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ORIGINAL ARTICLE

Electro-acupuncture at Acupoint ST36 Ameliorates Inflammation and Regulates Th1/Th2 Balance in Delayed-Type Hypersensitivity

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Abstract—Increasing evidence indicates anti-allergic and anti-inflammatory effects of electro-acupuncture (EA) therapy. However, its underlying mechanism on delayed-type hypersensitivity (DTH), a classic allergic inflammatory disease, still remains unclear. In this study, we aimed to explore the immunomodulatory mechanism of EA intervention in a mouse model of ovalbumin (OVA)-induced DTH. Mice were randomly divided into four groups: Control, OVA-DTH, DTH + EA, DTH + Sham. "Zusanli" acupoint (ST36) was used for DTH + EA, whereas a non-acupoint (localized 5 mm below the "Zusanli" acupoint) was selected for DTH + Sham. Footpad thickness was checked, and the infiltration of inflammatory cells was estimated by hematoxylin and eosin staining. Levels of IgG and IgE in serum of different groups and inflammatory cytokines in the supernatants from homogenized footpads, including IFN-γ, TNF-α, IL-4, and IL-5, were determined by ELISA. Cell proliferation of spleen lymphocytes was assayed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT). The frequency of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}4^+$ T cells was analyzed with flow cytometry. In addition, the mRNA and protein expression of T-bet and GATA-3 were evaluated by real-time PCR and

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Western blotting, respectively. Our data showed EA treatment at acupoint ST36 relieved the pathological progression of DTH responses via reduction in footpad swelling, infiltration of inflammatory cells, levels of IgG and IgE as well as decreased production of IFN-γ and TNF-α in homogenized footpad tissue. Moreover, detailed studies were performed revealing that EA attenuated the percentage of $CD4^+IFN\text{-}\gamma^+$ T cells and prevented Th cells differentiation into Th1 cells, and this results from inhibiting secretion of IFN-γ and suppressing expression of T-bet, an IFN-γ transcription factor. The results indicated that EA treatment improved Th1-mediated allergic skin inflammation via restoring Th1/Th2 balance by curbing Th1 differentiation. These findings suggested that EA at acupoint ST36 might be a useful and promising therapeutic for allergic inflammatory as well as Th1-mediated inflammation response.

KEY WORDS: electro-acupuncture; Zusanli; inflammation; Th1/Th2; delayed-type hypersensitivity.

INTRODUCTION

Acupuncture, a crucial composition of Traditional Chinese Medicine, is becoming more widely accepted and is becoming a popular alternative therapy in China and many Western countries [1]. According to the ancient oriental medical theory, acupuncture acts by thin needles stimulating acupoints at specific body sites on meridians to unlock the meridians and corresponding collaterals via the functional regulation of "Qi" and "Xue" [2]. When acupuncture needles are inserted into acupoints, manipulation such as twirling or lifting-thrusting needles is usually adopted to deport pathogens and support health, resulting from harmonizing and balancing the energy in the body [3].

Electro-acupuncture (EA) is a modified technique of acupuncture stimulated with a low-voltage electrical current [4]. The anti-inflammatory and anti-allergic properties of EA intervention at the "Zusanli" acupoint (ST36), the most commonly used acupoint for the purpose of immune strengthening, have been reported in recent studies. EA stimulation exerted a protective effect on rheumatoid arthritis, one of the Th1 dominant disorders linked to the induction of IFN-γ, which reduced arthritis incidence, prevented histological destruction of the joint and down-regulated serum levels of IFN-γ and TNF-α in collagen-induced arthritic mice [5]. In clinical studies, EA treatment was reported to be beneficial for allergic disorders, such as asthma, chronic urticaria and allergic rhinitis [6]. In DNP-KLH immunized mice, EA at ST36 significantly suppressed the elevated serum IgE levels by inhibiting Th2 cytokines, especially IL-4 instead of IFN-γ. Interestingly, the protective effect of EA was acupoint-specific since EA stimulation at a non-acupoint did not induce a significant change [7]. Therefore, EA intervention might have dual immunomodulatory effect in either Th1- or Th2-skewed conditions to maintain homeostasis.

It is well known that naïve $CD4^+$ T cells can be differentiated into two classic subpopulations, i.e., Th1 and Th2, based on their patterns of cytokine production [8]. Generally, Th1 cells produce IL-2, IFN-γ, and TNF-α that are primarily responsible for cell-mediated immunity including delayed-type hypersensitivity (DTH). In addition, Th2 cells produce IL-4, IL-5, and IL-13 that are mainly contributed to humoral immunity such as allergic responses [9]. Ovalbumin (OVA)-induced DTH is characterized by allergic reactions accompanying inflammatory progression involving T cells activation and production of Th1-type cytokines, such as IL-2, which is an initiator of T cell activation, and IFN-γ, which plays a role in the effect phase of DTH responses [10]. Although some studies have shown that EA treatment was effective in alleviating allergic diseases induced by OVA [11], but details mechanisms of EA-induced immunomodulation are still undetermined.

In the present study, our results demonstrate that EA treatment at acupoint ST36 ameliorates inflammation and regulates Th1/Th2 balance on OVA-induced DTH, while selectively affecting Th1 rather than Th2 immunity in DTH reactions. These findings suggest EA intervention at acupoint ST36 would be an alternative and effective therapy for treatment of allergic and inflammatory diseases.

