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Osthole attenuates mouse atopic dermatitis by inhibiting thymic stromal lymphopoietin production from keratinocytes

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Abstract

Atopic dermatitis is one of the most common skin diseases. Dysregulation of immune system and chronic inflammation were believed to be associated with atopic dermatitis. Osthole was reported to play important roles in anti-tumor and anti-inflammation. However, whether osthole has effects on atopic dermatitis remains unclear. In this present study, we explored the biological role of osthole in atopic dermatitis and the molecular mechanism. Atopic dermatitis was induced by 2,4-dinitrochlorobenzene. Pathological damage of ear was detected by H&E staining. IgE level in serum or thymic stromal lymphopoietin (TSLP) level in supernatant was detected by ELISA. Interleukin (IL)-4, IL-13 expression in CD4⁺ T cells were detected using flow cytometry. The expression levels of mRNA or protein levels were detected by RT-PCR or western blot. Osthole attenuated atopic dermatitis development in mouse model. Osthole inhibits Th2 cells response, but have no influence on Th1 or Th17 cells response in the skin. In mouse model, osthole treatment significantly inhibited atopic dermatitis via directly inhibiting TSLP expression levels in keratinocytes. Osthole treatment alleviates atopic dermatitis through directly down-regulating TSLP production from keratinocytes. Osthole may serve as a potential choice for atopic dermatitis treatment in clinic.

Keywords: osthole, atopic dermatitis, TSLP, keratinocytes

Introduction

Atopic dermatitis is one of the most common skin diseases, characterized by erythema, pruritus, dry skin, impaired epidermal barrier and abnormal immune responses (1, 2). In adults, the prevalence of atopic dermatitis is 1%-3%, while in child the prevalence is much higher

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(10%-20%) (3, 4). Atopic dermatitis progression leads to severe pruritus, which influences personal and familial life quality. The development of atopic dermatitis is associated with environmental factor and genetic factors (5). In clinic, atopic dermatitis diagnosis was based on associated signs and characteristic morphology. For atopic dermatitis therapy, there are various topical agents which are approved by Food and Drug Administration (6). Most of the agents have effects of anti-inflammation, or help to establish immune tolerance. However, the efficacy of these agents is limited. Therefore, how to increase the efficacy is urgently needed.

Osthole (7-methoxy-8-(3-methyl-2-butenyl) coumarin) was first isolated *Cnidium* plant and commonly used in Chinese medicine for improving immune system functions, attenuating rheumatic pain and relieving dampness (7). Increasing attention has been received because of the crucial activation of osthole in anti-inflammation, anti-tumor, anti-hepatitis and anti-allergic. In hepatocellular carcinoma mouse model, osthole treatment significantly improved the percentage and absolute number of CD4⁺ T and CD8⁺ T cells in spleen and HCC tissues. The serum interleukin-2 (IL-2) and tumor necrosis factor- α (TNF- α) also increased after osthole treatment. In addition, the proportion of regulatory T cells (Tregs, CD4⁺CD25⁺Foxp3⁺) in spleen significantly decreased, indicating the anti-tumor role of osthole in HCC (8). In the rat model of collagen induced arthritis, 40 mg/kg osthole administration significantly inhibited arthritic symptoms, evidenced by alleviated inflammatory cytokine levels (IL-1 β , TNF- α and IL-6), decreased serum oxidative stress (9). Moreover, osthole administration significantly inhibited cell proliferation and cell migration of synovial cell line SW982 (9). However, whether osthole has effects on atopic dermatitis remains unclear.

In the present study, we aim to explore the potentials of osthole against atopic dermatitis and to elucidate the underlying mechanisms of the protection of osthole.

Methods

Animals and cell line

BALB/c mice of 7 weeks were purchased from Vital River Laboratory Animal Technology (Beijing, China) and kept in animal facility with the temperature of 22°C to 24°C, and 12 hours-12 hours (light-dark) cycle. Animal studies are in accordance with the Guide for the Care and Use of Laboratory Animals (NIH). Animal protocols were approved by animal use and care committee of Quanzhou Medical College People's Hospital Affiliated.

Murine keratinocyte cell line MSC-P5 was purchased from Oxford Genetics. The MSC-P5 cell line was cultured in DMEM medium with 10% fetal bovine serum (FBS, Invitrogen, Pleasanton, CA), 100 units/ml penicillin and 100 g/ml streptomycin (both purchased from Invitrogen), at 37°C incubator with humidified 5% (v/v) atmosphere of CO₂.

Atopic dermatitis induction and osthole administration

To establish atopic dermatitis model, mice were first sensitized with 100 µl 1% 2,4-dinitrochlorobenzene (DNCB, Sigma, St. Louis, MO, dissolved with acetone/olive oil=4:1) on ear every 2 days for one week (Day0-Day7). One week later, the mice were administered with 100 µl 0.5% DNCB every 2 days for another 5 weeks (Day7-Day42). On Day 43, the mice were sacrificed for further detection. The vehicle (acetone/olive oil= 4:1) was applied as negative control with the same schedule as DNCB.

