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Chinese Journal of Natural Medicines 2019, **17**(7): 0525–0534

Chinese Journal of Natural Medicines

Chinese medicine *Yu-Ping-Feng-San* attenuates allergic inflammation by regulating epithelial derived pro-allergic cytokines

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Available online 20 July, 2019

[ABSTRACT] This study aimed to investigate the mechanisms of *Yu-Ping-Feng-San* (YPFS) on attenuating allergic inflammation in the initial stage of atopic dermatitis (AD). AD mouse model was established with fluorescein isothiocyanate (FITC) sensitization and elicitation. Epithelial barrier structure was observed with transmission electron microscope. The populations of dendritic cells (DCs) and group 2 innate lymphoid cells (ILC2s) were detected by flow cytometry. Human immortalized keratinocyte (HaCaT) cells were stimulated with Poly(I:C)/TNF-α *in vitro* to assess thymic stromal lymphopoietin (TSLP), interleukin (IL)-33 and nuclear factor-κB (NF-κB) levels or expressions by immunofluorescence, enzyme linked immunosorbent assay (ELISA) and western blot. In the initial stage of AD, ear swelling and infiltration of inflammatory cells in ear tissues were markedly attenuated with YPFS treatments. The damaged structures of ear epithelium and the increased levels of Th2-cytokines induced by FITC were significantly rescued in YPFS-treated mice. The production of pro-allergic cytokines, TSLP and IL-33, as well as the cell populations of their target cells DCs and ILC2s were decreased in AD model, respectively. Likewise, the levels of TSLP and IL-33 in Poly(I:C)/TNF-α-stimulated HaCaT cells showed the same results. Lower levels of p-NF-κB were detected with YPFS treatment, and the expressions of TSLP and IL-33 could be further decreased with inhibiting of NF-κB. Therefore, YPFS attenuates allergic inflammation in the initial stage of AD probably through regulating NF-κB-TSLP/IL-33 pathway, which may provide a novel effective target for the prevention and treatment of allergic diseases.

[KEY WORDS] *Yu-Ping-Feng-San*; Allergic inflammation; TSLP; IL-33; NF-κB

[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2019)07-0525-10

Introduction

The allergic disease is mainly attributed to the abnormal immune response, which has the features of continuous strike, slow occurrence or long-time stimulation [1]. Further, it may result in the tissue injury, thus causing more serious inflammatory diseases [2]. It has been shown that the clinical treatments, such as β receptor agonist and antihistamine, will be given at the effective stage of allergic inflammation [3]. However, they barely delay the pathological process of anaphylactic disease [4]. Glucocorticoids are commonly used in controlling the relapse of allergic diseases, like the treatment of asthma, while it is limited by side effects. Therefore, it is urgent to investigate a novel drug targeting allergic inflammation especially for reducing recurrence.

Yu-Ping-Feng-San (YPFS) is a classical traditional Chinese medicine, which has been widely applied for the allergy diseases, such as atopic dermatitis (AD), allergic asthma, allergic purpura, allergic rhinitis and allergic urticaria [5-10]. Its advantage lies in repairing the fundamental cause of disease, controlling the pathological process of the disease, improving

[Received on] 08-May-2019

[Research funding] This work was supported by the National Natural Science Foundation of China (Nos. 81473395, 81473390, and 81703733), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the Natural Science Foundation of Jiangsu Province (No. BK20141466), and Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of China (No. JKLPSE201603).

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These authors have no conflict of interest to declare.

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allergic constitution and reducing the recurrence. Clinical application of YPFS in allergic diseases can effectively reduce the recurrence and alleviate the pain [11], which suggest it may play an important role at the initial stage of disease. YPFS can play a role in the remission period of inflammation, rather than acting like a western medicine in the attack period. While the initial stage of allergic disease represents the early stage of inflammation and at this time the inflammation has not yet been triggered. Therefore, understanding the mechanism of YPFS in the initial stage of allergy is important for understanding its prevention of recurrence. YPFS has been reported to promote transformation of T lymphocytes [12] and maintain the balance of Th1/Th2 to prevent the allergic diseases. However, this is far from explaining the underlying mechanism of reducing allergy recurrence.

Allergic inflammation is a type of disease that caused by Th2 type immune response [13]. Accumulating evidences implied that thymic stromal lymphopoietin (TSLP) and interleukin (IL)-33, secreted from the epithelial cells (ECs) driven by external stimulus, can initiate Th2 type immune response [14]. Therefore, ECs not only play the role of the natural physical barrier, but also participate in the initiation phase of allergic diseases [15]. Since TSLPR induces dendritic cells (DCs) to express OX40 ligand, TSLP can create a micro-environment which allows Th2 cells differentiation. Additionally, it can directly initiate the differentiation of $CD4^+$T precursor cells into Th2 cells by binding to TSLPR [16]. Hence, TSLP is a crucial initiation switch for the allergic disease. IL-33 can advance the aggregation and activation of type 2 innate lymphoid cells (ILC2s), which is a type of immunological cell producing IL-5 and IL-13 through the ST2 receptor [17]. Nuclear factor-κB (NF-κB) also plays an important role in allergic inflammation and could increase the expressions of TSLP and IL-33, which induces more inflammatory cells recruitment and activation [18-19].

Existing studies of YPFS have focused on the control of inflammation, but less research has been conducted on unveiling its mechanism of recurrence reduction. We speculate that it might relate to its role in the early stage of allergic disease. Given that TSLP and IL-33 are key factors in triggering the allergic disease and YPFS is able to control the allergic inflammation effectively, especially reduce the recurrence, we put forward the hypothesis that YPFS might influence the TSLP/IL-33 related signaling in the initial stage of allergic diseases. In the present study, we investigated the anti-inflammatory role of YPFS in initial stage of AD and its underlying mechanisms.

