

ORIGINAL ARTICLE

# Involvement of the Negative Feedback of IL-33 Signaling in the Anti-Inflammatory Effect of Electro-acupuncture on Allergic Contact Dermatitis *via* Targeting MicroRNA-155 in Mast Cells

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**Abstract—** In this study, we aimed to investigate the effect of electro-acupuncture (EA) at the Zusanli acupoint (ST36) on interleukin (IL)-33-mediated mast cell activation. Firstly, 2,4-dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis (ACD) in rats was developed with or without EA treatment. Then, rat peritoneal mast cells (RPMCs) were obtained and cultured in the presence of IL-33. EA treatment relieved ear swelling and reduced mast cell infiltration in the local inflammation area with DNFB challenge, accompanying the decrement of IL-33 production. RPMCs isolated from ACD rats with EA treatment showed significant downregulation of IL-6, TNF- $\alpha$ , IL-13, and MCP-1 production following IL-33 stimulation. However, there was no obvious difference in surface ST2 receptor expression among different groups. In addition, EA selectively altered IL-33 signaling, suppressing p38 phosphorylation as well as NF- $\kappa$ B- and AP-1-mediated transcription but not Akt phosphorylation. Importantly, EA lowered microRNA (miR)-155 expression in the RPMCs, which presented a positive correlation with IL-33-induced IL-6 production. Furthermore, overexpression of miR-155 in the RPMCs was established following miR-155 mimic transfection. RPMCs with the overexpressed miR-155 displayed an obvious increment of inflammatory cytokine and abrogated the inhibitive effect of EA on NF- $\kappa$ B- and AP-1-regulated

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transcription in response to IL-33 compared with those without transfected-miR-155. These findings demonstrate EA treatment inhibits NF- $\kappa$ B and AP-1 activation as well as promotes the negative feedback regulation of IL-33 signaling *via* targeting miR-155 in mast cells, which contribute to the anti-inflammatory effect of EA on DNFB-induced ACD in rats.

**KEY WORDS:** electro-acupuncture; IL-33; microRNA-155; mast cell; inflammation; allergic contact dermatitis.

## INTRODUCTION

The prevention and treatment of allergic diseases are attracting growing research attention, as the prevalence of these illnesses is increasing in both developed and developing countries [1]. Allergic contact dermatitis (ACD), which is also known as delayed-type allergic response, is a T cell-mediated inflammatory skin disease triggered by repeated exposure to contact allergens [2]. Recently, mast cell (MC) has been shown to influence the process of ACD. Following interactions between antigen and IgE-bound Fc $\epsilon$ RI, Fc $\epsilon$ RI cross-linking elicits MC degranulation, including the release of histamine,  $\beta$ -hexosaminidase and proteases as well as cytokines and chemokines, accelerating recruitment of the circulating T cells, eosinophils, basophils, and neutrophils to the local inflammatory site, which together contribute to the pathology of ACD [3, 4].

Increasing evidence indicates that interleukin (IL)-33, a newly discovered alarmin of the IL-1 cytokine family, is an important regulator of MC function as activator [5, 6]. Generally, IL-33 promotes formation of a signaling complex between T1/ST2 and IL-1 receptor accessory protein, which initiates downstream signaling cascade culminating in NF- $\kappa$ B- and AP-1-dependent transcription of inflammatory cytokines and chemokines, such as IL-6, TNF- $\alpha$ , IL-13, and MCP-1 [7]. Accumulating study reported that IL-33 was elevated in asthma and atopic dermatitis, which was able to amplify IgE-induced MC degranulation, resulting in the exacerbation of allergic responses [8, 9]. IL-33-induced MC activation worsened airway hyperreactivity in the process of asthma [10]. Blocking IL-33 could lessen late-phase responses during antigen-induced passive cutaneous anaphylaxis, suggesting the involvement of IL-33-MC axis in the allergic skin disease and the rationale for targeting IL-33 in MC-regulated allergic disease [11].

Acupuncture, originally originated in ancient China, is becoming one of the most popularly used complementary medicine modalities in East and West countries. According to the theory of traditional Chinese medicine, acupuncture can modulate the immune system to improve health and well-being, *via* the insertion and manipulation of thin needles at acupuncture points of the body [12]. Electro-acupuncture (EA) is a modification of

conventional acupuncture combining electrical boost, which strengthens the stimulation by the needles at the specific acupoints through electrical impulses [13]. Our previous publication showed that EA treatment at the Zusanli acupoint (ST36) improved Th1-mediated allergic skin inflammation in the mouse model of ovalbumin (OVA)-induced delayed-type hypersensitivity (DTH), resulting from the restoration of Th1/Th2 balance by curbing Th1 differentiation [14]. Recent study reported that EA at ST36 ameliorated inflammation associated with 2,4-dinitrofluorobenzene (DNFB)-induced ACD in rats, which was related to trigger local IL-10 production and inhibit p38 MAPK activation [15]. However, modulation effect of EA on IL-33/ST2 signaling referred to ACD still remains inconclusive.

In this study, we evaluated the effect of EA at ST36 on DNFB-induced ACD in rats; observed the change of mast cell infiltration; investigated the expression of cytokines, NF- $\kappa$ B, AP-1, and microRNA-155 involved in IL-33-mediated mast cell response; and explored the underlying mechanisms of EA application in treating allergic inflammation such as ACD.

