

ORIGINAL ARTICLE

Electro-Acupuncture at *Zusanli* Acupoint (ST36) Suppresses Inflammation in Allergic Contact Dermatitis *Via* Triggering Local IL-10 Production and Inhibiting p38 MAPK Activation

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Abstract— Acupuncture has shown beneficial effect in the treatment of multiple dermatologic conditions including dermatitis, pruritus, urticaria, and hyperhidrosis; however, the detailed mechanisms are still kept unclear. This study aimed to investigate if electro-acupuncture (EA) treatment prevents 2,4-dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis (ACD) in rats and explore its underlying mechanisms. ACD was induced by sensitizing and challenging with DNFB topically. Rats were treated daily following bilateral subcutaneous stimulation of EA at *Zusanli* acupoint (ST36) for 1 week. Ear swelling and serum IgE levels were measured. The ear biopsies were obtained for histology. Inflammatory cytokines on the dermatological ear and local acupoint tissue were assayed. Spleen lymphocytes and the homogenized supernatant of local acupoint area were used to co-culture for flow cytology and immune analysis, respectively. EA treatment at ST36 notably inhibited ear swelling and inflammatory cell infiltration on DNFB-induced ACD. EA also decreased serum IgE concentrations and alleviated the production of inflammatory cytokines in dermatological ear. Additionally, EA treatment attenuated the percentage of CD4⁺IFN- γ ⁺

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and CD4⁺IL-4⁺ T cells associated with ACD. Interestingly, secretion of interleukin (IL)-10 in the local acupoint tissue following EA stimulation was increased and showed suppressive function when co-cultured with the spleen lymphocytes from DNFB group. Lastly, EA treatment demonstrably suppressed p38 MAPK activation in DNFB-treated rats. Our findings suggest that EA treatment at ST36 may ameliorate inflammation associated with DNFB-induced ACD *via* triggering local IL-10 production and inhibiting p38 MAPK activation, which provide an alternative and promising therapy for ACD.

KEY WORDS: electro-acupuncture; inflammation; Zusanli; allergic contact dermatitis; IL-10; p38 MAPK.

INTRODUCTION

Allergic contact dermatitis (ACD), characterized predominantly by erythema, edema, papules, and vesicles, followed by scaling and dryness, is a T cell-mediated delayed type hypersensitivity caused by skin repeated exposure to the haptens, such as 2,4-dinitrofluorobenzene (DNFB) [1]. Generally, ACD develops in two distinct phases, *i.e.*, sensitization and challenge (elicitation) phase. In the sensitization phase, a hapten is taken up and intaken to form immunogenic hapten-carrier complexes *via* binding to an endogenous protein in the skin. The hapten-carrier complex is uptaken and recognized by resident Langerhans cells and dermal dendritic cells (DCs). Then, they migrate to draining lymph nodes and present the haptenated peptides to naïve T cells which are subsequently activated. In the challenge (elicitation) phase, the hapten-specific effector T cells are activated by the repeated exposure of the skin to the same hapten, which induces tissue damage through the release of pro-inflammatory cytokines and by killing hapten-loaded keratinocytes [2]. Following a better understanding of the cellular and immune mechanisms on ACD, the different potential therapeutic approaches have been shown, including anti-inflammatory strategies, methods interfering with cell migration, and induction of the immune balance [3].

Up to now, many studies have indicated the therapeutic effects of acupuncture as a complementary medicine on dermatologic diseases such as pruritus, atopic dermatitis, urticaria, and herpes zoster [4–7]. As a crucial composition of traditional Chinese medicine, acupuncture is becoming accepted and popular alternative medical treatment in many Western countries [8]. Based on the ancient oriental medical theory of “Qi” and “Xue,” acupuncture acts through inserting and stimulating acupoints with thin needles, wherein it locates on specific body sites following the meridian [9]. In several studies, acupuncture has been shown to reduce experimental itch, allergen-induced basophil activation, and eczema in atopic dermatitis. Also, the

therapeutic efficacy of acupuncture in the treatment of atopic dermatitis has been proven in some clinical studies; however, its mechanisms of action remain poorly understood [10–13].

Electro-acupuncture (EA) is an improved technique of acupuncture stimulated with an intermittent or continuous low-voltage electrical current according to its practical application. Increasing evidence has shown its beneficial effects of EA intervention at *Zusanli* acupoint (ST36) on allergic disorders, such as asthma, chronic urticaria, and allergic rhinitis, and inflammatory diseases, such as experimental allergic encephalomyelitis, inflammatory bowel disease, and rheumatoid arthritis [4, 14–18]. Recent study reported that high-frequency EA alleviated pruritus of atopic dermatitis-like lesions in rats induced by capsaicin injection *via* the release of dynorphin [19]. However, the mechanisms underlying the local and systemic effects of EA treatment kept unclear and were needed to further elucidate.

In the present study, we investigated the effects of EA at *Zusanli* acupoint (ST36) on DNFB-induced ACD in rats and explored the molecular mechanisms involved in the therapeutic effect of EA treatment. Our results indicated that EA treatment reduced inflammation and suppressed DNFB-induced ACD *via* triggering local IL-10 production and inhibiting p38 MAPK activation, which afforded a new insight into the orchestration of the cellular and immune response for EA treatment on ACD.

MATERIALS AND METHODS

Animals

Male 8-week-old Sprague-Dawley rats were purchased from the Laboratory Animal Services Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China), and housed in specific pathogen-free conditions. All experiments were approved

by the Animal Care and Use Committee of Hubei University of Chinese Medicine (No. SYXK2012-0067) and performed according to the international ethical guidelines and National Institutes of Health Guide concerning the Care and Use of Laboratory Animals.

Groups

A schematic of the experimental procedure is shown in Fig. 1a. The rats were randomly divided into four groups: group I (control) not immunized ($n = 8$); group II (DNFB) untreated, sensitized, and challenged with DNFB for allergic contact dermatitis ($n = 8$); group III (EA + DNFB) electro-acupuncture (EA) treatment at bilateral *Zusanli* acupoint (ST36) for continuous 1 week, sensitized and challenged with DNFB ($n = 8$); group IV (sham + DNFB) EA treatment at a non-acupoint following the same stimulation as group III, sensitized and challenged with DNFB ($n = 8$).

Zusanli Acupoint (ST36) and Sham Acupoint

Zusanli acupoint ST36 is located 5 mm below the fibular head and lateral to the anterior tubercle of the tibia. Electrical stimulation was applied to bilateral ST36 via two needles with a Hans Acupoint Nerve Stimulator (HANS, Beijing, China). The needles (length 3.0 cm, diameter 0.20 mm) (Suzhou Medical Appliance Factory, Suzhou, China) were inserted perpendicular to the skin, 5 mm from each other, and about 5 mm to one side of the anterior tibial muscle. The non-acupoint (sham acupoint) localized 5 mm below the *Zusanli* acupoint. For group IV, EA was applied to bilateral non-acupoint with the same treatment as *Zusanli* acupoint, including needle insertion and electrical stimulation.

EA Treatment

The intensity of EA stimulation was determined to be the minimum voltage that caused moderate muscle contraction, and 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 for 7 consecutive days following experimental protocol between EA + DNFB group and sham + DNFB group.

2,4-Dinitrofluorobenzene-Induced Allergic Contact Dermatitis

Allergic contact dermatitis was induced by challenging the ear of previously sensitized rats with 2,4-

dinitrofluorobenzene (DNFB) (Sigma-Aldrich, St. Louis, MO, USA). Briefly, rats were sensitized by applying 25 μ l 0.5% DNFB (acetone/olive oil = 4:1) topically on shaved hind flank on day 0. On day 7, sensitized rats were challenged with 10- μ l 0.2% DNFB on the left ear. An identical volume of acetone/olive oil was painted on the right ear. The ear swelling test was used to measure DNFB-induced allergic contact dermatitis.

Ear Swelling Assay

Ear thicknesses were measured in a blinded fashion by comparing challenged (left) and unchallenged (right) ears using a thickness gauge (Digimatic caliper, Mitutoyo, Japan) at 24 h after the challenge. The magnitude of DNFB-induced contact dermatitis was determined as follows: ear swelling = thickness of DNFB-challenged ear (left) – thickness of unchallenged ear (right). The rats were then sacrificed, and their serum samples, spleens, ears, and local acupoint tissue were harvested for further experimentation.