Materials and Methods

Preparation of YPFS extract

YPFS is composed of Radix Astragali (RA; Inner Mongolia, China) purchased from Zhangzhou Fushun Pharmaceutical Co., Ltd. (20150601, Zhangzhou, China), Rhizoma Atractylodis Macrocephalae (RAM; Zhejiang, China) purchased from Shanxi Wanhui Pharmaceutical Co., Ltd. (150102, Shanxi, China), and Radix Saposhnikoviae (RS; Heilongjiang, China) purchased from the Herbal Decoction Slices division of Nanjing Pharmaceutical Company (20150601, Nanjing, China). Botanic identification was confirmed by Professor WANG Chun-Gen (Nanjing University of Chinese Medicine, China). Five hundred grams of YPFS (3 : 1 : 1) were immersed in 3.25 L lethanol : water (95 : 5, *V/V*) for 1 h and then refluxed for 2 h. The extraction process was repeated twice and the extracts were combined, filtered, and evaporated to dryness using a vacuum concentrator system (CH-9230; BUCHI Labortechnik, Flawil, Switzerland) at 60 °C. The extract was then subjected to analysis at a concentration of 1.5 g crude drug/g extract.

Animals and cells

Six to eight week-year-old BALB/c mice (18−22 g) were purchased from Beijing Vitalriver and Shanghai Slac Laboratory Animal Company. All animals were maintained at Nanjing University of Chinese Medicine under specific pathogen-free conditions at 18–25 °C and 50%–60% humidity. All procedures involving animals were approved by the Animal Care and Use Committee of Nanjing University of Chinese Medicine and strictly performed according to the Guide for the Care and Use of Laboratory Animals (ACU-04, 08-07-2013). HaCaT cells were a friendly gift from Model Animal Research Center of Nanjing University and cultured in MEM (HyClone, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO) at 37 °C and 5% CO_2.

Animal models

AD model and treatments: BALB/c mice were sensitized with 80 μL 1.5% fluorescein isothiocyanate (FITC, Sigma, St. Louis, MO, USA) solution (dissolved in acetone/dibutylphthalate 1 : 1) on the abdominal skin from days 1 to 2. Mice were elicited on the right ear with 20 μL 0.6% FITC solution on day 6. Acetone and dibutylphthalate was applied as vehicle control. YPFS (3.25, 6.5 g·kg^{-1}·bw, intragastrically, 6.5 g·kg^{-1} YPFS is consistent with the amount actually ingested by humans daily) and dexamethasone (Dex, 0.67 mg·kg^{-1}, i.p. Tianjin Kinyork Group Co., Ltd., Tianjin, China) were treated from day −1 to day 3 for 5 days. Ear thickness was measured 24 h after elicitation by thickness gauge (Harbin Measuring & Cutting Tool Group Co., Ltd., Harbin, China). Mice were sacrificed and a patch (8 mm diameter) was punched from left and right ears to calculate the ear weight. The right ear homogenate was applied for cytokines analysis. Histopathological changes of the ears were examined by H&E staining.

The initial stage of AD: BALB/c mice were topically sensitized with 20 μL 0.6% FITC solution on both ears on day 1 and 2. 20 μL acetone and dibutylphthalate was applied as vehicle control. Mice were treated once daily with YPFS (3.25, 6.5 g·kg^{-1}, intragastrically) and Dex (0.67 mg·kg^{-1}, i.p.) from 2 days before sensitization. The mice were sacrificed on day 3 (24 h after elicitation), and both ears were homogenated for cytokine and mRNA expression analysis. The draining

lymph nodes were isolated for Flow-cytometric analysis.

Electron microscopy

Mice ear tissues were fixed with 2.5% glutaraldehyde for more than 4 h, after washing with PBS, they were fixed in 1% OsO_4 for 1 h. The specimens were dehydrated by a graded series of ethanol (30%, 50%, 70%, 80%, 90% and 100%) for about 15 min. Afterwards, the specimens were placed in 1 : 1 mixture of absolute acetone and the resin for 1 h at room temperature, then transferred to 1 : 3 mixture of absolute acetone and resin for 3 h and to final resin for overnight. After that, specimens were placed in capsules contained embedding medium and heated at 70 °C for 48 h. The 70 nm of specimen sections were stained by acetate and alkaline lead citrate for 15 min. respectively and observed in transmission electron microscope (JEOL, Tokyo, Japan).

MTT assay

Cytotoxicity was determined with the MTT assay. HaCaT cells were seeded in 96-well plates (1×10^4 cells/well) and incubated for 12 h in the presence of indicated doses of YPFS. 20 μL MTT (5 mg·mL^{-1}, Sigma, Germany) stock solution was added to each well, and plates were incubated at 37 °C. After 4 h incubation, cells were lysed with dimethyl sulfoxide (DMSO, Sigma, Germany). Absorbance was measured at 490 nm using Synergy HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA).

Cell treatment in vitro

HaCaT cells were starved with serum-free medium overnight and were pre-treated with YPFS for 6 h or culture medium as a control before stimulated with Poly(I:C) (100 μg·mL^{-1}) (Sigma, Germany) or Poly(I:C) (100 μg·mL^{-1}) combined with TNF-α (20 ng·mL^{-1}) (R&D systems, Minneapolis, MN, USA) for 24 h. NF-κB inhibitor PDTC (20 μmol·L^{-1}, Beyotime Institute of Biotechnology, China) was added 2 h before Poly(I:C) andTNF-α stimulation in HaCaT.

ELISA assay

ELISA was used to measure the IL-4, IL-5, IL-9, IL-13, TSLP and IL-33 in the ear homogenate with the mouse ELISA Ready-SET-Go (eBioscience, Inc. San Diego, CA, USA), according to the manufacturer's instructions. Human IL-33 and TSLP in culture supernatants were measured by Human IL-33 Duoset ELISA kit (R&D system, USA) and human TSLP Ready-SET-Go kits (eBioscience, USA) respectively, according to the manufacturer's instructions. The cytokine levels in ear homogenate were calculated as: concentration of cytokine in the homogenate/total protein (pg·mg^{-1}).