Osthole was purchased from Sigma and dissolved according to the manufacturer's protocol. The mice were orally fed with 50 mg/kg osthole daily from Day7 to Day 43. Our pilot study suggests that this dose was the minimum dose to be effective against atopic dermatitis.

ELISA

The IgE levels in serum, and the levels of IL-4, IL-13 and thymic stromal lymphopoietin (TSLP) in supernatant were detected using commercial ELISA kit (eBioscience, San Diego, CA) according to the manufacturer's protocol.

Histology, ear thickness and total dermatitis score

Mice were sacrificed on Day 43. Then, ear tissues were fixed by 10% formalin, followed by embedded with paraffin. Total of 5 μ m sections were sent out to companies for hematoxylin eosin (H&E) staining.

To detect ear thickness, the randomly selected sections after H&E staining were measured by Image Pro Plus software under microscope.

Total dermatitis score is the sum of 4 individual scores: edema, erythema/hemorrhage, scaling/dryness and excoriation/erosion. For each index, the score was judged as follows: 0, no symptoms; 1, mild; 2, moderate; 3, severe. All detection was performed blindly.

RNA isolation and RT-PCR

RNeasy Mini Kit (Qiagen, Valencia, CA) was used to isolate mRNAs from tissues according to the manufacturer's protocol. Then, RNA concentrations were detected using NanoDrop Microvolume Spectrophotometers. Total of 500ng RNA was used for cDNA synthesis using iScript cDNA Synthesis Kits (Bio-Rad, Hercules, CA, USA). The primers used for RT-PCR are as follows: IL4: F 5'-CAC CCT CAA ACC ACT CTC TGA A-3', R 5'-CTT GGA AGC CCT ACA GAC GA-3'; IL5: F 5'-ATG AGA CGA TGA GGC TTC CT -3', R 5'- CCC ACG

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GAC AGT TTG ATT CT-3'; IL10: F 5' - AGT GTG TAT TGA GTC TGC TGG -3', R 5' - TTT CCA AGG AGT TGT TTC CG -3'; IL13: F 5' - AGC ATG GTA TGG AGT GTG GAC CTG -3', R 5' - CAG TTG CTT TGT GTA GCT GAG CAG-3'; IL17: F 5' - TGG GTT CTG CGG TAT TTG AC -3', R 5' - GCT GCT GTG TGA GGT TGA GC -3'; INF- γ , F 5' -TAT CTG GAG GAA CTG GCA AA-3', R 5' -ATC AGG TGT GAT TCA ATG ACG-3'; TSLP, F 5' - GGA GAT TTG AAA GGG GCT AAG -3', R 5' - TGG GCA GTG GTC ATT GAG -3'; IL-25, F 5' - TGG CAA TGA TCG TGG GAA CC-3', R 5' - GAG AGA TGG CCC TGC TGT TGA -3'; β -actin: F 5' - CGC TCT TCC AGC CTT CCT T -3', R 5' - CGT TGT TGG CAT ACA GGT CCT -3'. β -actin expression level was used as control. Relative expression levels of genes were detected by the $2^{-\Delta\Delta CT}$ method.

Western blot

Tissues were first lysed using radioimmunoprecipitation assay buffer, and quantified by bicinchoninic acid assay protein assay kit. Then, the lysates were electrophoresed onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. After separation, the proteins were transferred to membrane. Then, primary antibody for TSLP (1:500, Cell Signaling Technology, Danvers, MA) was used to incubate with the membranes at 4°C overnight, followed by blocking with 10% no-fat milk for 1 hour at room temperature. Then, the membrane was incubated with horseradish peroxidase-secondary antibody at room temperature 1 hour and detected by chemiluminescence reaction. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Cell Signaling Technology was used as control.

Flow cytometry

Total of 1×10^6 single cells were first stained using surface makers (CD45-FITC, CD3-PE, CD4-APC, Biolegend, San Diego, CA) at room temperature for 15 mins. Then, the cells were performed intracellular staining for IL4-PerCrCy5.5 and IL-13-Pacific blue for at least 1 hour at 4°C . The dose of antibodies used in this study is 0.2 mg per 1×10^6 cells. After staining, the cells were detected using flow cytometry.

CD4⁺ T-cell responses *in vitro*

Total of 3×10^5 CD4⁺ T cells isolated from spleen of BALB/c mice were added into 96-well plate. The CD4⁺ T cells were stimulated using anti-CD3/anti-CD28 (both 1 $\mu\text{g}/\text{ml}$) antibodies for 56 hours. Meanwhile, 2 $\mu\text{g}/\text{ml}$ of osthole was added into wells to detect the effects on CD4⁺ T cell responses. After culture, [3H]-thymidine incorporation was used to detect CD4⁺ T cell proliferation(10). The collected supernatant was used to IL-4 and IL-13 detection using ELISA.