## MATERIALS AND METHODS

### Animals

Male 7-week-old Sprague-Dawley rats (194  $\pm$  12 g) were purchased from Hubei Research Center of Laboratory Animals (Wuhan, China) and housed in specific pathogen-free conditions. All experiments were performed according to the standard of the Animal Care and Use Committee of Hubei University of Chinese Medicine concerning the Care and Use of Laboratory Animals (No. SYXK2012-0067).

### 2,4-Dinitrofluorobenzene-Induced Allergic Contact Dermatitis

Rats were sensitized by applying 25  $\mu$ l 0.5% DNFB (acetone/olive oil = 4:1) (Sigma-Aldrich, St. Louis, MO, USA) topically on shaved hind flank on days 1 and 2. After

Involvement of the negative feedback of IL-33 signaling in the anti-ACD effect of EA *via* targeting miR-155 in mast cells.

5 days, sensitized rats were challenged with 10  $\mu$ l 0.2% 2,4-dinitrofluorobenzene (DNFB) on the left ear to induce allergic contact dermatitis (ACD). On day 8, ear thicknesses were measured in a blinded fashion using a thickness gauge (Digimatic caliper, Mitutoyo, Japan) at 24 h after the challenge as previously described [15]. Then, rats were decapitated for ear sample collection and further experiments.

### Groups

Rats were randomly divided into four groups ( $n = 8$ , each group): Control group, not immunized; ACD group, sensitized and challenged with DNFB for the ACD model; EA + ACD group, EA treatment at the bilateral Zusanli acupoint (ST36, located 5 mm below the fibular head and lateral to the anterior tubercle of the tibia) following DNFB application; Sham + ACD group, EA treatment at a non-acupoint (Sham acupoint, localized 5 mm below the Zusanli acupoint) accompanying DNFB administration.

### EA Treatment

The needles (length 3.0 cm, diameter 0.20 mm) (Suzhou Medical Appliance Factory, Suzhou, China) were inserted perpendicularly to the skin, and about 5 mm to one side of the anterior tibial muscle. The intensity of EA stimulation was determined to be the minimum voltage that caused moderate muscle contraction with a Hans Acupoint Nerve Stimulator (HANS, Beijing, China). The following stimulus parameters were selected: a continuous wave at 2 Hz and 1 mA for 5 min, 2 Hz and 1.5 mA for 5 min, and 2 Hz and 2 mA for 20 min. EA stimulation was repeated from day 1 to day 7 between the EA + ACD group and Sham + ACD group.

### Toluidine Blue Staining

The left ear of rats was removed and fixed in 4% paraformaldehyde (Sigma-Aldrich). Fixed tissues were embedded in paraffin for 24 h and serially sectioned to a thickness of 5  $\mu$ m for histological analysis. Tissue sections were stained with toluidine blue for observing mast cells under a Nikon Eclipse Ti-S microscope (Nikon, Tokyo, Japan). Ten different fields of each section were counted manually and evaluated in a blinded fashion.

### Analysis of IL-33 Production

Ear samples of different groups were weighed and homogenized in 1 ml of tissue protein extraction reagent containing a protease inhibitor cocktail (Pierce, Rockford,

IL, USA). Homogenates were centrifuged at 12,000 $\times$ g for 15 min at 4 °C to obtain the supernatant. IL-33 was determined by enzyme-linked immunosorbent assay (ELISA) (M3300, R&D systems, Minneapolis, MN, USA) using a microplate reader (Bio-Rad Laboratories, Inc., Richmond, CA, USA). All experiments were done in triplicate.

### Isolation of Rat Peritoneal Mast Cells

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  $\mu$ g/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 $\times$ 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 [16]. MCs isolated by this procedure exceed 95% in purity as determined by light microscopy with toluidine blue staining. Approximately  $1 \times 10^6$  rat peritoneal mast cells (RPMCs) are usually harvested from one rat.

### Cytokine Measurement by ELISA

RPMCs of different groups were collected as above described and treated with 50 ng/mL of IL-33 for 24 h. Then, the supernatants were harvested for cytokines assay including IL-6, TNF- $\alpha$ , IL-13, and MCP-1 by ELISA kit (R&D systems) and measured using a microplate reader (Bio-Rad Laboratories). All experiments were done in triplicate.

### Western Blot Analysis

Cellular proteins were extracted using RIPA lysis buffer with protease and phosphatase inhibitor cocktail (Cell Signaling Technology Inc., Beverly, MA, USA). The supernatants were collected and protein concentration was analyzed using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). Proteins (20  $\mu$ g) were separated by electrophoresis and transferred onto polyvinylidene fluoride (PVDF). Blocking with 5% skim milk, the membranes were incubated with the primary antibodies (anti-Akt, #4685; anti-phospho-Akt, #9271; anti-P38, #9212; anti-phospho-P38, #4631; anti-phospho-c-Jun, #2361; anti-

IKK $\alpha$ , #9242; anti-IKK $\alpha/\beta$ , #2682; anti-phospho-IKK $\alpha$ , #2697; anti-GAPDH, #5174; anti-NF- $\kappa$ B p65, #3034; anti-Lamin B1, #13435) (Cell Signaling Technology) overnight at 4 °C and then incubated with the appropriate HRP-conjugated secondary antibody. The membranes were visualized by enhanced chemiluminescence detection (HP Scanjet 7400C) (Hewlett-Packard Co., Palo Alto, CA, USA). Optical density for each band was assessed using the ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA). Sample loading was normalized by quantities of GAPDH or Lamin B1 detected parallel.