Western blot analysis

HaCaT cells in 6-well plates were lysed with 100 μL/well RIPA (Beyotime, China) and phenylmethylsulfonyl fluoride (Biosharp, China) (100 : 1). Total protein level in the homogenates was examined by BCA kit (Beyotime, China). Twenty μg of protein of cell samples were electrophoresed in 10% SDS-AGE and transferred onto polyvinylidene fluoride membranes (Milipore, USA), after blocking with 5% BSA (Absin Bioscience Inc, China), the membranes were incubated with antibodies with p-NF-κB (p-p65, 1 : 1000, Cell Signaling Technology, USA), NF-κB (p65, 1 : 1000, Abcam, England), IL-33 (1 : 1000, Santa Cruz Biotechnology, USA) and GAPDH (1 : 1000, GeneTex, USA) at 4 °C overnight. Secondary peroxidase-linked goat anti-rabbit IgG or anti-mouse IgG (1 : 10 000, Santa Cruz Biotechnology, USA) were used and the membranes were detected by enhanced chemiluminescence (ECL kit; Millipore, USA).

Real-time qPCR

Total RNA was isolated from the ear tissue with TRIzol Raegent (Life Technologies, USA), according to the manufacturer's protocol, and reverse-transcribed to cDNA Synthesis Kit (Thermo Fisher Scientific, Inc. Waltam, MA, USA). The cDNA was amplified with the QuantiFast SYBR Green PCR Kit (Qiagen, Germany). The assays were performed in the iCycler iQ^{TM} Single-Color Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The primers were synthesized by invitrogen and the primer sequences were: GAPDH, 5'-GGTTGTCTCCTGCGA CTTCA-3' and 5'-TGGTCCAGGGTTTCTTACTCC-3'; TSLP, 5'-TACTATA CTCTCAATCCTATCCCTG-3' and 5'-ACTTCTTGTGCCAT TTCCTG-3'; IL-33, 5'-TCCAACTCCAAGA TTTCCCCG-3' and 5'-CATGCAGTAGACATGGCAGAA-3'; The results of RT-qPCR were normalized by using GAPDH as internal control and the expression of target mRNA was determined by the ΔΔCt method.

Detection of DCs and ILC2s by flow cytometry

The mice draining lymph nodes were removed and filtered through a 400 mesh sieve under aseptic conditions. The expressions of surface markers on the activated DCs (defined as CD11c$^+$CD40$^+$CD86$^+$) and ILC2s (defined as Lineage$^-$CD25$^+$ST2$^+$) in lymphocytes were then analyzed with flow cytometry. In order to show the percentage of ILC2 cells, the single-cell suspensions of the draining lymph nodes were stained with lineage markers and divided into Lin$^-$ and Lin$^+$ populations. Then, the cells in the Lin$^-$ populations that were doubly positive for CD25 and ST2 were gated and analyzed. The proportions of CD25$^+$ST2$^+$ in Lin$^-$ of draining lymph nodes are given as the proportions of ILC2. Also, for DCs, cells were first gated with CD11c$^+$ cells and then double-stained with CD40 and CD86, the proportions of CD40$^+$CD86$^+$ in CD11c$^+$ of draining lymph nodes are given as the proportions of DCs. FITC-conjugated anti-mouse CD11c antibody, PE-conjugated anti-mouse CD25 antibody, PE-conjugated anti-mouse CD40 antibody, APC-conjugated anti-mouse CD86 antibody, APC-conjugated anti-mouse ST2/IL33R antibody and FITC-conjugated anti-mouse Lineage Cocktail with Isotype Ctrl antibody were purchased from Biolegend (San Diego, CA, USA). The results were analyzed with the AccuriTM C6 flow cytometer and FASDive software (BD Biosciences, San Jose, CA, USA).

Immunofluorescence assay

The coverslips with HaCaT cells were fixed in 4% ice-cold paraformaldehyde (PFA) for 30 min and were washed

with PBS then blocked with 1.5% goat serum for 1 h at 37 °C. The cells were probed with 4 mg·mL^{-1} rabbit monoclonal antibody to TSLP and IL-33 (Santa Cruz Biotechnology, CA, USA) at 4 °C overnight. After repeated washing with PBS, the cells were probed with 1 mg·mL^{-1} goat anti-rabbit IgG conjugated to FITC (Santa Cruz Biotechnology, CA, USA) at a concentration of 5 mg·mL^{-1} for 2 h at 37 °C and then treated with 4', 6-diamidino-2-phenylindole (DAPI, Santa Cruz Biotechnology, CA, USA). The labeled sections were viewed with fluorescence confocal microscopy (Zeiss, Germany).

Statistical analysis

The data were expressed as mean ± SD. Multiple groups' comparisons were analyzed by one-way analysis of variance, and Dunnett's test was used for comparison between two groups, with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

Results

The quality control of YPFS extract was confirmed with HPLC fingerprint assessment [20]. As previously reported, there were 32 main peaks in the fingerprint of YPFS extract at 254 nm and the HPLC peaks of eight components were identified by comparing their retention times with those of the standards under the same chromatographic. They were identified as cimifugin, prim-*O*-glucosylcimifugin, 4′-*O*-*β*-glucopyranosyl-5-*O*-methylvisammiol, formononetin-7-*O*-*β*-D-glucoside, *sec*-*O*-glucosylcimifugin, calycosin-7-glucoside, galycosin and formononetin in YPFS extract.

YPFS treated in the initial stage inhibited FITC-induced Th2 cell-mediated AD in mice

We have previously reported that YPFS treatment through the whole model (day 1 to 6) could effectively attenuate the allergic inflammation [21], which suggested its anti-inflammation effect. However, as a clinical drug used to reduce the recurrence of disease, YPFS may play a role of preventing or alleviating allergic inflammation when initial exposed to allergens. So in this study we examined the effects of YPFS in the initial stage of AD. The results showed that YPFS (3.25 and 6.5 g·kg^{-1}), only applied in the initial stage, significantly decreased the levels of ear swelling and weight (Fig. 1B), so as the levels of IL-4, IL-5, IL-9 and IL-13 (Fig. 1C) compared with those in model group.

