

Astragaloside IV prophylactic administration relieves recurrent allergic atopic dermatitis

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Abstract

Background

Atopic dermatitis (AD) is a common allergic inflammatory skin disease with high relapse rates and recurring severity. However, the underlying pathogenesis of AD recurrence is still unclear. Our previous study reported Astragaloside IV (AS-IV) administration in sensitization stage ameliorated the allergic inflammation of AD. In this study, we aimed to evaluate the efficacy of AS-IV which prophylactic applied in remission phase of AD and to investigate the underlying mechanisms.

Methods

The efficacy of AS-IV with prophylactic administration in remission phase of AD was evaluated in a fluorescein isothiocyanate (FITC)-induced AD relapse (AD-Re) model. Production of IL-4, IL-5, IL-13, IFN- γ in mice ear tissues, IgE in serum were measured by ELISAs. Pharmacokinetic profile of AS-IV was assessed by HPLC-MS. Protein and gene expression levels of TLRs and NF- κ B were detected by WB and qRT-PCR, respectively.

Results: Prophylactic administration in remission phase of AD, AS-IV attenuated ear swelling, inflammatory cell infiltration, IgE production of ear homogenates in AD-Re mice. Levels of T helper (Th)₂ cytokines including IL-4, IL-5, IL-13 but not Th₁ cytokine IFN- γ in AD-Re mice were inhibited remarkably with AS-IV preventively treatment. In addition, a different pharmacokinetic profile of AS-IV was observed as applied in remission phase. Moreover, AS-IV prophylactic administration decreased the gene expression of *NF- κ B* and *TLR8* in AD-Re mice. Consistently, the proteins expression of TLR8, TIRAP and MyD88 in ear homogenates also reduced obviously in AS-IV treated mice.

Conclusions: Our finding indicated the disordered TLR8-mediated NF- κ B pathway may play an important role in the pathogenesis of AD recurrence. AS-IV prophylactic administration in remission phase could regulate TLR8-mediated NF- κ B pathway to against AD recurrence. These findings further improved our understanding of the pathogenesis of AD recurrence and the pharmacological effects of AS-IV.

Background

Atopic dermatitis (AD) is a common allergic inflammatory skin disease, with a lifetime prevalence of up to 20% and substantial effects on quality of life [1]. Characteristic features of AD include intense pruritus and a chronic or chronically relapsing course [2]. It has been recognized the innate and adaptive immune systems play dynamic interrelated roles in the pathogenesis of AD, which can in turn favour epidermal barrier disruption in AD. Current treatments for AD include topical moisturizers and anti-inflammatory agents (such as corticosteroids, anti-histamines, leukotriene regulators, as well as novel immunomodulators). These approaches successfully control the symptoms and inflammation of AD, but barely reduce the recurrence rate of AD along with the inevitable side-effects. Therefore, we need to

improve understanding the mechanisms of AD recurrence and develop effective prophylactic drugs to against AD relapse.

Astragaloside IV (AS-IV), a natural saponin abundant in *Astragalus membranaceus*, possesses multiple pharmacological effects including anti-inflammatory, anti-fibrotic, anti-tumor, neuronal and cardiac protection effects via numerous signaling pathways [3–7]. Accumulated evidence recently points to the important role of AS-IV in regulating Toll-like receptors (TLRs)-mediated NF- κ B pathway to exert its pharmacological effects [8–10]. Yu-Ping-Feng-San (YPFS), a well-known traditional Chinese medicine (TCM), is clinically applied for allergic disorders with reduced relapse rates and recurring severity [11, 12]. Our previous study elucidated that YPFS attenuated recurrent allergic inflammation of AD by repairing epithelial barrier defects in remission phase [13]. Furthermore, by means of bioactive components screening, we found AS-IV as a potent bioactive component of YPFS, could ameliorate the allergic inflammation by inhibiting alarmin cytokines such as thymic stromal lymphopoietin (TSLP) and IL-33 in sensitization stage of AD [14]. However, whether AS-IV could also effectively alleviate the recurrent allergic inflammation of AD was still unknown.

In this study, we compared the efficacy of AS-IV with three commonly applied medicines including Dexamethasone (Dex), Montelukast (Mon), and Loratadine (Lor) in a fluorescein isothiocyanate (FITC)-induced AD relapse (AD-Re) model. By investigating the pharmacokinetics profiles of AS-IV, and its effect on TLRs-mediated NF- κ B signalling pathway, we attempted to unravel the underlying mechanism of AS-IV with prophylactic administration against AD recurrence.

Methods

Reagents

AS-IV was purchased from Nanjing Zelang Med-Tech Co., Ltd (QZ-0811102; Nanjing, China; purity \geq 99%). Dexamethasone was purchased from TianYao Pharmaceutical Co., Ltd (41303101; Hubei, China). Montelukast and Loratadine were from MSD Pharmaceutical Co., Ltd (K004567; Hangzhou, China) and XianLin BaoYa Pharmaceutical Co., Ltd (12CRXF1018; Shanghai, China), respectively.