MATERIALS AND METHODS

Materials

OVA was chromatographically purified and purchased from Worthington (Lakewood, NJ, USA). Unless mentioned, all the other common reagents were obtained from Sigma Aldrich (St. Louis, MO, USA).

Animals

Female C57BL/6 mice weighing 18–20 g, supplied by the Laboratory Animal Services Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China), were acclimated in an air-conditioned room (23 ± 0.5 °C, 12 h light/dark cycle) with free access to food and water. All experiments were approved by the Institutional Ethics Committee of Hubei University of Chinese Medicine and performed in accordance with the international ethical guidelines and National Institutes of Health Guide concerning the Care and Use of Laboratory Animals.

Induction of Delayed-Type Hypersensitivity

Mice were sensitized following a subcutaneous injection of OVA (50 μg in 50 μL saline) emulsified with 50 μL complete Freund's adjuvant (CFA). Two injections were given to each mouse, one in each hind flank. After 6 days, mice were re-challenged in the left footpad with 200 μg heat-aggregated OVA in 20 μL PBS and in the right footpad with PBS only. Twenty-four hours after re-challenge, footpad thickness was measured with a digital caliper (LS Starrett Company, Athol, MA, USA). The magnitude of OVA-induced DTH response was determined as follows: Footpad swelling = Footpad thickness of OVA-injected footpad − Footpad thickness of PBS-injected footpad. The mice were then sacrificed, and their serum samples, spleens, and footpad tissue were harvested for further experimentation.

Groups

A schematic of the experimental procedure is shown in Fig. 1a. The animals were randomly divided into four groups ($n = 8$, each group): group I (Control), not immunized; group II (OVA-DTH), immunized, untreated, and re-challenged with OVA for DTH model; group III (DTH + EA), immunized, treated with EA at bilateral "Zusanli" acupoint (ST36) for continuous 7 days, and re-challenged; group IV (DTH + Sham), immunized, treated with EA at a non-acupoint following the same stimulation as group III, and re-challenged.

"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 localized 5 mm below the "Zusanli" acupoint (ST36). For group IV, EA was applied to bilateral non-acupoint with the same treatment as "Zusanli" acupoint (ST36), including needles insertion and electrical stimulation.

EA Stimulation

The intensity of 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 were repeated for seven consecutive days following experimental protocol between DTH + EA group and DTH + Sham group.

Histological Examination of Footpad

Footpads of mice were fixed with 10% neutral buffered formalin for 2 days, and then immersed in a rapid decalcifier solution (Cryostat, CM3050S) (Leica Microsystems, Nussloch, Germany) for 3 days. The tissue specimens were embedded in paraffin, cut into 5 μm section, and stained with hematoxylin and eosin (H&E) for routine histopathology. Pictures were observed using a Nikon Eclipse Ti-S microscope (Nikon, Tokyo, Japan). All sections were randomized and evaluated by two trained observers who were blinded to the groups.

Preparation of Spleen Lymphocytes

Spleen mononuclear cells 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 as spleen lymphocytes. For ConA stimulation assay, spleen lymphocytes (2×10^5) were incubated in the presence of 5 mg/mL ConA for 24 h, and the supernatant was collected for the cytokine detection.

MTT Assay

Spleen lymphocytes (5×10^5) were separated and incubated for 24 h in the presence of OVA (100 μg/mL).

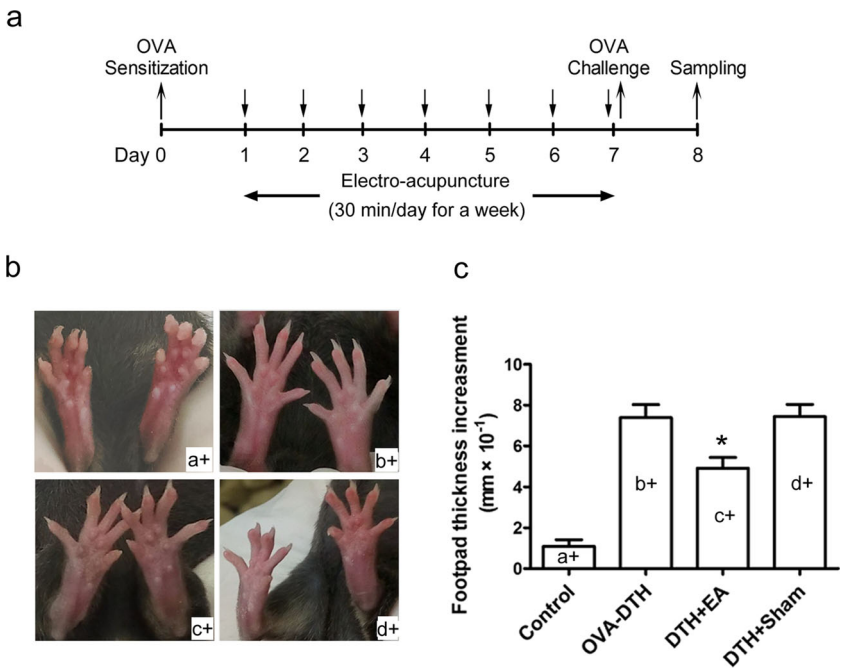

Fig. 1. EA treatment at acupoint ST36 inhibits the allergic response in mice model of OVA-induced DTH. **a** Schematic diagram of the experimental procedure. The total experiment took 9 days. C57BL/6 mice were immunized with OVA at beginning of the experiment and boosted after 6 days. "Zusanli" acupoint (ST36) immunization group was treated with EA for 1 week. **b** Photographs of the footpad swelling and **c** footpad thickness increment were shown in mice model of OVA-induced DTH with or without EA treatment. *a*+, control group; *b*+, OVA-DTH group; *c*+, DTH + EA group; *d*+, DTH + Sham group. Data are presented as mean ± SD ($n = 8$). *$P < 0.05$, comparison with the OVA-DTH group.