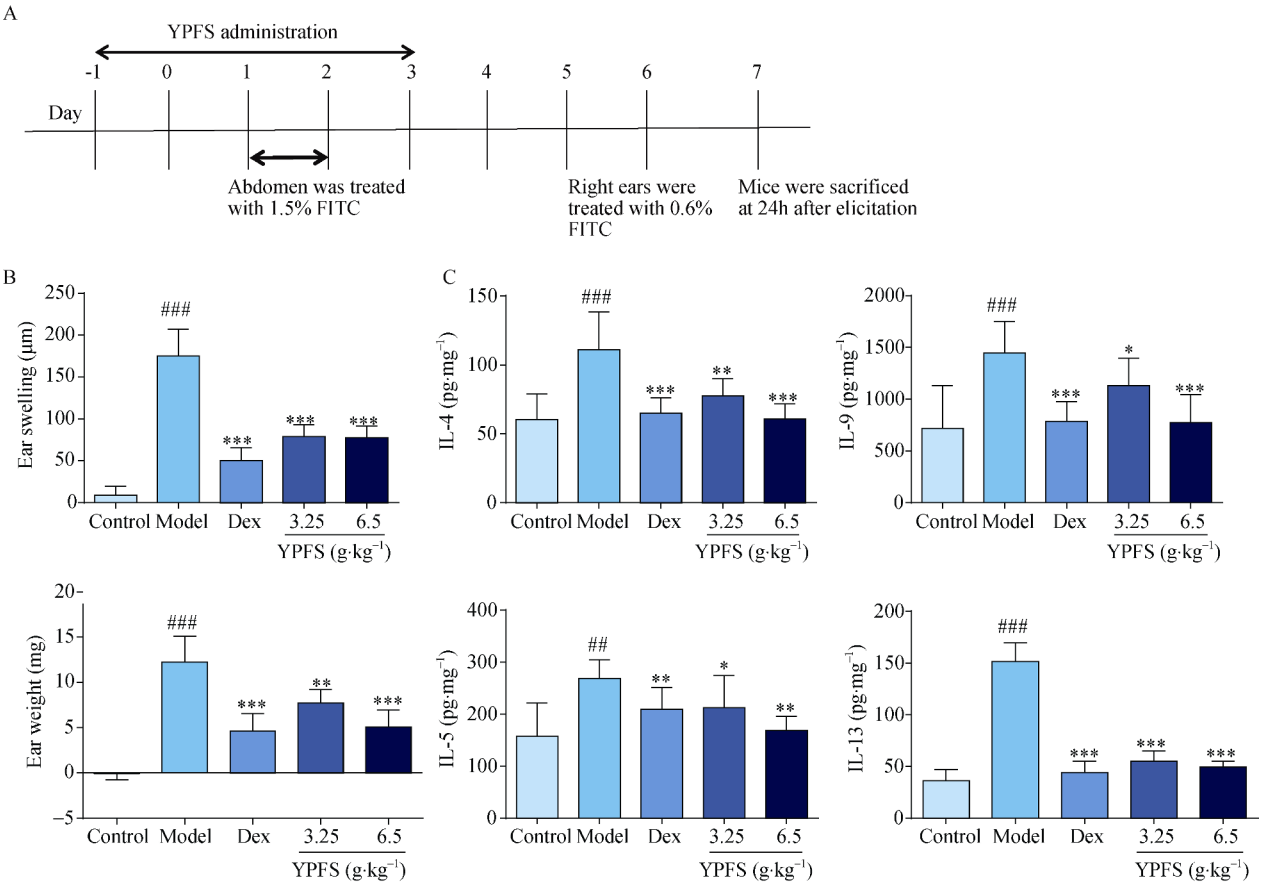


Fig. 1 Effect of YPFS extract treated in the initial stage of AD model. (A) Flow chart of YPFS extract administered to the murine AD model. (B) Effect of YPFS extract on ear swelling and ear weight in the AD murine model. (C) Cytokines IL-4, IL-5, IL-9 and IL-13 were analyzed by ELISA and total protein was determined by BCA kit in ear homogenates. (Mean ± SD, $n = 8$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* model). All the experiments were performed in triplicates

YPFS treated in the initial stage of AD repaired epithelium integrity

ECs played important roles and may have morphological changes in allergic inflammation. We observed the pathological changes of mice ear tissues in AD model with YPFS treatment. Histological analysis demonstrated that the marked thickening of epidermis and dermis, accompanied by increased infiltration of inflammatory cells were improved significantly in YPFS-treated mice (Fig. 2A). Furthermore, observation by electron microscopy indicated that the epithelial cells in control group were arranged regularly and connected with each other tightly while the tight junction of ear epithelial cells in model group was fuzzier with larger gap and the number of desmosomes was reduced, and the cleavage and dissociation phenomena were observed. However, YPFS (6.5 g·kg^{-1}) treatment showed a significant improvement in the epithelium barrier integrity with smaller inter-cellular connection gap (Fig. 2B).

