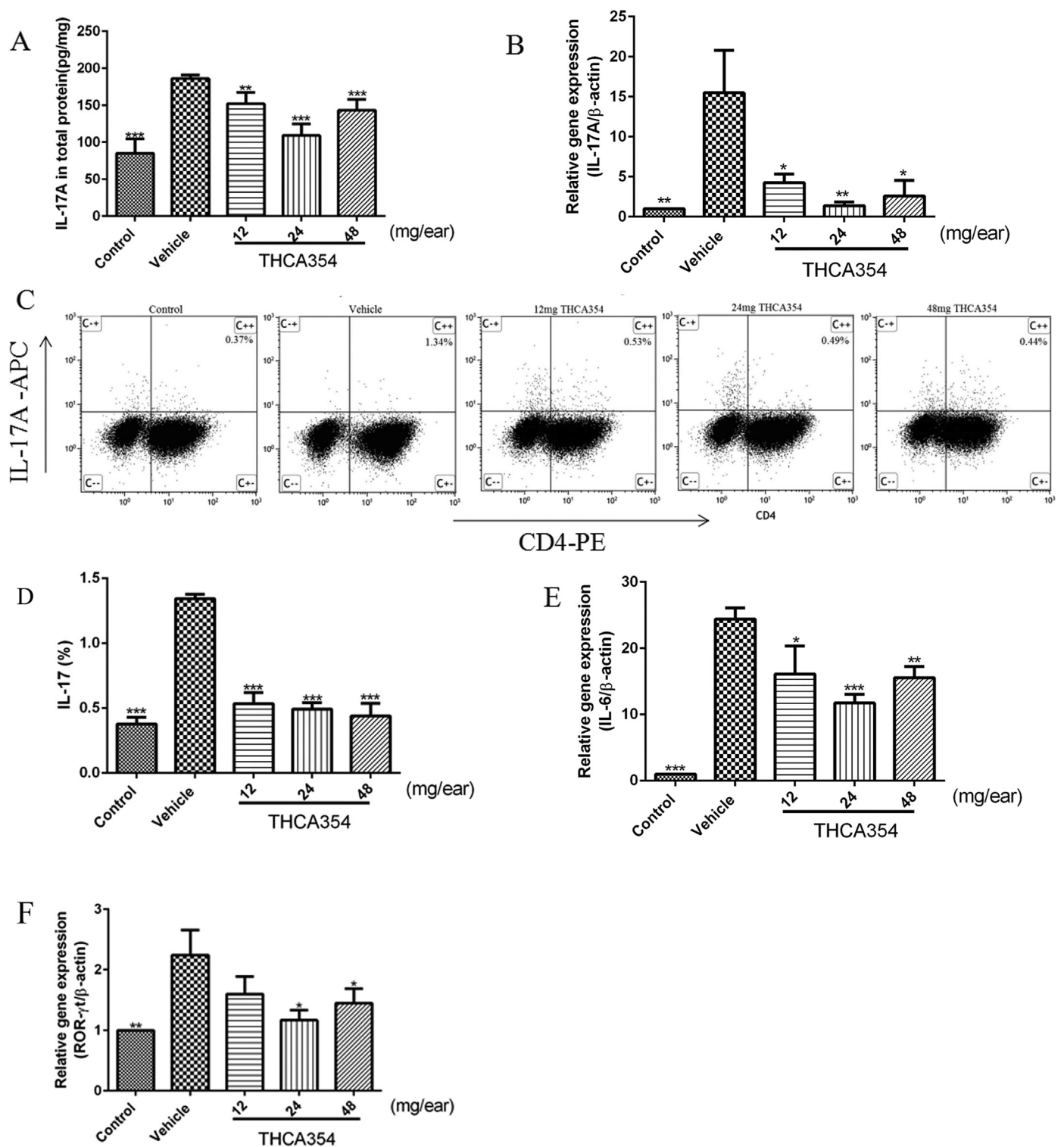


Fig. 6. Effects of THCA354 gel on Th17 in DNFB-induced ACD. (A) The level of IL-17 by ELISA; (B) the expression of IL-17 at the mRNA level; (C) representative FACS plots for CD4+IL-17A+ T cells; (D) the percentages of CD4+IL-17A+ T cells (Th1) were analyzed by flow cytometry; (E) the expression of IL-6 at the mRNA level; (F) the expression of ROR-γt at the mRNA level. Data were shown as the means $\pm$ S.D. * P < 0.05, ** P < 0.01, *** P < 0.001, vs. the vehicle group.

