

Original Article

Yu-Ping-Feng-San ameliorates recurrent allergic inflammation of atopic dermatitis by repairing tight junction defects of the epithelial barrier

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ARTICLE INFO

Key words:

Yu-Ping-Feng-San
Atopic dermatitis
Allergic inflammation relapse
Epithelial barrier
Tight junctions

ABSTRACT

Background: Atopic dermatitis (AD) is a common allergic inflammatory skin disease, concomitant with a high relapse rate. *Yu-Ping-Feng-San* (YPFS), a well-known Chinese herbal decoction, reduces the AD relapse rate and recurring severity incidence. However, the underlying mechanism of YPFS on resisting AD recurrence is still unknown and further study is needed.

Purpose: To evaluate the effects of YPFS on recurrent allergic inflammation of AD in a murine model and to investigate the underlying mechanisms *in vivo* and *ex vivo*.

Methods: A fluorescein isothiocyanate (FITC)-induced AD relapsing mouse model was established to study the effects of YPFS and three active components, claycosin, formononetin, and cimifugin, on recurrent allergic inflammation *in vivo*. Histological analyses of ear tissue inflammation were evaluated by hematoxylin and eosin staining. Production of interleukin (IL)-4, IL-5, IL-13, and interferon-gamma in mice ear tissues, IgE in serum, and thymic stromal lymphopoietin (TSLP) in cell cultures were measured by ELISAs. Tight junction (TJ) expression was detected by immunohistochemistry and western blots. Epithelial barrier integrity was observed with electron microscopy, transepithelial electric resistance (TER), and paracellular flux measurements. HaCaT cells were utilized for *ex vivo* cellular analyses.

Results: In the recurrent phase of AD, YPFS exhibited both short- and long-term anti-allergic inflammatory efficacy with reduced ear tissue inflammation and decreased IL-4, IL-5, IL-13, and IgE production. The three active components, claycosin, formononetin, and cimifugin, showed similar effects as YPFS. Stimulus-induced decreased TER and increased FITC-dextran flux in air-liquid interface cultures of HaCaT cells were significantly repaired by YPFS and the three active components. Notably, the upregulated TJ (CLDN-1 and occludin) expression of epithelium was observed only with YPFS and the three components-treated mice as opposed to the result using conventional anti-allergy medicines. Restored TJ expression by YPFS three components was also detectable in the remission phase of AD. Moreover, decreased TJ expression influenced the effects of YPFS on epithelial cells-derived TSLP production.

Conclusions: YPFS ameliorated recurrent allergic inflammation of AD by repairing TJ defects of epithelial barriers. Intervening epithelial barrier functions could be a preventive and therapeutic approach for recurrent allergic inflammation of AD.

Abbreviations: AD, atopic dermatitis; ALI, air-liquid interface; CLDN, claudin; Dex, dexamethasone; ECs, epithelial cells; FD4, FITC-dextran 4 kDa; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; IHC, immunohistochemistry; Lor, Loratadine; MS, mass spectrometry; Mon, Montelukast; RA, Radix Astragali; RAM, Rhizoma Atractylodis Macrocephalae; RS, Radix Saposhnikovia; TER, transepithelial electric resistance; Th2, T helper type 2 cell; TJs, tight junctions; TSLP, thymic stromal lymphopoietin; WB, western blot; YPFS, Yu-Ping-Feng-San

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<https://doi.org/10.1016/j.phymed.2018.09.190>

Received 10 February 2018; Received in revised form 4 August 2018; Accepted 17 September 2018

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Introduction

Atopic dermatitis (AD) is the most common allergic inflammatory skin disease and is mainly mediated by type 2 immunity. It is characterized by overexpression of T helper type 2 (Th2) cytokines, such as interleukin (IL)–4, IL-5, and IL-13 as well as by IgE production (Czarnowicki et al., 2014; Schmitt et al., 2013). Although conventional anti-allergy medications including glucocorticosteroids (dexamethasone), anti-histamines (Loratadine), and leukotriene regulators (Montelukast), as well as novel immunomodulators clinically achieve symptom and inflammation control (Boguniewicz and Leung, 2015; Gangwar et al., 2017), the high relapse rate is an inevitable challenge to developing effective approaches for AD treatment (Ong, 2014). Recent novel insights into the genetics and pathophysiology of AD point to an important role of structural abnormalities in the epidermis during the development of AD (Boguniewicz and Leung, 2011; Lambrecht and Hammad, 2012). Barrier epithelial cells (ECs) represent the first line of defense against allergens. Impaired epithelial barrier contributes to the increased susceptibility to allergens and evokes Th2-mediated immunity (Hammad and Lambrecht, 2015). Therefore, an intact, healthy epithelial barrier is critical for preventing AD. The integrity of the epithelial barrier depends on apical tight junctions (TJs) and adherens junctions (Wawrzyniak et al., 2017). TJs consist of transmembrane proteins, including claudins (CLDNs) and occludin. The sufficient CLDNs and occludin expression of ECs has been found is essential for maintaining barrier structural integrity and inhibiting allergy-related inflammation in allergic disorders (De Benedetto et al., 2011; Park et al., 2013).

Yu-Ping-Feng-San (YPFS), a Chinese herbal decoction also known as *Jade-Screen Powder*, consists of *Radix Astragali* (RA), *Rhizoma Atractylodis Macrocephalae* (RAM), and *Radix Saposhnikovia* (RS). Clinically, YPFS is widely used for allergic disorders, with few adverse reactions (Chan et al., 2016; Zhong et al., 2013). Notably, it has been found to reduce the relapse rates and recurring severity (Fang et al., 1983; Song et al., 2016). Our previous study found that YPFS attenuated Th2-mediated allergic inflammation by inhibiting alarmin cytokines, thymic stromal lymphopoietin (TSLP) and IL-33 production of ECs (Hong et al., 2014). We further identified several bioactive components, including astragaloside IV, calycosin, formononetin, and cimifugin, either by using respiratory ECs combined with HPLC-MS approaches or serum pharmacochemical analyses (Shan et al., 2014). We also reported that astragaloside IV, cimifugin, and calycosin had immunosuppressive and barrier protection effects in the initial stage of AD (Bao et al., 2016; Tao et al., 2017; Wang et al., 2017). However, the underlying mechanism of YPFS for resisting allergy recurrence is still unknown and further inquiry is therefore needed.