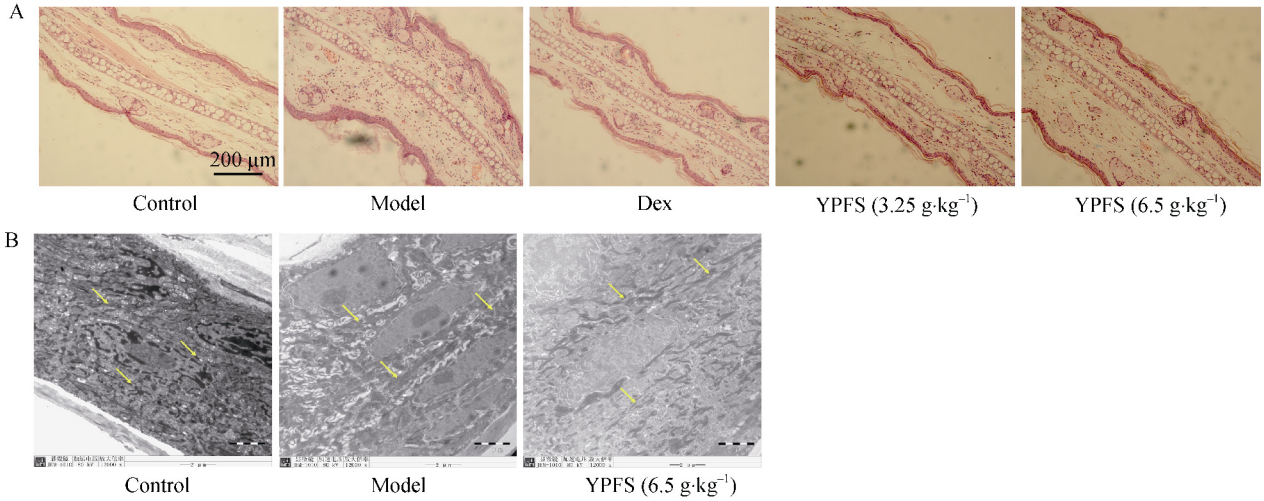
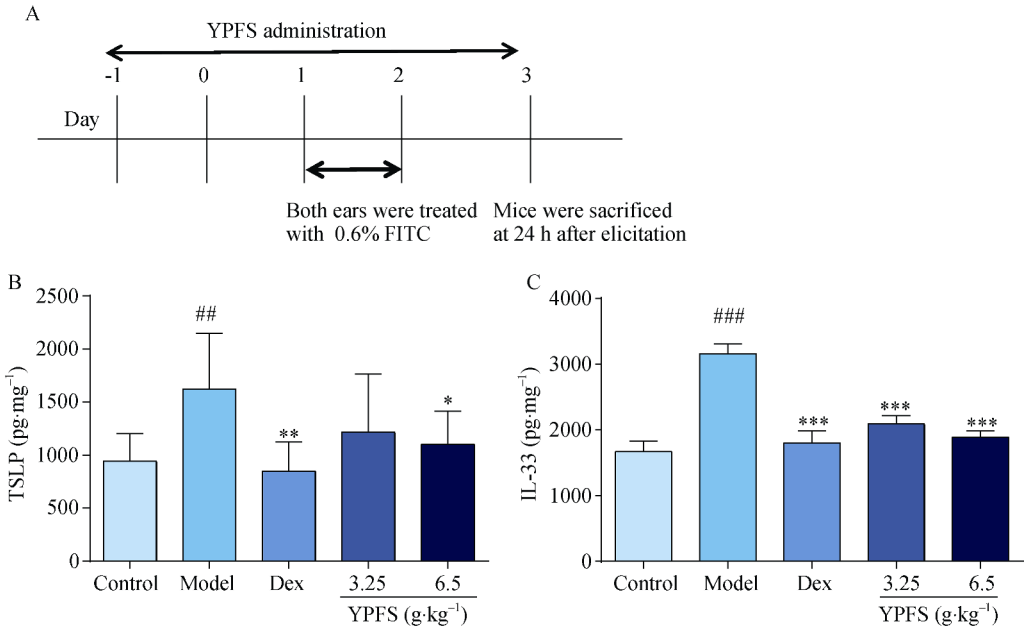


Fig. 2 Effect of YPFS extract on ECs structural integrity. (A) Effect of YPFS extract on the histopathological changes of ear skin sections with hematoxylin/eosin staining (magnification: × 200). (B) Effect of YPFS on the junction of ear skin epithelium detected with electron microscopy (magnification: × 12 000). Arrows showed tight junctions and desmosomes in ECs

YPFS reduced the production of TSLP and IL-33 in the initial stage of AD

As the alarmin cytokines, TSLP and IL-33 secreted from epithelial cells could induce Th2 cells recruitment and inflammation. To observe the effects of YPFS on TSLP and IL-33 production, we established the initial stage of AD model. It showed that the levels of TSLP and IL-33 were highly increased in model compared with those in control group, while decreased significantly with YPFS treatment (Figs. 3B and 3C). The mRNA expressions of TSLP and IL-33 were also downregulated by YPFS treatment (Figs. 3D and 3E).



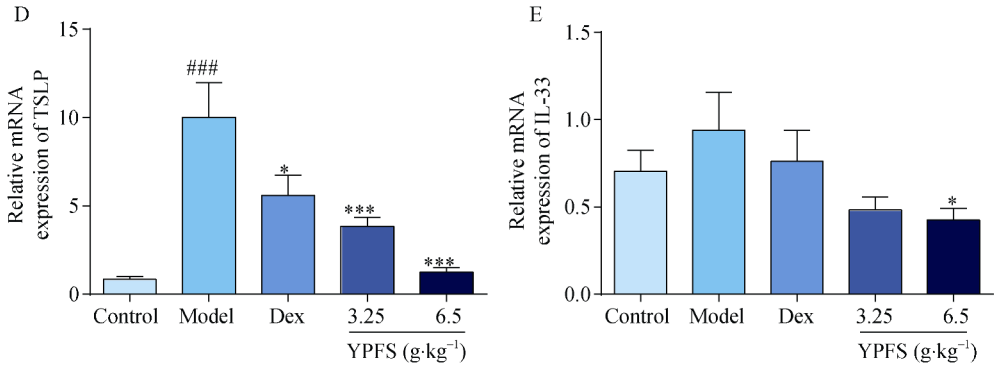


Fig. 3 Effect of YPFS extract on TSLP and IL-33 protein and mRNA expression in the initial stage of AD model. (A) Flow chart of FITC-induced mouse model of initial-stage AD and the administration of YPFS extract. (B, D) TSLP and IL-33 in ear homogenates were analyzed by ELISA and total protein was determined by BCA kit. (C, E) The relative mRNA expression of TSLP and IL-33. Mean ± SD, n = 8, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* normal; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* model. All the experiments were performed in triplicates

YPFS reduced the proportions of DCs and ILC2s in draining lymph nodes in the initial stage of AD model

