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IL-37 is protective in allergic contact dermatitis through mast cell inhibition

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ABSTRACT

Allergic contact dermatitis (ACD), characterized predominantly by erythema, vesiculation, and pruritus, is a T cell-mediated skin inflammatory condition. Among immune cells involved in ACD, mast cells (MCs) play an essential role in its pathogenesis. As an inhibitor of proinflammatory IL-1 family members, interleukin 37 (IL-37) has been shown to ameliorate inflammatory responses in various allergic diseases. In this study, we assessed the immunomodulatory effect of IL-37 on allergic inflammation using a 2,4-dinitrofluorobenzene (DNFB)-induced ACD rat model and isolated rat peritoneal mast cells (RPMCs). Systematic application of IL-37 significantly relieved ear swelling, reduced inflammatory cell infiltration, decreased inflammatory cytokine production (TNF-α, IL-1β, IFN-γ, and IL-13), inhibited MC recruitment, lowered IgE levels, and reduced IL-33 production in the local ear tissues with DNFB challenge. Additionally, RPMCs isolated from ACD rats with IL-37 intervention showed downregulation of IL-6, TNF-α, IL-13, and MCP-1 production following IL-33 stimulation, and reduction of β-hexosaminidase and histamine release under DNP-IgE/HSA treatment. Moreover, IL-37 treatment also significantly restrained NF-κB activation and P38 phosphorylation in ACD RPMCs. SIS3, a specific Smad3 inhibitor, abolished the suppressive effects of IL-37 on MC-mediated allergic inflammation, suggesting the participation of Smad3 in the anti-ACD effect of IL-37. These findings indicated that IL-37 protects against IL-33-regulated MC inflammatory responses via inhibition of NF-κB and P38 MAPK activation accompanying the regulation of Smad3 in rats with ACD.

1. Introduction

Allergic contact dermatitis (ACD) is a frequent skin inflammatory disease that is caused by repeated exposure to contact allergens, which generally need to be linked by a protein in the epidermis to constitute a new antigenic determinant, eliciting activation of the immune system [1]. Among immune cells involved in ACD, including keratinocytes, dendritic cells (DCs), neutrophils, and T cells, mast cells (MCs) have been indicated to play a fundamental role in its pathogenesis [2]. On one hand, increased MCs are recruited to dermis upon allergen stimulation and are favorable to activation of keratinocytes, infiltration of neutrophils, maturation and migration of DCs during sensitization stage [3]. On the other hand, the contact allergens trigger MC degranulation, inducing the immediate release of preformed granules containing histamine, proteases, and proteoglycans [4]. Additionally, the MCs also secrete late proinflammatory mediators, such as interleukin-4 (IL-4), IL-5, IL-6, IL-13, TNF-α, and chemokines, such as CCL2, CCL3, and CCL4 [5]. Due to its essential role in ACD, inhibition of MC activation (degranulation and inflammatory cytokine production), is considered as a promising therapeutic strategy for the skin inflammatory disease.

As a novel anti-inflammatory cytokine, IL-37 is a newly discovered member of proinflammatory IL-1 family and has displayed its immunomodulative effects in allergic and inflammatory disease [6,7]. Exogenous IL-37 functions by forming a complex with IL-18Rα and IL-1R8 extracellularly, and endogenous IL-37 translocates into nucleus upon assembling with mothers against decapentaplegic homolog 3 (Smad3) intracellularly, thus regulating gene transcription and signaling pathway activation [8]. So far, five structurally related isoforms

Abbreviations: IL-37, interleukin 37; MCs, mast cells; RPMCs, rat peritoneal mast cells; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; Anti-DNP IgE, anti-dinitrophenyl immunoglobulin E; DNP-HSA, dinitrophenyl-human serum albumin

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of human IL-37 have been identified, IL-37 a-e, but IL-37b is the most mentioned and reported [9]. A previous study revealed that compared with wild-type mice, IL-37tg mice showed fewer contact hypersensitivity to DNFB application, such as ear swelling and inflammatory cytokine production, owing to tolerance of DCs expressing IL-37 thereby impairing activation of effector T cells and induction of Treg cells [10]. However, the effects and mechanisms of systemic administration of IL-37 on MC activation in rats with ACD remain largely unknown.

In this study, we investigated the anti-inflammatory effect of IL-37 using an ACD rat model. Our results revealed that IL-37 attenuated DNFB-induced ACD in rats and inhibited MC-mediated inflammatory responses by restraining NF-κB and P38 activation with the involvement of Smad3.

2. Materials and methods

2.1. Animals

Six-week-old female Sprague-Dawley rats (weighing 180 ± 20 g) were purchased from Hubei Research Center of Laboratory Animals (Wuhan, China). Rats were housed in a specific pathogen-free facility under a 12-h light/dark cycle (temperature 24 ± 2 ˚C, relative humidity 50 ± 5%) at the Animal Care Facility of Hubei University of Chinese Medicine (Wuhan, China). Food and water were provided ad libitum throughout the experiments. Animal care and use were approved by the Animal Care and Use Committee of Hubei University of Chinese Medicine (No.: SYXK2017-0067) and also complied with the National Institutes of Health guide for the care and use of Laboratory animals.

2.2. 2,4-dinitrofluorobenzene (DNFB)-induced ACD model

On day 0, hind flank of rats was shaved. Then, the rats were sensitized with 25 μL 0.5% DNFB (acetone : olive oil = 4 : 1) (Sigma-Aldrich, St. Louis, MO, USA) topically on day 1 and 2. After 5 days, the left ears were challenged by 10 μL 0.2% DNFB to induce ACD.

2.3. Groups

Rats were randomly divided into five groups (n = 8 per group): Control group, ACD group, IL-37 + ACD group (3 μg recombinant human IL-37b was injected intraperitoneally from day 1 to day 7 during induction of ACD), SIS3 + ACD group (2 mg/kg SIS3 was intraperitoneally applied from day 1 to day 7 following the ACD process), and SIS3 + IL-37 + ACD group (2 mg/kg SIS3 was intraperitoneally administrated for 7 consecutive days accompanying the same treatment as in IL-37 + ACD group). Both IL-37b (7585-IL-025/CF) and SIS3 (5291/50) were purchased from R&D Systems Inc. (Minneapolis, MN, USA).