Material and methods

Preparation of YPFS

All crude drugs including RA (the dry root of *Astragalus membranaceus* var. *mongolicus*), RAM (the dry rhizome of *Atractylis macrocephala* Koidz), and RS (the dry root of *Saposhnikovia divaricate*) were purchased from Nanjing Pharmaceutical Company (China). Botanical identifications were authenticated by Professor Gu Wei (Nanjing University of Chinese Medicine, China). Preparation of YPFS extracts was performed as described previously (Shen et al., 2014). Briefly, 500 g herbal mixture (RA: RAM: RS in a 3:1:1 weight ratio) were extracted twice with boiling water and further evaporated to 1.5 g crude drug/g extracts, and stored at -20°C . The details regarding the HPLC fingerprint of YPFS extracts and main marker compound identifications were published previously (Shen et al., 2014).

Reagents

Cimifugin was purchased from National Institutes for Food And Drug Control (20120511; Shanghai, China; purity $\geq 99\%$). Formononetin and calycosin were from Wanxiang Hengyuan Technology Co., Ltd (201302; Tianjin, China; purity $\geq 98\%$), and Mark Biotechnology Co., Ltd (201309; Tianjin, China; purity $\geq 98.75\%$), respectively. Dexamethasone (Dex) was from TianYao Pharmaceutical Co., Ltd (41303101; Hubei, China). Loratadine (Lor) and Montelukast (Mon) were purchased from XianLin BaoYa Pharmaceutical Co., Ltd (12CRXF1018; Shanghai, China) and MSD Pharmaceutical Co., Ltd (K004567; Hangzhou, China), respectively.

Experimental AD relapsing model and medications

BALB/c mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). Mice were raised at Nanjing University of Chinese Medicine under specific pathogen-free conditions. After acclimatization for 3 days, mice abdomens were shaved with an area of approximately $3 \times 3 \text{ cm}^2$ on day 0, topically sensitized with 1.5% FITC (Sigma-Aldrich, St. Louis, MO, USA) in 80 μl of acetone and dibutylphthalate (1:1; vehicle) solution on days 1 and 2, and the right ear was treated with 20 μl of 0.6% FITC solution on day 6. From day 7 (24 h after initial elicitation) to day 14, ear swelling was measured with a thickness gauge (7301; Mitutoyo, Kawasaki, Japan). Mice were preventively treated once daily with YPFS (1.625, 3.25, or 6.5 g/kg), cimifugin (3.125 or 12.5 mg/kg), formononetin (0.4 or 10 mg/kg), calycosin (0.4 or 10 mg/kg), Lor (1.3 mg/kg), or Mon (1.3 mg/kg) intragastrically (i.g.), or Dex (0.67 mg/kg) intraperitoneally (i.p.) for 10 days. Medications were terminated at indicated time points before re-challenge with 0.6% FITC on the right ear, to induce AD relapse. Ear swelling was calculated as the thickness difference between the left and right ear. Mice were then sacrificed and samples taken. All procedures and animal use 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.

Determination of cytokines and IgE production

Production of IL-4, IL-5, IL-13, and interferon (IFN)-gamma in mice ear tissue homogenates and IgE in serum were measured by ELISAs (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. TSLP levels in HaCaT culture medium were assessed using a human TSLP ELISA kit (eBioscience).

Histology and immunohistochemistry

Ear tissues were fixed in 10% formalin immediately after mice were euthanized. Paraffin-embedded sections (4 μm) of the ear tissues were stained with hematoxylin and eosin (H&E). CLDN-1 and occludin expressions in ear tissues were evaluated by immunohistochemistry (IHC) staining using CLDN-1 and occludin antibodies (1:1000 dilution; Abcam, Cambridge, MA, USA), and observed with optical microscopy (Axion A1; Carl Zeiss AG, Oberkochen, Germany).

Electron microscopy

Ear tissue specimens were prepared according to the procedure described previously (Wang et al., 2017) and observed with transmission electron microscope (JEOL, Tokyo, Japan).

Transepithelial electric resistance and paracellular flux measurements

HaCaT cells (5×10^4 cells per well) were seeded on Transwell® inserts (Costar, Corning, NY, USA) precoated with rat tail collagen for air-liquid interface (ALI) cultures (Wawrzyniak et al., 2017). Epithelial

integrity was evaluated by using transepithelial electric resistance (TER) measurements with an EVOM2 (WPI, Sarasota, FL, USA) and results were presented as $\Omega \times \text{cm}^2$. Once TER reached $700 \Omega \times \text{cm}^2$, the cells were treated with YPFS (10 or 100 $\mu\text{g}/\text{ml}$), cimifugin (1 μM), formononetin (10 μM), or calycosin (10 μM) for 6 h and then stimulated with tumor necrosis factor (TNF)- α (20 ng/ml) plus Poly(I: C) (100 $\mu\text{g}/\text{ml}$) for 12 h. Permeability of the polarized HaCaT cell monolayers was assessed by adding FITC-dextran (4 kDa; FD4; 2 mg/ml; Sigma-Aldrich), apically. After incubation for 2 h, FD4 in the basolateral medium was assessed with a fluorescence reader, at 480 nm.

Western blots

Western blots were performed as described previously (Wang et al., 2017) by using primary antibodies against mouse CLDN-1, CLDN1, occludin (1:1000 dilution; Abcam), or GAPDH (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA).

CLDN-1 siRNA transfection

HaCaT cell transfections with CLDN-1 siRNA (Transheep, Shanghai, China) were performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) (Wang et al., 2017). After 48 h transfection, cells were treated with YPFS (100 $\mu\text{g}/\text{ml}$) for 6 h and then stimulated with TNF- α (20 ng/ml) plus Poly(I:C) (100 $\mu\text{l}/\text{ml}$) simultaneously for further 12 h. The sequence of CLDN-1 siRNA was CGAAATGAGCATTTGAGAT.

Statistical analysis

Data are expressed as the mean $\pm$ SEM. One-way ANOVA analysis was used for multiple group comparisons and the unpaired two-tailed Student's *t*-test was used for comparison between two groups, using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Values of *P* < 0.05 indicated statistical significance.