MTT working solution (0.5 mg/mL) was added, and the plates were incubated for an additional 4 h at 37 °C. After centrifugation, the medium was replaced with dimethyl sulfoxide. The absorbance of each well at 570 nm was measured with a plate reader (Thermo Scientific, Waltham, MA, USA).

Enzyme-Linked Immunosorbent Assay

At the end of the experiment, sera of mice in different groups were collected. Anti-OVA IgG titers and the level of IgE were determined by enzyme-linked immunosorbent assay (ELISA) analysis (Life Technologies, Grand Island, NY, USA) using a micro-plate reader (Thermo Scientific).

Footpads were mechanically homogenized, centrifuged, and supernatants collected for cytokine determination. The expression levels of IFN-γ, TNF-α, IL-4, and IL-5 were detected using the ELISA kit (R&D cystems, Minneapolis, MN, USA) according to the manufacturer's protocol. All experiments were done in triplicate.

Flow Cytometry Analysis

Spleen lymphocytes were prepared as described above. Cells were stained with the following anti-mouse antibodies (Biolegend, San Diego, CA, USA): anti-CD3-FITC (clone 17A2), anti-CD4-PE-Cy5 (clone RM4-5), anti-IFN-γ-PE (clone XMG1.2), and anti-IL-4-PE (clone 11B11). For intracellular staining, cells were stimulated for 4–6 h with 1 μg/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 permeabilized with Fix/Permbuffer (Biolegend) and finally incubated with antibodies against intracellular cytokines. Cells were analyzed on a FACS LSRII system (BD Bioscience, San Diego, CA, USA).

Quantitative Real-Time PCR Analysis

RNA from spleen lymphocytes was extracted and purified using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using the

QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time PCR of cDNA was performed using the forward (F) and reverse (R) primer sequences: T-bet: F, 5′-TGCCTGCAGTGCTTCTAACA-3′; R, 5′-TGCCCCGCTTCCTCTCCAACCAA-3′; GATA-3: F, 5′-GAAGGCATCCAGACCCGAAAC-3′; R, 5′-ACCCATGGCGGTGACCATGC-3′; IFN-γ: F, 5′-AGCGGCTGACTGAACTCAGATTGTAG-3′; R, 5′-GTCGCTTCGTTGATCACAA-3′; IL-4: F, 5′-TCAACCCCCAGCTAGTTGTC-3′; R, 5′-TGTTCTTCGTTGCTGTGAGG-3′; GAPDH: F, 5′-AACTTTGGCATTGTGGAAGG-3′; R, 5′-ACACATTGGGGGTAGGAACA-3′. Amplification was performed in a Rotor-Gene Q 2plex System (Qiagen) under the following conditions: 10 min at 95 °C, then 45 cycles of 10 s at 95 °C and 15 s at 60 °C. Relative levels of mRNA, including T-bet, GATA-3, IFN-γ, and IL-4, were examined using Sybr green real-time quantitative reverse transcription-PCR (qRT-PCR) (Applied Biosystems, Foster City, CA, USA) and normalized to levels of GAPDH mRNA. "Fold-induction" of each mRNA species was calculated as follows: d threshold cycle (dCt) = (Ct of target mRNA) − (Ct of GAPDH); ddCt = (dCt of mRNA in target gene) − (dCt of mRNA in control gene); fold-induction = 2^{-ddCt}.

Western Blot Analysis

Spleen lymphocytes were obtained and lyzed 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 after denaturation at 95 °C, and then transferred onto polyvinylidene fluoride (PVDF). After blocked with 5% skim milk, membranes were incubated with the desired primary rabbit anti-mouse antibodies against T-bet, GATA-3, or GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) 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 ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA). Sample loading was normalized by quantities of GAPDH detected parallel.

Statistical Analysis

Data are presented as mean ± standard deviation (SD) and analyzed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Student's t test was used to compare data between two groups. One-way ANOVA with Tukey post hoc test was used to compare three or more groups. All P values are two-tailed and $P < 0.05$ was considered statistically difference.

RESULTS

EA Treatment Inhibits the Allergic Progression in OVA-Induced DTH

To explore the effect of EA treatment at acupoint ST36 on the allergic progression of OVA-induced DTH, we firstly assayed the severity of allergic inflammation through measuring the footpad thickness increment (Fig. 1b). As shown in Fig. 1c, OVA-challenge on day 7 induced a significant amplification of footpad swelling compared with that in the control group. Compared to the OVA-DTH group, EA intervention at ST36 led to a decline in footpad thickness. Additionally, EA treatment also significantly inhibited the OVA-specific antibody and IgE levels in serum (Fig. 2a, b), which further suggested the anti-allergic effect of EA on OVA-induced DTH.