displays that the gene expressions of IL-10 and TGF-β in the THCA354 gel groups were significantly increased compared with the vehicle group. Next, we examined the frequency of CD4+CD25+Foxp3+ Tregs cells and the gene expression of Foxp3 by FACS and RT-PCR, respectively. The percentage of CD4+CD25+Foxp3+ Tregs cells (Fig. 7C and D) and the gene expression of Foxp3 (Fig. 7E) in the THCA354 gel groups were significantly higher compared with the

vehicle group. These findings indicated that THCA354 gel could promote production of Tregs, leading to inhibited ACD response.

4. Discussion

The pathological process of ACD is mainly a T-cell-mediated DTH. Although there is some controversy about the nature of the ACD

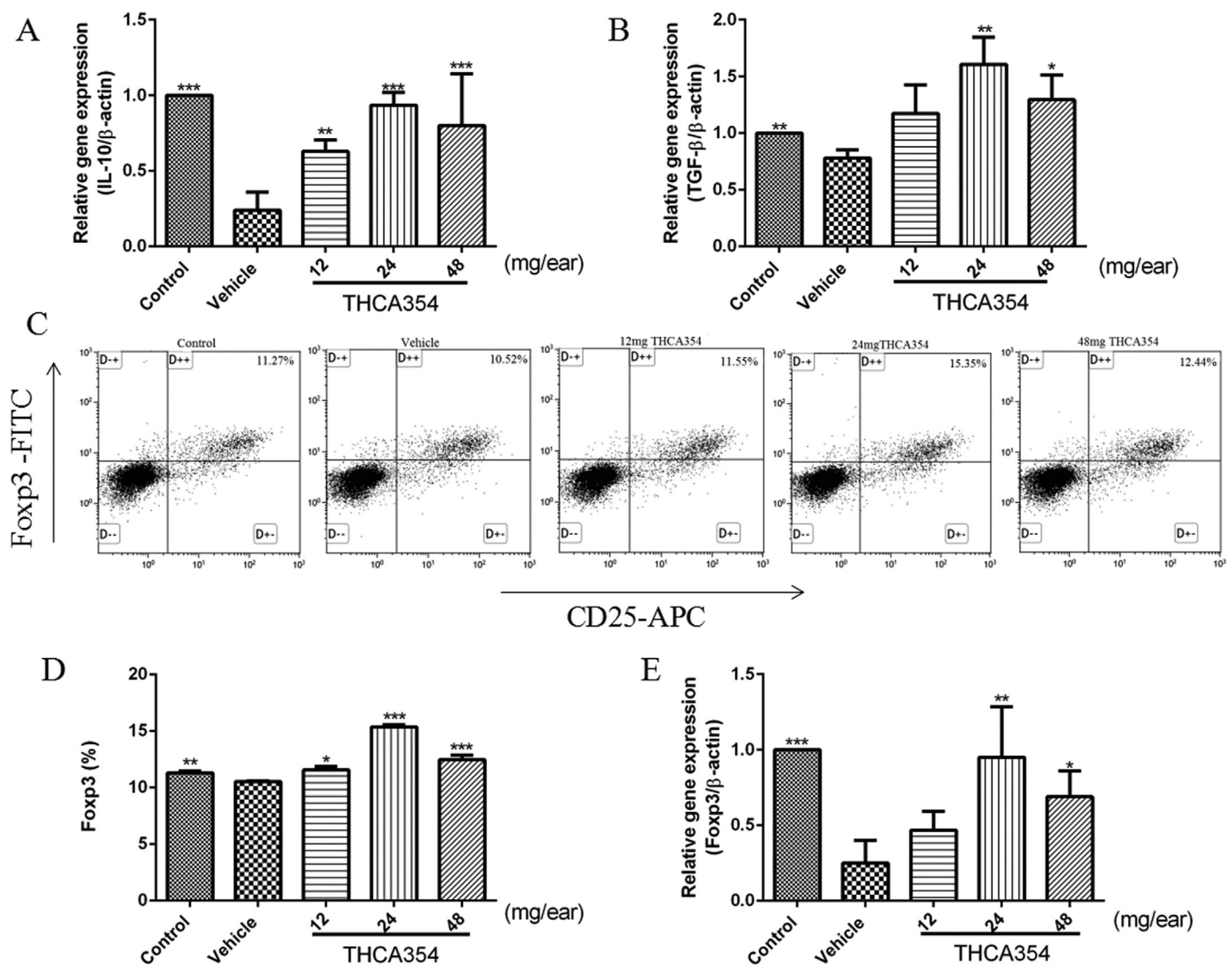


Fig. 7. Effects of THCA354 gel on Treg in DNFB-induced ACD. (A) The expression of IL-10 at the mRNA level; (B) the expression of TGF- β at the mRNA level; (C) representative FACS plots for CD25+Foxp3+ T cells; (D) the percentages of CD25+Foxp3+ T cells (Th1) were analyzed by flow cytometry; (E) the expression of Foxp3 at the mRNA level. Data were shown as the means $\pm$ S.D. * P < 0.05, ** P < 0.01, *** P < 0.001, vs. the vehicle group.

response, according to a dominant concept, it is mainly mediated by Th1 cell subsets in the DNFB-induced ACD mouse model [21,22]. In contrast, the induction of antigen-specific Th2 responses is associated with inhibition of inflammation in animal models of ACD. In addition, ACD is closely related to imbalanced secretion of inflammatory and anti-inflammatory cytokines. Therefore, changing the secretion and release of key cytokines during the inflammatory response and correcting the imbalanced Th1/Th2 cytokine pattern can be an effective strategy for the treatment of ACD. Th1-type cytokines, such as IFN- γ and TNF- α , are involved as mediators initiating the progression of allergic reactions. IL-4 produced by Th2 cells negatively regulates the ACD response, as evidenced by more pronounced inflammation in IL-4 deficient mice [23]. ACD in response to dinitrofluorobenzene is significantly enhanced in mice lacking STAT6 (signal transduction and transcription activator of