Results

YPFS attenuated recurrent allergic inflammation in an AD relapse model

A murine AD relapsing model was established as outlined (Fig. 1A). Medications were started from day 14 and terminated at 1 h before final challenge on day 23 (Fig. 1A), as the components in serum from YPFS-treated mice could be detected 1 h after administration (Shen et al., 2014). Ear swelling in the untreated model group reached the peak value at 6 h after final challenge (Fig. 1B), indicating the FITC-induced AD relapse model was successfully established. In the recurrent phase of AD, Dex, Mon, and Lor, which were used as the positive control drugs (conventional drug groups), as well as YPFS remarkably suppressed ear swelling (Fig. 1B and C) compared to the untreated model group. These results suggested that preventive administration of YPFS and conventional anti-allergy medicines, which were terminated a short time prior to allergen exposure, effectively attenuated allergic inflammation in the recurrent phase of AD.

YPFS showed long-term anti-recurrent allergic inflammation efficacy in the AD relapse model

To further determine whether YPFS could maintain long-term anti-recurrent inflammation efficacy, medications were terminated at 24 h before rechallenge on day 24 (Fig. 2A); except Lor₂, which was delivered only after initial allergic inflammation had occurred (from day 7 to day 11, to mimic clinical application). Ear swelling of the mice increased robustly at 8 h after final challenge and persisted to 12 h (Fig. 2B). Conventional drugs (Dex, Mon, and Lor₁) showed poor efficacy on prevention of ear swelling and inflammatory cell infiltration in the recurrent phase of AD (Fig. 2C and D). In addition, conventional

drugs had no effect on IL-5 and IL-13 production in ear homogenates and IgE level in serum in the recurrent phase of AD (Fig. 2F–H), except for IL-4 production was inhibited by Mon and Lor₁ (Fig. 2E). On the contrary, ear swelling and inflammatory cell infiltration at the recurrent phase of AD was notably reduced with YPFS treatment (6.5 and 1.625 g/kg) compared to the untreated model group (Fig. 2B–D). Consistently, the amounts of IL-4, IL-5, and IL-13 in ear homogenates and IgE in serum were significantly decreased in the YPFS-treated mice (6.5 g/kg) (Fig. 2E–H). All medications showed no significant effects on IFN- γ production (Fig. 2I). Although Lor₂ remarkably decreased ear swelling of AD after initial allergic responses (Fig. 2J), it had no effect in the recurrent phase of AD (Fig. 2B–I). These observations suggested YPFS had long-term anti-recurrent allergic inflammation efficacy in the AD relapse mouse model.

Epithelial barrier integrity and TJ expression were regulated by YPFS and its active components in the recurrent phase of AD

Previously, we screened several active components of YPFS that inhibited allergic inflammation in the initial stage of AD, including cimifugin, formononetin, and calycosin (Shen et al., 2014). In the current study, to evaluate the effects of these active components on recurrent allergic inflammation of AD, mice were treated with cimifugin (3.125 or 12.5 mg/kg), formononetin (0.4 or 10 mg/kg), calycosin (0.4 or 10 mg/kg), or in parallel with the conventional drugs (Dex, Lor, and Mon). Medications were terminated 48 h before rechallenge (Fig. 3A). Consistent with the abovementioned findings, in the recurrent phase of AD, mice ear swelling and AD relapse-related inflammatory cell infiltration of ear tissues were significantly reduced in formononetin- or calycosin-treated mice, as well as at high doses of cimifugin (Fig. 3B–D). In contrast to Dex, all tested drugs had no obvious effects on spleen and thymus indices (Fig. 3E and F).

As a disturbed epithelial barrier may lead to increased susceptibility of ECs to allergens and could evoke allergic inflammation, we then investigated the effects of YPFS and the three active components, cimifugin, formononetin, and calycosin, on epithelial integrity in the recurrent phase of AD. In the untreated model mice and conventional drug-treated groups, either extensive separated gaps or loose epithelial connections between ECs were observed (Fig. 4A). On the contrary, the three active components, cimifugin, formononetin, and calycosin, ameliorated the pathological status of ECs by reducing intercellular gaps and increasing epithelial connections (Fig. 4A). Moreover, YPFS and the three active components remarkably increased TER and reduced FD4 permeability in ALI cultures of HaCaT cells compared with those of TNF- α and Poly(I: C) co-stimulation groups (Fig. 4B and C). In addition, both CLDN-1 and occludin expression in the cimifugin-, formononetin-, and calycosin-treated ear tissues were comparable to that of the normal control group as assessed by IHC analyses (Fig. 4D). Although, Dex, Mon, or Lor slightly increased CLDN-1 expression similar to the three active components, they showed poor efficacy on occludin expression (Fig. 4D). Consistently, increased CLDN-1 and occludin expression of mice ear skin were also notable with YPFS (6.5 g/kg) treatment (Fig. 4E). These results suggested that YPFS and the three active components, cimifugin, formononetin, and calycosin, effectively suppressed recurrent allergic inflammation of AD, partially through regulating epithelial barrier integrity and TJ expressions of ECs.

YPFS active components repaired TJ defects in the remission phase of AD

We further assessed TJ expressions of ECs after treatments with YPFS active components, cimifugin, formononetin, and calycosin, at the remission phase of AD, i.e., sampling on day 25 without rechallenge (Fig. 5A). Electron microscopy showed that the three active components improved the pathological status of ECs by reducing intercellular gaps and increasing epithelial connections, when compared to the untreated and Dex-treated mice (Fig. 5B). Moreover, restored CLDN-1 and