2.4. Evaluation of ear swelling

Ear thicknesses were measured using a thickness gauge (Digimatic caliper, Mitutoyo, Japan) at 24 h after the challenge of DNFB (Day 8). Ear swelling increment = thickness of DNFB-challenged ear (left) − thickness of unchallenged ear (right).

2.5. Histological analysis

The left ears were removed and fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich). Fixed tissues were embedded in paraffin for 24 h and serially sectioned to a thickness of 5 μm for histological analysis. Tissue sections were stained with hematoxylin and eosin (H&E), and then evaluated independently by 2 evaluators in a blinded fashion under a light microscope (BX41, Olympus, Tokyo, Japan). For analysis of inflammatory cell infiltration, five sections per rat was examined and ten visual fields of each sample were randomly selected to count.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The left ear samples were collected and homogenized in 1 mL of tissue protein extraction reagent (P0013K, Beyotime Institute of Biotechnology, Shanghai, China). Homogenates were then centrifuged at 12,000 × g for 15 min at 4 ˚C to obtain the supernatant. Cytokine production, including TNF-α (ab100785), IL-1β (ab100768), IFN-γ (ab46107), IL-13 (ab100766), IL-33 (ab236714), and IgE (ab157736), were detected by the ELISA kits (Abcam, Cambridge, MA, USA) using a Multiskan MK3 microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. All the experiments were done in triplicate.

2.7. Toluidine blue staining

The left ears of rats were removed, fixed, embedded and serially sectioned to a thickness of 5 μm for histological analysis. Tissue sections were stained with toluidine blue and observed under a Nikon Eclipse Ti-S microscope (Nikon, Tokyo, Japan). Ten different fields of each section were selected, and the number of metachromatic cells were counted manually in five sections per rat. Lastly, the results from eight rats were averaged.

2.8. Isolation of rat peritoneal mast cells (RPMCs)

At the end of the experiment, rats were anesthetized with 7% Chloral hydrate ip (0.5 mL/100 g). Twenty milliliters of DMEM with 10% serum containing penicillin (100 U/mL), streptomycin (100 μg/mL) and heparin (5 U/mL) were injected into the peritoneal cavity, followed by gentle massage and recovery of fluid into cooled polypropylene tubes. After the lavage was completed, the rats were euthanized with cervical dislocation. The cells were centrifuged at 400 × g for 15 min at room temperature and washed twice with serum-free DMEM. Cell pellets were resuspended in 1 mL serum-free DMEM, and macrophages were separated from MCs by differential centrifugation using a Percoll solution as described before. MCs isolated by this procedure exceed 95% in purity as determined by light microscopy with toluidine blue staining [11]. Approximately 1×10^6 RPMCs are usually harvested from one rat.

2.9. Assay of IL-33-induced inflammatory cytokine production

Isolated RPMCs (2×10^5) were cultured and stimulated with IL-33 (50 ng/mL) (NBP2-35248, Novus Biological, Littleton, Colorado, USA). After 8 h, cytokine productions in the supernatants were measured by ELISA and statistically analyzed, including IL-6 (ab100772), TNF-α (ab100785), IL-13 (ab100766), and MCP-1 (ab219045) (Abcam). All the experiments were done in triplicate.

2.10. β-hexosaminidase and histamine release

RPMCs were cultured in 12-well plates at a density of 2×10^5 cells/well and incubated overnight with anti-dinitrophenyl immunoglobulin E (Anti-DNP IgE, 500 ng/mL) (Sigma-Aldrich), followed by stimulation with dinitrophenyl-human serum albumin (DNP-HSA, 100 ng/mL) (Sigma-Aldrich) for 4 h. Then, the cells were centrifugated at 150 × g for 10 min at 4 ˚C. The supernatant (50 μL) was transferred to 96-well plates and incubated with an equal volume of substrate solution (1 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide in 0.1 M citrate buffer, pH = 4.5) for 1 h at 37 ˚C. The cells were lysed with 0.5% Triton X-100 (50 μL) and lysates were collected to measure total β-hexosaminidase activity. The reaction was stopped by adding 150 μL stop solution (0.1 M Na_2CO_3-$NaHCO_3$, pH = 10). The absorbance was measured at

405 nm. β-hexosaminidase release (%) = ($OD_{supernatant}$ − $OD_{control}$)/(OD_{total} − $OD_{control}$) × 100%.

Histamine concentration in culture supernatant of RPMCs were detected using an ELISA kit (KT-60094) (Kamiya Biomedical, Seattle, WA, USA) according to the manufacturer's protocols.

2.11. Signal transduction inhibitors assay

Signal transduction inhibitors, such as BAY 11-7085 (NF-κB inhibitor, 5 μM) (ab141574, Abcam), SB203580 (P38 inhibitor, 10 μM) (#5633S), PD98059 (ERK inhibitor, 10 μM) (#9900L), and SP600125 (JNK inhibitor, 10 μM) (#8177S) (Cell Signaling Technology Inc., Beverly, Massachusetts, USA). Inhibitors were added to culture 2 h prior to stimulation with IL-33 (50 ng/mL), respectively. After 8 h, the supernatants were collected for ELISA assay.

2.12. Quantitative real-time PCR (qRT-PCR)

Total RNA were obtained from the RPMCs using TRizol reagent (Invitrogen, Carlsbad, CA, USA). RNA (50 ng) was used for primer-specific reverse transcription (RT) using the RT reagent Kit (Applied Biosystems Life Technologies, Foster City, CA, USA) following the manufacturer's instructions. Real-time PCR was performed on the 7300 Sequence Detection System using a SYBR-Green Master PCR Mix (Applied Biosystems). Amplification conditions: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 1 min. The primer sequences were: ST2-F CGCCTGTTCAGTGGTTTA, ST2-R TGGTTCCGTTCTCCGTGT; Smad3-F TGGACGCAGGTTCTCCAAAC, Smad3-R CCGGCTCGCAGTAGGTAAC; GAPDH-F GGCCCCTCTGGAAA GCTGTG, GAPDH-R CCGCCTGCTTCACCACCTTCT. To calculate the relative expression level of the products, the $2^{-\Delta\Delta Ct}$ method was used and results were normalized to GAPDH.