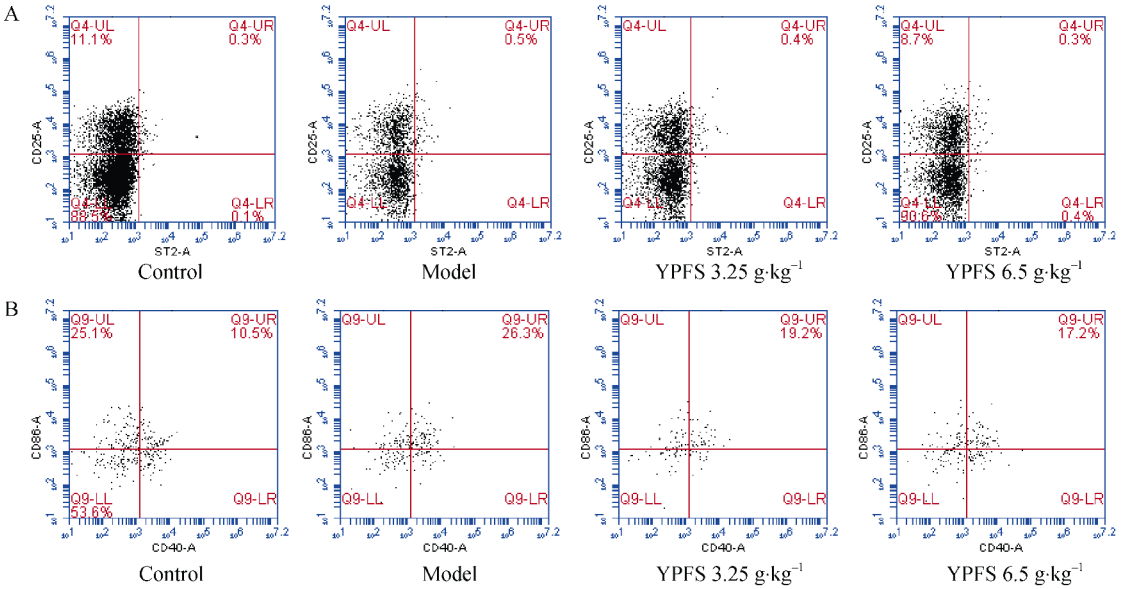
TSLP and IL-33 could active DCs and ILC2s respectively to initial Th2-mediated immune response, so we identified the impacts of YPFS on the proportions of DCs and ILC2s. We analyzed the draining lymph nodes from mice in the initial stage of AD model. Increased numbers of DCs (CD11c+CD40+CD86+) and ILC2s (Lineage−CD25+ST2+) in the model group were detected, while YPFS moderately reduced the proportions of DCs and significantly decreased the proportions of ILC2s (Fig. 4).

YPFS reduced TSLP and IL-33 production in epithelial cells in vitro

We have found YPFS significantly inhibited allergic inflammation in the initial stage of AD *in vivo*, and the mechanisms may correlate with epithelium-derived cytokines. Therefore, we then explored whether YPFS had effects on TSLP and IL-33 production in HaCaT cells. Immunofluorescence assay of HaCaT cells showed YPFS significantly decreased the expressions of TSLP and IL-33 (Figs. 5A and 5B). Moreover, the levels of these two cytokines in culture supernatant of HaCaT cells showed the similar results (Figs. 5C and 5D). To exclude the cytotoxicity of YPFS on the cell proliferation, we detected the effect of YPFS on cell viability of HaCaT cells. Results showed that YPFS had no notable effect on HaCaT proliferation in 24 h (Fig. 5E).

YPFS reduced the expressions of key pro-allergic cytokines TSLP and IL-33 through inhibiting NF-κB activation

Although YPFS had a significant effect in the initial stage of AD model and could weaken the expressions of TSLP and IL-33 to alleviate inflammation, we did not know how it works. NF-κB could bind the promoter of TSLP and is a transcription factor which is primarily involved in the synthesis of alarmin cytokines. In view of NF-κB is highly related with TSLP and IL-33 in allergic inflammation, we then explored whether YPFS could inhibit the activation of NF-κB to play



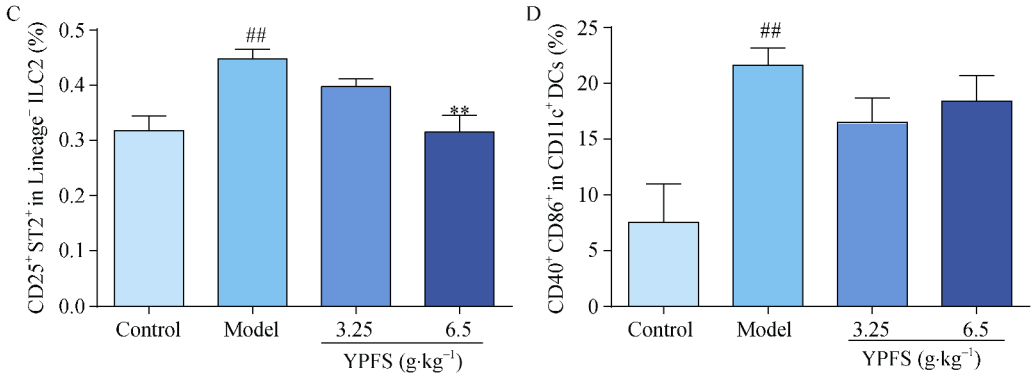


Fig. 4 Effects of YPFS extract on the numbers and maturation of DCs and ILC2s in the lymph nodes of FITC-induced AD mouse model in the initial-stage. (A and B), the results of flow cytometry on DCs ($CD11c^+CD40^+CD86^+$) and ILC2 ($Lineage^-CD25^+ST2^+$) in lymphocytes. (C and D), proportions of DCs ($CD11c^+CD40^+CD86^+$) and ILC2 ($Lineage^-CD25^+ST2^+$) in lymphocytes (Mean ± SD, n = 8, $^{\#\#}P < 0.01$ *vs* control; $^{**}P < 0.01$ *vs* model). All the experiments were performed in triplicates