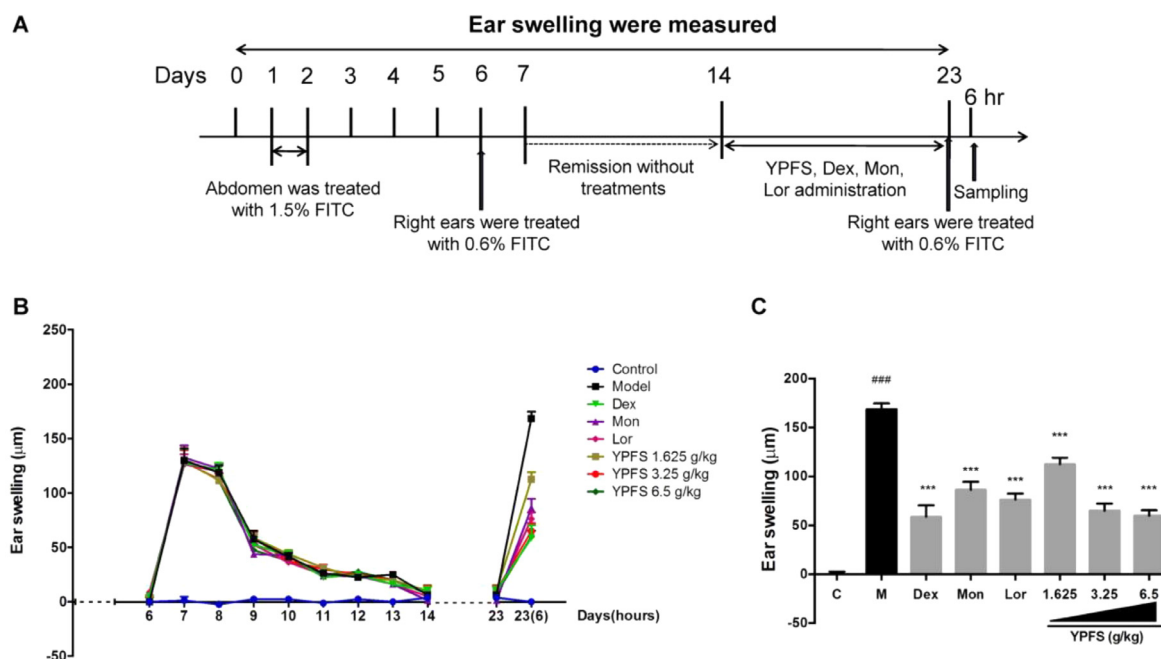


Fig. 1. YPFS attenuates recurrent allergic inflammation in an AD relapsing mouse model. (A) Overview of FITC-induced AD relapsing mouse model and medications used: YPFS (1.625, 3.25, or 6.5 g/kg), Lor (1.3 mg/kg), or Mon (1.3 mg/kg) i.g., or Dex (0.67 mg/kg) i.p. for 10 days. (B) Ear swelling was measured from day 6 to day 14, and day 23 pre-/post-final challenge at indicated time points. (C) Ear swelling was measured at 6 h after FITC rechallenge on day 23. Data show the mean $\pm$ SEM, $n = 8$, ### $P < 0.001$ vs normal control; *** $P < 0.001$ vs untreated model group.

occludin expressions of ear epithelium were only observed after treatment with the three active components, cimifugin, formononetin, and claycosin (Fig. 5C). Western blots confirmed that the three active components increased CLDN-1 and occludin expression in the remission phase of AD, but only a mild effect was observed for CLDN-1 levels (Fig. 5D), as opposed to the effects shown after Dex, Mon, and Lor treatment (Fig. 5D). These observations suggested that the three active components of YPFS, cimifugin, formononetin, and claycosin, repaired the disrupted TJ expressions of ECs in the remission phase of AD, and this could help to form an integrity barrier to prevent AD recurrence.

Impaired TJ expression altered the effect of YPFS on EC-derived TSLP production

The high level of EC-derived TSLP expression leads to chronic Th2-mediated inflammation (Omori-Miyake and Ziegler, 2012), which is highly correlated with the severity of AD (Yoo et al., 2005). In the current study, to further confirm that TJ impairment leads to increased susceptibility of ECs in the AD relapse model, and to further clarify the recovery effects of YPFS on TJs, CLDN-1 siRNA was employed to interfere with TJ expression of ECs. Cell transfection efficiency was confirmed by western blots (Fig. 6A). In response to cell transfection with CLDN-1 siRNA, TSLP production increased notably with both TNF- α and Poly(I:C) co-stimulation either present or absent in the culture medium. Addition of YPFS (100 μ l/ml) significantly inhibited stimulus-induced TSLP production (Fig. 6B). However, antagonized TSLP production by YPFS was remarkably abrogated in CLDN-1 siRNA transfected HaCaT cells (Fig. 6B). These results indicated that decreased TJ expression influenced the effects of YPFS on ECs-derived TSLP production.

Discussion

YPFS is widely used to treat allergic disorders to reduce relapse rates and lessen recurring severity. However, the underlying mechanism of YPFS on resisting allergy recurrence is still unknown. In the current

study, we confirmed that YPFS effectively ameliorated recurring inflammation in a murine AD relapse model. Unlike conventional anti-allergy medicines (Dex, Mon, and Lor), which suppressed short-term recurrent allergic inflammation, YPFS showed long-term efficacy in alleviating the recurring severity of AD. In addition, as the key active components of YPFS, cimifugin, formononetin, and claycosin showed similar anti-allergic inflammation effects in the AD relapse model.

Recent evidence suggests the disturbance of the epithelial barrier as a common feature in many allergic inflammatory disorders including food allergy, asthma, and AD (Kuo et al., 2013; Mocsai et al., 2014; Ring et al., 2012). Defective barrier function is known to favor the penetration of allergens into the dermis, and accounts for the Th2-mediated immune responses observed in AD. Considering the defensive barrier properties of YPFS, we evaluated its effects and those of cimifugin, formononetin, and claycosin on epithelial integrity in an AD relapse model. The results showed that YPFS and the three active components cimifugin, formononetin, and claycosin improved the allergen-induced pathological status of ECs and restored their impaired TER and paracellular permeability. In addition, in the AD relapse model, YPFS and the three abovementioned components, remarkably increased TJ (CLDN-1 and occludin) expression in mice ear epithelium. Previously, we have shown that cimifugin suppressed allergic inflammation by reducing epithelial-derived TSLP and IL-33 levels via regulating TJs in the initial phase of AD (Wang et al., 2017). In the present study, we demonstrated recovery of barrier integrity and TJ expression after treating with the three active components of YPFS, not only in the recurrent phase of AD, but also in the remission phase prior to rechallenge, to help form an intact “defensive barrier” against allergy recurrence. In addition, we found that decreasing TJ expression by using CLDN-1 siRNA also prevented the effects of YPFS on inhibiting TSLP production of ECs. These results were consistent with recent studies that highlighted the importance of sufficient TJ expression for maintaining epithelial barrier integrity to improve allergic sensitization (De Benedetto et al., 2011; Park et al., 2013; Salimi et al., 2013). Combined with our previous studies of YPFS, we suggest that in different phases of AD, YPFS may act through different mechanisms to