2.13. Cytosolic protein and nuclear protein extraction

RPMCs were collected for protein extraction on ice using a Nuclear Protein and Cytosolic Protein Extraction Kit (P0028) (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, cells were washed with cold PBS, resuspended in the protein extraction buffer A containing 1 mM PMSF (ST506) (Beyotime Institute of Biotechnology), and vortexed for 30 s at the highest speed. After incubated on ice for 15 min, the protein extraction buffer B was added, vortexed for 30 s, incubated on ice for 1 min, and then centrifugated at 12,000 × g for 5 min at 4 °C. The supernatants containing extracted cytoplasmic protein were completely drawn into a pre-cooled plastic tube. Next, remained precipitation was resuspended with the extraction buffer for nuclear protein, incubated on ice for 30 min (vortexed for 30 s every 2 min), and centrifugated at 12,000 g for 10 min at 4 °C. Then, the supernatants containing extracted nuclear protein were completely drawn into a pre-cooled plastic tube.

2.14. Western blot

Extracted supernatant of cytosolic protein nuclear protein were separately quantitated for protein concentration with a BCA Protein Assay kit (P0010) (Beyotime Institute of Biotechnology). Samples (30 μg) were loaded and separated by 10% serum dodecyl sulfate–polyacrylamide gels (SDS-PAGE). Then, the gels were electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat dry milk for 2 h at room temperature, the membranes were incubated with the primary antibodies against IκBα (9242), GAPDH (5174), NF-κB p65 (3034), Lamin B1 (13435) (Cell Signaling Technology Inc., Beverly, Massachusetts, USA), p-IKKα/β (ab194528), IKKα (ab32041), p-P38 (ab4822), P38 (ab27986), p-ERK (ab4819), ERK (ab17942), p-JNK (ab76572), and JNK (ab208035) (Abcam) overnight at 4 °C. Subsequently, the horseradish peroxidase-conjugated secondary antibody (ab6721) (Abcam) were used to incubate the membranes for 2 h at room temperature. Peroxidase-labeled protein bands were detected by enhanced chemiluminescence (ECL) kit (32106) (Pierce Biotechnology/Thermo Fisher Scientific, Rockford, IL, USA) and the protein intensity was analyzed with ImageJ Version 1.52 software (National Institutes of Health, Bethesda, MD, USA). Sample loading was normalized by quantities of GAPDH or Lamin B1 detected parallel.

2.15. Statistical analysis

Data were presented as means ± standard deviation (SD) and analyzed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). Curve estimation and linear regression analyses were performed for correlation analysis. Comparisons between two groups were done using an unpaired Student *t* test, and comparisons among multiple groups were done using one-way ANOVA by Tukey's *post hoc* test. $P < 0.05$ indicated statistical significance.

3. Results

3.1. IL-37 attenuates DNFB-induced ACD in rats

According to the experimental schedule (Fig. 1A), rats were sensitized and challenged with DNFB to induce ACD. Compared with untreated Control, edema and erythema were clearly visible on the left ear of ACD rats (Fig. 1B). Systemic administration of IL-37 significantly attenuated the ear swelling and also decreased the ear thickness increment compared with ACD group (Fig. 1C). Histological analysis showed that DNFB painting induced hyperplasia of subcutaneous tissue and attracted notable infiltration of inflammatory cells, but the administration of IL-37 clearly inhibited this induction (Fig. 1D). In addition, IL-37 treatment decreased the thickness of dermis and reduced the number of inflammatory cells recruited to the ear skin (Fig. 1E and F).

3.2. IL-37 plays an immunoregulatory role in rats with ACD

Under IL-37 treatment, levels of inflammatory cytokines (TNF-α, IL-1β, IFN-γ, and IL-13) in homogenate supernatant of the ear samples were evidently decreased compared with ACD group (Fig. 2A-D). Furthermore, IL-37 notably limited the enrollment of MCs, lowered the levels of IgE, and decreased the production of IL-33 (Fig. 2E-H). Correlation analysis showed that there was a positive correlation between MC infiltration and the levels of IgE in ACD group ($r = 0.7450$, $P = 0.0339$) (Fig. 2I). Simultaneously, a positive correlation between the number of MC infiltration and IL-33 production in ACD group also was presented ($r = 0.8506$, $P = 0.0074$) (Fig. 2J).

3.3. IL-37 suppresses IL-33-induced cytokine production in RPMCs

Considering the relationship between MC infiltration and IL-33 production as shown above, it was possible that IL-33-induced inflammation associated with MC activation contributed to the inflammatory processes of ACD. So, we further assessed the effect of IL-37 on IL-33-induced cytokine production using RPMCs. As shown in Fig. 3A-D, IL-37 clearly suppressed the production of proinflammatory cytokines (IL-6, TNF-α, IL-13, and MCP-1) induced by IL-33 in comparison with the ACD group.

3.4. IL-37 inhibits MC degranulation

β-hexosaminidase and histamine, both important markers of MC degranulation [12], were evaluated for if IL-37 inhibited MC degranulation in RPMCs in response to IgE/antigen stimulation. Compared to the ACD group, IL-37 treatment significantly inhibited β-