IL-4) [24]. Our results indicated that treatment with THCA354 gel inhibited IFN- γ production in DNFB-induced ACD mouse ear tissue and mandibular lymphocytes, but promoted IL-4 secretion. T-bet is a transcription factor of Th1 cells and a specific marker reflecting Th1-releasing cytokines. GATA-3 is selectively expressed on Th2 cells, and it is the only specific transcription factor that can regulate the synthesis of Th2-type cytokines. Studies have shown that T-bet can inhibit the expression of GATA-3 and induce the

differentiation of mature Th2 cells to Th1 cells, while GATA-3 can inhibit T-bet and promote the development of Th2. Therefore, regulation of T-bet/GATA-3 expression can control the Th1/Th2 balance upstream of Th1/Th2 cell differentiation, overcoming the one-sidedness of the single cytokine effect [25]. This study showed that THCA354 gel not only significantly inhibited the expression of T-bet, but also promoted the expression of GATA-3, thereby affecting the differentiation of Th1/Th2 cells. These results indicated that THCA354 gel can significantly improve the Th1/Th2 immune imbalance, resulting in attenuated inflammatory response.

Some studies have shown that the process of ACD immune response is not only the activation of effector T cells, but also accompanied by the formation of Tregs. Moreover, the low function of Tregs can even become an important cause of ACD. Tregs are a subset of T cells that control autoimmune responses in vivo. As the most important regulator of Tregs, Foxp3 plays an important role in the formation and function of Tregs [26,27]. CD4+CD25+Foxp3+ Tregs inhibit inflammation through a combination of secretory and surface-bound factors and promotes peripheral tolerance or hyporesponsiveness to self and foreign antigens [28]. Besides, Tregs can also mediate ACD responses by secreting inhibitory cell factors, such as IL-10 and TGF- β . The results of this study showed that THCA354 gel could induce the generation of

CD4+CD25+Foxp3+ Tregs cells and increase the expressions of Foxp3, IL-10 and TGF- β at the mRNA level. These results demonstrated that THCA354 gel played an immunosuppressive role by inducing Tregs.