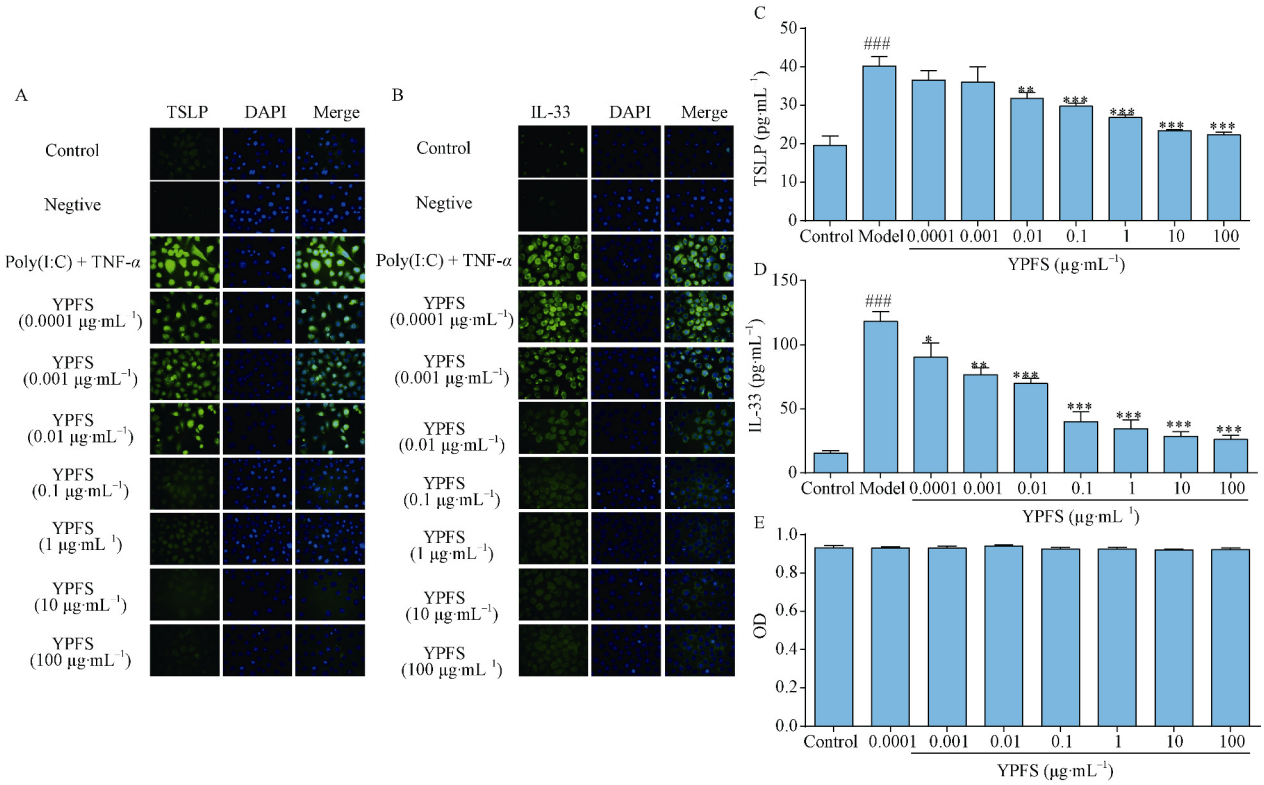


Fig. 5 Effect of YPFS extract in HaCaT, TSLP and IL-33 expressions were examined by immunofluorescence assay in HaCaT (A and B). The levels of TSLP and IL-33 in the cell culture supernatants were measured by ELISAs in HaCaT (C and D). Effect of YPFS extract on HaCaT cell proliferation for 24 h (E). Mean ± SD, n = 3, $^{\#\#\#}P < 0.001$ *vs* normal; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* model. All the experiments were performed in triplicates

an anti-inflammation role. As shown in Figs. 6A and 6B, after stimulating with Poly(I:C) combined with TNF-α, the expressions of p-p65, IL-33 and TSLP were markedly increased. Addition of PDTC, an effective NF-κB inhibitor, could decrease the expressions of TSLP and IL-33 under the stimulation in HaCaT cells. Furthermore, with YPFS additional pre-treatment, p-p65 could be further decreased, as well as TSLP and IL-33 (Figs. 6C and 6D). Interestingly, compared with cells treated with YPFS alone under the stimulation circumstance, those with PDTC combined with YPFS treatment had a moderate decrease, which suggested that YPFS may modulate TSLP and IL-33 expression through NF-κB signaling, however, together with some other unknown pathways.

Discussion

A large number of studies have proved that the key mechanisms of allergic dermatitis are immune abnormality and Th1/Th2 imbalance [22-24]. At present, accumulating evidences