Dendritic cells (DCs) migration assay

Mouse ear skin was split, and then cultured in 24 well plate. A table of sieves with epidermis was used to support mouse ear skin for migration. Then, the tables were transported into new wells daily. Cells migrated through the table on day 2 and day 3 were collected and counted. DCs migration was calculated in per mg tissues.

Statistical analysis

All data are from three independent experiments. Data presented as mean $\pm$ SEM. from three independent experiments. The statistical significance was detected using one-way ANOVA or Student's t-test. $P < 0.05$ was considered as significant.

Results

Osthole inhibited mouse atopic dermatitis development

To explore the effect of osthole on atopic dermatitis development, we first established atopic dermatitis model using Balb/c mice. As shown in Fig. 1A, the mice were first treated by 1% DNCB for 7 days, followed by 0.5% DNCB treatment during Day7 to Day 42. Osthole administration was performed during Day7 to Day 42. It was noted that no obvious side-effect was observed in the osthole-treated mice, including weight loss and death. H&E staining in Fig. 1B and statistics in Fig. 1C showed that DNCB treatment significantly increased ear thickness as compared with Normal control, indicating successful establishment of atopic dermatitis model. Osthole treatment obviously decreased ear thickness in DNCB-induced atopic dermatitis (Fig. 1B and 1C). The increased dermatitis scores after DNCB treatment were inhibited after osthole treatment (Fig. 1D).

Serum IgE levels were reported to be increased in 80% of atopic dermatitis patients, which promoted us to detect serum IgE levels after osthole treatment. As shown in Fig. 1E, IgE levels from DNCB mice were much higher than normal control, while significantly decreased after osthole treatment. These data suggested that osthole inhibited mouse atopic dermatitis development.

Osthole inhibits Th2 cells response

To explore the mechanism of how osthole inhibits atopic dermatitis development, we detected Th2 (T-helper 2) inflammation cytokine levels in ear, as Th2 cells play crucial roles in early stage of atopic dermatitis. As shown in Fig. 2A, the mRNA expression levels of IL-4 and IL-13 significantly increased in DNCB-induced atopic dermatitis, while decreased after osthole treatment. Another Th2 inflammation cytokine IL-5 mRNA level showed a trend to decrease, but without significance.

We further detected IL-4⁺CD4⁺ T and IL-13⁺CD4⁺ T cells using flow cytometry in ear. The frequency of IL-13⁺ or IL-4⁺ in CD4⁺ T cells significantly decreased after osthole treatment (Fig. 2B and 2C). These data indicated that osthole inhibits Th2 cells response in atopic dermatitis mouse model.

Osthole has no effects on Th1 and Th17 cells response

Th1 and Th17 cell responses were reported to be important at late stage of atopic dermatitis. We therefore detected the influence of osthole on Th1 and Th17 cell responses. As shown in Fig. 3A, the mRNA expression levels of IFN- γ , IL-17A and IL-10 significantly increased in DNCB-induced atopic dermatitis. However, osthole treatment showed no influence on their expression levels, although slightly, but not significant increase of IL-17A after osthole treatment, indicating the specific effect of osthole on Th2 cell response in the mouse model.

To further explore how osthole inhibited atopic dermatitis, we isolated CD4⁺ T cells and co-cultured with osthole *in vitro*. As shown in Fig. 3B, osthole had no influence on CD4⁺ T cell proliferation, which is detected by [3H]-thymidine incorporation assay. Moreover, the concentration of IL-4 and IL-13 in supernatant showed no difference after osthole treatment

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(Fig. 3C, 3D). These results indicated that osthole inhibited atopic dermatitis not through regulating CD4⁺ T cell proliferation, or cytokine secretion.

TSLP in keratinocytes was down-regulated after osthole treatment

Given the results that osthole inhibited atopic dermatitis via regulating Th2 cell response, we next detected the role of osthole in Th2 cell polarization. DC were crucial for Th2 cell polarization, which promoted us to detect DC number and function after osthole treatment. There was no difference of migrated cells after (Fig. 4A), or DCs number (Fig. 4B) after osthole treatment, indicating that DCs had no influence on Th2 cell polarization in this mouse model.

TSLP and IL-25 secreted by keratinocytes were widely reported to promote Th2 cell polarization. Accordingly, we detected TSLP and IL-25 levels. After osthole treatment, the mRNA expression levels of TSLP significantly decreased in DNCB-induced atopic dermatitis model. However, IL-25 mRNA levels showed no difference after osthole treatment (Fig. 4C). TSLP level secreted by keratinocytes *in vitro* was much lower after osthole treatment (Fig. 4D). Moreover, TSLP protein level detected by Western blot obviously decreased after osthole treatment (Fig. 4E). These data indicated that osthole regulated Th2 polarization via inhibiting TSLP expression.

Osthole alleviates atopic dermatitis via directly inhibiting TSLP from keratinocytes

To detect the effect of osthole on keratinocytes was directly or indirectly, we performed osthole treatment to mouse keratinocyte cell line MSC-P5. Osthole treatment significantly decreased

TSLP concentration in supernatant, which induced by poly I:C stimulation (supplementary Fig. S1A), indicating the direct role of osthole in keratinocytes.