In addition, it has been reported that Th17 cells are involved in the pathogenesis of ACD by stimulating the expressions of proinflammatory cytokines and chemokines. IL-17 is a major effector of Th17 cells, which can amplify inflammatory responses and promote the development of allergic diseases by promoting the release of pro-inflammatory cytokines. In contrast, ROR- γ t plays an important role in the differentiation of Th17 cell subsets [6]. IL-6 is an important proinflammatory cytokine [29]. It is generally accepted that IL-6 and TGF- β are key cytokines that induce the differentiation of naive CD4+ T cells into Th17 cells [30]. Th17 cells can interact with Tregs in differentiation and function. TGF- β induces the differentiation of naive T cells into Tregs and suppresses the immune response. In the presence of IL-6, TGF- β promotes the production and secretion of IL-17 by Th17 cells, leading to enhanced inflammation reaction. However, with the decreased IL-6 level, the increased TGF- β level can maintain effector cell functionality through the expansion and maintenance of Tregs, and can thus effectively control the pathogenesis of ACD. Therefore, regulating the balance between Th17/Treg may provide a new approach for the effective treatment of ACD. The results of this experiment showed that THCA354 gel significantly inhibited the production of ROR- γ t and IL-17 in ACD mice, and up-regulated TGF- β , thereby further promoting the production of Treg cells. On the other hand, THCA354 gel could also inhibit the level of IL-6. With the decrease of IL-6, the increased TGF- β could also extend and maintain Treg cells, and maintain the function of the effector cells in the appropriate state, thus effectively controlling the pathogenesis of ACD.

Taken together, the destruction of immune balance between Th1/Th2 cells, Treg/Th17 cells and their related cytokines played an important role in the pathogenesis and development of ACD. In this study, we established a DNFB-induced ACD model and found that treatment with THCA354 gel not only inhibited DNFB-induced ear swelling in mice, but also reduced inflammatory cells recruited to inflamed ear tissue. Moreover, the reduction of ACD response was accompanied by decreased levels of IFN- γ and IL-17 as well as reduced expressions of T-bet and ROR- γ t. Meanwhile, THCA354 gel reduced the CD4+ cells expressing IFN- γ and IL-17 in the LNs of mice. In addition, THCA354 gel could induce the production of CD4+CD25+Foxp3+ cells and IL-4-expressing CD4+ cells, increase the expressions of IL-4, Foxp3, IL-10, TGF- β and GATA-3 at the mRNA level. Therefore, THCA354 gel could be used as a potential drug candidate for the treatment of ACD by altering the immune balance of Th1/Th2/Th17/Tregs.