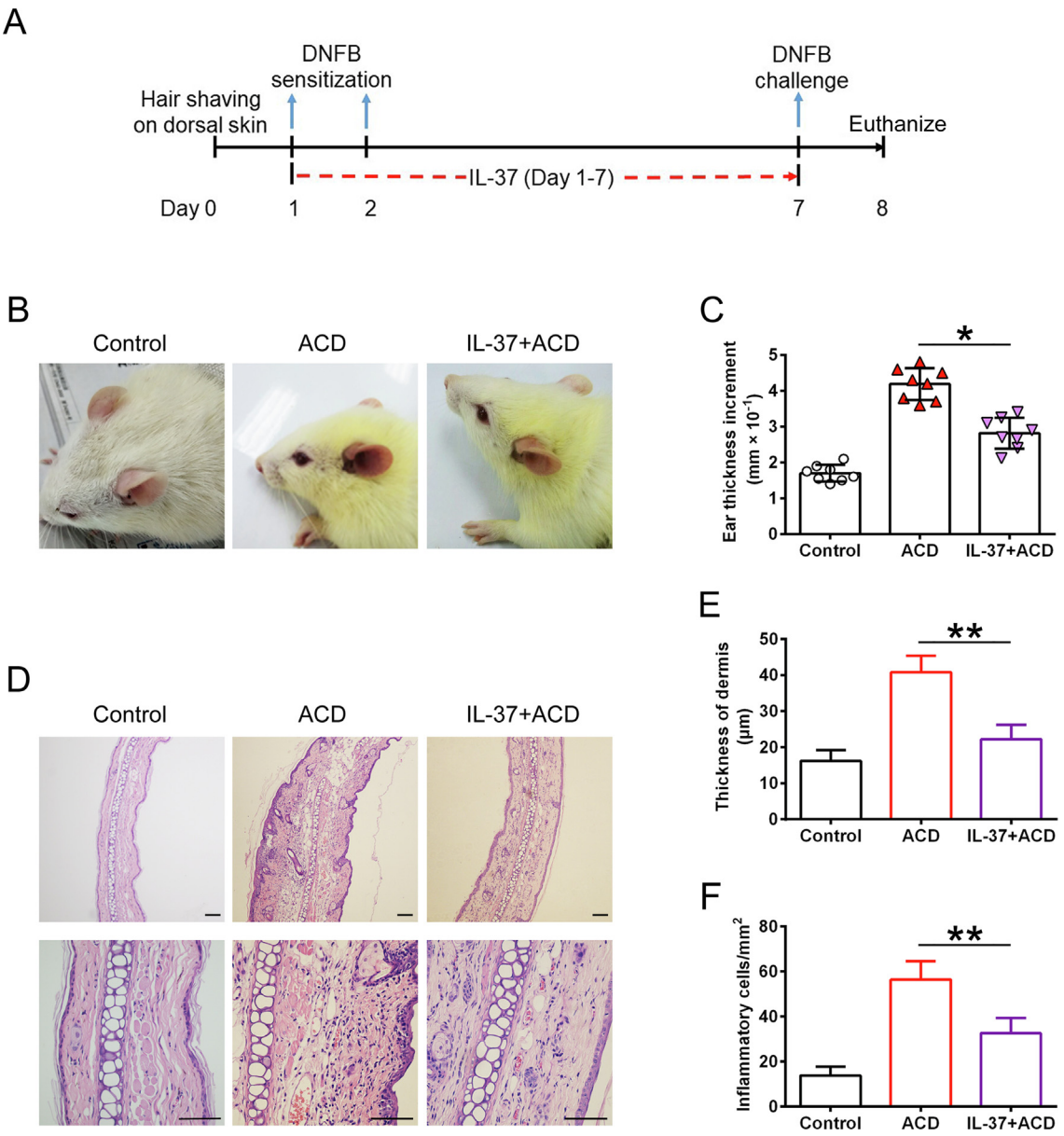

Fig. 1. IL-37 ameliorates DNFB-induced ACD in rats. (A) Experimental schedule for the induction of ACD by DNFB application and IL-37 treatment. (B) Photographs of ear swelling and (C) ear thickness increment in the different groups were shown, respectively. (D) Ear samples were obtained and stained using hematoxylin and eosin. Histological changes in DNFB-challenged ears of rats with or without IL-37 treatment were shown. Scale bar = 20 μm. (E) The thickness of dermis and (F) the number of inflammatory cells in ear tissue sections were statistically analyzed. Data are presented as mean ± SD from three independent experiments (n = 8) (*$P < 0.05$ and **$P < 0.01$).

hexosaminidase and histamine release in the DNP-IgE/HAS-stimulated ACD RPMCs (Fig. 4A and B).

3.5. Involvement of NF-κB and P38 MAPK signaling in IL-33-induced MC inflammation

As shown in Fig. 5A and B, both NF-κB and P38 inhibitor showed antagonistic effects on IL-33-induced MC inflammatory responses, where the production of IL-6 and TNF-α were significantly decreased. However, ERK and JNK inhibitors had no effect on inflammatory cytokine production. These findings suggest the involvement of NF-κB and P38 MAPK signaling in IL-33-induced MC inflammation.

3.6. NF-κB activation and P38 MAPK phosphorylation are affected by IL-37

IL-37 showed little effect on ST2 and Smad3 expression (Fig. 6A and B), which suggested that the inhibition of IL-33 signaling was involved in the anti-inflammatory effect of IL-37 on ACD. RPMCs obtained from ACD group showed up-regulation of cytosolic phosphorylated IKK and nuclear NF-κB (p65), while down-regulation of cytosolic IκB (Fig. 7A-D). Compared with Control group, increasing phosphorylation of cytosolic P38 was presented in the RPMCs obtained from ACD rats, but not ERK and JNK (Fig. 7E-H). However, the administration of IL-37 inhibited the increase of cytosolic phosphorylated IKK, suppressed the activation of nuclear NF-κB (p65), and decreased the levels of phosphorylated P38 compared with the ACD group (Fig. 7).