Experimental AD relapsing model and medication

Male BALB/c mice of 6-8 weeks old were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (SCXK-2012-0002, Shanghai, China). Mice were maintained at Nanjing University of Chinese Medicine under specific pathogen-free conditions. All procedures and 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. The FITC-induced AD-Re mouse model was established as reported previously [13]. Briefly, mice abdomens were shaved with an area of approximately $3 \times 3 \text{cm}^2$ on day 0, topically sensitized with 80 μl of 1.5% FITC (Sigma-Aldrich, St. Louis, MO, USA) solution on days 1 and 2, and the right ear was treated with 20 μl of 0.6% FITC solution on day 6 (elicitation). The initial allergic inflammation was established on day 7 (24 h after elicitation). From day

7 to day 14, mice were given no treatment, allowing inflammation to subside naturally, which was confirmed by the ear swelling measurement with a thickness gauge (7301; Mitutoyo, Kawasaki, Japan). Mice were then preventively treated with AS-IV (12.5, 25, 50 mg/kg/day, i.g.), Lor (1.3 mg/kg/day, i.g.), Mon (1.3 mg/kg/day, i.g.), or Dex (0.67 mg/kg/day, i.p.) for 10 days in the remission phase of AD-Re. 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 ears. Mice were then sacrificed and samples taken. Thymus index was calculated as the ratio of the weight of thymus glands to the body weight of mice.

Determination of cytokines and IgE production

Production of IL-4, IL-5, IL-13, and IFN- γ in mice ear tissue homogenates and IgE in serum were measured by ELISAs (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Histology

Ear tissue was fixed in 10% formalin immediately after mice were euthanized. Paraffin-embedded sections (4 μ m) of the ear tissue were stained with hematoxylin and eosin (H&E) for histology analysis.

Pharmacokinetic study of AS-IV *in vivo*

To evaluate the pharmacokinetics of AS-IV in the remission of AD-Re model, male BALB/c mice of 6-8 weeks old were divided into two groups (Control and AD-Re). Control: mice were treated with AS-IV (25mg/kg/day, i.g.) for 10 days; AD-Re: mice were treated with AS-IV (25mg/kg/day, i.g.) from day 14 to day 23 for 10 days in the remission phase of AD-Re. Blood samples were collected into heparinized tubes *via* the *oculi chorioideae* vein at 0.167, 0.333, 0.667, 1, 2, 4, 8, 12 and 24 h after the final administration (4 animals/group were collected for each time point). The blood samples were centrifuged at 4000 rpm for 15 min, and the plasma samples obtained were stored at -20 °C until the analysis.

HPLC-MS determination of AS-IV

The determination of AS-IV was performed on the Waters ZQ 2000 LC/MS Waters 2695 HPLC System with 2996 PDA Detector (Waters, USA). The chromatographic analysis of AS-IV was performed on a Waters Chrom-matrix GP-C₁₈ ODS column (4.6×250 mm, 5 μ) at 30 °C. The mobile phase was methanol and water (75:25, v:v) at a flow rate of 0.2 mL/min. The positive ion electrospray ionization mode was used for mass spectrometry. The precursor ion and product ion are m/z 807.2→627.2⁺ for AS-IV and m/z 493.8→368.9⁺ for glibenclamide, respectively. The collision energy for AS-IV and glibenclamide was 53 and 14 eV, respectively. The MS conditions were optimized as follows: ion spray voltage, 4600V; nebulizer gas pressure (N₂), 15 psi; drying gas flow (N₂), 10 L/min; Desolvation Temp, 360 °C.

Western blots

Ear tissue lysates were subjected to SDS-PAGE and Western blots analysis with the use of anti-TLR4 (1:1000 dilution; Santa Cruz Biotechnology), anti-TLR8 (1:1000 dilution; MultiSciences), anti-MyD88 (1:1000 dilution; Cell Signaling Technology), anti-TIRAP (1:1000 dilution; GeneTex), and anti-GAPDH (1:1000 dilution; Cell Signaling Technology) antibodies followed by horseradish peroxidase-electrochemiluminescence (HRP-ECL) detection (Millipore).