Histological Analysis

The left ear was 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 μ m for histological analysis. Tissue sections were stained with hematoxylin and eosin (H&E) and examined for general morphology. Pictures were captured using a Nikon Eclipse Ti-S microscope (Nikon, Tokyo, Japan). All sections were randomized and evaluated in a blinded fashion.

Analysis of IgE Production in Serum

Total serum IgE levels were measured by enzyme-linked immunosorbent assay (ELISA). Blood samples were collected 24 h after the last application of DNFB and IgE concentrations in serum was quantified using IgE ELISA kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instruction.

Measurement of Cytokine Production

Cytokine levels were determined by ELISA. Ear samples and local acupoint tissue (about 1×1 cm) were weighed (100 mg) and homogenized in 1 ml of tissue protein extraction reagent containing a protease inhibitor cocktail (Pierce, Rockford, IL, USA), respectively. Homogenates were then centrifuged at $12000 \times g$ for 15 min at

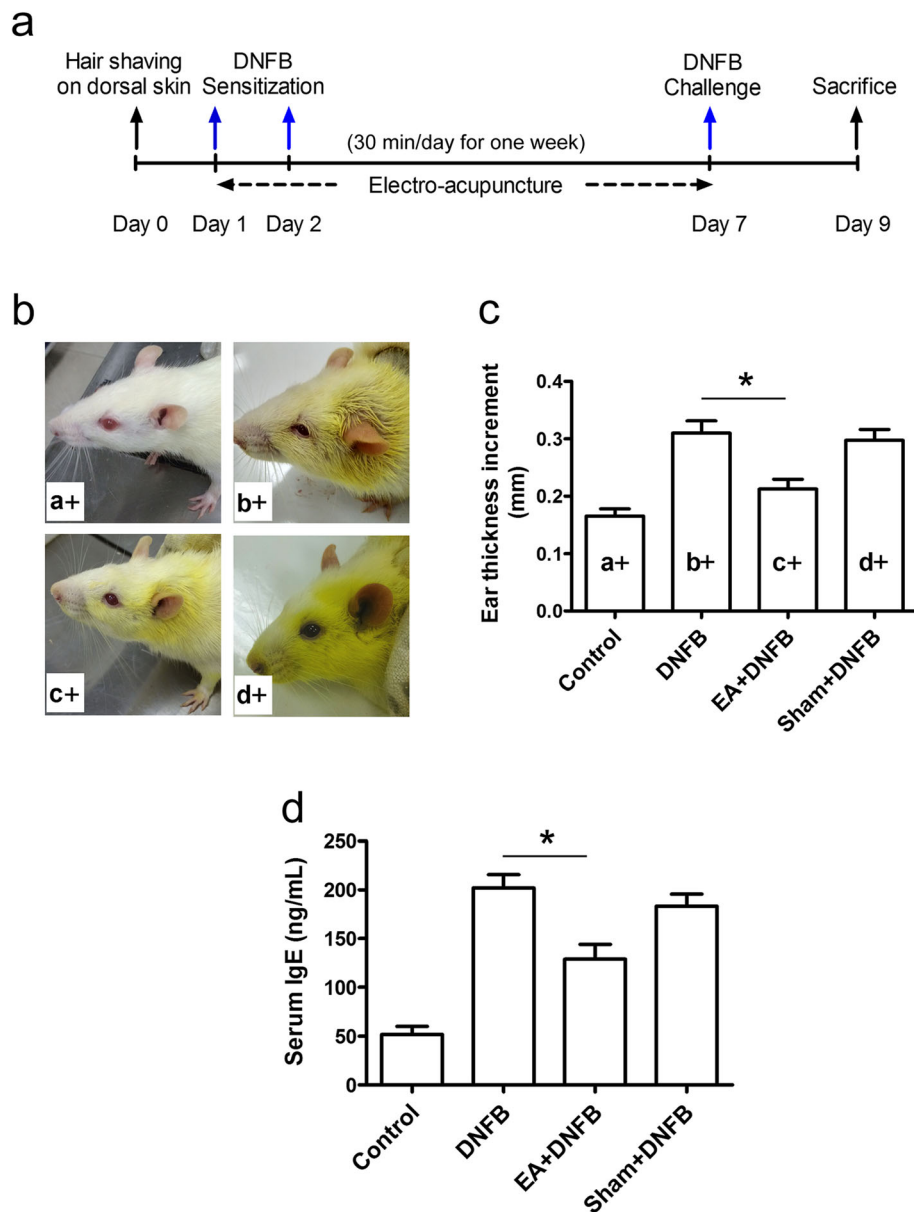


Fig. 1. EA treatment at *Zusanli* acupoint attenuates DNFB-induced ACD in rats. **a** Experimental schedule for the induction of allergic contact dermatitis by DNFB administration and EA treatment. **b** Photographs of ear swelling and **c** ear thickness increment were shown in rat model of DNFB-induced ACD with or without EA treatment. **d** Effects of EA treatment on serum IgE levels. *a+*: control, *b+*: DNFB-induced ACD, *c+*: EA + DNFB, *d+*: sham + DNFB. Data are presented as mean $\pm$ SD ($n = 8$) (* $P < 0.05$).

4 °C to obtain the supernatant. Cytokine levels of IFN- γ , TNF- α , IL-1 β , IL-4, IL-5, TGF- β 1, and IL-10 were determined by ELISA analysis (R&D systems, Minneapolis, MN, USA) using a micro-plate reader (Thermo Scientific, Waltham, MA, USA). All experiments were done in triplicate.

Preparation of Spleen Lymphocytes

Spleen lymphocytes were isolated by density gradient centrifugation (2000 rpm/min for 30 min) (Ficoll-Hypaque density 1.077 g/ml) (Sigma-Aldrich) and incubated overnight at 37 °C in complete RPMI-1640 medium containing 10% fetal bovine serum and antibiotics (100 units/ml

streptomycin and penicillin) (Gibco, Grand Island, NY, USA). Next day, non-adherent cells were harvested for spleen lymphocytes.

Flow Cytometry Analysis

Spleen lymphocytes were prepared and stained with the following anti-rat antibodies: anti-CD3-FITC (clone IF4) (Biolegend, San Diego, CA, USA), anti-IFN- γ -PE (clone DB-1) (Biolegend), anti-IL-4-PE (clone OX-81) (Biolegend), and anti-CD4-PE-Cy5 (clone OX-35) (BD PharMingen, San Diego, CA, USA). For intracellular staining, cells were stimulated for 5 h with 1 μ g/mL ionomycin and 50 ng/mL phorbol myristate acetate (PMA) in the presence of Brefeldin A (GolgiStop, Biolegend). After staining with antibodies against surface markers, cells were fixed with IC Fixation Buffer (Biolegend), permeabilized using 1 $\times$ Permeabilization Buffer (Biolegend), and finally incubated with antibodies against intracellular cytokines. Lastly, the cells were analyzed on a FACS LSRII system (BD Bioscience, San Diego, CA, USA).

Immunofluorescent Staining

We performed immunofluorescent staining to evaluate IL-10 co-localization and macrophages (F4/80). The sections fixed by 4% PFA were washed 3 times with PBS. The slides were simultaneously incubated with the primary antibodies anti-IL-10 (Abcam, Cambridge, MA, USA) and anti-F4/80 (Abcam). For labeling, the primary antibodies were detected with FITC-conjugated secondary antibody (Invitrogen) and Cy3-conjugated secondary (Invitrogen). Lastly, the sections were stained with DAPI (Invitrogen) and examined under a fluorescence microscope (Olympus BX50, Tokyo, Japan). IL-10 positive macrophages were counted in six randomly selected non-overlapping areas of per high-power field and represented as the percentage of 100 macrophages per slides.