### Real-Time RT-PCR

Total RNA was extracted from RPMCs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with the TaqMan MicroRNA Reverse Transcription Kit, according to the manufacturer's instructions (Applied Biosystems Life Technologies, Foster City, CA, USA). To analyze miR-155 expression, qRT-PCR was performed using the 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) with TaqMan MicroRNA Assay Kit (ABI). The following forward (F) and reverse (R) primer sequences were used: ST2: F, 5'-CGGAACCACTGGGAAACCT-3'; R, 5'-ATCCTAGC TGGCCTCTTGT-3'; GAPDH, F: 5'-CACGGCAAGTTC AACGGCACA-3', R: 5'-GCGGCATGTCAGATCCAC AACG-3'. Amplification was performed under the following conditions, 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The relative ST2 mRNA levels were normalized to GAPDH for mRNA analysis. MiR-155: F, 5'-CGGCGGTAAATGCTAATTGTGAT-3'; R, 5'-GTGCAGGGTCCGAGGT-3'; U6: F, 5'-CTCGCTTC GGCAGCACATA-3'; R, 5'-CGCTTCACGAATTTGCGT GTCAT-3'. The expression of U6 was used as endogenous control for miRNA analysis. Data were analyzed by the  $2^{-\Delta\Delta C_t}$  method.

### Transfection

RPMCs were transfected with miR-155 mimic (miR-155 group) and negative control (NC group) (GenePharma Co., Ltd., Shanghai, China) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The final concentration of oligonucleotides was 50 nM. After 24 h of incubation, the cells were processed for further experiments.

### Statistical Analysis

All data were presented as the mean  $\pm$  standard deviation (SD). Results were analyzed with the SPSS 19.0

software (SPSS Inc., Chicago, IL, USA). For comparisons of multiple samples to the control group, one-way analysis of variance (ANOVA) was used. For comparisons of two groups, a Student *t* test was used. Curve estimation and linear regression analyses were performed for correlation analysis.  $P < 0.05$  indicated statistical significance.

## RESULTS

### EA treatment reduces mast cell infiltration and IL-33 production in the rats with DNFB-induced ACD

Following the experimental schedule (Fig. 1a), ear swelling was measured and the thickness increment was used to assay the severity of ACD. As shown in Fig. 1b, EA treatment at the ST36 acupoint led to an incremental decline in ear thickness. Additionally, repeated-DNFB administration induced the visible enrollment of MC in the ear dermis. However, EA treatment significantly reduced MC infiltration compared with those in the ACD group (Fig. 1c, d). Comparison with the ACD group, the concentration of IL-33 was significantly declined in the EA + ACD group (Fig. 1e). Correlation analysis showed that there was a positive correlation between MC infiltration and IL-33 production in the ACD group ( $r = 0.8887$ ,  $P = 0.0032$ ) (Fig. 1f). These results suggest that the inhibition of mast cell recruitment to local inflammation, accompanying the downregulation of IL-33 production, is involved in the anti-ACD effect of EA.

### EA Attenuates Inflammatory Cytokine Production in IL-33-Activated Mast Cells but Has Little Effect on ST2 Expression

RPMCs obtained from the EA + ACD group showed obvious decrement of IL-6, TNF- $\alpha$ , IL-13, and MCP-1 production, which was different from the significant increment of cytokine in the ACD group (Fig. 2). A possible explanation for the suppressive effect of EA on ACD is that EA might curb ST2 expression, which is needed for MC responses to IL-33 as receptor. However, there was no obvious difference in ST2 expression among the four groups (Fig. 3). These data reinforced our rationale to investigate the effect of EA on IL-33 signaling.

### EA Suppresses IL-33-Mediated NF- $\kappa$ B and c-Jun Activation

IL-33 signaling is conveyed by protein phosphorylation involved in the signaling cascade, leading to the