Quantitative real-time PCR

Gene expression of *TLR2*, *TLR3*, *TLR4*, *TLR8*, *TLR9* and *NF- κ B* in ear tissue were detected by quantitative real-time PCR as described previously [15]. The oligonucleotide sequences of primers (GenScript Biotech Corp, Nanjing, China) applied were 5'-ATCAGTCCCAAAGTCTAAAGTCG-3' (S) and 5'-ATGCCAGCTTCTTCATCGGT-3' (AS) for *TLR2*, 5'-AACGGTTCCTTCTCCTATCTCC-3' (S) and 5'-CACTTTGCTTAGTAAATGCTCGC-3' (AS) for *TLR3*, 5'-CTGTCTTACTACACCGCTATTTGG-3' (S) and 5'-ATGGGAAGAAAACCTTTACAG-3' for *TLR9*, 5'-TCTCTATGACCTGGACGACTCTT-3' (S) and 5'-GCTCATACGGTTTCCCATTTAGT-3' (AS) for *NF- κ B*; 5'-TGGTTTACACGTCCATCGGT-3' (S) and 5'-ATCAATGGTCACATCACATAGTCC-3' (AS) for *TLR4*; 5'-CTCCAGTATTTTCTCACCTTCA-3' (S) and 5'-GATTATGGCTCAGTAGCAGTGTCTC-3' (AS) for *TLR8*; 5'-GGTTGTCTCCTGCGACTTCA-3' (S) and 5'-TGGTCCAGGGTTTCTTACTCC-3' (AS) for *GAPDH*.

Statistical analysis

Data are expressed as means \pm standard deviations (SD). One-way ANOVA analysis was used for multiple groups comparisons and the unpaired two-tailed Student's *t*-test was used for comparison between two groups, using GraphPad Prism 7 (GraphPad Software, CA, USA). A statistical value of $p < 0.05$ was considered significant.

Results

1. AS-IV attenuated recurrent allergic inflammation in AD relapse model

Based on the well-established FITC-induced AD-Re model [13], we firstly evaluate the effects of AS-IV on allergic inflammation relapsing as outlined (Fig 1a). Briefly, the initial AD allergic inflammation was established on day 7 (Fig 1b). From day 7 to day 14, mice were given no treatment, allowing the allergic inflammation to subside naturally, and AD mice were then divided into 7 groups for further indicated medication treatments on day 14 (Fig.1C). AS-IV (12.5, 25, 50 mg/kg), Lor (1.3 mg/kg), Mon (1.3 mg/kg), or Dex (0.67 mg/kg) were administered from day 14 in remission phase and terminated at 1 h before re-challenged on day 23 (Fig 1a). Ear swelling in AD-Re group increased remarkably at 6 h after re-challenge on day 23 (Fig 1d). Three commonly applied drugs Dex, Mon, and Lor, as well as three doses of AS-IV (12.5, 25, 50 mg/kg) significantly suppressed ear swelling and inflammatory cell infiltration in recurrent phase of AD compared to the untreated AD-Re group (Fig 1d and 1e). These results suggested that preventive administration of AS-IV and conventional medicines, which terminated with a short time prior to allergen re-exposure, effectively attenuated recurrent AD allergic inflammation.

2. AS-IV exhibited advantages in anti-recurrent allergic inflammation

Clinically, allergic inflammation relapsing occurs unpredictably, we then identified whether AS-IV administrated in remission phase of AD-Re could maintain a long-term anti-recurrent allergic inflammation efficacy. Medications were administrated as described above and terminated at 24 h before final re-challenge on day 24 (Fig 2a). Ear swelling of AD-Re mice was measured at 4, 8 and 12 h after final re-challenge. As shown in Fig 2b, ear swelling of AD-Re mice increased dramatically at 12 h after final challenge. Three conventional drugs (Dex, Mon, and Lor) showed barely effect in AD-Re mice (Fig 2b). On the contrary, AS-IV (12.5, 25, 50 mg/kg) alleviated ear swelling and inflammatory cell infiltration notably compared to AD-Re mice (Fig 2b and 2c). In addition, serum levels of IgE in recurrent phase of AD also decreased significantly in AS-IV (25, 50 mg/kg) treated mice (Fig 2d). However, in contrast to Dex, AS-IV with three doses (12.5, 25, 50 mg/kg) showed no obvious effect on thymus index (Fig 2e). These results indicated AS-IV administration in remission phase of AD exhibited a superior effect in maintaining remission compared with conventional drugs.

3. AS-IV inhibited Th2 cytokines production in AD relapse model

Since AD is mainly mediated by type 2 immunity, the effects of AS-IV on T helper 2 (Th2) cytokines (including IL-4, IL-5, and IL-13) production from AD-Re mice were then evaluated. As shown in Fig 2f, IL-4 levels of ear homogenates reduced significantly with Dex or Lor treatment compared with AD-Re mice, but not as potent as AS-IV (25, 50 mg/kg) being capable to decrease to a similar level with control. Furthermore, both IL-5 and IL-13 levels of ear homogenates attenuated remarkably with AS-IV (25, 50 mg/kg) administration compared to AD-Re mice (Fig 2g and 2h). In contrast with AS-IV, three conventional medicines showed no evident efficacy (Fig 2g and 2h). In addition, none of these medications showed notable effect on IFN- γ production (Fig 2i), which is a typical Th1 cytokine. These results indicated AS-IV preventive administration inhibited Th2 cytokines production in AD-Re model.