IFN- γ Enzyme-Linked Immunospot Assay

We performed enzyme-linked immunospot (ELISPOT) experiments by using a rat IFN- γ ELISPOT kit (BD Biosciences) following the manufacturer's protocol. Spleen lymphocytes (5×10^5 cells) isolated from the rat with DNFB-induced contact dermatitis (group II) were prepared and cultured in well with or without the supernatant obtained from the rat following EA treatment at acupoint ST36 (group III), respectively. After incubation at 37 °C for 24 h, the number of IFN- γ spot-forming cells

were visualized and counted using an automated ELISPOT plate reader (CTL ImmunoSpot S5 Core Analyzer; Cellular Technology, Ltd., Shaker Heights, OH, USA), then analyzed with CTL Version 4.0 Academic Software (Cellular Technology Ltd.). Blocking studies were performed by detecting secreted IFN- γ in the presence of neutralizing antibodies against IL-10 (0.5 μ g/ml) (Abcam).

Western Blot Analysis

Spleen lymphocytes were obtained and lysed with lysis buffer containing 1 mM PMSF and a cocktail of protease and phosphatase inhibitors (Cell Signaling Technology Inc., Beverly, Massachusetts, USA). The supernatants were analyzed for protein concentration using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). Proteins were separated by electrophoresis and then transferred onto polyvinylidene fluoride (PVDF). After being blocked with 5% skim milk, membranes were incubated with the desired primary rabbit anti-rat antibodies against p-ERK, p-p38, p-JNK, or GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4 °C and then incubated with the appropriate HRP-conjugated goat anti-rabbit secondary antibody (Santa Cruz). 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 ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA). Sample loading was normalized by quantities of GAPDH detected parallel.

Statistical Analysis

Results were presented as the mean $\pm$ standard deviation (SD). Data were analyzed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Significance was assessed by unpaired two-tailed Student's *t* test or analysis of variance (ANOVA). *P* < 0.05 was considered to indicate a statistically significant difference.

RESULTS

EA Treatment Attenuates the Allergic Progression of DNFB-Induced ACD

Firstly, we investigated the effects of EA treatment at ST36 on the allergic progression of DNFB-induced ACD. Following the experimental schedule, ear swelling increment was used to assay the severity of the allergic reactions (Fig. 1a, b). As shown in Fig. 1c, DNFB challenge on day 7

induced a significant amplification in ear swelling compared with control. In comparison to DNFB-induced contact hypersensitivity, EA treatment at ST36 led to an incremental decline in ear thickness. In addition, EA treatment also significantly inhibited IgE levels in serum (Fig. 1d), suggesting the anti-allergic effect of EA on DNFB-induced ACD in rats.

EA Treatment Inhibits DNFB-Induced Inflammatory Cell Infiltration in the Ear Skin

Next, we observed the inflammatory cell infiltration in dermis of the ear skin. As shown in Fig. 2a, DNFB-challenge incurred significant infiltration of the inflammatory cells into the ear dermis. Conversely, EA treatment evidently inhibited increment in thickness of the ear epidermis caused by DNFB application and suppressed the number of inflammatory cells recruited to the ear dermis (Fig. 2b, c).

EA Treatment Ameliorates Inflammation Associated with DNFB-Induced ACD

We further investigated the effect of EA at ST36 on the production of inflammatory cytokines in the ear skin. Interestingly, the levels of Th1-type cytokines, such as IFN- γ , TNF- α , and IL-1 β , were found to be highly decreased on homogenate supernatant of the ear skin following EA treatment compared with those without EA intervention (Fig. 3a–c). Simultaneously, classic Th2-type cytokines such as IL-4 and IL-5, but not IL-10, showed the same declining trend following EA treatment (Fig. 3d–f).

EA Plays an Immunoregulatory Role on DNFB-Induced ACD

To clarify the possible immunological mechanisms involved in the protective effect of EA treatment on DNFB-induced ACD, we examined the change of CD4⁺ T cells by FACS. In comparison to control, DNFB

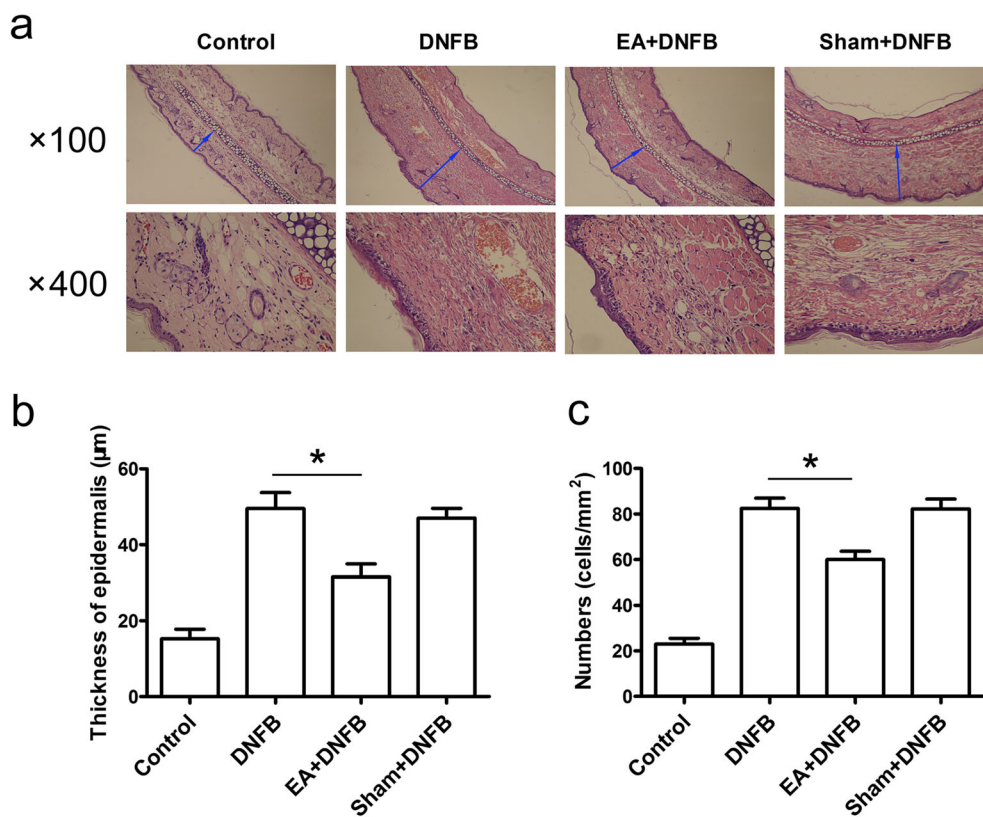


Fig. 2. EA treatment decreases inflammatory cell infiltration of ears from the rats with DNFB-induced ACD. **a** Histological changes and inflammatory cell infiltration in DNFB-challenged ears with or without EA treatment. Ears obtained from different groups were stained using hematoxylin and eosin (H&E), $\times 100$ (up) and $\times 400$ (down). **b** The ear swelling and **c** inflammatory cell densities in the dermis were statistically analyzed, respectively. Data are presented as mean $\pm$ SD ($n = 8$) (* $P < 0.05$).