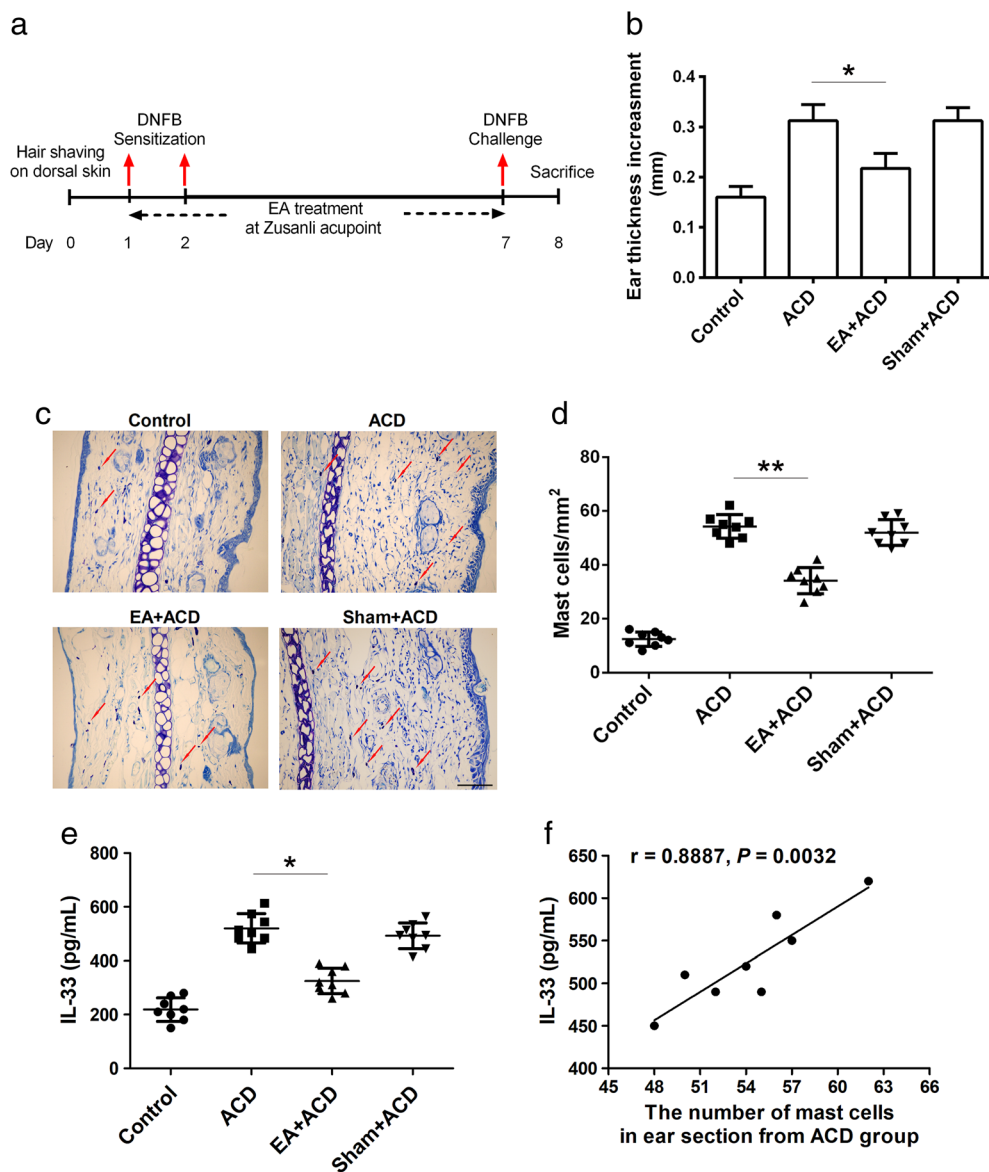


**Fig. 1.** EA treatment inhibits mast cell infiltration and suppresses IL-33 production in ear samples of the rats with DNFB-induced ACD. **a** Experimental schedule for DNFB-induced ACD following EA treatment. **b** Ear thickness increment was shown among four groups. **c** Ear samples obtained from different groups were sectioned and stained using toluidine blue to observe mast cell infiltration. Red arrows indicate the mast cell. Scar bar = 20  $\mu$ m. **d** The number of mast cells in ear sections from the different groups was statistically analyzed. **e** Ear tissues of different groups were homogenized and the supernatant were collected to detect IL-33 production. **f** Correlation analysis between the number of mast cells in ear sections and the levels of IL-33 in homogenate supernatant of the ear samples from ACD group. Data are presented as mean  $\pm$  SD ( $n = 8$ ) (\* $P < 0.05$  and \*\* $P < 0.01$ ).

activation of Akt and MAPKs that ultimately promotes the transcriptional activities of NF- $\kappa$ B and AP-1. RPMCs obtained from the ACD group showed upregulated expressions of cytosolic phosphorylated IKK and nuclear NF- $\kappa$ B (p65), while downregulated expressions of cytosolic I $\kappa$ B (Fig. 4a). Compared with the control group, increasing

cytosolic phosphorylation of p38 and nuclear c-Jun activation were presented, but not Akt (Fig. 4b). In contrast to the ACD group, cytosolic p38 and nuclear c-Jun phosphorylation as well as NF- $\kappa$ B (p65) activation were remarkably inhibited in the RPMCs obtained from the EA + ACD group (Fig. 4).