To further confirm the direct role of osthole in keratinocytes, we applied osthole treatment to epidermis directly in DNCB-induced atopic dermatitis mouse model (supplementary Fig. S1B). TSLP mRNA expression levels in keratinocytes that isolated from ear significantly decreased after osthole treatment (supplementary Fig. S1C). Moreover, ear thickness significantly decreased after osthole administration to epidermis directly (supplementary Fig. S1D and S1E). These data indicated that osthole alleviates atopic dermatitis via directly inhibiting TSLP from keratinocytes.

Discussion

Atopic dermatitis is one of the most common skin diseases. Inflammation is associated with and plays crucial role in the development of atopic dermatitis. As osthole was widely reported to be effective on anti-inflammation, we therefor explored the role of osthole in atopic dermatitis. Atopic dermatitis mouse model was first established using DNCB, as evidenced by increased ear thickness. Osthole treatment significantly inhibited atopic dermatitis via directly inhibiting TSLP production from keratinocytes. This study links atopic dermatitis, inflammation and the related gene expression, indicating that osthole may serve as the potential choice for atopic dermatitis treatment clinically.

DNCB was commonly used to induce atopic dermatitis. Repeatedly DNCB administration to mouse skin leads to thick epidermis and hyperkeratosis. In addition, inflammation and lymphocytes infiltration were also found after DNCB explosion, which is similar to the symptoms of human atopic dermatitis (11). We successfully established atopic dermatitis

mouse model by administrated DNCB every 2 days for 5 weeks. Osthole treatment by oral administration or directly applied to epidermis significantly inhibited atopic dermatitis.

Inflammation plays important roles during the development of atopic dermatitis. There is a balance of Th1 and Th2 response regulated by cytokines under normal conditions. However, the imbalance of Th1 and Th2 response, actually the switch from Th1 to Th2 was found in atopic dermatitis progression (12, 13). In clinic, Th2 response in alveolar was involved in the poor control (14). Th2 cytokines such as IL-4, IL-5, IL-13 were reported to be increased during atopic dermatitis development (13). IL-4 is the most important cytokine to induce Th2 response, while IL-5 was believed to be crucial for the differentiation and recruitment of eosinophil (15). IL-13 plays more important roles in inducing pathological symptoms and eosinophil infiltration (16). In atopic dermatitis lesions, the cells expressing IL4, IL-5 and IL-13 significantly increased as compared with normal skin (17, 18). Moreover, the significance of Th2 response in atopic dermatitis was demonstrated by the evidence that IL-4 associated genes including CCL17 and periostin were greatly correlated with atopic dermatitis severity (19, 20). Our results showed that the mRNA expression levels of IL-4, IL-5 and IL-13 significantly increased in DNCB-induced atopic dermatitis mouse model, indicating the importance of Th2 response in atopic dermatitis, which is consistent with previous studies. Osthole treatment inhibited IL-4 and IL-13 mRNA levels and CD4⁺IL-4⁺, CD4⁺IL-13⁺ cells, while IL-5 level showed the trend to decrease without significance. These data indicated that osthole inhibited Th2 response in atopic dermatitis.

Th1 and Th17 response were also reported to be important in at the late stage of atopic dermatitis (21), where the related cytokines such as IFN- γ , IL-17A and IL-10 showed significant elevation. We found that the mRNA expression levels of IFN- γ and IL-17A increased after DNCB challenge, while no great significance of IL-10 level was observed.

Moreover, osthole treatment showed no influence on IFN- γ , IL-17A and IL-10 expression levels. These data suggested that osthole attenuates atopic dermatitis by inhibiting Th2 response, but not Th1 or Th17 response.

TSLP is a cytokine that expressed skin, lung and gut, especially in epidermal keratinocytes (22). The profound role of TSLP is regulating Th2 cytokines (IL-4, IL-5 and IL-13) production by mediating DC cell polarization. T cell proliferation upon T cell receptor activation was also promoted by TSLP. In addition, TSLP play crucial roles in the differentiation and expansion of B cells. All the characters confer the crucial role of TSLP in regulating Th2 associated inflammation. In the skin lesions of atopic dermatitis and the epithelial cells of asthma, TSLP expression levels were reported to be increased (23, 24). Even in the early stage of breast cancer, TSLP inhibited breast cancer development by regulating Th2-dominant immune response (25), indicating the novel role of TSLP in tumor development. In our study, we found that TSLP levels in MSC-P5 cell line significantly increased after poly I:C stimulation, while decreased after osthole treatment. In the DNCB-induced atopic dermatitis mouse model, TSLP mRNA and protein levels greatly decreased after osthole treatment by oral administration or directly applied to epidermis, indicating that osthole attenuates mouse atopic dermatitis by inhibiting TSLP production of keratinocytes.