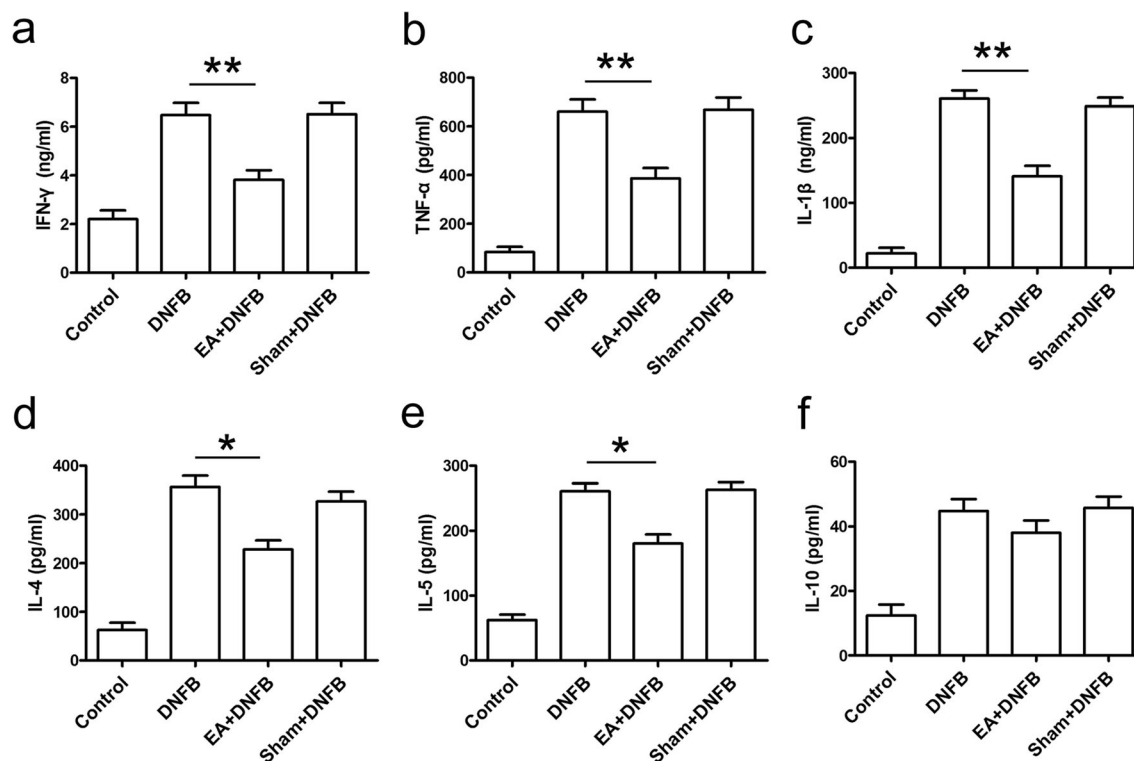


Fig. 3. EA treatment reduces inflammatory cytokine production of ears from the rats with DNFB-induced ACD. The levels of Th1-type cytokines including **a** IFN- γ , **b** TNF- α , and **c** IL-1 β , and Th2-type cytokines including **d** IL-4, **e** IL-5, and **f** IL-10 in the ears of rats were measured by ELISA and statistically analyzed, respectively. All experiments were done in triplicate. Data are presented as mean $\pm$ SD ($n = 8$) (* $P < 0.05$ and ** $P < 0.01$).

application induced a significant increment of the percentage of CD4⁺IFN- γ ⁺ and CD4⁺IL-4⁺ T cells (Fig. 4a, c). In line with the above results, EA at ST36 obviously curbed the immune activation of CD4⁺ T cells on DNFB-induced ACD (Fig. 4b, d). These results suggested that EA treatment at ST36 may play an immunoregulatory role to suppress CD4⁺ T cell activation, eventually resulting in reduction in the levels of intracellular factors including IFN- γ and IL-4.

EA Treatment at ST36 Increases IL-10 Production of Local Acupoint on DNFB-Induced ACD

As shown in Fig. 5, DNFB administration induced cytokine production on homogenate supernatant of local *Zusanli* acupoint, including IFN- γ , IL-4, TGF- β 1, and IL-10. Consistent with the previous results, EA treatment markedly restricted pro-inflammation cytokine secretion such as IFN- γ , compared with those on DNFB-induced ACD without EA intervention (Fig. 5a). However, there were no significant differences in the IL-4 and TGF- β 1 production between two groups (Fig. 5b, c). Importantly,

EA treatment at ST36 increased IL-10 production of local acupoint (Fig. 5d).

Furthermore, the homogenate supernatant from local *Zusanli* acupoint with EA treatment showed inhibitive function when co-cultured with T cells isolated from rats with single DNFB administration. Interestingly, neutralizing anti-IL-10 antibody (0.5 μ g/ml) could reverse the inhibitive effect of the homogenate supernatant from rats with EA treatment at ST36 according to ELISPOT assay (Fig. 6), suggesting that EA executed its immunoregulatory role associated with local IL-10 production.

Macrophages Contribute to IL-10 Production of Local Acupoint Following EA Treatment.

Next, we explored which cell subtype contributes to the local IL-10 production. Representative immunolabeling staining of local acupoint tissues indicated that the number of IL-10 positive cells co-localized with macrophages, identified by its antigen marker F4/80, showed no difference between the DNFB and sham + DNFB group (Fig. 7a). However, EA treatment significantly increased IL-10

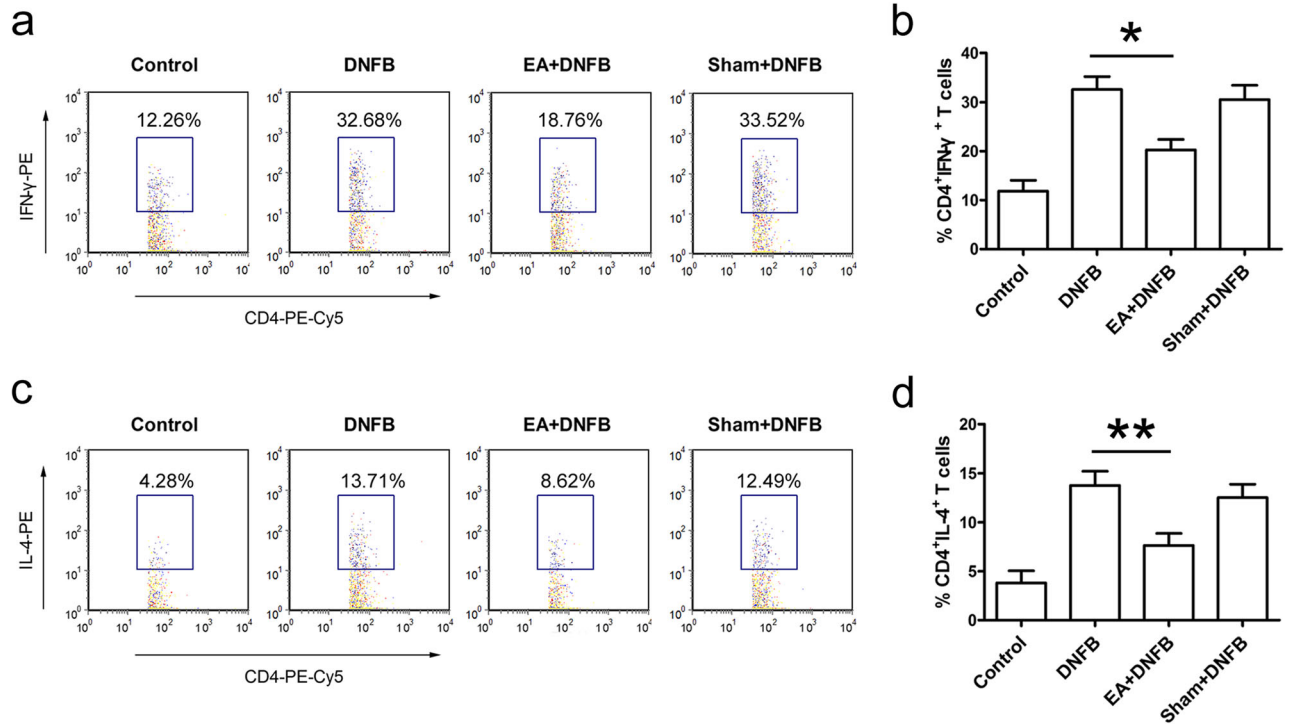


Fig. 4. EA treatment inhibits inflammation associated with DNFB-induced ACD. **a** Representative FACS plots for CD4⁺IFN-γ⁺ and **c** CD4⁺IL-4⁺ T cells in spleens from the rats of different group, respectively. **b, d** Statistical analysis of the percentage of CD4⁺IFN-γ⁺ and CD4⁺IL-4⁺ T cells, respectively. Data are presented as mean ± SD ($n = 8$) (* $P < 0.05$ and ** $P < 0.01$).

production of macrophages (IL-10⁺F4/80⁺ macrophages) (Fig. 7b), suggesting that macrophages contributed to IL-10 production of local acupoint following EA treatment.

Inhibition of p38 MAPK Signaling Was Involved in Its Anti-ACD Effect of EA Treatment at ST36

To determine the details of the molecular mechanisms, we detected protein expression of the MAPK signaling pathway including p-ERK, p-JNK, and p-p38 by Western blotting. In comparison to control group, DNFB administration increased protein expression of p-ERK, p-JNK, and p-p38 (Fig. 8a). However, it was worth noting that only p-p38 expression was mediated in response to EA treatment at ST36, but not p-ERK and p-JNK (Fig. 8b).