EA Treatment Attenuates Infiltration of Inflammatory Cells in the Footpad Skin

Next, we observed infiltration of inflammatory cells in the dermis of footpad skin. As shown in Fig. 3a, OVA-challenge induced significant infiltration of inflammatory cells into the dermis of the footpad tissue in DTH group. However, EA stimulation attenuated cell infiltration triggered by OVA. The degree of infiltration was reflected by the epidermal thickness of the footpad skin and the number of inflammatory cells. EA treatment markedly suppressed the increment in thickness of the epidermis induced by OVA and restrained the number of inflammatory cells recruited to the area compared to the OVA-DTH group (Fig. 3b, c).

EA Ameliorates Inflammation Mediated by Th1 Cells in OVA-Induced DTH

To investigate the effect of EA treatment on the production of inflammatory cytokines in footpad tissue, the levels of IFN-γ, TNF-α, IL-4, and IL-5 were

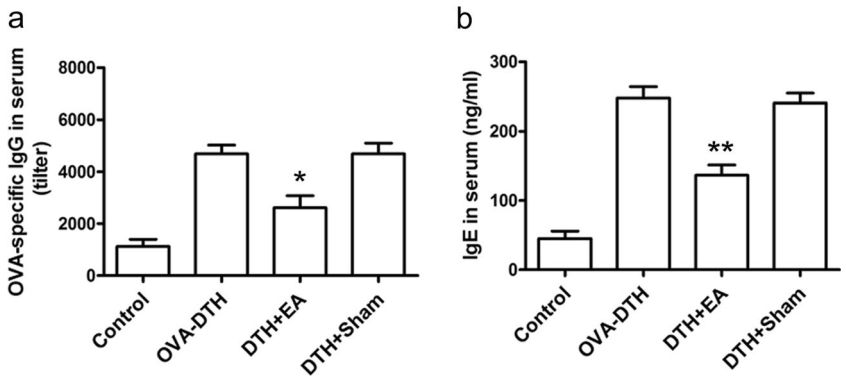

Fig. 2. Effect of EA intervention on the serum IgG and IgE levels. **a** Effect of EA on serum OVA-specific antibody and **b** IgE levels in OVA-induced DTH responses. Data are presented as mean ± SD ($n = 8$). *$P < 0.05$ and **$P < 0.01$, comparison with the OVA-DTH group.