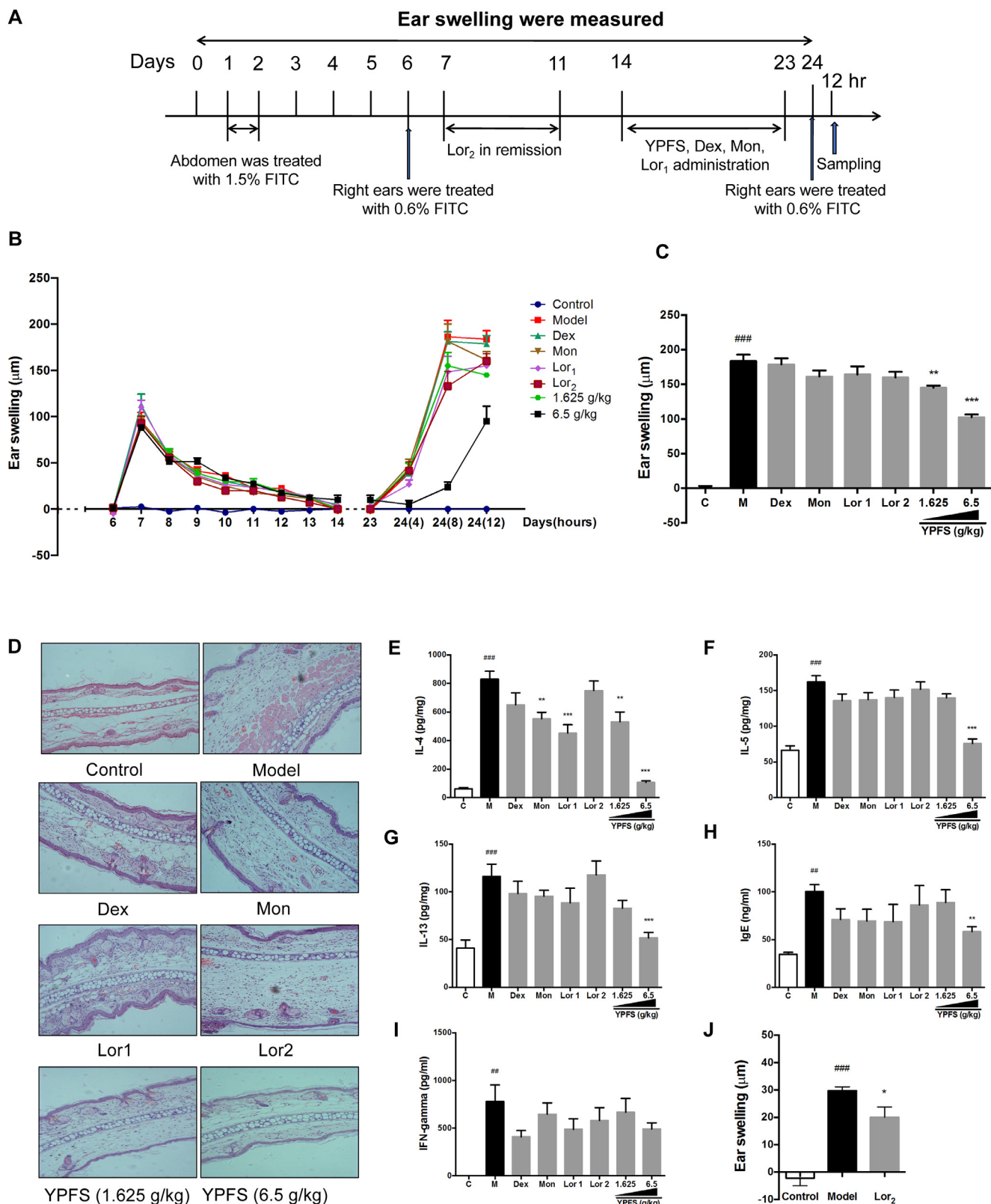


Fig. 2. YPFS exhibits long-term, anti-recurrent allergic inflammation efficacy in an AD relapsing mouse model. (A) Overview of FITC-induced AD relapsing mouse model and medications used: YPFS (1.625 or 6.5 g/kg), Lor (Lor₁ and Lor₂, 1.3 mg/kg), Mon (1.3 mg/kg) i.g., or Dex (0.67 mg/kg) i.p. for 10 days. (B) Ear swelling was measured from day 6 to day 14, and day 23 to day 24 pre-/post-final challenge at indicated time points. (C) Ear swelling was measured at 12 h after FITC rechallenge on day 24. (D) H&E staining of ear tissue from FITC-induced AD relapsing mouse model ($n = 6$; magnification: $\times 200$). (E–I) Cytokine production of ear homogenates and serum IgE levels in the recurrent phase of AD were measured by ELISA assays on day 24. (J) Ear swelling in the Lor₂-treated group was measured on day 11. Data are the mean $\pm$ SEM, $n = 8$ –32. $^{*}P < 0.01$, $^{***}P < 0.001$ vs normal control; $^{**}P < 0.01$, $^{***}P < 0.001$ vs untreated model group.