Lastly, different signaling inhibitors including SB203580, PD98059, and SP600125 were used, respectively. Considerably, p38 inhibitor SB203580 could definitely abrogate the suppressive effect prompted by EA treatment at ST36, leading to the improved frequency of IFN-γ positive spots, but not ERK inhibitor (PD98059) or JNK inhibitor (SP600125) (Fig. 9).

DISCUSSION

This study was designed to investigate the anti-ACD effect of EA treatment at *Zusanli* acupoint (ST36). EA treatment reduced allergy and inflammation associated with ACD induced by DNFB, which attributed to IL-10 production in the local acupoint and inhibition of p38 MAPK activation. These findings suggest that EA stimulation can be used as an alternative and promising therapy on patients with allergic skin diseases such as ACD or atopic dermatitis.

As we all know, ACD is defined as a hapten-specific T cell-mediated delayed-type hypersensitivity, which is induced by an allergic reaction to some chemical (hapten) in contact with the skin. The allergic condition causes itching and inflammation of the skin manifested by varying degrees of erythema, edema, and vesiculation [20]. DNFB is a well-known hapten for its ability to cause allergic inflammation in ACD, which was reported to elevate Th1 response but after repeated exposure can shift from the Th1 to the Th2 response or mixed response [21, 22]. In this study, sensitization of dorsal skin with the repeated topical

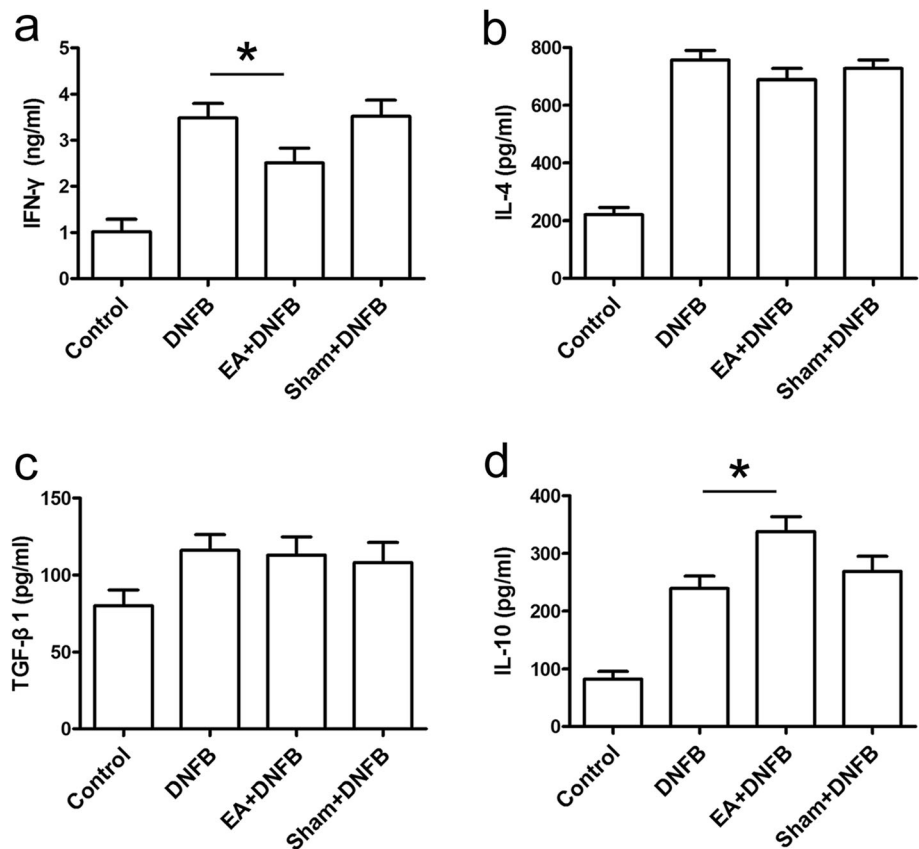


Fig. 5. Cytokine change in homogenate supernatant of local acupuncture area (ST36). The levels of cytokines, including **a** IFN- γ , **b** IL-4, **c** TGF- β 1, and **d** IL-10, in homogenate supernatant of local acupuncture area (ST36), were measured by ELISA and statistically analyzed, respectively. All experiments were done in triplicate. Data are presented as mean $\pm$ SD ($n = 8$) (* $P < 0.05$).

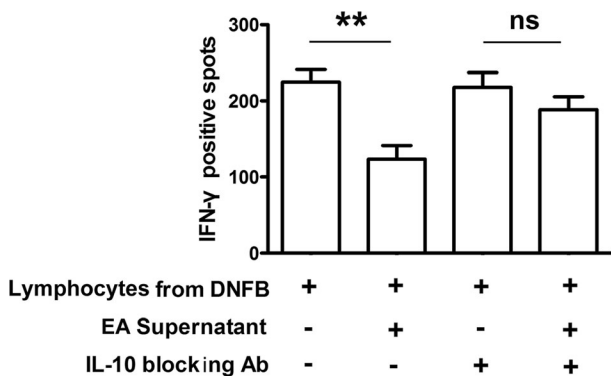


Fig. 6. IL-10 is involved in anti-ACD effect of EA stimulation. Spleen lymphocytes (1×10^5 cells/well) from rats with DNFB-induced ACD were obtained and co-cultured with 100 μ l homogenate supernatant of acupoint ST36 in the presence or absence of IL-10 antibody (0.5 μ g/ml) for 24 h. Then, the IFN- γ ELISPOT assays were performed in duplicate for detecting the number of IFN- γ^+ cells. Data are presented as mean $\pm$ SD ($n = 3$) (** $P < 0.01$).

application of DNFB and subsequent challenge of the ear resulted in ear swelling within 24 h, ear inflammation, the elevated levels of IgE in serum and allergic inflammation. EA treatment reduced erythema and edema of ear, serum IgE levels (Fig. 1), and inflammatory cell infiltration (Fig. 2). Additionally, EA treatment also attenuated the allergic inflammation of ear tissue, where Th1- and Th2-type cytokines were inhibited, including IFN- γ , TNF- α , IL-1 β , IL-4, and IL-5, but not IL-10 (Fig. 3). These findings suggested that EA treatment effectively ameliorated allergic inflammation of ACD induced by DNFB. Further, we observed the change of CD4⁺IFN- γ^+ and CD4⁺IL-4⁺ T cells in spleen following EA intervention, which contributed to the systemic inflammatory response to indicate the effect of EA treatment at ST36 acupoint. Decreasing IFN- γ and IL-4 production of T cells (Fig. 4), biomarker of Th1 and Th2, strongly suggested the anti-allergic and anti-inflammatory effects of EA treatment on

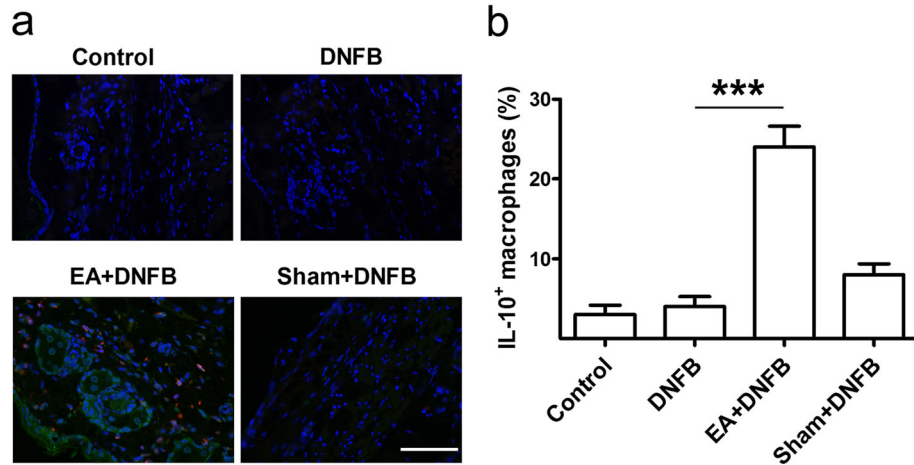


Fig. 7. Macrophages contribute to IL-10 production of local acupoint following EA treatment. **a** Representative fluorescence images of local acupoint tissues stained with F4/80 (macrophage marker, red), IL-10 (green), and DAPI (cell nuclei, blue). Scale bar is 50 μ m. **b** Statistical analysis of the percentage of IL-10⁺ macrophages in local acupoint tissues of different group. Data are presented as mean $\pm$ SD ($n = 8$) (** $P < 0.001$).