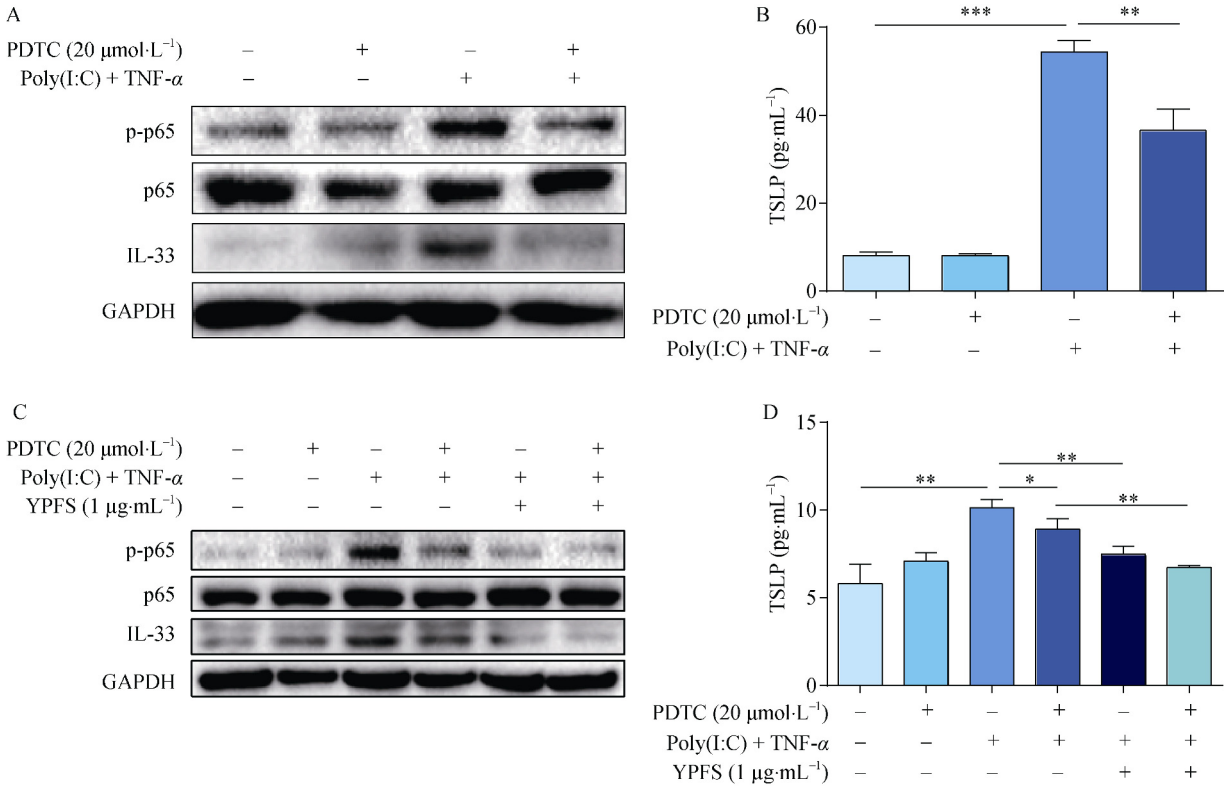


Fig. 6 YPFS decreased the expressions of initial key factors *via* inhibiting NF-*κ*B activation under the stimulation of Poly(I:C) combined with TNF-*α*. (A, B), Effect of PDTC on the expressions of p-p65, p65, IL-33 and TSLP in HaCaT cells by Western blot and ELISA assays. (C, D), YPFS reduced the expressions of p-p65 and initial key factors in HaCaT cells by Western blot and ELISA assays. Mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. All the experiments were performed in triplicates

have focused on the changes of the inflammatory cells and their associated inflammatory factors, which are related to the progression and severity of allergic diseases [25]. However, few studies hit on the changes in the early stage of AD (a symptomatic stage). YPFS is a classical Chinese herbal formula, which is widely used in clinical allergic diseases and could reduce allergy relapse, whereas underlying mechanism in the initial stage has not been identified.

FITC-induced Th2 AD mice model can well clarify the pathogenesis of AD, which can be used to evaluate the efficacy of new drugs. In the present study, the mechanism of YPFS on the allergic inflammation was explored in this model and the results showed that YPFS only treated in the initial stage can eventually inhibit Th2 type immune responses. YPFS considerably inhibited the swelling and improved the pathological changes of the ear tissue in mice, including edema thickening and inflammatory cells infiltrations. In addition, it significantly decreased the expressions of Th2 cytokines. These results indicated the effect of YPFS on anti-allergy in the early stage of sensitization. Further, the effect of YPFS on reducing allergy relapse may be related to its role in the initial stage of allergic disease.

EC is a key cell that involved in the initiation phase of allergic diseases. Epithelial integrity depends on tight junction, adhesion junction, desmosome junction and other structural proteins, which play an important role in maintaining cell polarity and integrality. Defects in epithelial junction structures are vital factors to trigger allergic diseases. Patients with allergic diseases showed impaired integrity of the epithelial connection [26-27]. Interestingly, we demonstrated that YPFS could alleviate the allergic disease by reducing separated gaps among epithelial cells and maintaining the structural integrity of epithelial cell, which may contribute to alleviate the allergic inflammation.

EC used to be considered as a physical barrier, but now accumulating evidences showed that it can also play a key role in stimulation and regulation the process of allergic inflammation by secreting IL-33 and TSLP [28-29]. TSLP and IL-33 are both important factors to initiate Th2 type immune response, which can further influence the Th1/Th2 balance and effect on lymphocytes, dendritic cells, regulatory T cells, eosinophils and mast cells [30-31]. TSLP is an IL-17-like cytokine, which can promote the differentiation of B cells and proliferation of T cells. TSLP can trigger T cells differentiation into Th2 cells through upregulating OX40L on human DCs surface. In addition, experiments proved that TSLP could directly alter T cells in mice, promote Th2 differentiation through inducing the transcription of IL-4, which might contribute to allergic inflammation [16]. IL-33 is a newly discovered member of IL-1 family. It can strongly modulate allergic inflammation by binding to its receptor ST2 and promote the

aggregation and activation of ILC2s [17]. Moreover, IL-33 has been reported as a dominant cytokine, which can promote ILC2s response and inflammation in allergic disease models. Thus, EC-derived TSLP and IL-33, together with DCs and ILC2s may play important roles in allergic inflammation.

In the present study, we demonstrated that the levels of TSLP and IL-33 were markedly downregulated by YPFS in the early stage of AD. Further, YPFS differentially reduced the proportions of DCs and ILC2 cells in mouse lymph nodes, which are target cells of TSLP and IL-33, respectively. Given that TSLP and IL-33 are mainly derived from epithelial cell, we studied the effect of YPFS on IL-33 and TSLP production in HaCaT cells *ex vivo*. We found that YPFS could notably reduce TSLP and IL-33 production, which were consistent with results *in vivo*. These implied that YPFS might inhibit the expressions of these pro-allergy key promoters (TSLP and IL-33) in the initial stage of AD. Overall, YPFS might prevent and alleviate the allergic diseases by regulating the expressions of TSLP and IL-33.

NF-κB is an important transcription factor which could bind to TSLP gene promoter and play the role of regulation in allergic inflammation [32]. Many researches showed that TSLP initiated the allergic inflammation *via* NF-κB-dependent signaling pathways [33-34]. We found that TSLP and IL-33 could be inhibited when epithelium were pre-treated with PDTC. In addition, YPFS could decrease the activation of NF-κB, which showed as same effect as PDTC, even the expressions of TSLP and IL-33 could be further reduced. It may suggest YPFS could control the allergic inflammation through NF-κB-TSLP/IL-33 pathway. However, we noticed that the expressions of TSLP and IL-33 in the treatment of YPFS were lower than treated with PDTC alone under the stimulation of Poly(I:C) combined with TNF-α in HaCaT, which hinted that there may be some other mechanisms involved in the anti-inflammation procedure of YPFS.

Conclusions

This study indicated that YPFS could significantly inhibit the allergic inflammation *via* its effect on the early stage of AD and regulate the pro-allergic cytokines TSLP and IL-33 through the NF-κB-dependent pathway. It may provide a novel effective target for the prevention and treatment of allergic diseases. However, a comprehensive and in-depth understanding of the mechanism of YPFS in the treatment of allergic diseases still needs to be further explored.

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Cite this article as: WANG Xiao-Tong, LIU Hai-Liang, YU Xi, WANG Can, GUI Li-Li, WANG Xiao-Yu, HUA Yong-Qing, ZHENG Jie, HONG Min. Chinese medicine *Yu-Ping-Feng-San* attenuates allergic inflammation by regulating epithelial derived pro-allergic cytokines [J]. *Chin J Nat Med*, 2019, **17**(7): 525-534.