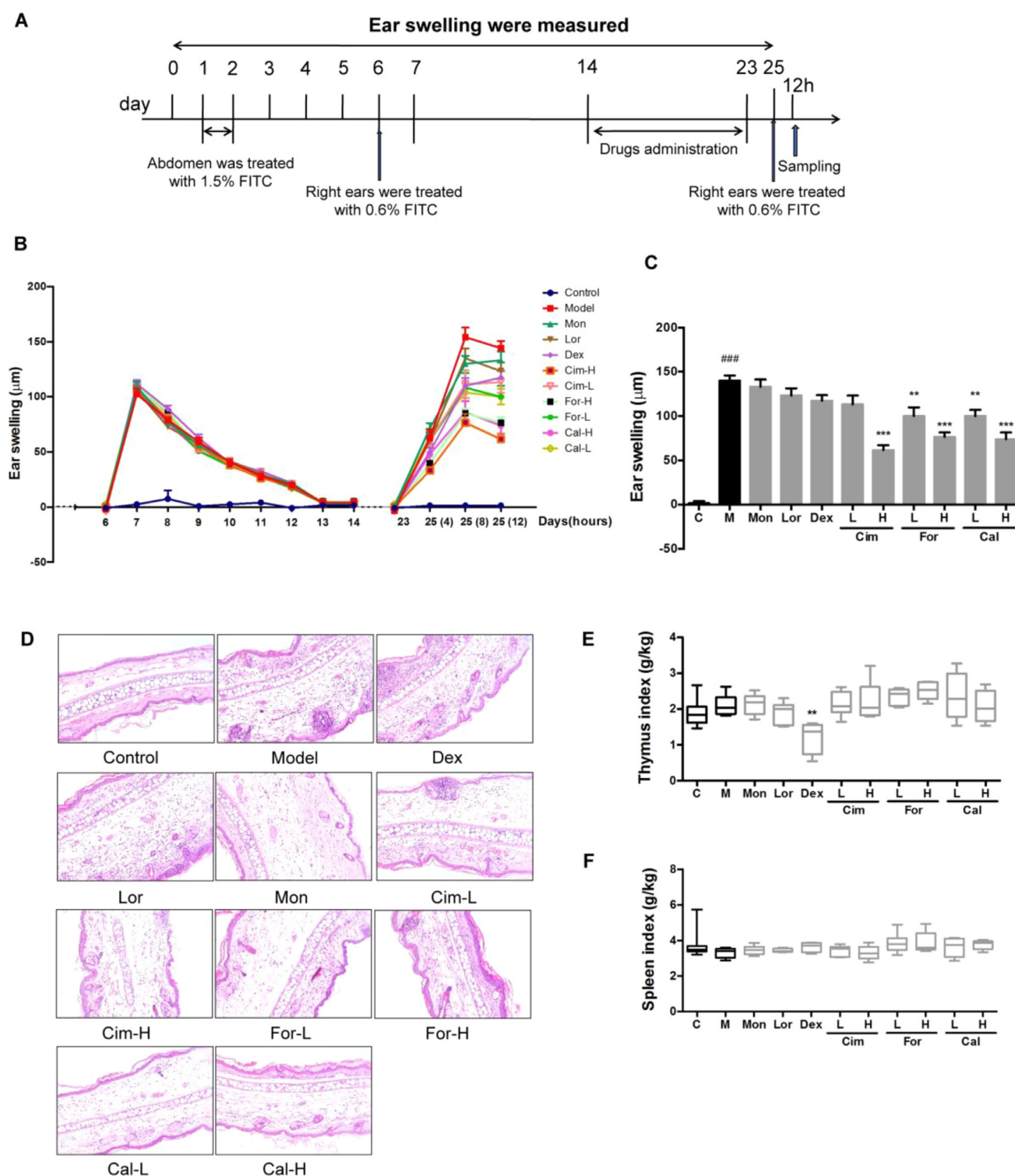


Fig. 3. YPFS active components suppress allergic inflammation in the recurrent phase of AD. (A) Overview of FITC-induced AD relapsing mouse model and preventive medications for 10 days (Cim-L, 3.125 mg/kg; Cim-H, 12.5 mg/kg; For-L, 0.4 mg/kg; For-H, 10 mg/kg; Cal-L, 0.4 mg/kg; Cal-H, 10 mg/kg; Lor, 1.3 mg/kg; Mon, 1.3 mg/kg, i.g.; or Dex, 0.67 mg/kg, i.p.) (B) Ear swelling was measured from day 6 to day 14, and day 23 to day 25 pre-/post-final challenge at indicated time points. (C) Ear swelling was measured at 12 h after FITC rechallenge on day 25. (D) H&E staining of ear tissue from FITC-induced AD relapsing mice ($n = 6-8$; magnification: $\times 200$). (E–F) Thymus and spleen indices were assessed at 12 h after FITC rechallenge on day 25. Data are the mean $\pm$ SEM, $n = 8$; ### $P < 0.001$ vs normal control; ** $P < 0.01$, *** $P < 0.001$ vs untreated model group.

abrogate allergic inflammation. In the initial phase of AD, YPFS attenuated Th2-mediated allergic inflammation primarily through reducing the alarmin cytokines such as TSLP and IL-33, as reported previously (Hong et al., 2014; Tao et al., 2017; Wang et al., 2017). However, in the

remission phase and recurrent phase of AD, as presented in the present study, we found that YPFS, and especially its active components, cimifugin, formononetin, and claycosin, acted mainly through regulating epithelial barrier integrity and TJ expression, to ameliorate the