ACD. A recent study also showed the effects of high-frequency EA at six acupoints, including bilateral Feishu (BL13), unilateral (right) Quchi (LI11), unilateral (left) Zusanli (ST36), Xuehai (SP10), and Sanyinjiao (SP6), on capsaicin-induced model of atopic dermatitis in rats. The finding indicated that EA treatment induced significant change in the dermatitis score and no significant difference in other indicator, which was associated with the dynorphin secretion [19]. The study emphasized the anti-inflammatory of EA stimulation *via* evoking neurological changes rather than immunological and histological changes. Conversely, our study focused on the immune response such as IFN- γ secretion, the production of IgE,

and the changes of CD4⁺ T cells, suggesting its immunoregulatory effects of EA treatment as previous studies reported.

Although acupuncture treatment has been increasingly used in atopic dermatitis, with several clinical studies demonstrating the effectiveness of acupuncture, preclinical studies, especially *in vivo* studies, investigating the mechanism of action for acupuncture is lacking [10–13, 23–25]. Our previous study indicated that EA treatment at acupoint ST36 could ameliorate inflammation associated with ovalbumin-induced delayed-type hypersensitivity *via* restoring Th1/Th2 balance by curbing Th1 differentiation [26]. This study provided more evidence to elucidate the

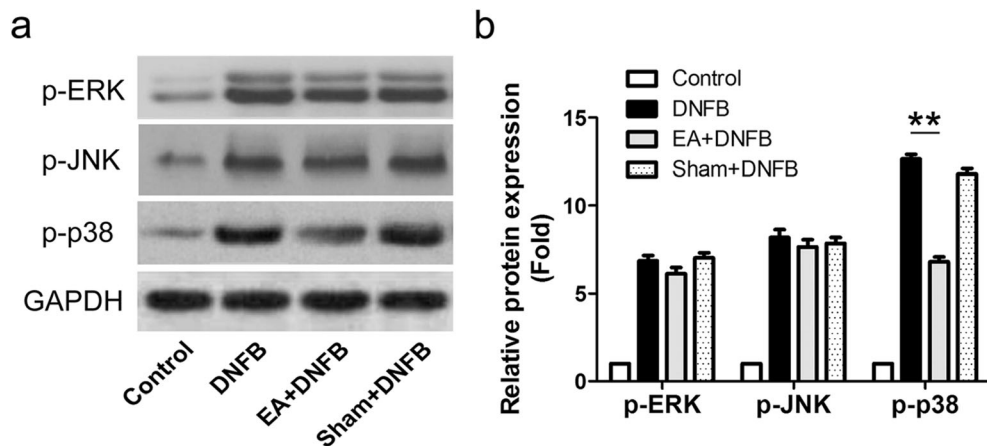


Fig. 8. Effects of EA treatment at ST36 on MAPK signaling pathway. Spleen lymphocytes from rats of different group were obtained. **a** The protein expressions including p-ERK, p-JNK, and p-p38 were performed by Western blotting, and a representative of eight independent experiments was shown. **b** Statistical analysis of protein expression including p-ERK, p-JNK, and p-p38 was shown. Data are presented as mean $\pm$ SD ($n = 8$) (** $P < 0.01$).

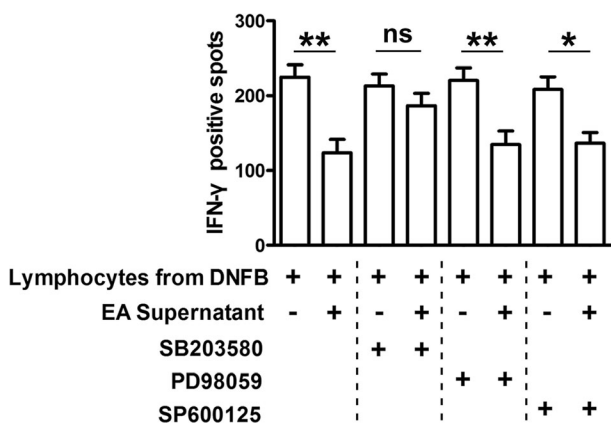


Fig. 9. Inhibiting p38 MAPK activation is associated with anti-ACD effect of EA. Spleen lymphocytes (1×10^5 cells/well) from rats with DNFB-induced ACD were obtained and pretreated with inhibitors including SB203580, PD98059, and SP600125 for 30 min, respectively. Then, the cells were co-cultured with 100 μ l homogenate supernatant of acupoint ST36. After 24 h, the IFN- γ ELISPOT assays were performed in duplicate for detecting the number of IFN- γ ⁺ cells. Data are presented as mean $\pm$ SD ($n = 3$) (* $P < 0.05$ and ** $P < 0.01$).

anti-inflammatory and anti-allergic effects of EA at ST36 acupoint, which was consistent with the previous results. In the current study, EA treatment triggered local IL-10 production at ST36 acupoint compared with those in DNFB group (Fig. 5). However, there was no significant difference for IL-10 production in serum among groups with DNFB application (data not shown). Accumulating evidence indicated that acupuncture treatment was able to induce IL-10 production. EA applied to bilateral ST36 acupoint promoted an anti-inflammatory action on the 2, 4, 6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice, mediated by increase of IL-10 and decrease of iNOS expression [27]. Unilateral manual acupuncture at the SP6 acupoint elicited pronounced anti-inflammatory actions in mice submitted to carrageenan-induced peritonitis, which was associated with increased IL-10 levels in peritoneal fluid [28]. In addition, local IL-10 released from M2 macrophages was important for analgesic and anti-inflammatory effects of acupuncture at SP6 in a model of inflammatory muscle pain, resulting from phenotypic switch of muscle macrophages with reduced M1 macrophages and an increase of M2 macrophages [29].

Macrophages, which can respond differently to different environmental signals, are generally separated into two subsets, that is, classical activation (more pro-inflammatory, M1 macrophages) and alternative activation (more anti-inflammatory and pro-healing, M2 macrophages) [30]. As a central negative regulator of the immune

responses, IL-10 can be produced by several cell types within the immune system, including macrophages [31–34]. Many studies also emphasized the role of IL-10 in macrophage polarization. IL-10/IL-10R axis was required for mycobacterial heat shock protein 70-induced M2-like polarization in the murine macrophages [35]. Lentiviral delivery of IL-10 to macrophages represented a promising strategy for directing and sustaining macrophage polarization toward M2 phenotype in order to local immune responses [36]. In this study, IL-10 blocking antibody assay provided further evidence for its possible role of IL-10 in shifting macrophage polarization toward to M2 subsets (Fig. 6). Moreover, EA treatment significantly increased IL-10 production of macrophages (IL-10⁺F4/80⁺ macrophages) (Fig. 7), suggesting that macrophages contributed to IL-10 production of local acupoint following EA stimulation at ST36. We speculated that repeated EA stimulation at ST36 affected and changed the local micro-environment, such as the balance of Th1 and Th2 cytokines, accompanying with DNFB-induced inflammation. Conversely, local up-regulation of IL-10 and down-regulation of IFN- γ will influence development and differentiation of immune cell surrounded in acupoint, such as macrophages, DCs, Langerhans cells, and keratinocytes [37–39]. In addition, EA treatment at ST36 may affect local cholinergic anti-inflammatory, which mediated by acetylcholine (ACh) via the vagus nerve binding to $\alpha 7$ -nicotinic receptors ($\alpha 7$ nAChR) on macrophages [40, 41]. It was also possible that EA treatment affected the hypothalamus-pituitary-adrenal (HPA) axis, regulating the local and systemic responses including immune cells, cytokines, IgE, nitric oxide, neuropeptide, opioid neuropeptide, and NF- κ B activation, etc. [42–45].

Mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase1/2 (ERK1/2), and p38 MAPK, are mediators of various cellular signaling pathways in response to EA stimulation exerting its biological function [46]. In a rodent model of surgical trauma, p38 MAPK activation was required for EA restoration of CD4⁺/CD8⁺ homeostasis and cytokine expression [47]. A previous study evaluated the effects of acupuncture at meridian point LI11 on 1-chloro-2,4-dinitrochlorobenzene (DNCB)-induced ACD, which was related to suppression of NF- κ B and MAP kinase including ERK1/2, JNK, and p38 [48]. Utilizing the fetal mouse skin DC line, DNFB application activated ERK1/2 and p38 MAPK signaling pathways, suggesting that this strong sensitizer induced phenotypic changes that may represent an early activation state of the DCs associated with

the sensitization phase of ACD [49]. In contrast, our findings showed that ERK and JNK activation seemed to have no significant difference in the presence or absence of EA stimulation; however, EA treatment suppressed p38 MAPK activation (Fig. 8). Also, the inhibitor assay of MAPK signaling pathway suggested that p38 MAPK activation contributed to the anti-ACD effect of EA treatment at ST36 acupoint (Fig. 9). Further studies are needed to clarify the detailed mechanisms associated with p38 MAPK activation such as upstream and downstream signaling molecules.

There were some limitations in the present study that should be mentioned. Firstly, we did not investigate the anti-ACD effect of EA treatment using IL-10 knock out rat, which will provide more evidences to support the therapeutic role of IL-10 triggered by EA stimulation in local acupoint. Secondly, future investigations will focus on fully understanding the roles of each change in gene expression upon the regulation of EA treatment.

In conclusion, the present study demonstrates that EA treatment at *Zusanli* acupoint (ST36) reduces inflammation and suppresses ACD via triggering local IL-10 production and inhibiting p38 MAPK activation. Our results may represent a novel profile for its protective mechanisms of EA treatment on DNFB-induced ACD, suggesting that EA could be a potential therapeutic candidate for ACD or atopic dermatitis.

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Author's Contribution. Z.G.W., N.N.Y., and Z.B.C. designed the study, Z.G.W. and T.Y. finished experiments and wrote the manuscript. M.L., Y.S.G., and C.H.C. performed partial research. C.W.H. and Q.W. analyzed the data. Z.B.C. helped revise the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

All experiments were approved by the Animal Care and Use Committee of Hubei University of Chinese Medicine (No. SYXK2012-0067) and performed according to the international ethical guidelines and National Institutes of Health Guide concerning the Care and Use of Laboratory Animals.

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

REFERENCES

1. Peng, W., and N. Novak. 2015. Pathogenesis of atopic dermatitis. *Clinical and Experimental Allergy* 45: 566–574.
2. Kaplan, D.H., B.Z. Igyártó, and A.A. Gaspari. 2012. Early immune events in the induction of allergic contact dermatitis. *Nature Reviews. Immunology* 12: 114–124.
3. Schlapbach, C., and D. Simon. 2014. Update on skin allergy. *Allergy* 69: 1571–1581.
4. Han, J.B., C.W. Kim, B. Sun, S.K. Kim, M.G. Lee, D.S. Park, et al. 2008. The antipruritic effect of acupuncture on serotonin-evoked itch in rats. *Acupunct Electrother Res* 33: 145–156.
5. Sur, B., B. Lee, M. Yeom, J.H. Hong, S. Kwon, S.T. Kim, et al. 2016. Bee venom acupuncture alleviates trimellitic anhydride-induced atopic dermatitis-like skin lesions in mice. *BMC Complementary and Alternative Medicine* 16: 38.
6. Yao, Q., Y. Ye, X. Liu, Z. Qin, and Z. Liu. 2015. Acupuncture for patients with chronic urticaria: A systematic review protocol. *BMJ Open* 5: e007704.
7. Ursini, T., M. Tontodonati, L. Manzoli, E. Polilli, C. Rebuzzi, G. Congedo, et al. 2011. Acupuncture for the treatment of severe acute pain in herpes zoster: Results of a nested, open-label, randomized trial in the VZV pain study. *BMC Complementary and Alternative Medicine* 11: 46.
8. Tan, H.Y., G.B. Lenon, A.L. Zhang, and C.C. Xue. 2015. Efficacy of acupuncture in the management of atopic dermatitis: A systematic review. *Clinical and Experimental Dermatology* 40: 711–715.
9. Shu, Q., H. Wang, D. Litscher, S. Wu, L. Chen, I. Gaischek, et al. 2016. Acupuncture and Moxibustion have different effects on fatigue by regulating the autonomic nervous system: A pilot controlled clinical trial. *Scientific Reports* 6: 37846.
10. Pfab, F., M.T. Kirchner, J. Huss-Marp, T. Schuster, P.C. Schalock, J. Fuqin, et al. 2012. Acupuncture compared with oral antihistamine for type I hypersensitivity itch and skin response in adults with atopic dermatitis: A patient- and examiner-blinded, randomized, placebo-controlled, crossover trial. *Allergy* 67: 566–573.
11. Pfab, F., J. Huss-Marp, A. Gatti, J. Fuqin, G.I. Athanasiadis, D. Irnich, et al. 2010. Influence of acupuncture on type I hypersensitivity itch and the wheal and flare response in adults with atopic eczema - a blinded, randomized, placebo-controlled, crossover trial. *Allergy* 65: 903–910.
12. Pfab, F., G.I. Athanasiadis, J. Huss-Marp, J. Fuqin, B. Heuser, L. Cifuentes, et al. 2011. Effect of acupuncture on allergen-induced basophil activation in patients with atopic eczema: A pilot trial. *Journal of Alternative and Complementary Medicine* 17: 309–314.
13. Lee, K.C., A. Keyes, J.R. Hensley, J.R. Gordon, M.J. Kwasny, D.P. West, et al. 2012. Effectiveness of acupressure on pruritus and lichenification associated with atopic dermatitis: A pilot trial. *Acupuncture in Medicine* 30: 8–11.
14. Kwon, Y., S.H. Sohn, G. Lee, Y. Kim, H. Lee, M. Shin, et al. 2012. Electroacupuncture attenuates ovalbumin-induced allergic asthma via modulating CD4(+)CD25(+) regulatory T cells. *Evidence-based Complementary and Alternative Medicine* 2012: 647308.
15. Kim, A.R., J.Y. Choi, J.I. Kim, S.Y. Jung, and S.M. Choi. 2011. Acupuncture treatment of a patient with persistent allergic rhinitis