In summary, our study explored the role of osthole in atopic dermatitis, and the mechanism how osthole regulated atopic dermatitis development. One of the limitations in the current study is the relatively small number of animals used. Future study may examine the effects of osthole in much more animals. Osthole inhibits Th2 cells response, but have no influence on Th1 or Th17 cells response or DC migration, which may be through directly inhibiting TSLP expression levels in keratinocytes. To the best of our knowledge, this is the first study to report the role of osthole in atopic dermatitis.

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Conclusion

Osthole treatment alleviates atopic dermatitis through directly down-regulating TSLP production from keratinocytes. Osthole may serve as a potential choice for atopic dermatitis treatment in clinic.

Author Contribution

XPF: performed the research; analyzed the data

CHH: designed the research study; wrote the paper

All authors have read and approved the final manuscript.

Acknowledgements

None.

Conflict of interest

None.

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Figure legends

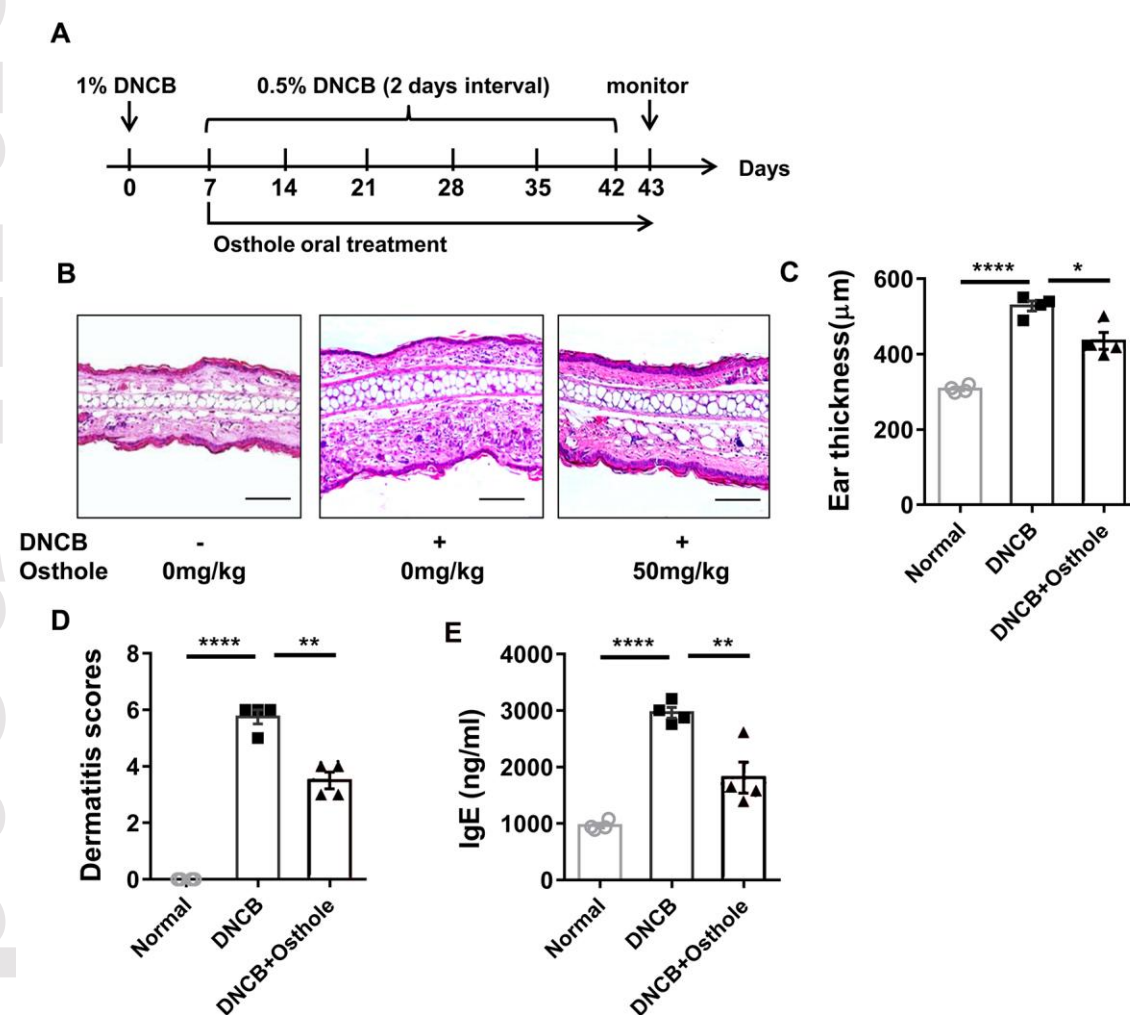
Fig. 1. Osthole prevents the development of mouse atopic dermatitis. (A) Schematic diagram of the study protocol. 50mg/kg of osthole was orally administered to DNCB-treated Balb/c mice. (B) H&E staining of the ears and from groups of mice on day 43. Scale bar, 200 μ m. (C) Ear thickness was determined on day 43. (D) The dermatitis score of (B). (E) Serum level of IgE was measured by ELISA. Data are representative of three or more independent experiments with ≥ 4 mice per group. Data are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