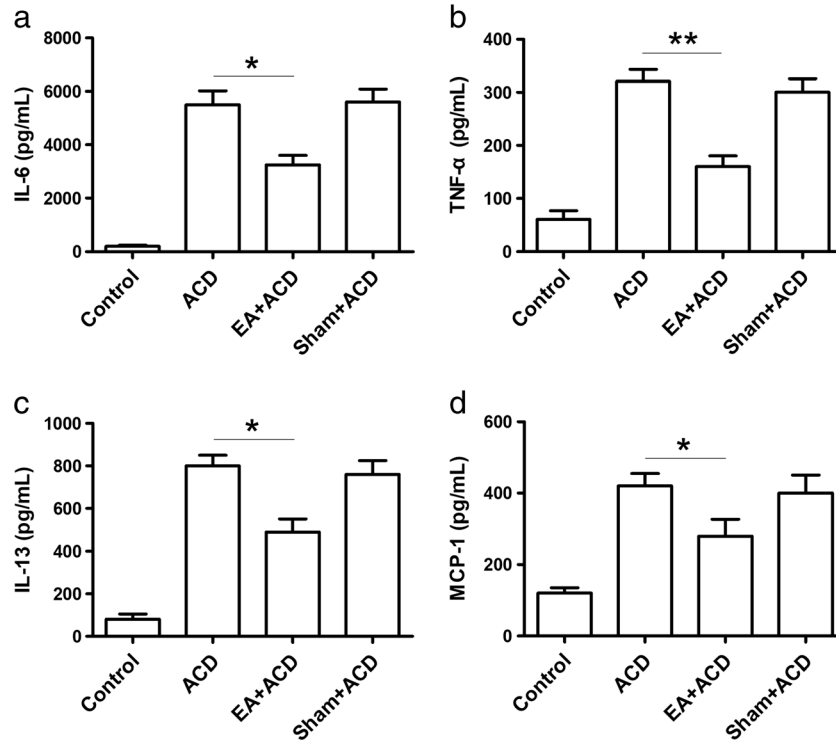


**Fig. 2.** IL-33-induced cytokine production in the peritoneal mast cells. RPMCs of different groups were isolated and treated with IL-33 (50 ng/mL). After 24 h, supernatants were collected for cytokines analysis by ELISA, including **a** IL-6, **b** TNF- $\alpha$ , **c** IL-13, and **d** MCP-1. Data are presented as mean  $\pm$  SD ( $n = 8$ ) (\* $P < 0.05$  and \*\* $P < 0.01$ ).

### EA Treatment Restrains miR-155 Expression in the Peritoneal Mast Cells

MiR-155 has been indicated that plays a crucial role in MC activation and IL-33 signaling in allergic inflammation. As shown in Fig. 5a, miR-155 expression was

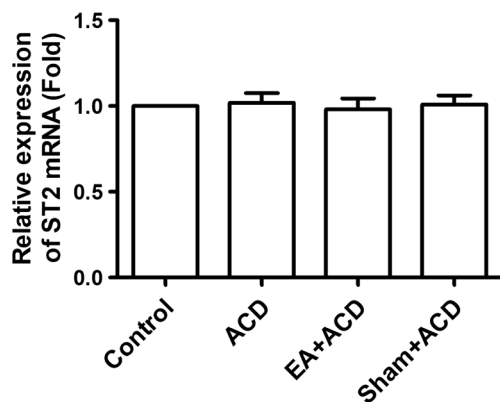


**Fig. 3.** ST2 receptor expression in the peritoneal mast cells. RPMCs isolated from different groups were stimulated with IL-33 (50 ng/mL) for 24 h, and the cells were collected. ST2 expression in mRNA level was measured by real-time PCR. Data are presented as mean  $\pm$  SD ( $n = 8$ ).

apparently decreased in the EA + ACD group than that in the ACD group (Fig. 5b). Simultaneously, correlation analysis revealed that there was a positive correlation between the miR-155 expression and IL-6 production induced by IL-33 in the ACD group ( $r = 0.9127$ ,  $P = 0.0016$ ) (Fig. 5c).

### MiR-155 Expression Is Associated with the Anti-Inflammatory Effect of EA on ACD

As shown in Fig. 6, miR-155 expression was significantly upregulated following transfection with miR-155 mimic (miR-155 group) compared with its negative control (NC group). RPMCs with the overexpressed miR-155 showed increased production of inflammatory cytokines such as IL-6 and TNF- $\alpha$ , which were decreased in RPMCs from the EA + ACD group without transfected-miR-155 (Fig. 7a, b). Importantly, the transfection of miR-155 significantly abrogated the inhibitive effect of EA on NF- $\kappa$ B- and AP-1-regulated transcription in response to IL-33 compared with those in the absence of miR-155 transfection (Fig. 7c, d), suggesting miR-155 is involved in the anti-ACD effect of EA.

Involvement of the negative feedback of IL-33 signaling in the anti-ACD effect of EA *via* targeting miR-155 in mast cells.

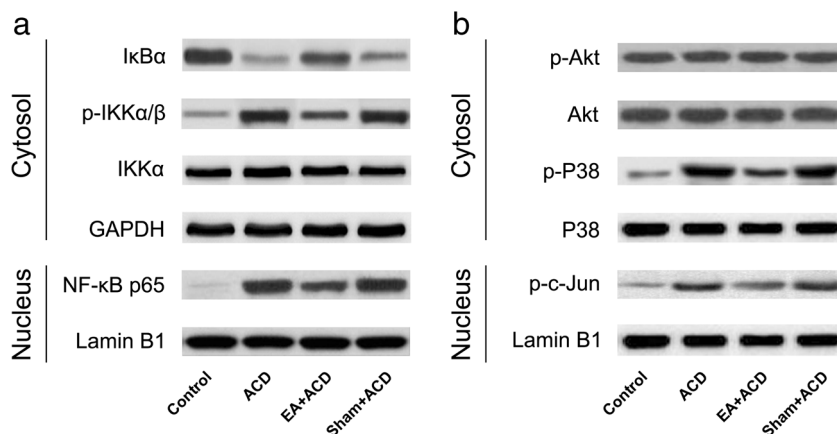


**Fig. 4.** Inhibition of NF- $\kappa$ B and c-Jun signaling is associated with anti-inflammatory effect of EA on ACD. RPMCs isolated from different groups were treated with IL-33 (50 ng/mL) for 1 h, and the cells were collected. **a** Expression of cytosolic I $\kappa$ B $\alpha$ , p-IKK $\alpha$ / $\beta$ , IKK $\alpha$ , and nuclear NF- $\kappa$ B (p65) was measured by Western blotting. **b** Expression of cytosolic Akt, p-Akt, P38, p-P38, and nuclear p-c-Jun was assessed by Western blotting. GAPDH and Lamin B1 were used to confirm equal sample loading, respectively. Representative results were shown. Data are presented as mean  $\pm$  SD from at least three independent experiments.