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References

- [1] Y. Hirasaki, C. Iwamura, M. Yamashita, T. Ito, M. Kitajima, K. Shinoda, et al., Repressor of GATA negatively regulates murine contact hypersensitivity through the inhibition of type-2 allergic responses, *Clin. Immunol.* 139 (3) (2011) 267–276.
- [2] SLJBHASHC. GAUTAM JMNC, MARK G. EDINGER ART, L3T4 (CD4⁺) cells that mediate contact sensitivity to trinitrochlorobenzene express I-A determinants, *Cell. Immunol.* 135 (1991) 27–41.
- [3] M. A. VADAS JFAP, Ly and Ia antigen phenotypes of T cells involved in delayed-type hypersensitivity and in suppression, *J. Exp. Med.* 144 (1976) 10–19.
- [4] DANIEL J. CHER TRM, Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by Th1 clones, *J. Immunol.* 138 (1987) 3688–3694.
- [5] J.J. O'Shea, W.E. Paul, Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells, *Science* 327 (5969) (2010) 1098–1102 (2010-02-26).
- [6] T. Korn, E. Bettelli, M. Oukka, V.K. Kuchroo, IL-17 and Th17 cells, *27* (1) (2009) 485–517.
- [7] H. Park, Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y. Wang, et al., A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17, *Nat. Immunol.* 6 (11) (2005) 1133–1141.
- [8] Susumu Nakae YK, Aya Nambu KSMI, Ikuo Homma KS, Masahide Asano AYI, Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses, *Immunity* 17 (2002) 375–387.
- [9] Tetsuya Honda GESG, Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis, *J. Invest. Dermatol.* 133 (2013) 303–315.
- [10] D. Shi, X. Li, D. Li, Q. Zhao, Y. Shen, H. Yan, et al., Oral administration of paeoniflorin attenuates allergic contact dermatitis by inhibiting dendritic cell migration and Th1 and Th17 differentiation in a mouse model, *Int. Immunopharmacol.* 25 (2) (2015) 432–439.
- [11] Sergio Faroni De Andrade C MLAE, Valdir Cechinel Filho C RN, Evaluation of the antitumor activity of *Maytenus robusta* (Celastraceae) in different experimental ulcer models, *J. Ethnopharmacol.* 113 (2) (2007) 252–257.
- [12] J. Xiao, H. Zhai, Y. Yao, C. Wang, W. Jiang, C. Zhang, et al., Chrysin attenuates experimental autoimmune neuritis by suppressing immuno-inflammatory responses, *Neuroscience* 262 (2014) 156–164.
- [13] M.H. Siard, K.E. McMurry, A.A. Adams, Effects of polyphenols including curcuminoids, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene on lymphocyte pro-inflammatory cytokine production of senior horses in vitro, *Vet. Immunol. Immunopathol.* 173 (2016) 50–59.
- [14] CAO Wen LHHQ, Mengjun ZH, Anti-inflammation effect of (E)-phenethyl 3-(3, 5-dihydroxy-4-isopropylphenyl) acrylate and the preliminary mechanism, *Chin. J. Immunol.* 31 (01) (2015) 12–16 (2015-01-01).
- [15] M. Zemelka-Wiącek, M. Majewska-Szczepanik, W. Pyrczak, M. Szczepanik, Complementary methods for contact hypersensitivity (CHS) evaluation in mice, *J. Immunol. Methods* 387 (1–2) (2013) 270–275.
- [16] Estelle Bettelli YCWG, Kuchroo HLWW, Resveratrol induces apoptosis, influences IL-6 and exerts immunomodulatory effect on mouse lymphocytic leukemia both in vitro and in vivo, *Nature* 441 (11) (2006).
- [17] Jesse M. Damsker AMHA, Th1 and Th17 cells_ adversaries and collaborators, *Ann. N. Y. Acad. Sci.* 1 (1183) (2010) 211–221.
- [18] T. Tanaka, M. Narazaki, T. Kishimoto, IL-6 in inflammation, immunity, and disease, *CSH Perspect. Biol.* 6 (10) (2014) a16295(2014-10-01).
- [19] Gershon RK KK, Cell interactions in the induction of tolerance: the role of thymic lymphocytes, *Immunology* 18 (5) (1970) 723–735.
- [20] M. Kianmehr, D. Haghmorad, R. Nosratabadi, A. Rezaei, A. Alavinezhad, M.H. Boskabady, The effect of *Zataria multiflora* on Th1/Th2 and Th17/T regulatory in a mouse model of allergic asthma, *Front. Pharmacol.* 8 (2017) (2017-08-07).
- [21] A.H. Wagner, I. Wittjen, T. Stojanovic, P. Middel, J.G. Meingassner, M. Hecker, Signatur and activator of transcription 1 decoy oligodeoxynucleotide suppression of contact hypersensitivity, *J. Allergy Clin. Immunol.* 121 (1) (2008) 158–165.
- [22] S.F. Martin, Immunological mechanisms in allergic contact dermatitis, *Curr. Opin. Allergy Clin. Immunol.* 15 (2) (2015) 124–130.
- [23] Hui Xu NDAF, T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon gamma-producing (Tc1) effector CD8+ T cells and interleukin (IL) 4/IL-10-producing (Th2) negative regulatory CD4+ T cells, *J. Exp. Med.* 183 (3) (1996) 1001–1012 (1996-03-01).
- [24] K. Takeshita, Essential role of MHC II-independent CD4+ T cells, IL-4 and STAT6 in contact hypersensitivity induced by fluorescein isothiocyanate in the mouse, *Int. Immunol.* 16 (5) (2004) 685–695 2004-03-29.
- [25] T KTSH, Th2 cells predominate in idiopathic steroid-sensitive nephrotic syndrome, *Clin. Exp. Nephrol.* 14 (6) (2010) 578–583.
- [26] Hori S NTSS, Control of regulatory T cell development by the transcription factor Foxp3, *Science* 5609 (299) (2003) 1057–1061.
- [27] S.C. Balmert, C. Donahue, J.R. Vu, G. Erdos, L.D. Falo, S.R. Little, In vivo induction of regulatory T cells promotes allergen tolerance and suppresses allergic contact dermatitis, *J. Control. Release* 261 (2017) 223–233.
- [28] Dario A. A. Vignali LWCA, How regulatory T cells work, *Immunology* 8 (2008) 523–532.
- [29] W. Niedbala, J.C. Alves-Filho, S.Y. Fukada, S.M. Vieira, A. Mitani, F. Sonogo, et al., Regulation of type 17 helper T-cell function by nitric oxide during inflammation, *Proc. Natl. Acad. Sci.* 108 (22) (2011) 9220–9225 (2011-05-31).
- [30] Xuexian O. Yang BPPR, T helper 17 lineage differentiation is programmed by orphan nuclear receptors RORa and RORg, *Immunity* 28 (1) (2007) 29–39.