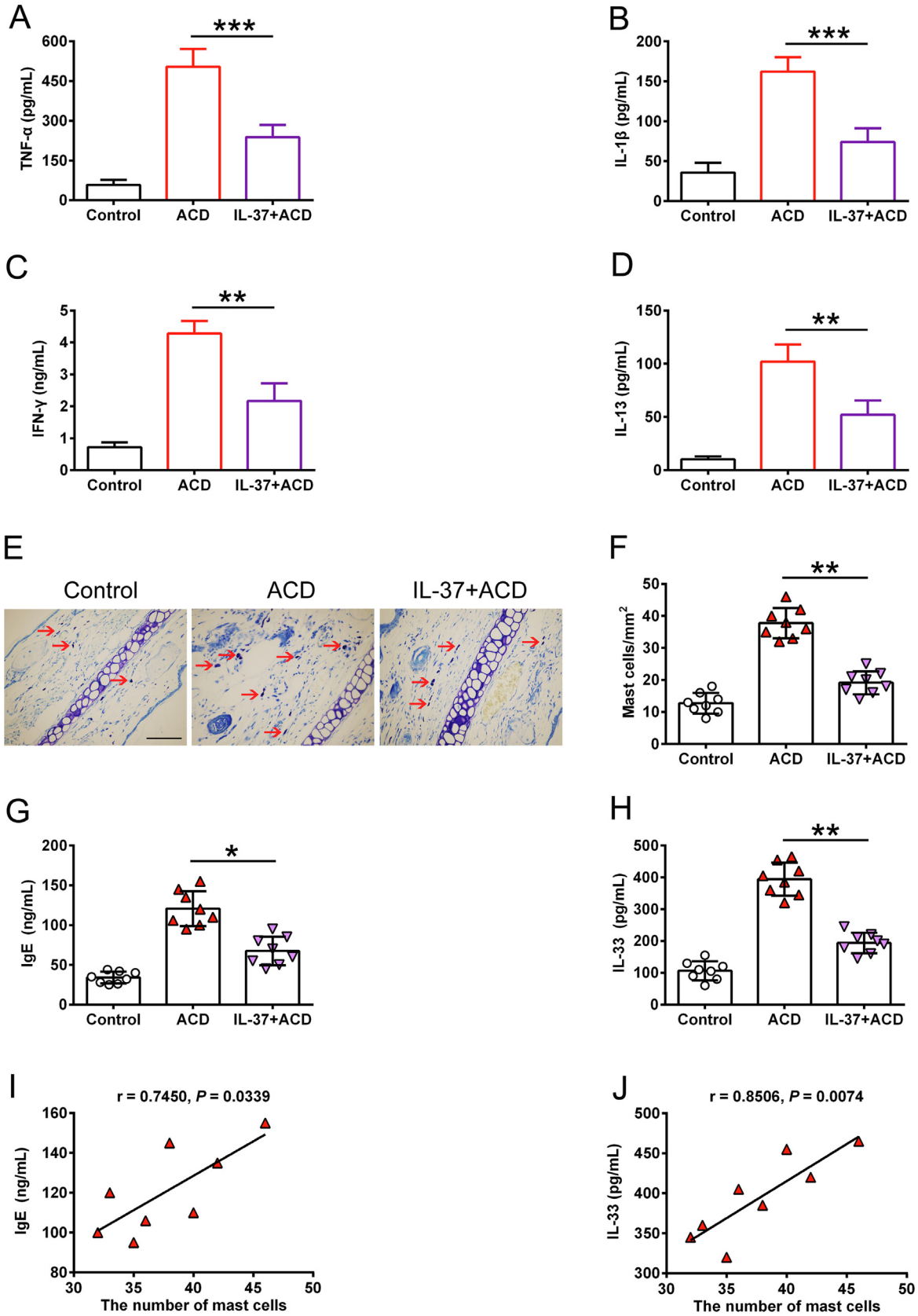

Fig. 2. IL-37 inhibits MC infiltration and decreases IL-33 production. (A-D) Inflammatory cytokine production in the homogenate supernatant of ear tissues was measured by ELISA and statistically analyzed, such as TNF-α, IL-1β, IFN-γ, and IL-13. (E) Ear samples were collected, sectioned and stained with toluidine blue to observe MC infiltration. Red arrows indicate the MCs. Scar bar = 20 μm. (F) The number of MCs in ear tissue sections from the different groups was statistically analyzed. (G, H) The levels of IgE and IL-33 in the homogenate supernatant of ear tissues were determined by ELISA and statistically analyzed, respectively. (I, J) Correlation analysis between the number of MCs in ear sections and the levels of IgE (IL-33) in homogenate supernatant of the ear samples from the ACD group, separately. Data are presented as mean ± SD from three independent experiments (n = 8) (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.01$).

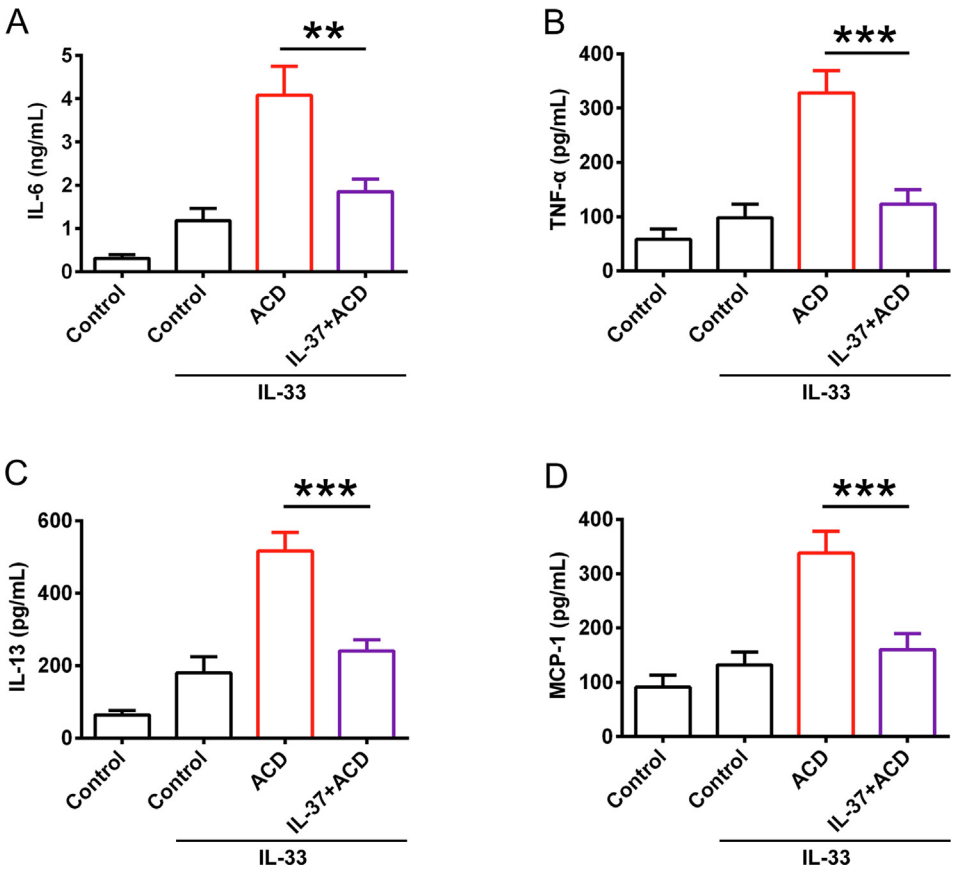

Fig. 3. IL-37 suppresses IL-33-induced cytokine production in the RPMCs. RPMCs (2×10^5) of the different groups were isolated and stimulated with IL-33 (50 ng/mL). After culture for 8 h, the concentrations of cytokines in the supernatants were measured by ELISA and statistically analyzed, including (A) IL-6, (B) TNF-α, (C) IL-13, and (D) MCP-1. Data are presented as mean ± SD from at least three independent experiments (n = 8) (**$P < 0.01$ and ***$P < 0.001$).