4. Pharmacokinetics of AS-IV in remission phase of AD relapse model

Previously, we have found that in remission phase of AD, although the allergic inflammation subsided, the underlying pathological changes were still existed [13]. We next evaluated the pharmacokinetic characters of AS-IV (25 mg/kg) with prophylactic administration in remission phase from AD-Re mice compared with control mice. As shown in Tab 1, the T_{max} of AS-IV in control group and in remission phase of AD-Re group were both reached at 2 h. However, the $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ of AS-IV in remission phase of AD-Re group increased significantly compared with control group ($p < 0.05$). Consistently, the C_{max} of AS-IV was raised in AD-Re mice with a statistical difference compared with control (Fig 3 and Tab 1). In addition, the CLz/F of AS-IV in remission phase of AD-Re mice decreased notably as opposed to control mice ($p < 0.05$). The different pharmacokinetic profiles of AS-IV between AD-Re and control indicated that the pathological changes still existed in remission phase of AD, which confirmed with our previous findings. It also suggested that AS-IV effectively attenuated the recurrent allergic inflammation

might due to its regulation of certain underlying pathological processes of AD rather than drug retention, since the blood concentration of AS-IV had dropped to 1.661 ± 1.83 ng/ml after 12 h administration.

5. AS-IV regulated TLR8-mediated NF- κ B pathway in AD relapse model

In view of the pharmacokinetic profiles of AS-IV in remission phase of AD-Re, we then speculated that AS-IV administration in remission phase might regulate certain potential pathological process of AD. It has been widely recognized that alterations of TLRs play a potent role in susceptibility to AD. Therefore, we assessed the gene expression of TLRs in ear tissue with AS-IV (25 mg/kg) treatment in remission phase of AD-Re before final re-challenge (Fig 4a). The gene expression of *TLR2* in ear tissue was down-regulated obviously with AS-IV treatment compared with AD-Re group, however no significant changes between AD-Re and control mice (Fig 4b). In addition, gene expression of *TLR3*, *TLR4* and *TLR9* in ear homogenates showed no detectable changes (Fig 4c, 4d, and 4f). Notably, gene expression of *NF- κ B* in ear homogenates of AD-Re group increased significantly compared with control group, which decreased dramatically with AS-IV treatment (Fig 4g). Consistently, the gene expression and protein level of TLR8 increased in AD-Re group, but reduced obviously with AS-IV treatment (Fig 4e and 5h). Moreover, protein levels of TIRAP and MyD88 in ear tissue also reduced obviously in AS-IV treated mice (Fig 4h). These results suggested that AS-IV administration in remission phase of AD could regulate TLR8-mediated NF- κ B pathway to against recurrence.

Discussion

Although topical corticosteroids as well as non-specific immunosuppressive drugs control the symptoms and inflammation successfully, the recurrence of AD is still a clinical challenge. One of the reasons may due to the underlying pathogenesis of AD recurrence is still unclear. YPFS as a potent TCM against allergic disorder, applied in remission phase has been proven efficient through thousands of years of Chinese history [11, 12]. Revealing the pharmacological mechanism of YPFS against AD recurrence, may help us to improve understanding the mechanisms of AD recurrence. We previously found that in remission phase of AD, although the major symptoms and inflammation subsided, the pathological changes still existed, especially the deficiency in tight junction protein (occludin and CLDN1) [13].

AS-IV has been shown to be effective in relieving allergic asthma [16, 17]. As a representative bioactive compound of YPFS, we reported AS-IV ameliorated allergic inflammation in sensitization stage of AD by inhibiting TSLP and IL-33 production [14]. In this study, we firstly investigated the effect of AS-IV on anti-AD recurrence. Medications in remission phase of AD and re-exposure to allergens in a short time, AS-IV and conventional clinical drugs (Dex, Mon and Lor) alleviated the recurrent inflammation of AD significantly. However, with prolonged drug withdrawal, only AS-IV attenuated ear swelling and inflammatory cell infiltration as well as IgE production in recurrent phase of AD. AS-IV has been reported to down-regulate inflammatory cytokines such as TNF- α , IL-1 β , and TGF- β [18]. However, the immune response in AD is skewed towards Th2-mediated pathway and can in turn favour epidermal barrier disruption [1]. Therefore, we then investigated the effects of AS-IV on Th2 cytokines production in AD-Re.

Unlike Dex, Mon, and Lor, AS-IV could remarkably damp the IL-4, IL-5, and IL-13 levels of ear homogenates in AD-Re mice, but showed feeble effect on IFN- γ production. These indicated AS-IV could specifically decrease Th2 cytokines production in AD-Re model.