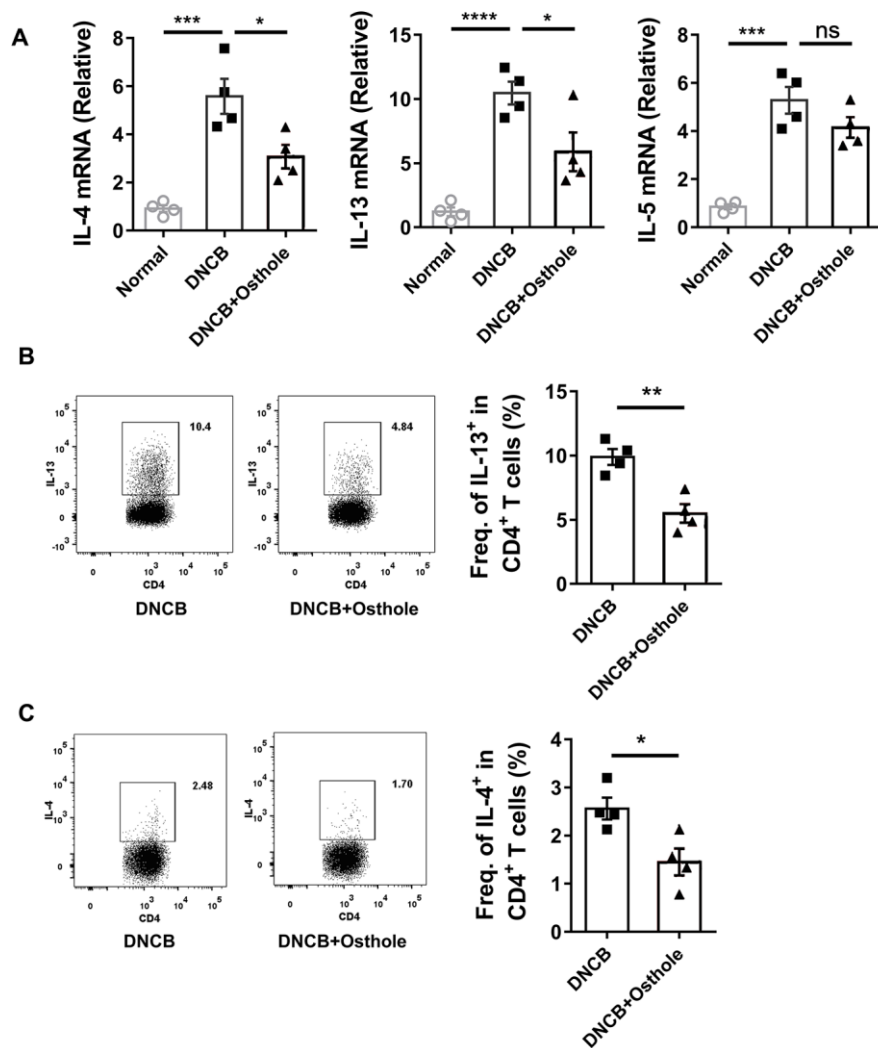
Fig. 2. Osthole inhibits Th2 cells response in the skin. (A-C) 50mg/kg of osthole was orally administered to DNCB-treated Balb/c mice. The mRNA of the ears from each group on day 43 was obtained and the expressions of IL-4, IL-13 and IL-5 were analyzed by Real-time PCR. (B) The IL-13 expression on CD4⁺ T cells (Gating on CD45⁺CD3⁺ single cells) was analyzed by FACS. (C) The IL-4 expression on CD4⁺ T cells (Gating on CD45⁺CD3⁺ single cells) was analyzed by FACS. Data are representative of three or more independent experiments with ≥ 4 mice per group. Data are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