## DISCUSSION

According to what we know, few studies focus on the relationship between acupuncture and miR-155. A recent research showed that acupuncture at the Zusanli (ST36), Zhongwan (CV12), and Pishu (BL20) acupoints downregulated the expression of miR-155, miR-21, and NF- $\kappa$ B p65 in rats with chronic atrophic gastritis [17]. In this study, EA treatment at ST36 restrained miR-155 expression in mast cells, which was associated with the negative feedback of IL-33 signaling, together contributing to the anti-inflammatory effect of EA on DNFB-induced ACD in rats.

MC is known as an important regulator participated in the allergic diseases, such as asthma, allergic rhinitis,

atopic dermatitis, and anaphylaxis [18]. As newly emerging MC activator, IL-33 attracts lots of attention due to its cross-interaction in regulating MC-related allergy and inflammation [19]. On the one hand, MCs express ST2 and IL-33 *in vitro* as well as Fc $\epsilon$ RI-induced MC activation increases IL-33 mRNA levels, although very low IL-33 expression in unstimulated MCs [20]. On the other hand, IL-33 also intensifies classic IgE-mediated MC activation by increasing the concentration of circulating IgE and synergizing Fc $\epsilon$ RI signaling in the passive cutaneous anaphylaxis [11, 21]. In addition, IL-33/ST2 signaling on both human and murine MCs has been shown to enhance their survival through upregulation of B cell lymphoma-X large in the peritoneum [22]. In view of the bidirectional

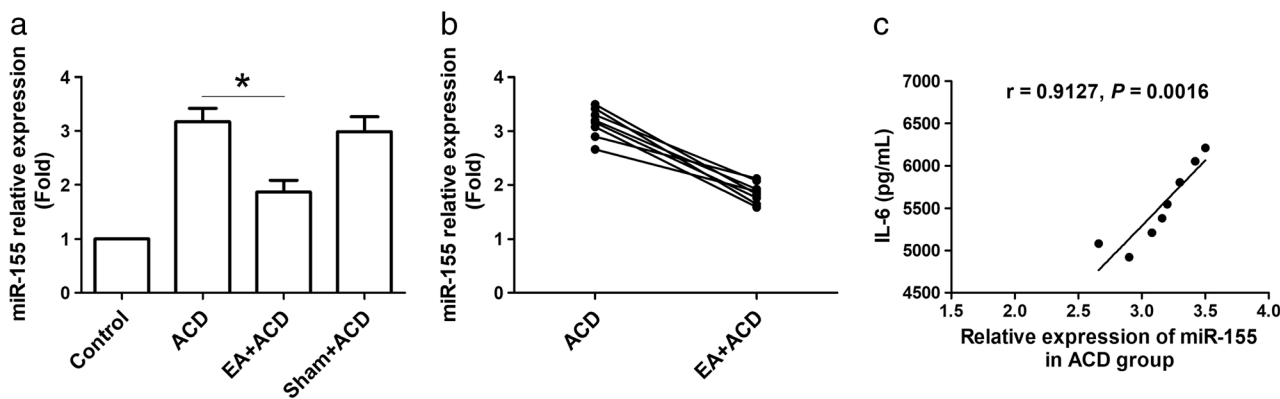


**Fig. 5.** Relationship between miR-155 expression and IL-33-induced cytokine production in peritoneal mast cells. **a, b** RPMCs of different group were isolated and miR-155 expression was estimated by real-time PCR. Simultaneously, RPMCs were treated with IL-33 (50 ng/mL) for 24 h. Then, the levels of IL-6 in supernatants were detected by ELISA. **c** Correlation analysis between miR-155 expression and IL-6 production in the ACD group. Data are presented as mean  $\pm$  SD ( $n = 8$ ) (\* $P < 0.05$ ).

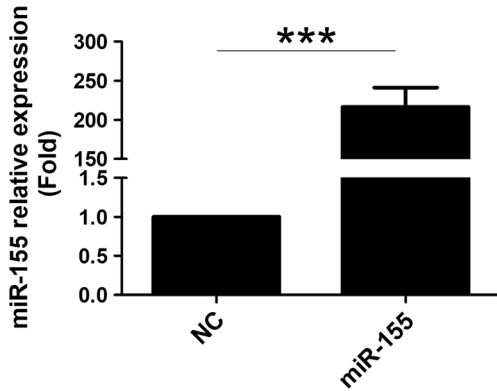


**Fig. 6.** miR-155 is overexpressed in the transfected mast cells. RPMCs obtained from the EA + ACD group were transfected with miR-155 mimic (miR-155) or its negative control (NC). After 24 h of transfection, miR-155 expression was examined by real-time PCR. Data are presented as mean  $\pm$  SD ( $n = 5$ ) (\*\* $P < 0.001$ ).

modulation between MC and IL-33, our results also indicated that EA treatment inhibited MC infiltration and downregulated IL-33 production (Fig. 1), but had little effect on ST2 receptor expression in spite of IL-33 induced cytokines productions were declined (Figs. 2 and 3). These findings suggest that inhibition of IL-33-mediated MC activation may play a role in the anti-ACD effect of EA treatment.