Recently, HPLC-MS becomes a powerful tool for qualitative and quantitative analysis of pharmaceutical ingredients. As a special saponin, AS-IV has low gastrointestinal tract absorption and bioavailability in rat and dog [19, 20]. Interestingly, we found a different pharmacokinetic profile of AS-IV (administration in remission phase) between AD-Re and control mice. It has been pointed out that the disease status can modify the pharmacokinetic characters of drugs [21]. Therefore, our results further confirmed the pathological changes still existed in remission phase of AD. It also suggested that AS-IV attenuated the recurrent allergic inflammation might due to its regulation of certain underlying pathological processes in remission phase of AD rather than drug retention.

Impairment of epidermal barrier function, for instance, due to deficiency in the structural proteins, have been recognized contributing to AD aetiology and clinical manifestation. We previously found the defects of tight junction protein including occludin and CLDN1 were observed in remission phase of AD. However, AS-IV appeared had barely effects on CLDN1 and occludin (data not shown) expression in AD-Re. In addition, TLRs play a fundamental role in detecting invading pathogens or damage and initiating the innate immune system of mammalian cells. Alterations of TLRs play a potent role in susceptibility to AD [22]. AS-IV presented a wide range of pharmacological effects through multiple pathways, however, most of them are related to TLR4-mediated NF- κ B signalling pathways [8–10]. Therefore, we assessed several TLRs gene expression of epithelium in remission phase of AD-Re, and evaluated the effects of AS-IV on TLRs expression. As shown in Fig. 4, no obvious changes were observed in terms of *TLR2*, *TLR3*, *TLR4* and *TLR9* gene expression in remission phase of AD. In contrast, both gene expression and protein level of TLR8 increased in AD-Re group, but down-regulated with AS-IV treatment. Consistently, protein levels of TIRAP and MyD88, the adapter proteins of TLR8 in ear tissue also reduced obviously with AS-IV treatment. Moreover, the downstream transcription factor NF- κ B showed a similar trend as TLR8. TLR8 was initially considered to be inactive in mice [23]. Until recently, the potential role of TLR8 in the generation of a critical immune response against bacterial infection and cancer has just begun to be uncovered [24]. Our finding indicated TLR8-mediated NF- κ B pathway may also play an important role in AD recurrence pathogenesis. AS-IV administration in remission phase of AD could regulate TLR8-mediated NF- κ B pathway to against recurrence.

Conclusions

AS-IV administration in remission phase of AD attenuated the recurrent allergic inflammation of AD, along with inhibited Th2 cytokines production and presented a different pharmacokinetic profile. Through regulating TLR8-mediated NF- κ B pathway might be the potential mechanism of AS-IV against AD recurrence. These results further improved our understanding of the pathogenesis of AD recurrence and the pharmacological effects of AS-IV.

Abbreviations

AD, atopic dermatitis; AS-IV, Astragaloside IV; TCM, traditional Chinese medicine; Dex, Dexamethasone; Mon, Montelukast; Lor, Loratadine; FITC, fluorescein isothiocyanate; AD-Re, atopic dermatitis relapse; Th, T helper; TLR, Toll-like receptor; TIRAP, Toll-interleukin1 receptor (TIR) domain containing adaptor protein; MyD88, myeloid differentiation factor 88; YPFS, Yu-Ping-Feng-San; TSLP, thymic stromal lymphopoietin

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors confirm that there are no conflicts of interest.

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Authors' contributions

JZ and MH designed the experiments. XY and XW performed the experiments and collected data; JZ, XY, and XW prepared the figures and analyzed the data. HF assisted with data analysis. The manuscript was written, revised, and edited by JZ and MH.

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Not applicable.

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Table

Tab 1 Main pharmacokinetic parameters of AS-IV in remission phase of AD relapse model
(mean±SD, n=4)

Parameters	Unit	Control	AD-Re
AUC _(0-t)	μg/L×h	81.398±18.931	146.131±49.263*
AUC _(0-∞)	μg/L×h	82.55±20.165	148.687±50.085*
t _{1/2}	h	2.96±1.214	3.225±1.872
T _{max}	h	2±0	2±0
CL _{Z/F}	L/h/kg	317.771±82.035	184.686±66.2*
V _{Z/F}	L/kg	1296.364±361.63	825.039±527.105
C _{max}	μg/L	15.267±3.333	34.581±15.069*

* $p < 0.05$ indicates significant differences from the control.