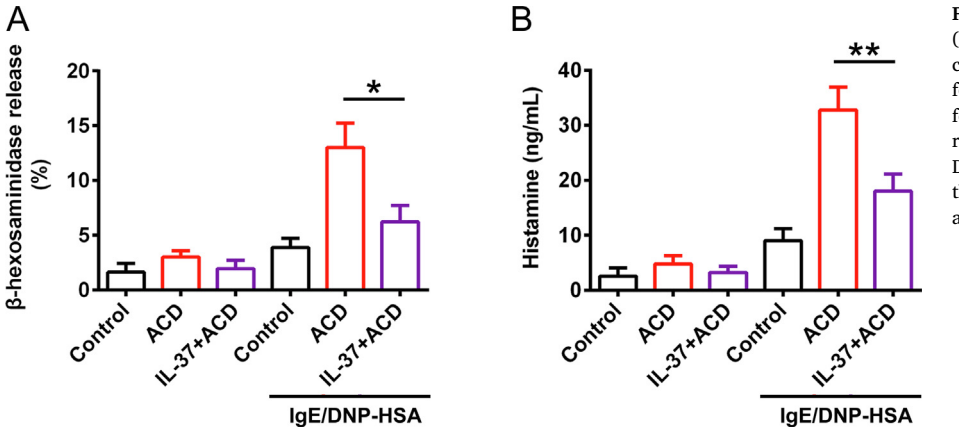

Fig. 4. IL-37 hampers MC degranulation. RPMCs (2×10^5) of the different groups were isolated and cultured with anti-DNP IgE (500 ng/mL) overnight, followed by stimulation with DNP-HSA (100 ng/mL) for 4 h. (A) β-hexosaminidase and (B) histamine release were detected and statistically analyzed. Data are presented as mean ± SD from at least three independent experiments (n = 8) (*$P < 0.05$ and **$P < 0.01$).

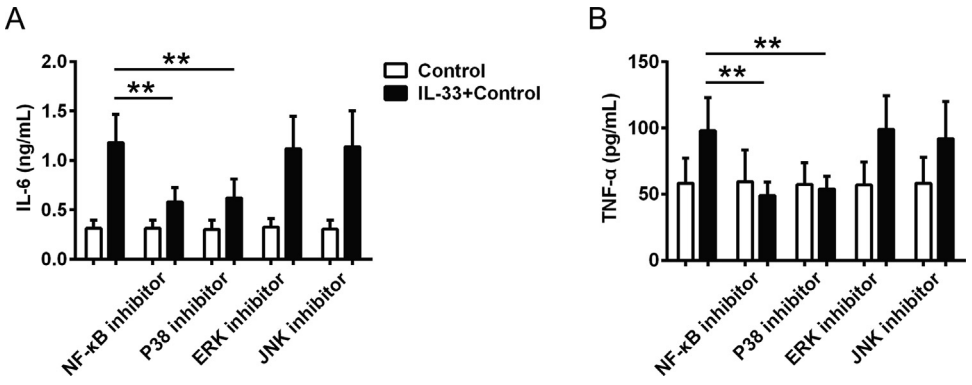

Fig. 5. Involvement of NF-κB and P38 MAPK signaling in IL-33-induced MC inflammatory responses. RPMCs (2×10^5) in control group were isolated and pretreated with different signal transduction inhibitors for 2 h, such as NF-κB inhibitor (BAY 11-7085, 5 μM), P38 inhibitor (SB203580, 10 μM), ERK inhibitor (PD98059, 10 μM), and JNK inhibitor (SP600125, 10 μM), separately. Then, the cells were activated with IL-33 (50 ng/mL) for 8 h. (A) The production of IL-6 and (B) TNF-α in the supernatants was measured by ELISA and statistically analyzed. Data are presented as mean ± SD from at least three independent experiments (n = 8) (**$P < 0.01$).

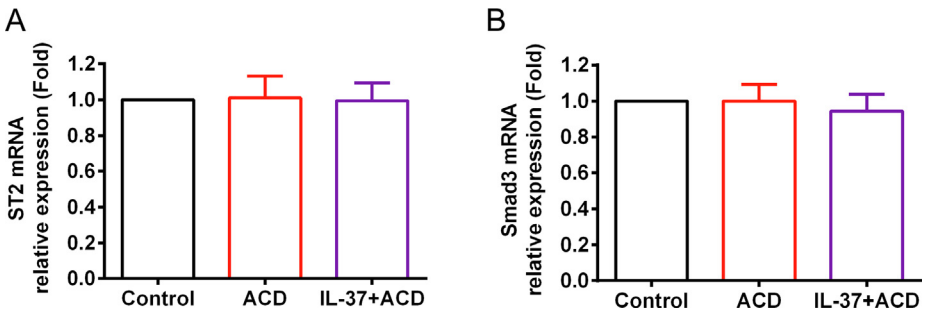

Fig. 6. IL-37 has no detectable effects on ST2 and Smad3 expression. Isolated RPMCs (2×10^5) were stimulated with IL-33 (50 ng/mL) for 8 h, and then the cells were collected. (A) ST2 and (B) Smad3 expression at mRNA level were assayed by Real-time PCR. Data are presented as mean ± SD from at least three independent experiments (n = 8).

3.7. Inhibition of Smad3 partly impaired IL-37-mediated anti-ACD effect

SIS3 is a specific inhibitor of Smad3-regulated cellular signaling, which interacts with IL-37 to exert its corresponding function [13]. When ACD rats were administrated both IL-37 and SIS3, ELISA assay demonstrated that inhibition of Smad3 partly impaired the suppressive effects of IL-37 on IL-33-induced inflammatory cytokine production (IL-6 and TNF-α) in the RPMCs (Fig. 8A and B). In line with the result, β-hexosaminidase and histamine release were both higher in the ACD rats treated with SIS3 + IL-37 than those in the IL-37 + ACD group, but still less than the levels in the ACD group (Fig. 8C and D).

4. Discussion

Increasing evidence has indicated the pivotal role of IL-37 in limiting excessive inflammatory responses in allergic diseases [14–16]. Recombinant human IL-37 protein significantly reduced ovalbumin (OVA)-induced airway hyperresponsiveness, inflammatory cell infiltration, and levels of IL-4, IL-6 and IL-13 in asthmatic mice [15]. Utilizing a house dust mite allergic rhinitis murine model, IL-37 relieved allergic inflammation, such as reduction of the allergic symptom scores and IgE levels as well as decrement of IL-4, IL-5, IL-13, and IL-17 production [16]. These studies suggest anti-inflammatory properties of IL-37 in allergic conditions. However, few studies focused on the effect of systemic administration of IL-37 on ACD. In this study, we found that IL-37 alleviated inflammation involved in ACD symptoms, such as ear swelling (Fig. 1B and C), neutrophil infiltration (Fig. 1D-F), and inflammatory cytokine production (TNF-α, IL-1β, IFN-γ, and IL-13) (Fig. 2A-D). To the best of our current knowledge, it is the first report on the effect of direct administration of IL-37 in rats with DBFB-induced ACD. These findings further confirm the anti-ACD potential of IL-37, which could be used as an inhibitor in skin inflammatory responses.