- complicated by rhinosinusitis and asthma. *Evidence-based Complementary and Alternative Medicine* 2011: 798081.
16. Liu, Y., H. Wang, X. Wang, L. Mu, Q. Kong, D. Wang, et al. 2013. The mechanism of effective electroacupuncture on T cell response in rats with experimental autoimmune encephalomyelitis. *PLoS One* 8: e51573.
 17. Ho, T.Y., H.Y. Lo, D.C. Chao, C.C. Li, J.J. Liu, C. Lin, et al. 2014. Electroacupuncture improves trinitrobenzene sulfonic acid-induced colitis, evaluated by transcriptomic study. *Evidence-based Complementary and Alternative Medicine* 2014: 942196.
 18. Dong ZQ, Zhu J, Lu DZ, Chen Q and Xu YL. 2016. Effect of Electroacupuncture in “Zusanli” and “Kunlun” Acupoints on TLR4 Signaling Pathway of Adjuvant Arthritis Rats. *American Journal Therapeutics*.
 19. Jung, D.L., S.D. Lee, I.H. Choi, H.S. Na, and S.U. Hong. 2014. Effects of electroacupuncture on capsaicin-induced model of atopic dermatitis in rats. *Journal of Dermatological Science* 74: 23–30.
 20. Honda, T., G. Egawa, S. Grabbe, and K. Kabashima. 2013. Update of immune events in the murine contact hypersensitivity model: Toward the understanding of allergic contact dermatitis. *The Journal of Investigative Dermatology* 133: 303–315.
 21. Hussain, Z., H. Katas, M.C. Mohd Amin, and E. Kumolosasi. 2014. Efficient immuno-modulation of TH1/TH2 biomarkers in 2,4-dinitrofluorobenzene-induced atopic dermatitis: Nanocarrier-mediated transcutaneous co-delivery of anti-inflammatory and antioxidant drugs. *PLoS One* 9: e113143.
 22. Kim, M.S., Y.G. Hur, W.G. Kim, B.W. Park, K.S. Ahn, J.J. Kim, et al. 2011. Inhibitory effect of *Platycodon grandiflorum* on T(H)1 and T(H)2 immune responses in a murine model of 2,4-dinitrofluorobenzene-induced atopic dermatitis-like skin lesions. *Annals of Allergy, Asthma & Immunology* 106: 54–61.
 23. Kim, J.I., M.S. Lee, S.Y. Jung, J.Y. Choi, S. Lee, J.M. Ko, et al. 2009. Acupuncture for persistent allergic rhinitis: A multi-centre, randomised, controlled trial protocol. *Trials* 10: 54.
 24. Chan, I.H., and D.F. Murrell. 2016. Itch management: Physical approaches (UV phototherapy, acupuncture). *Current Problems in Dermatology* 50: 54–63.
 25. Jeon, Y.C., and H.B. Lee. 2016. Treatment of an adult patient with atopic dermatitis using Traditional Korean medicine, especially Sam acupuncture. *Journal of Acupuncture and Meridian Studies* 9: 322–324.
 26. Wang, Z., T. Chen, M. Long, L. Chen, L. Wang, N. Yin, et al. 2017. Electro-acupuncture at Acupoint ST36 ameliorates inflammation and regulates Th1/Th2 balance in delayed-type hypersensitivity. *Inflammation* 40: 422–434.
 27. Goes, A.C., F.M. Pinto, G.C. Fernandes, J.S. Barbosa, E.S. Correia, R.A. Ribeiro, et al. 2014. Electroacupuncture ameliorates experimental colitis induced by TNBS through activation of interleukin-10 and inhibition of iNOS in mice. *Acta Cirúrgica Brasileira* 29: 787–793.
 28. da Silva, M.D., G. Guginski, M.F. Werner, C.H. Baggio, R. Marcon, and A.R. Santos. 2011. Involvement of interleukin-10 in the anti-inflammatory effect of Sanyinjiao (SP6) acupuncture in a mouse model of peritonitis. *Evidence-based Complementary and Alternative Medicine* 2011: 217946.
 29. da Silva, M.D., F. Bobinski, K.L. Sato, S.J. Kolker, K.A. Sluka, and A.R. Santos. 2015. IL-10 cytokine released from M2 macrophages is crucial for analgesic and anti-inflammatory effects of acupuncture in a model of inflammatory muscle pain. *Molecular Neurobiology* 51: 19–31.
 30. Mosser, D.M., and J.P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nature Reviews. Immunology* 8: 958–969.
 31. Howes, A., C. Taubert, S. Blankley, N. Spink, X. Wu, C.M. Graham, et al. 2016. Differential production of type I IFN determines the reciprocal levels of IL-10 and Proinflammatory cytokines produced by C57BL/6 and BALB/c macrophages. *Journal of Immunology* 197: 2838–2853.
 32. Kobayashi, M., F. Usui, T. Karasawa, A. Kawashima, H. Kimura, Y. Mizushima, et al. 2016. NLRP3 deficiency reduces macrophage interleukin-10 production and enhances the susceptibility to doxorubicin-induced cardiotoxicity. *Scientific Reports* 6: 26489.
 33. Na, Y.R., D. Jung, G.J. Gu, A.R. Jang, Y.H. Suh, and S.H. Seok. 2015. The early synthesis of p35 and activation of CDK5 in LPS-stimulated macrophages suppresses interleukin-10 production. *Science Signaling* 8: ra121.
 34. Dibra, D., and S. Li. 2013. The cell-to-cell coordination between activated T cells and CpG-stimulated macrophages synergistically induce elevated levels of IL-10 via NF- κ B, STAT3, and CD40/CD154. *Cell Communication and Signaling: CCS* 11: 95.
 35. Lopes, R.L., T.J. Borges, R.F. Zanin, and C. Bonorino. 2016. IL-10 is required for polarization of macrophages to M2-like phenotype by mycobacterial DnaK (heat shock protein 70). *Cytokine* 85: 123–129.
 36. Boehler, R.M., R. Kuo, S. Shin, A.G. Goodman, M.A. Pilecki, R.M. Gower, et al. 2014. Lentivirus delivery of IL-10 to promote and sustain macrophage polarization towards an anti-inflammatory phenotype. *Biotechnology and Bioengineering* 111: 1210–1221.
 37. Gros, E., S. Petzold, L. Maintz, T. Bieber, and N. Novak. 2011. Reduced IFN- γ receptor expression and attenuated IFN- γ response by dendritic cells in patients with atopic dermatitis. *The Journal of Allergy and Clinical Immunology* 128: 1015–1021.
 38. Matsui, K., and A. Nishikawa. 2013. Peptidoglycan-induced T helper 2 immune response in mice involves interleukin-10 secretion from Langerhans cells. *Microbiology and Immunology* 57: 130–138.
 39. Meehansan, J., H. Tsuda, M. Komine, S. Tominaga, and M. Ohtsuki. Regulation of IL-33 expression by IFN- γ and tumor necrosis factor- α in normal human epidermal keratinocytes. *The Journal of Investigative Dermatology* 132: 2593–2600.
 40. Hu, S., M.H. Du, H.M. Luo, H. Wang, Y. Lv, L. Ma, et al. 2013. Electroacupuncture at Zusanli (ST36) prevents intestinal barrier and remote organ dysfunction following gut ischemia through activating the cholinergic anti-inflammatory-dependent mechanism. *Evidence-based Complementary and Alternative Medicine* 2013: 592127.
 41. Song, Q., S. Hu, H. Wang, Y. Lv, X. Shi, Z. Sheng, et al. 2014. Electroacupuncture at Zusanli point (ST36) attenuates pro-inflammatory cytokine release and organ dysfunction by activating cholinergic anti-inflammatory pathway in rat with endotoxin challenge. *African Journal of Traditional, Complementary, and Alternative Medicines* 11: 469–474.
 42. Feng, Y., J. Johansson, R. Shao, L. Mannerås, J. Fernandez-Rodríguez, H. Billig, et al. 2009. Hypothalamic neuroendocrine functions in rats with dihydrotestosterone-induced polycystic ovary syndrome: Effects of low-frequency electro-acupuncture. *PLoS One* 4: e6638.
 43. Le, J.J., T. Yi, L. Qi, J. Li, L. Shao, and J.C. Dong. 2016. Electroacupuncture regulate hypothalamic-pituitary-adrenal axis and enhance hippocampal serotonin system in a rat model of depression. *Neuroscience Letters* 615: 66–71.
 44. Torres-Rosas, R., G. Yehia, G. Peña, P. Mishra, M. del Rocio Thompson-Bonilla, M.A. Moreno-Eutimio, et al. 2014. Dopamine mediates vagal modulation of the immune system by electroacupuncture. *Nature Medicine* 20: 291–295.
 45. Liu, F., J. Fang, X. Shao, Y. Liang, Y. Wu, and Y. Jin. 2014. Electroacupuncture exerts an anti-inflammatory effect in a rat tissue chamber model of inflammation via suppression of NF- κ B activation. *Acupuncture in Medicine* 32: 340–345.

46. Yang, Q., Y.D. Xie, M.X. Zhang, B. Huang, C. Zhang, H.Y. Li, et al. 2014. Effect of electroacupuncture stimulation at Zusanli acupoint (ST36) on gastric motility: Possible through PKC and MAPK signal transduction pathways. *BMC Complementary and Alternative Medicine* 14: 137.
47. Li, M., K. Gu, M. Chi, X. Sun, J. Gu, and G. Wang. 2014. p38 mitogen-activated protein kinase is required for electroacupuncture restoration of CD4+/CD8+ homeostasis and cytokine expression in a rodent model of surgical trauma. *Cell Biochemistry and Biophysics* 70: 1213–1218.
48. Park, J.Y., H.J. Park, Y.Y. Choi, M.H. Kim, S.N. Kim, and W.M. Yang. 2013. Effects of acupuncture on 1-chloro-2, 4-dinitrochlorobenzene-induced atopic dermatitis. *Evidence-based Complementary and Alternative Medicine* 2013: 982095.
49. Trompezinski, S., C. Migdal, M. Tailhardat, B. Le Varlet, P. Courtellemont, M. Haftek, et al. 2008. Characterization of early events involved in human dendritic cell maturation induced by sensitizers: Cross talk between MAPK signalling pathways. *Toxicology and Applied Pharmacology* 230: 397–406.