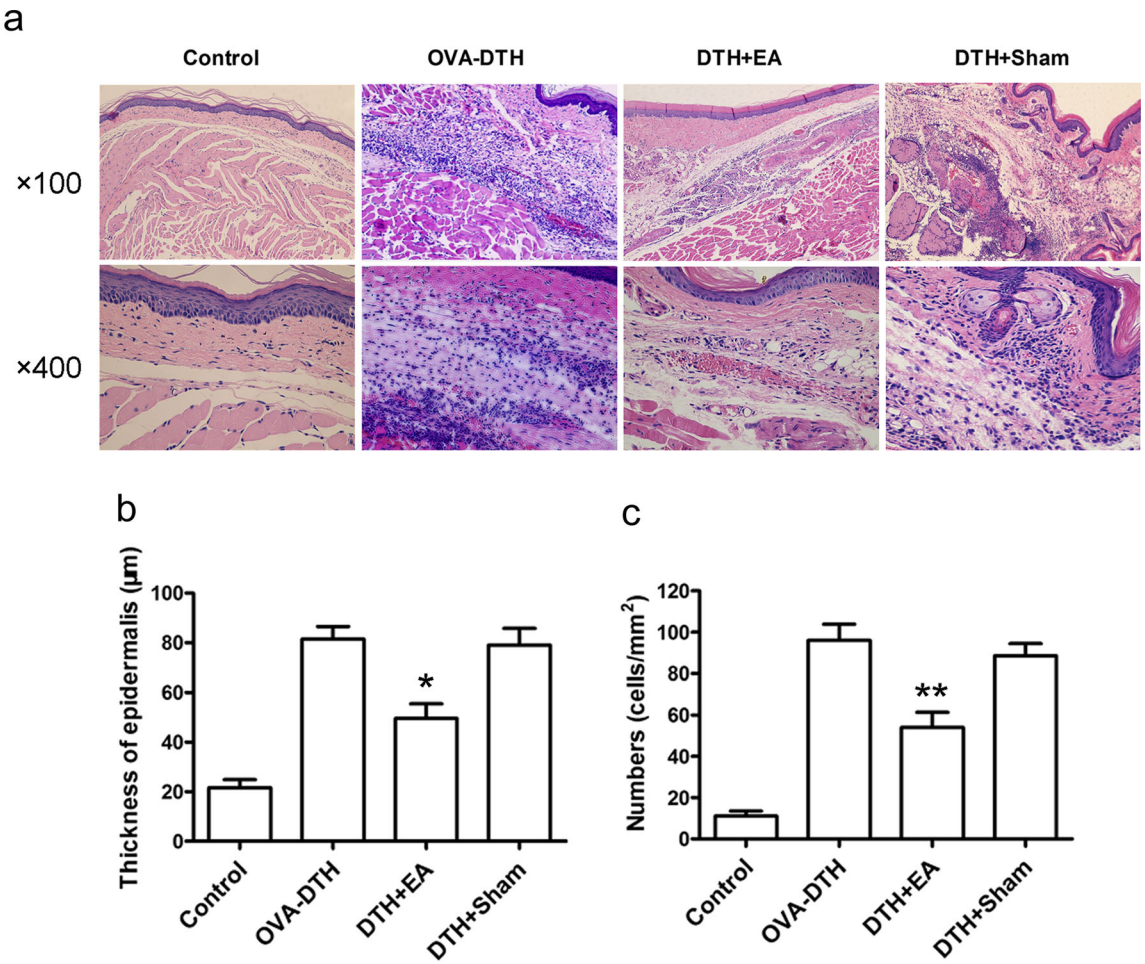

Fig. 3. EA treatment decreases inflammatory cell infiltration of footpad in DTH responses. **a** Histological changes and inflammatory cell infiltration in OVA-challenged footpads with or without EA treatment. Footpad skins of C57BL/6 mice obtained from different groups were stained using hematoxylin and eosin (H&E). ×100 (*up*) and ×400 (*down*). **b** The epidermal thickness of footpad skin and **c** inflammatory cells densities in the dermis were statistically analyzed, respectively. Data are presented as mean ± SD ($n = 8$). *$P < 0.05$ and **$P < 0.01$, comparison with the OVA-DTH group.

measured by ELISA. Interestingly, the levels of Th1-type cytokines, such as IFN-γ and TNF-α, were found to be highly decreased in footpad tissue following treatment with EA compared to the DTH group (Fig. 4a, b). However, similar levels of IL-4 and IL-5, the classic Th2-type cytokines, were shown among OVA-challenge groups (Fig. 4c, d).

When isolated spleen lymphocytes were cultured in the presence of ConA, ELISA detection showed the levels of IFN-γ were increased in the OVA-DTH group (Fig. 5a). However, the secreted IFN-γ was clearly lowered in the DTH + EA group, but not IL-4 (data not shown). In addition, the MTT assay indicated EA treatment significantly prompted cell proliferation compared with the OVA-DTH group (Fig. 5b). These results suggest that EA intervention could ameliorate inflammation mediated by Th1 cells in OVA-induced DTH responses, eventually resulting in the reducing levels of Th1 cytokines including IFN-γ and TNF-α.

The Protective Effect of EA Intervention Associated with Regulating Th1/Th2 Balance

To further determine the possible immunological mechanism involved in the protective effect of EA on OVA-induced DTH, we examined the cellular phenotypes by FACS, i.e., Th1 and Th2 cells. Compared to the control group, there was a significant amplification in the frequency of $CD4^+IFN\text{-}\gamma^+$ T cells in the OVA-DTH group (Fig. 6a). Consistent with the above results, EA treatment at ST36 reduced the percentage of $CD4^+IFN\text{-}\gamma^+$ T cells. On the other hand, there were no significant differences in the $CD4^+IL\text{-}4^+$ T cells between the two groups (Fig. 6b, c). Due to the change in the amount of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}4^+$ T cells, the Th1/Th2 ratio (i.e., ratio of the amount of $CD4^+IFN\text{-}\gamma^+$ T cells/the amount of $CD4^+IL\text{-}4^+$ T cells) in the DTH mice treated with EA was reduced to near normal level (Fig. 6d), suggesting the protective effect of EA intervention was associated with recovering a Th1/Th2 balance.