Figures

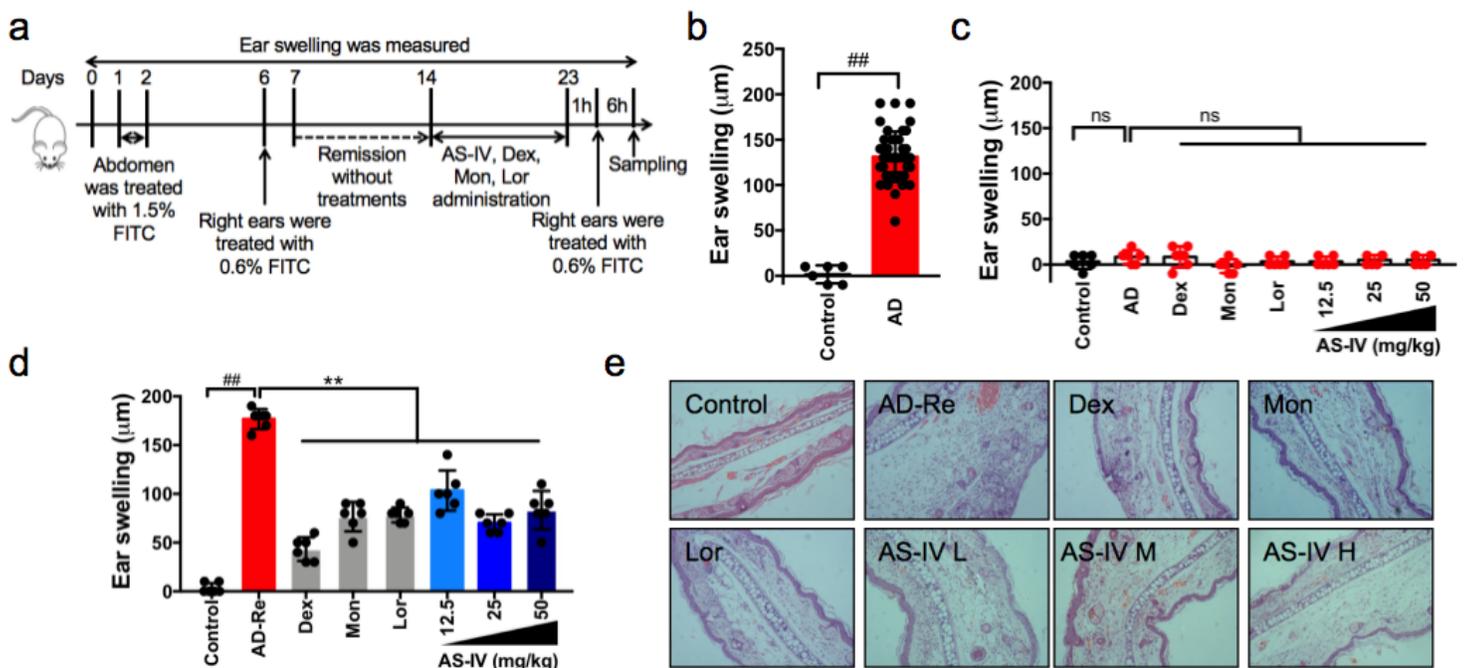


Figure 1

AS-IV attenuated recurrent allergic inflammation in AD-Re model (a) Overview of FITC-induced AD relapsing mouse model (AD-Re) and medications used: AS-IV (L=12.5, M=25, H=50 mg/kg/day, i.g.), Lor (1.3 mg/kg/day, i.g.), Mon (1.3 mg/kg/day, i.g.), or Dex (0.67 mg/kg/day, i.p.) for 10 days. (b) Ear swelling was measured at 24 h after FITC elicitation on day 7 (control group n=6, AD group n=42). (c) Ear swelling was measured at on day 14, AD group was divided into 7 groups for further different medication treatments (n=6/group). (d) Ear swelling was measured at 6 h after FITC rechallenge on day 23

(n=6/group). (e) H&E staining of ear tissue from FITC-induced AD-Re mice with indicated medication treatments and control mice (n=6/group, magnification: × 200). (## p<0.01 vs control group, ** p<0.01 vs AD-Re).