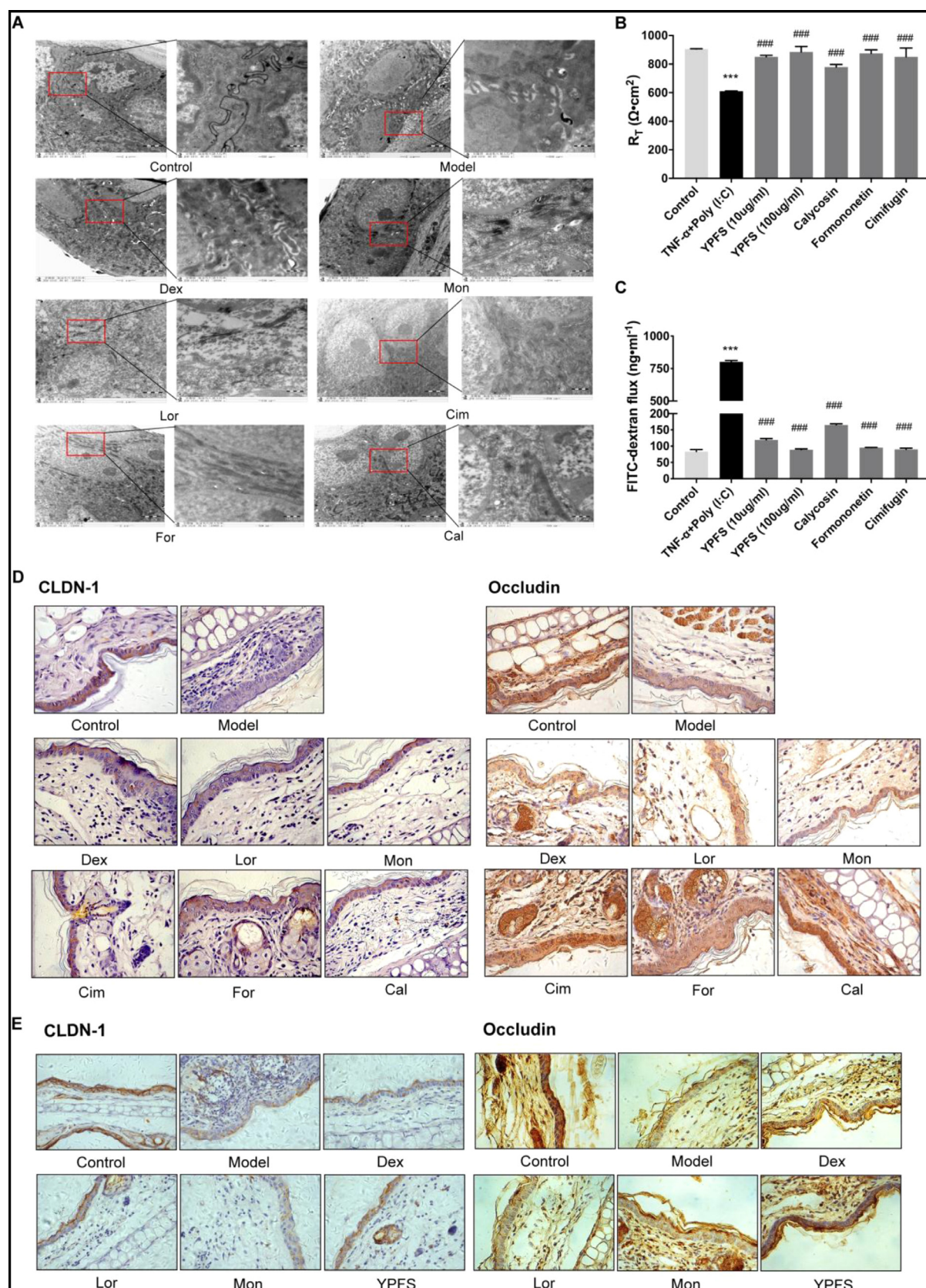


Fig. 4. YPFS and active components regulate epithelial integrity and tight junction expression of ECs in the recurrent phase of AD. (A) Electron microscopy observation of ear tissues. The red squares indicate junction gaps of epithelium ($n = 3$, magnification: $\times 12,000$; $\times 40,000$). (B–C) Transepithelial electrical resistance (TER; R_T) measurements (B) and FITC-dextran flux assays (C) in air-liquid interface cultures of HaCaT cells treated with YPFS (10 or 100 $\mu\text{g}/\text{ml}$), cimifugin (1 μM), formononetin (10 μM), and calycosin (10 μM) for 6 h, and then stimulated with TNF- α (20 ng/ml) + Poly(I:C) (100 $\mu\text{g}/\text{ml}$) for a further 12 h. Data are the mean $\pm$ SD, $n = 3$; ### $P < 0.001$ vs normal control; *** $P < 0.001$ vs TNF- α + Poly(I:C). (D–E) IHC analysis of CLDN-1 and occludin expression ($n = 6$, magnification: $\times 630$) of mice ear epithelium with indicated treatments. Preventive medications for 10 days were as follows: Cim, 12.5 mg/kg ; For, 10 mg/kg ; Cal, 10 mg/kg ; YPFS, 6.5 g/kg ; Lor, 1.3 mg/kg ; Mon, 1.3 mg/kg , i.g.; or Dex, 0.67 mg/kg , i.p. Data are representatives of three independent experiments.