Including the regulation of IgE-mediated allergic responses, inflammatory activity of MCs can be induced via the binding of antigen to various cell surface receptors, such as Toll-like receptors, IL-1 receptors [17], and neurotransmitter receptors, without the participation of IgE, which is associated with inflammatory cytokine and chemokine release in inflammatory diseases [18,19]. Although ACD is a T cell-mediated skin inflammatory condition, keratinocytes, neutrophils, and MCs are major factors to generate the inflammation in the elicitation stage [20]. The depletion or absence of MCs before sensitization hampered the migration and maturation of $CD8^+$ T cells, leading to an intense attenuation of ACD [21]. DNFB-repeated painting incurred recruitment of MCs accompanying aggregation of IL-33 in the ear dermis (Fig. 2E, F, H and J), which in turn provoked and promoted amplification of inflammatory cytokines once MCs were in an IL-33 environment, such as IL-6, TNF-α, IL-13, and MCP-1 (Fig. 3), as our previous publications demonstrated [22,23]. Meanwhile, increased levels of IgE were presented in rats with ACD following the increase in the number of MCs (Fig. 2G and I), together with repeated application of contact allergens, which boosted MC degranulation, accelerating β-hexosaminidase and histamine release (Fig. 4). These findings highlight the immunoregulatory role of IL-37 in MC activation, including inhibition of IL-33-induced MC inflammation and suppression of MC degranulation,

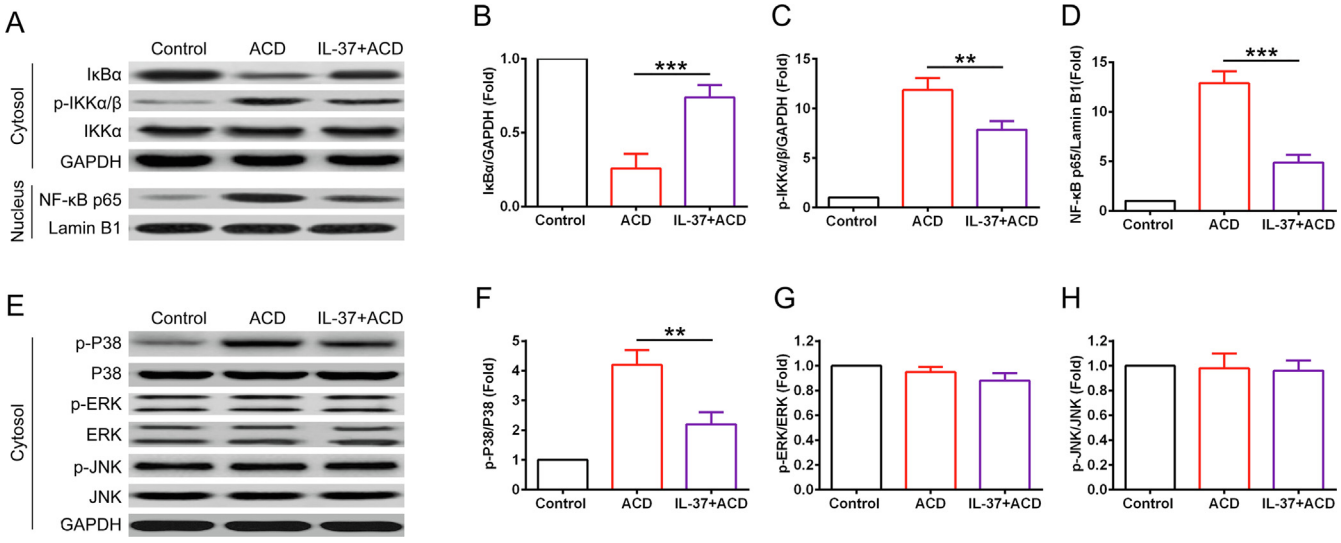

Fig. 7. NF-κB activation and P38 MAPK phosphorylation are affected by IL-37. RPMCs (2×10^5) of the different groups were isolated and collected. (A-D) Protein levels of cytosolic IκBα, p-IKKα/β, and IKKα as well as nuclear NF-κB (p65) were assayed by Western blot and statistically analyzed. (E-H) The expression of cytosolic proteins and phosphorylation levels were assayed by Western blot and statistically analyzed, such as P38, p-P38, ERK, p-ERK, JNK, and p-JNK. GAPDH and Lamin B1 were used to confirm equal sample loading, respectively. Representative results were shown. Data are presented as mean ± SD from at least three independent experiments (n = 8) (**$P < 0.01$ and ***$P < 0.001$).