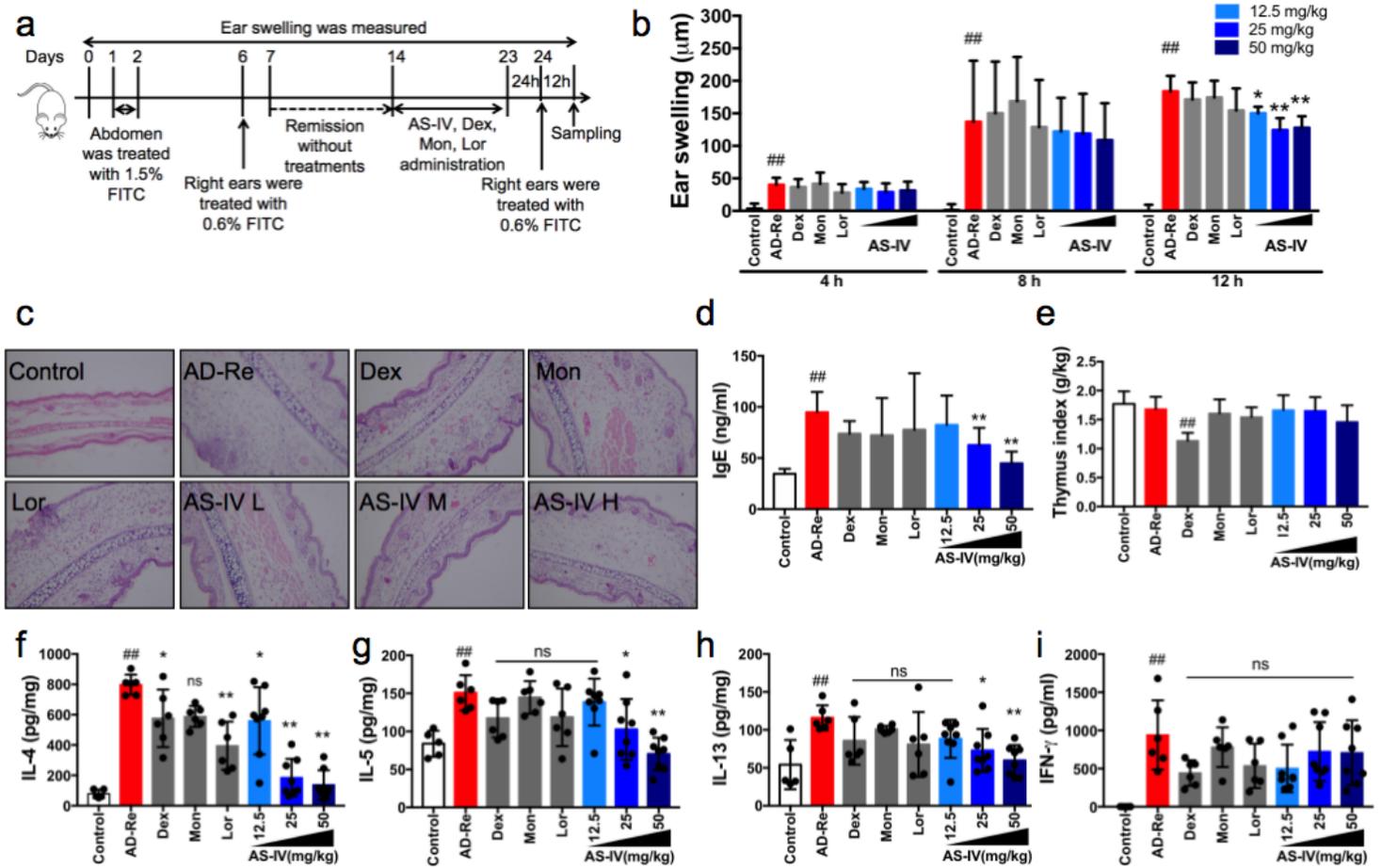


Figure 2

AS-IV exhibited advantages in resisting allergic inflammation recurrence (a) Overview of FITC-induced AD-Re mice and medications used: AS-IV (12.5, 25, 50 mg/kg/day, i.g.), Lor (1.3 mg/kg/day, i.g.), Mon (1.3 mg/kg/day, i.g.), or Dex (0.67 mg/kg/day, i.p.) for 10 days. (b) Ear swelling was measured at 4, 8 and 12 h after FITC rechallenge on day 24. (c) H&E staining of ear tissue from FITC-induced AD-Re mice with indicated treatments and control mice were observed at 12 h after final challenge (AS-IV: L=12.5 mg/kg, M=25 mg/kg, H=50 mg/kg, n=5-8/group, magnification: × 200). (d) Serum IgE level was evaluated at 12 h after FITC challenge. (e) Thymus index was assessed at 12 h after FITC final challenge. Cytokines production was evaluated at 12 h after FITC challenge (f) IL-4, (g) IL-5, (h) IL-13, (i) IFN-γ (n=5-8/group, ## p<0.01 vs control group, *p<0.05, ** p<0.01 vs AD-Re).

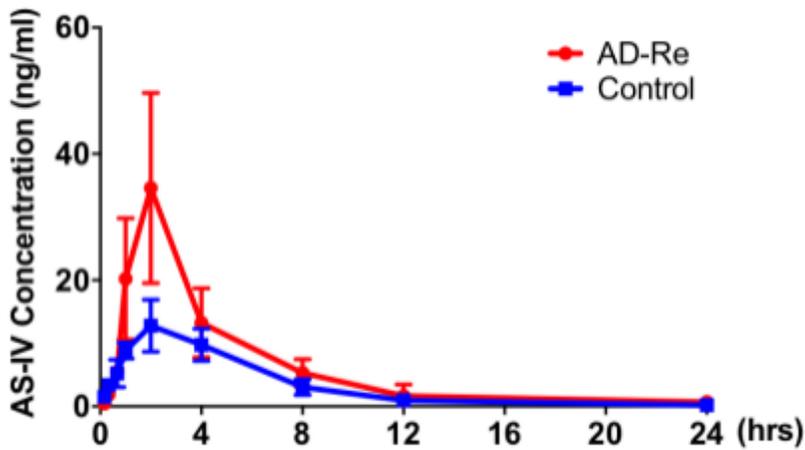


Figure 3

The pharmacokinetic profiles of AS-IV in remission phase of AD-Re model. The pharmacokinetic profiles of AS-IV (25 mg/kg/day) at indicated time point after preventively administration for 10 days in remission phase of AD relapse model (n=4 in each group / time point).