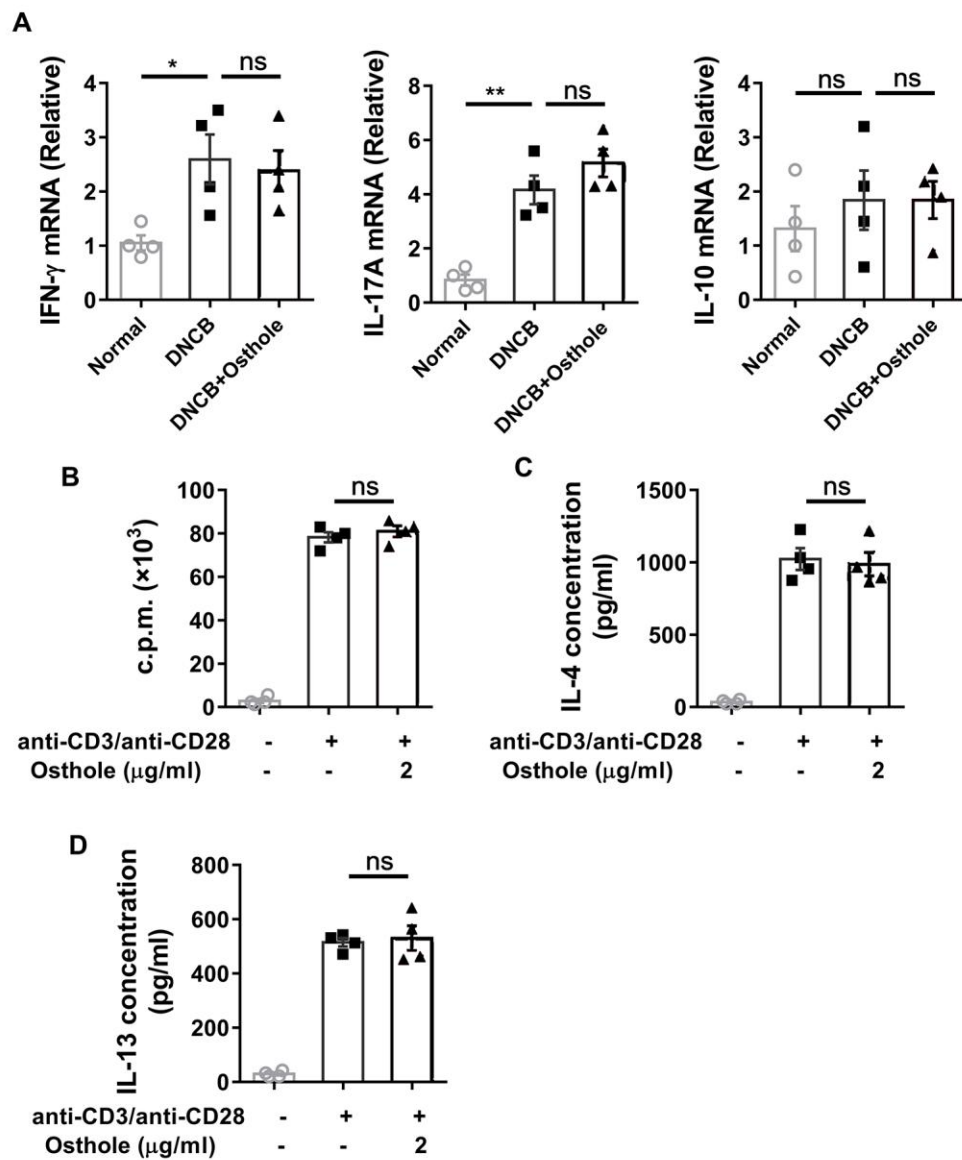
Fig. 3. Osthole does not influence Th1 and Th17 cells response in the skin. (A) 50 mg/kg of osthole was orally administered to DNCB-treated Balb/c mice. The mice were harvested 24 hours after the last challenge. The mRNA of the ears from each group was obtained and the expressions of IFN- γ , IL-17A and IL-10 were analyzed by Real-time PCR. **(B-D)** Total of 3×10^5 cells/well T cells were treated with 2 μ g/ml of osthole and then stimulated with anti-CD3 (1 μ g/ml)/anti-CD28 (1 μ g/ml) antibodies for 56 hours. T cells stimulated with anti-CD3/anti-CD28 alone serve as the control. Proliferative ability was determined using a [3H]-thymidine incorporation assay **(B)**. The IL-4 **(C)** and IL-13 **(D)** concentration was analyzed by ELISA. Data are representative of three or more independent experiments with ≥ 4 mice per group. Data are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