IL-33/ST2 signaling has been recently implicated in AD, as mice receiving topically applied OVA, house dust mites, or staphylococcal enterotoxin B led to upregulation of both ST2 and IL-33 expression [23, 24]. Patients with AD present higher IL-33 expression in their skin lesions compared with healthy controls [25]. Using a mouse model of poison ivy-induced ACD, neutralizing antibodies against IL-33 or ST2 reduced scratching behavior and skin inflammation, suggesting IL-33/ST2 signaling may represent a therapeutic approach in ACD [26]. 2, 4-dinitrochlorobenzene (DNCB)-induced AD-like mice treated with anti-mouse IL-33 antibody ( $\alpha$ IL-33Ab) showed improved AD-like symptoms. Additionally, eosinophils and MCs infiltration and serum IgE levels were also significantly decreased by  $\alpha$ IL-33Ab [27]. The activation of IL-33/ST2 signaling activation elicits subsequent MyD88/(IL-1R-associated kinase) IRAK-regulated signaling cascade, and leads to either the NF- $\kappa$ B or MAPK pathway being activated, which promotes inflammatory cytokine release [28]. Dexamethasone suppressed IL-33-induced cytokine production in mouse bone marrow-derived MCs (BMMCs), resulting from the antagonistic effect of AP-1- and NF- $\kappa$ B-mediated transcriptional activity [29]. In addition, flavonoids and didox (3,4-dihydroxybenzohydroxamic acid) also inhibited IL-33-

induced activation of NF- $\kappa$ B followed by suppression of phosphorylation of IKK, which was attributed to degradation of I $\kappa$ B $\alpha$  and nuclear localization of NF- $\kappa$ B [30, 31]. In line with the above results, our data confirmed that RPMCs isolated from EA + ACD group showed the decrement of cytosolic p38 and nuclear c-Jun phosphorylation as well as NF- $\kappa$ B (p65) activation (Fig. 4), accompanying inhibition of miR-155 expression in the peritoneal mast cells (Fig. 5). Correlation analysis suggested that the miR-155 expression was associated with IL-33 signaling cascade in MC response.

MicroRNAs (miRNAs) are a major class of short, non-coding, single-stranded RNA molecules, which regulate transcription repression or degradation by targeting the 3'UTR or the coding sequences of specific mRNAs [32]. In recent years, miRNA-155 (miR-155) also has emerged as a crucial regulator in several immunologic and inflammatory disorders, such as atopic dermatitis, allergic airway inflammation, passive cutaneous anaphylaxis, and rheumatoid arthritis [33–36]. A previous study showed miR-155 contributed to the regulating of allergic airway inflammation by modulating Th2 responses through the transcription factor PU.1 [37]. MiR-155<sup>-/-</sup> mice displayed enhanced-anaphylaxis reactions. Simultaneously, selective blockade of the PI3K $\gamma$  pathway inhibited degranulation in miR-155<sup>-/-</sup> BMMCs, suggesting miR-155 controlled MC activation *via* the PI3K $\gamma$  pathway [38]. Another study demonstrated that type 2 innate lymphoid cells (ILC2s) and IL-33 signaling were regulated by miR-155 in allergic airway inflammation, in which miR-155 suppression contributed to the inhibitory effect of IL-33-mediated mast cell inflammatory responses following lactic acid treatment [39]. Consistent with the above reports, our results implied that overexpression of miR-155 abolished the suppressive effect of EA on IL-33-induced cytokine production and the activation of NF- $\kappa$ B and AP-1 (Fig. 6 and Fig. 7).

Based on the ancient oriental medical theory, acupuncture is used not only as an effective treatment but also to prevent illness and improve health. EA, an application of electrical current on metal needles, is a modified technique of acupuncture treatment. Increasing evidence indicates that EA at the Zusanli (ST36) acupoint is effective for the management and treatment of immune-related diseases attributed to its immunomodulatory effect [12]. EA treatment at the ST36 acupoints of rats could restore the balance to the Th1/Th2/Th17/Treg T helper cell responses on myelin basic protein-induced experimental autoimmune encephalomyelitis [40]. In addition, EA at ST36 acupuncture point showed the anti-inflammatory effect on lipopolysaccharide (LPS)-induced cytokines productions, including