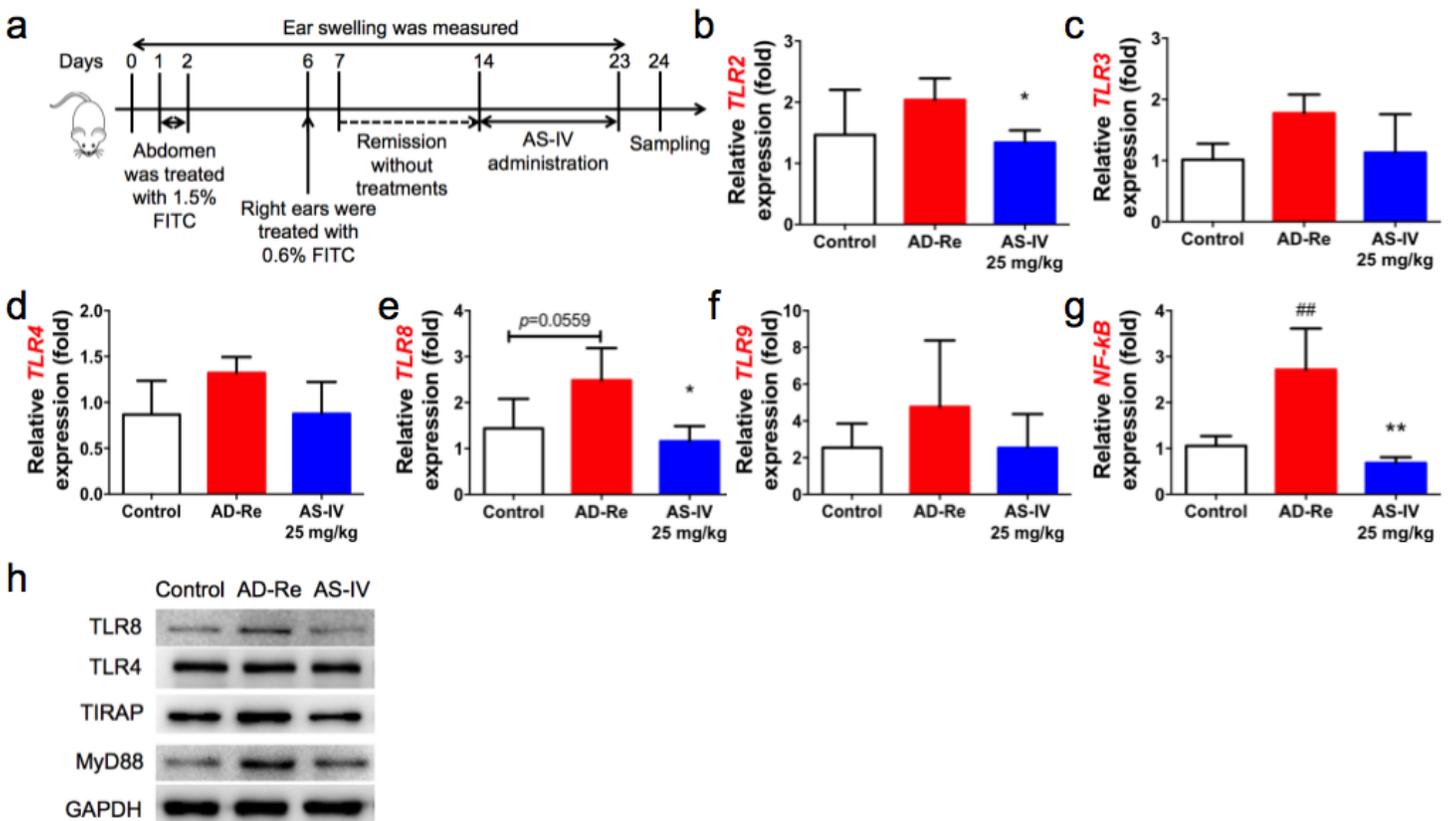


Figure 4

AS-IV regulated TLR8-mediated NF-kB in AD-Re model (a) Overview of FITC-induced AD-Re without re-challenge and AS-IV medication (25 mg/kg) in remission phase for 10 days. (b-g) Gene expression of TLR2, TLR3, TLR4, TLR8, TLR9 and NF-kB in ear tissue were detected by quantitative real-time PCR. (n=4,

p<0.01 vs control group, *p<0.05, ** p<0.01 vs AD-Re group). (h) Protein levels of TLR8, TLR4, TIRAP and MyD88 in ear tissue of AD-Re were analyzed by western blotting.