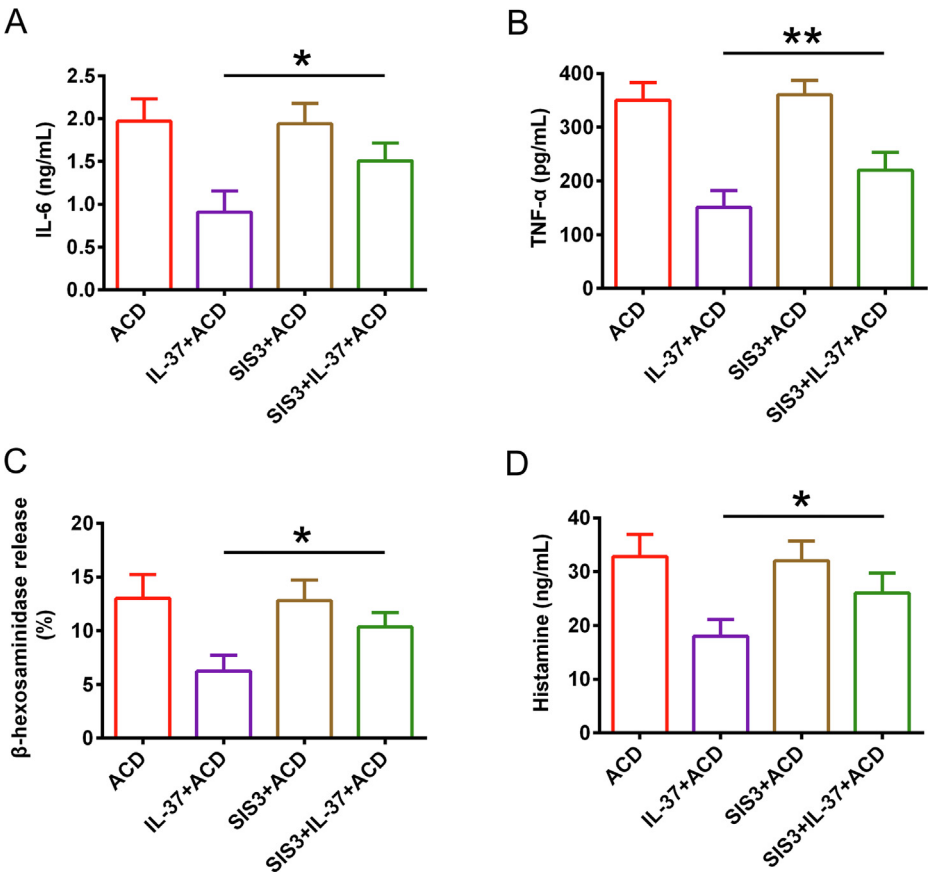

Fig. 8. Involvement of Smad3 in the anti-ACD effect of IL-37. ACD rats were administrated with IL-37 in the presence or absence of SIS3 (Smad3 inhibitor). (A) The isolated RPMCs (2×10^5) were treated with IL-33 (50 ng/mL) for 8 h, secreted IL-6 and (B) TNF-α in the supernatants were measured by ELISA and statistically analyzed. In additionally, the cells were cultured with anti-DNP IgE (500 ng/mL) overnight, followed by stimulation with DNP-HSA (100 ng/mL) for 4 h. (C) β-hexosaminidase and (D) histamine release were measured and statistically analyzed. Data are presented as mean ± SD from at least three independent experiments (n = 8) (*$P < 0.05$ and **$P < 0.01$).

which strongly suggest that negative regulation of MC activation contributes to the anti-ACD effect of IL-37.

Increasing evidence indicates that MCs can be activated once antigen cross-linking of IgE binds to the high-affinity receptor (FcεRI), triggering the intracellular cascade reactions, such as phosphorylation of Syk tyrosine kinase and Ca^{2+} influx as well as activation of protein kinase C and NF-κB transcription [24]. Some previous studies indicated that IL-33-induced mast cell inflammation is associated with the regulation of NF-κB and P38 MAPK signaling pathways [23,25,26]. In line with these reports, our result also showed the involvement of NF-κB and P38 MAPK signaling in IL-33-induced MC inflammation (Fig. 5). Because IL-37 had little effect on ST2 and Smad3 expression (Fig. 6A and B), which suggested that the inhibition of IL-33 signaling might participate in the anti-inflammatory effect of IL-37 on ACD. In this study, IL-37 treatment curbed NF-κB P38 activation and obstructed P38 phosphorylation (Fig. 7). These findings provided a new evidence to support the immunosuppressive effect of IL-37 on allergic inflammation as mentioned in a previous study, where IL-37 notably up-regulated IκB expression and down-regulated levels of NF-κB p65, phospho-NF-κB p65, STAT3 and phospho-STAT3 both in OVA-induced mice [15]. Further studies are needed to illuminate the concerns if other signaling pathway (such as Akt, SIGIRR, TLRs, and TGF-β1) or transcription factors (such as GATA-3, ROR-gt, T-bet, and Foxp3) participate in the immunomodulatory effect of IL-37 on ACD.

IL-37 is mainly produced by macrophages and has emerged as an important inhibitor of immune and inflammatory responses [27], which exerts its suppressive function in inflammation via the IL-1R8 receptor-mediated extracellular pathway and the Smad3-mediated intracellular pathway [28–30]. Extracellular IL-37 binds to the IL-18Rα and exploits IL-1R8 to form the tripartite complex IL-37b-IL-18Rα-IL-1R8, leading to the activation of a multifaceted intracellular anti-inflammatory program [31,32]. Besides, intracellular IL-37 translocates to the nucleus and then binds to Smad3 to regulate the expression of a variety of different cytokines and chemokines [33,34]. As a specific inhibitor of Smad3, SIS3 possesses the ability to affect the anti-inflammatory effect of IL-37 through blocking activation of Smad3 [35,36]. IL-37d has the anti-inflammatory characteristics similar to IL-37b, interacting with Smad3 and promoting its nuclear translocation. However, IL-37d exerted its inhibitory effects in a Smad3-dependent manner [33]. In contrast, SIS3 treatment and Smad3 knockdown partly reversed the inhibitory function of IL-37b [35–37], which indicated that IL-37b suppressed inflammatory cytokine production in a Smad3-independent manner. A recent study revealed that inhibition of Smad3 partly attenuated IL-37-mediated anti-inflammatory effects in autoimmune myositis Lewis model rats [38]. In this study, the anti-inflammatory activity of IL-37 was inhibited in the presence of SIS3 (Fig. 8), which suggested the regulation of Smad3 was involved in the immunomodulatory effect of IL-37. Further studies referred to IL-1R8 and IL-18Rα knockdown would clarify the details.

5. Conclusions

Taken together, systemic administration of IL-37 attenuated ACD symptoms, such as ear swelling, neutrophil infiltration, inflammatory cytokine production, MC recruitment, IgE and IL-33 release. Additionally, IL-37 modulated MC activation, including inflammation in response to IL-33 stimulation and degranulation following IgE/antigen treatment. Moreover, IL-37 inhibited the activation of NF-κB and phosphorylation of P38 MAPK with the participation of Smad3. These findings suggest that IL-37 can be considered as an alternative treatment for allergic diseases associated with inflammatory processes.

CRediT authorship contribution statement

Weihua Li: Conceptualization, Methodology, Investigation. **Fengmin Ding:** Resources, Investigation. **Yi Zhai:** Investigation,

Formal analysis. **Wenting Tao:** Data curation, Software. **Jing Bi:** Investigation. **Hong Fan:** Investigation. **Nina Yin:** Investigation. **Zhigang Wang:** Conceptualization, Methodology, Supervision, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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