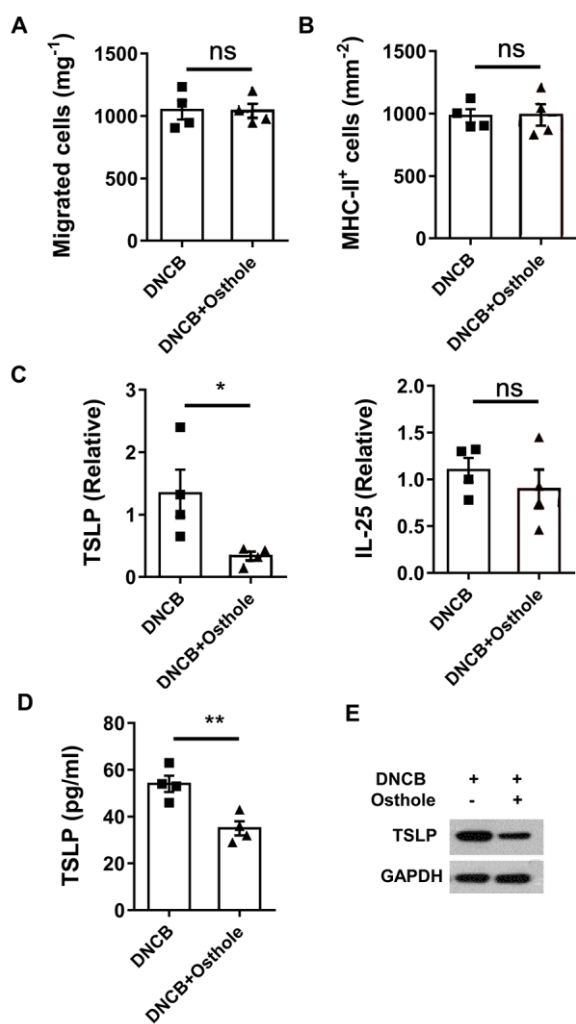
Fig. 4. TSLP production by keratinocytes is down-regulated after osthole treatment. (A) Balb/c mice were sensitized and challenged with DNCB and orally administrated with osthole as previously. 24 hours after the last challenge, dorsal ear halves were cultivated for 24 h. Explants were transferred daily into new wells, totally 3 days. Migrated cells were pooled for each explant and counted. **(B)** Balb/c mice were sensitized and challenged with DNCB and orally administrated with osthole as previously. One day after the last challenge, mice were harvested and epidermis was stained for MHC II⁺ cells and density were determined microscopically by means of a calibrated grid. **(C)** The mice were harvested 24 hours after the last challenge. The mRNA of the ears from each group was obtained and the expressions of TSLP and IL-25 were analyzed by Real-time PCR. **(D)** Keratinocytes were isolated from mice and *in vitro* stimulated with Poly I:C (2 μ g/ml) for 24 h. The level of TSLP was determined by ELISA. **(E)** The expression of TLSP by keratinocytes was determined by Western Blot. Data

are representative of three or more independent experiments with ≥ 4 mice per group. Data are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.









Supplementary Materials

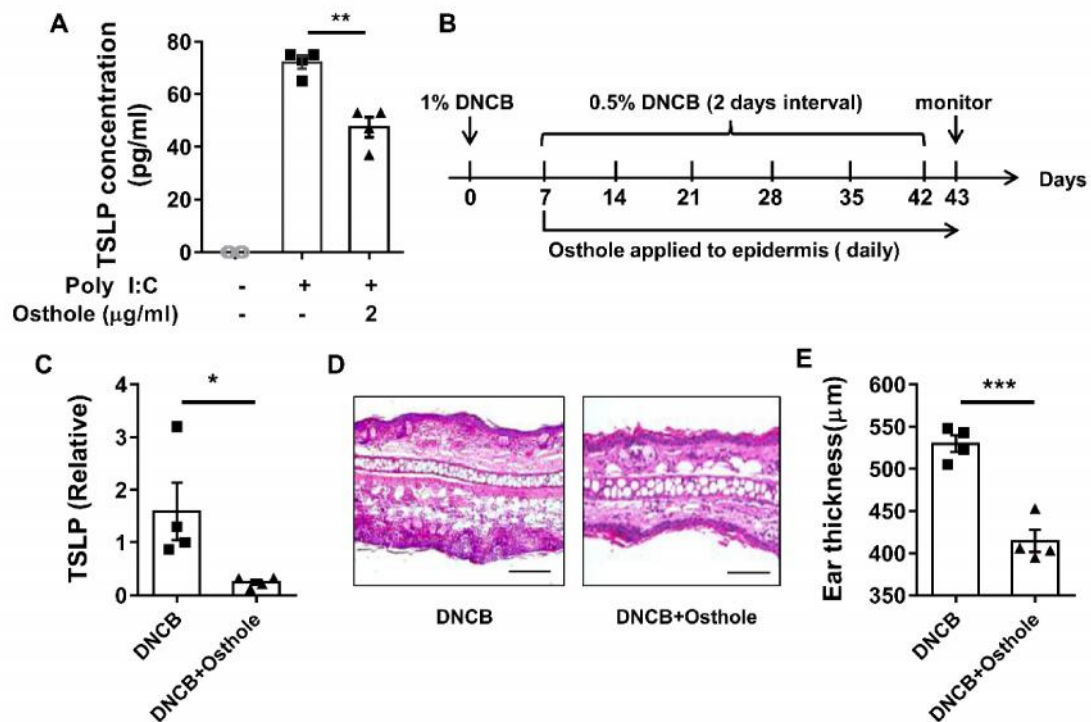


Fig. S1. Osteole treatment alleviates atopic dermatitis through directly down-regulating TSLP production from keratinocytes. (A) The murine keratinocyte cell line MSC were stimulated with poly I:C (1µg/ml) with or without osteole (2µg/ml) for 48 h the level of TSLP was determined by ELISA. (B) Schematic diagram of the study protocol. 50mg/kg of osteole was daily applied to epidermis of DNCB-treated Balb/c mice. (C) The mice were harvested 24 hours after the last challenge (at day 43). Keratinocytes were isolated and the mRNA was obtained and the expressions of TSLP were analyzed by Real-time PCR. (D) H&E staining of the ears and from groups of mice on day 43. Scale bar, 200 µm. (E) Ear thickness was determined on day 43. Data are representative of three or more independent experiments with ≥ 4 mice per group. Data are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.