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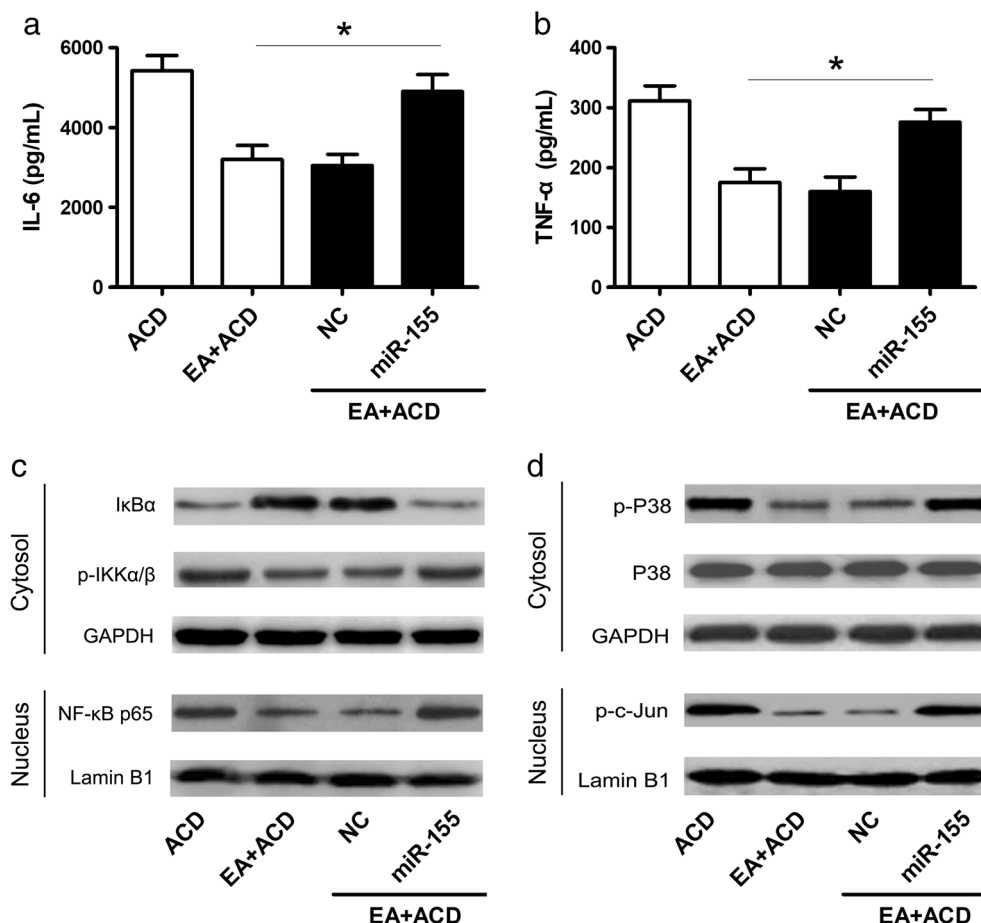


**Fig. 7.** miR-155 is involved in the anti-inflammatory effect of EA on ACD. RPMCs from the EA + ACD group were transfected with miR-155 mimic (miR-155) or its negative control (NC). **a, b** After 24 h of transfection, IL-33 (50 ng/mL) was added for additional 24 h. IL-6 and TNF-α production in the supernatants of the ACD group, EA + ACD group, EA + ACD group with transfected miR-155 or its negative control were detected by ELISA. **c, d** After 24 h of transfection, the cells were activated with IL-33 (50 ng/mL) for 1 h. Then, the cells were collected. Expression of cytosolic IκBα, p-IKKα/β, P38, p-P38 as well as nuclear NF-κB (p65) and p-c-Jun was assessed by Western blotting. 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 (\**P* < 0.05).

TNF, monocyte chemotactic protein-1 (MCP-1), IL-6, and INF-γ. However, EA with a wooden toothpick or stimulation of a non-acupuncture point did not inhibit cytokine levels [41]. In line with these previous reports, our results also confirmed that EA stimulation at the ST36 acupoint was effective to attenuate DNFB-induced ACD compared

to Sham treatment (non-ST36 acupoint). On the one hand, there are increased structures of capillaries, sympathetic nerve endings, dermal papillas, and electrolyte embedded sites with condensed gap junctions in the epidermis at the ST36 acupoint, so that they possess different electrical potentials than nearby sites [42]. On the other hand, the

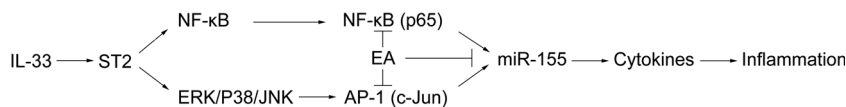


**Fig. 8.** Proposed mechanism for the anti-inflammatory effect of EA on ACD. EA treatment inhibits activation of NF-κB and AP-1 (c-Jun) as well as prompts negative feedback regulation of IL-33 signaling *via* targeting miR-155 in mast cells, which contribute to the anti-inflammatory effect of EA at ST36 on DNFB-induced ACD.

ST36 acupoint shows immunomodulatory potential as one of the most important acupuncture point associated with the regulation of nerve-immune-endocrine network [43].

Taken together, our study demonstrates that EA treatment inhibits the activation of NF- $\kappa$ B and AP-1 as well as prompts negative feedback regulation of IL-33 signaling *via* targeting miR-155 in mast cells, which contribute to the anti-inflammatory effect of EA on DNFB-induced ACD (Fig. 8). Although these findings provide fundamental insight into how EA at ST36 affects mast cell responses, more studies should be conducted in the future work to clarify the detailed mechanisms in response to EA treatment on ACD.

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## AUTHOR CONTRIBUTION

Zhigang Wang, Tao Yi, and Zebin Chen designed the study; Zhigang Wang and Tao Yi performed the experiment and wrote the manuscript; Man Long contributed to the rat model; Fengmin Ding analyzed the data; and Lichen Ouyang and Zebin Chen helped to revise the manuscript.

## COMPLIANCE WITH ETHICAL STANDARDS

All experiments were performed according to the standard of the Animal Care and Use Committee of Hubei University of Chinese Medicine concerning the Care and Use of Laboratory Animals (No. SYXK2012-0067).

**Conflict of Interest Statement.** The authors declare that they have no conflicts of interest.

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