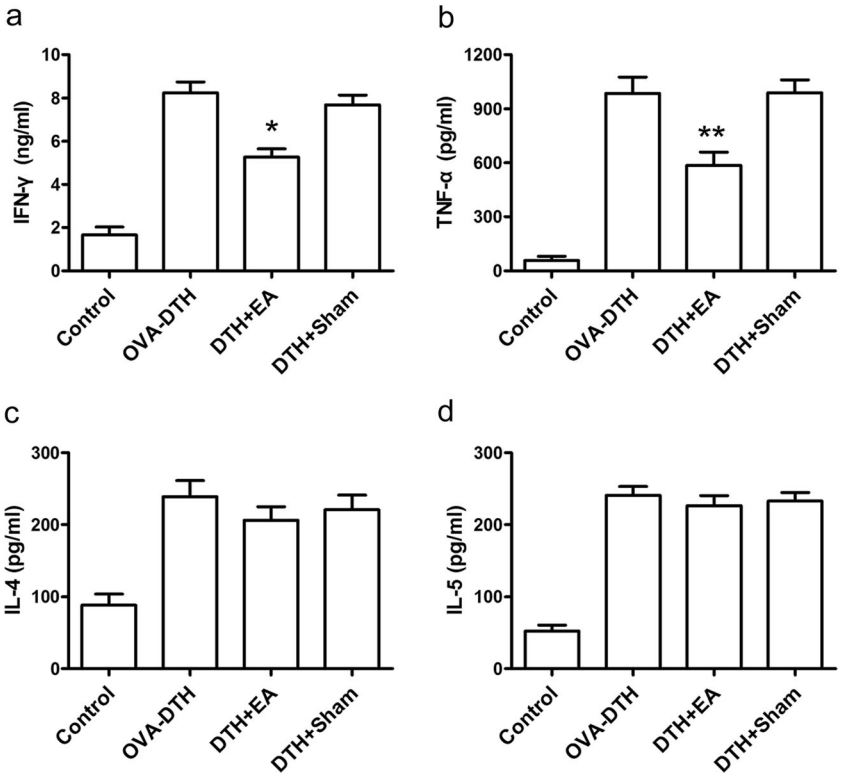

Fig. 4. EA intervention ameliorates inflammation connected with OVA-induced DTH. The levels of Th1-type cytokines including IFN-γ (**a**) and TNF-α (**b**), and Th2-type cytokines such as IL-4 (**c**) and IL-5 (**d**) in the homogenized footpads of mice were measured by ELISA and statistically analyzed. All experiments were done in triplicate. Data are presented as mean ± SD ($n = 8$). *$P < 0.05$ and **$P < 0.01$, comparison with the OVA-DTH group.

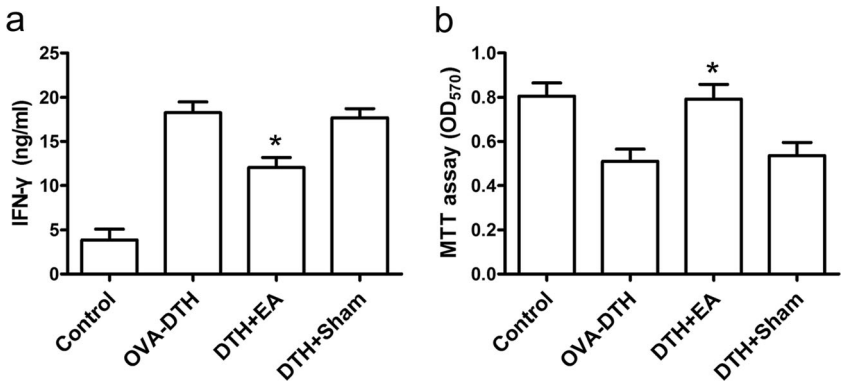

Fig. 5. EA treatment reduces IFN-γ production by Th1 cells. **a** Spleen lymphocytes were cultured for 24 h in the presence of 5 mg/mL ConA, and IFN-γ production in the supernatant was quantified by ELISA. **b** Spleen lymphocytes were cultured for 24 h in the presence of 50 μg/mL OVA, and cell proliferation was analyzed by MTT assay. All experiments were done in triplicate. Data are presented as mean ± SD ($n = 8$). *$P < 0.05$, comparison with the OVA-DTH group.