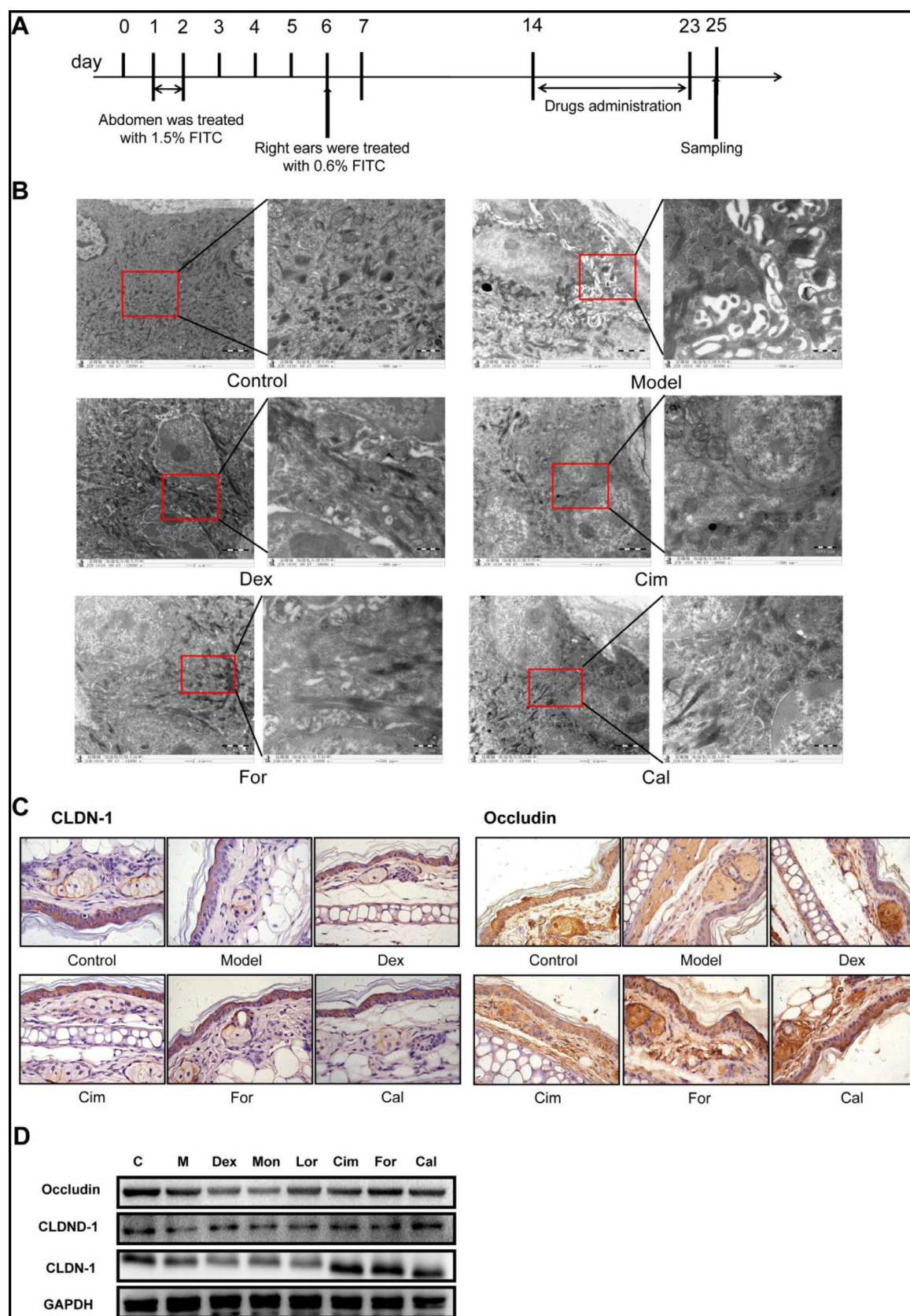


Fig. 5. YPFS active components restore TJ expression of ECs in the remission phase of AD. (A) Overview of FITC-induced AD relapsing mouse model establishment. Ear tissues were collected on day 25 without rechallenge. (B) Electron microscopy observation of ear tissues. The red squares indicate junction gaps of epithelium ($n = 3$, magnification: $\times 12,000$; $\times 40,000$). (C) IHC staining of CLDN-1 and occludin expression in ear epithelium after indicated treatments ($n = 6$, magnification: $\times 630$). (D) Occludin, CLDN-1, and CLDN-1 expressions in ear epithelium were analyzed by western blotting. Preventive medications were as follows for 10 days: Cim, 12.5 mg/kg; For, 10 mg/kg; Cal, 10 mg/kg; Lor, 1.3 mg/kg; Mon, 1.3 mg/kg, i.g.; or Dex, 0.67 mg/kg, i.p. The data are representative of three independent experiments.

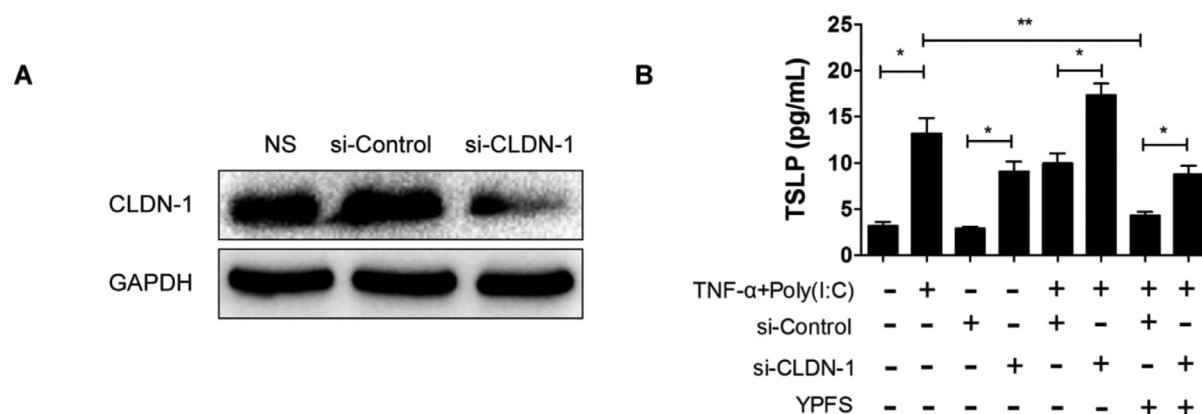


Fig. 6. Decreased TJ expression alters the effect of YPFS on EC-derived TSLP production. (A) Cell transfection efficiency of CLDN-1 siRNA in HaCaT cells was analyzed by western blotting. (B) TSLP production in HaCaT cells was measured by ELISAs. Cells were treated with YPFS (100 μ g/ml) for 6 h and stimulated with TNF- α (20 ng/ml) + Poly(I:C) (100 μ g/ml) for a further 12 h. The sequence of CLDN-1 siRNA was CGAAATGACATTGAGAT. Data are the mean $\pm$ SEM, $n = 3$; * $P < 0.05$; ** $P < 0.01$. The data are representative of two independent experiments.

recurring severity of allergy. The effects were notably different from that of conventional anti-allergy medicines (Dex, Lor, and Mon). Therefore, our results highlighted the repair function of epithelial barrier of YPFS, in addition to its immunomodulatory effects in anti-allergic inflammation.

In summary, we demonstrated that YPFS exhibited both short- and long-term anti-inflammatory efficacies in an AD relapse model, with reduced ear tissue inflammation and decreased IL-4, IL-5, IL-13, and IgE production. The three active components, cimifugin, formononetin, and claycosin, also had similar effects to that of YPFS. The decreased TER and increased FITC-dextran flux in ALI cell cultures of ECs were significantly repaired by YPFS, and by cimifugin, formononetin, and claycosin treatments. Notably, in mice, the upregulated TJ expression of the epithelium was observed only with YPFS and the three active components, as opposed to the effects of conventional anti-allergy medicines. Restored TJ expression by cimifugin, formononetin, and claycosin treatments was also detectable in the remission phase of AD. In addition, a disturbance of TJ expression also prevented the effects of YPFS on inhibiting ECs-derived TSLP production.

Conclusions

We demonstrated the critical role of having sufficient TJ expression in ECs for maintaining barrier structural integrity and inhibiting allergic inflammation recurrence. Restoring barrier function of ECs is therefore a potential preventive and therapeutic strategy for the treatment of allergic disorders.

Conflict of interest

The authors confirm that there are no conflicts of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81473395 and 81373549), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the Natural Science Foundation of Jiangsu Province (BK20141466), and the Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica (JKLPSE201603).

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