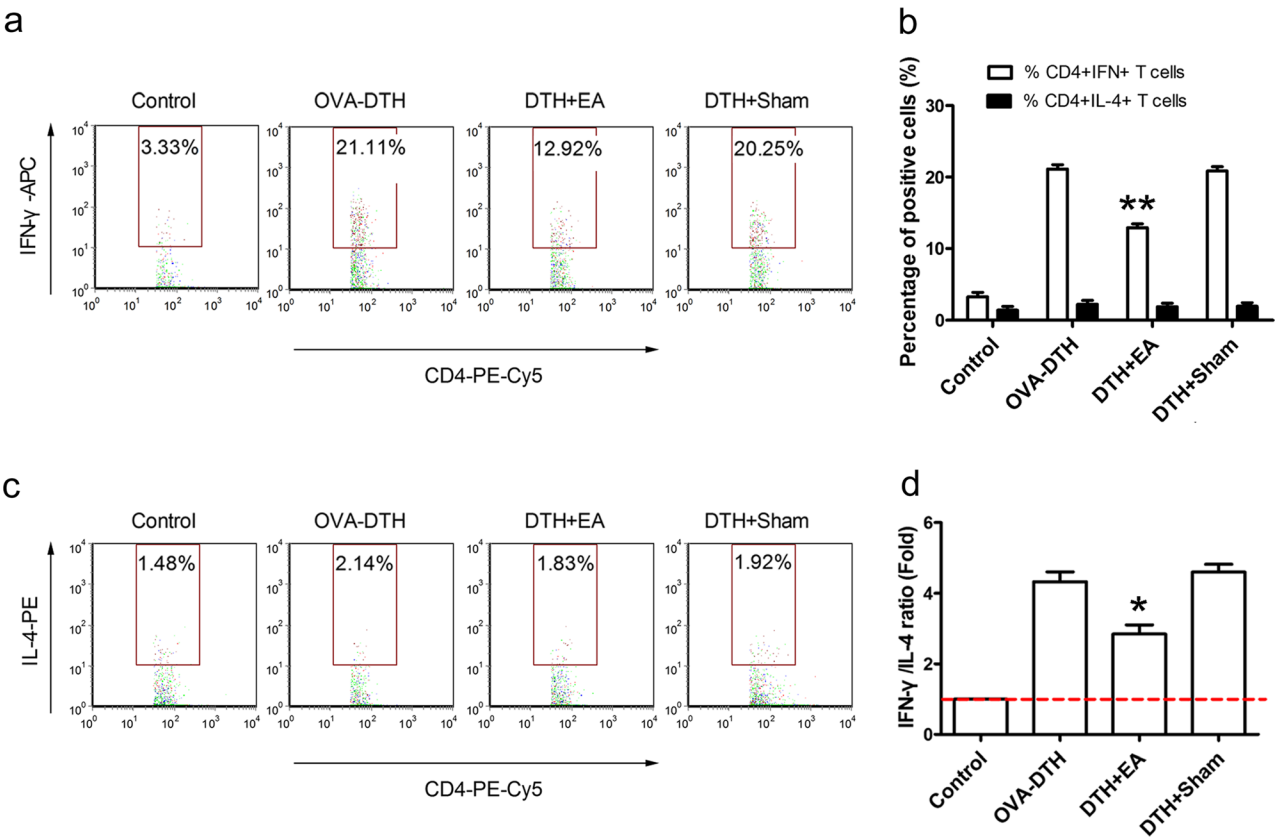

Fig. 6. EA treatment regulates the ratio of Th1/Th2 in OVA-induced DTH. **a** Representative FACS plots for $CD4^+IFN\text{-}\gamma^+$ T cells and **c** $CD4^+IL\text{-}4^+$ T cells in spleens from the mice of different group, respectively. **b** Statistical analysis of the percentage of $CD4^+IFN\text{-}\gamma^+$ T cells and $CD4^+IL\text{-}4^+$ T cells, respectively. **d** The ratio of Th1/Th2 (relative expression of $CD4^+IFN\text{-}\gamma^+/CD4^+IL\text{-}4^+$) in the spleen. Data are presented as mean ± SD ($n = 8$). *$P < 0.05$ and **$P < 0.01$, comparison with the OVA-DTH group.

Effect of EA Treatment on Transcription Factors Involved in Th1 and Th2 Differentiation

Lastly, we investigated the effect of EA on transcription factors such as T-bet and GATA-3, which directly or indirectly regulate Th1 and Th2 differentiation. Compared to the OVA-DTH group, EA treatment weakened the mRNA expression of T-bet by approximately 50%, whereas there was little effect for GATA-3 (Fig. 7a–c). Simultaneously, the expression of IFN-γ and IL-4 mRNA also showed the same tendency as above results (Fig. 7d–f). Furthermore, the enhancing activity of T-bet protein was shown in the OVA-DTH group compared with that in the control group, and EA intervention significantly reduced the level of T-bet protein but not GATA-3 (Fig. 8). These results suggest that EA treatment at ST36 might modulate an immune shift of Th from Th1 polarization to Th1/Th2 balance via the decreased mRNA and protein levels of T-bet, resulting in the reduction of IFN-γ secretion.

DISCUSSION

Basic principles of Chinese therapeutics emphasize adjusting and harmonizing the internal environment to promote stability. In this study, we investigate the anti-allergic and anti-inflammatory activities of EA treatment at acupoint ST36 and its potential mechanisms on OVA-induced DTH according to modern medical knowledge with a focus on the cross regulation of neuron-endocrine-immune interactions.

EA Treatment at Acupoint ST36 Attenuates Inflammation Associated with DTH Via Suppressing Th1 Response

As we all know, OVA is routinely used to trigger T cell-dependent immune reactions involving Th1-polarized skin inflammation such as DTH, and Th2-polarized allergic diseases such as asthma by activating B cells and modulating IgG and IgE secretion [12]. Although Th17

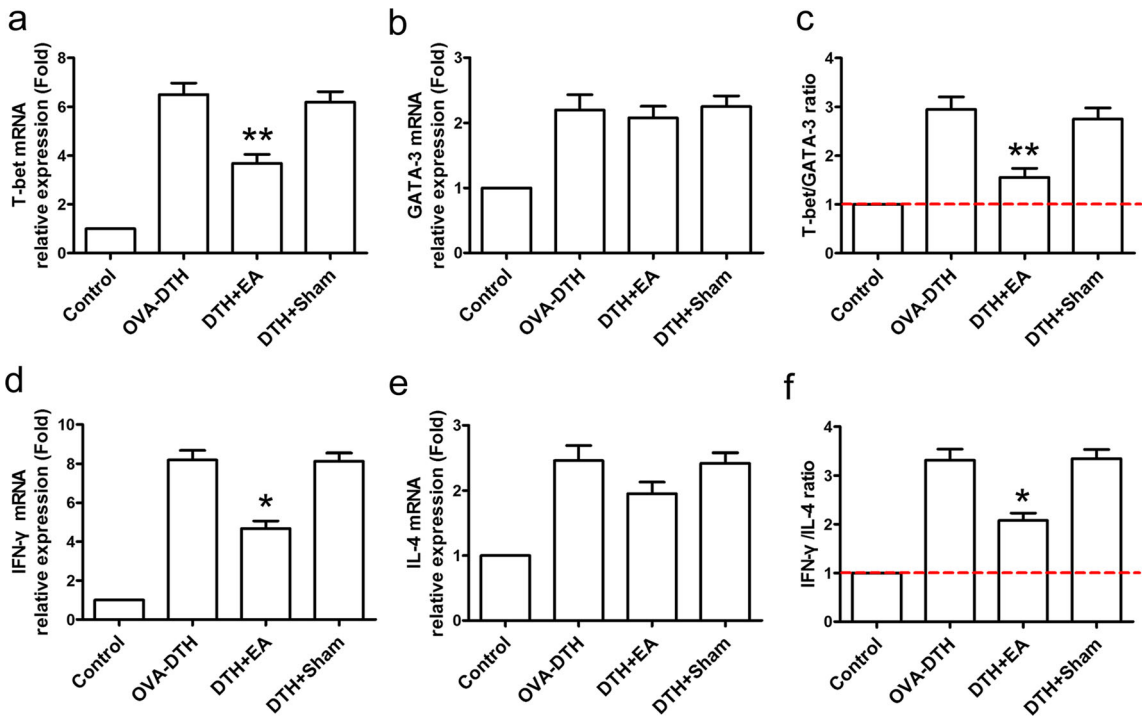

Fig. 7. Effect of EA treatment on the mRNA expression of transcription factors involved in Th1 and Th2 differentiation. Spleen lymphocytes from mice in different groups were obtained and total RNA isolated. **a** Quantitative real-time PCR of T-bet and **b** GATA-3 were performed, and **c** the ratio of T-bet/GATA-3 was shown. **d** Quantitative real-time PCR of IFN-γ and **e** IL-4 were performed and **f** ratio of IFN-γ/IL-4 is shown. Data are presented as mean ± SD ($n = 8$). *$P < 0.05$ and **$P < 0.01$, comparison with the OVA-DTH group.

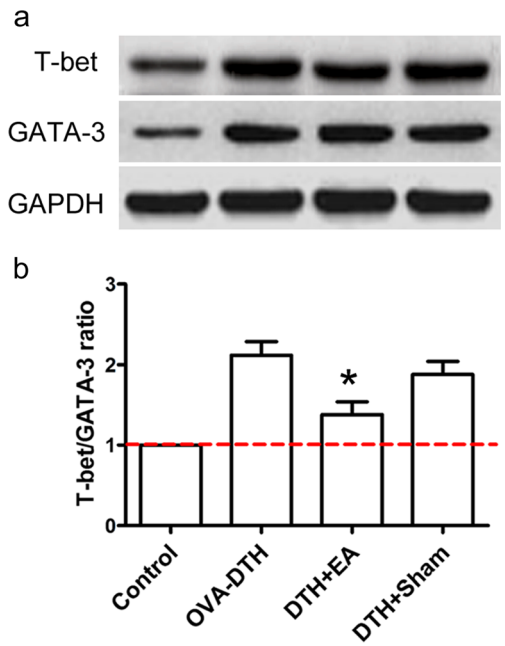

Fig. 8. Effect of EA treatment on the protein expression of transcription factors involved in Th1 and Th2 differentiation. Spleen lymphocytes from mice in different groups were obtained and proteins were isolated and quantitatively analyzed. **a** The protein expression including T-bet and GATA-3 was performed by Western blotting, and a representative of eight independent experiments was shown. **b** Statistical analysis of the ratio of T-bet/GATA-3 was shown. Data are presented as mean ± SD ($n = 8$). *$P < 0.05$, comparison with the OVA-DTH group.

and Th2 cell subsets have been shown to contribute to the overall pathogenesis, DTH is majorly attributed to Th1 cells-driven inflammation accompanying the allergic process in response to specific antigens at specific sites such as ear or footpad, and includes infiltration of inflammatory cells, upregulation of IgE, and increased expression of pro-inflammatory Th1 cytokines [13–15]. The severity of inflammation can be evaluated using footpad or ear thickness measurement. In the present study, EA treatment at ST36 not only inhibited OVA-induced footpad swelling (Fig. 1) but also reduced the inflammatory cells recruited to inflamed footpad tissue (Fig. 3). Skin inflammation is known to result from the production of pro-inflammatory Th1-type cytokines, such as IFN-γ and TNF-α, which are involved as mediators initiating the progression of allergic reactions [16]. Our results also indicated that the levels of OVA-specific IgG and IgE in serum were decreased following EA stimulation at ST36 (Fig. 2), which suggests an anti-allergic effect of EA treatment on DTH responses. Importantly, EA interaction suppressed the production of IFN-γ and TNF-α in the footpads of OVA-induced DTH mice, but not the production of IL-4 or IL-5 (Fig. 4), and also lowered the production of IFN-γ in spleen lymphocytes (Fig. 5). Thus, EA treatment had effects on cellular and humoral immunity triggered in OVA-induced DTH, and this was confirmed by the reduced production of IgE and pro-inflammatory Th1-type cytokines including IFN-γ and TNF-α. These results also indicated that EA treatment constricted inflammation associated with OVA-induced DTH via negatively modulating Th1 polarization, and that the biological basis of the anti-inflammatory effect appeared to selectively affect Th1 rather than Th2 immunity in DTH reactions.

Regulation of Th1/Th2 Balance Is Involved in the Anti-allergic and Anti-inflammatory Effects of EA

Studies have reported that EA intervention was effective for various immune-related diseases including allergic disorders, infections, inflammatory diseases, and autoimmune diseases, where T cells over-activation or unbalanced Th1-/Th2-type immune responses played a pivotal role co-occurred with an induced immune shift [17–19]. A previous study showed the beneficial anti-inflammatory and immunomodulatory activity of EA stimulation on asthma was related to regulation of the balance of the Th1/Th2 response and to reduction of leukotriene B4 (LTB4) and nitric oxide (NO), where EA increased IL-1 and IFN-γ and decreased IL-4, IL-10, NO, and LTB4 in the bronchoalveolar lavage and pulmonary tissue compared to the asthma group without EA treatment [20]. In depressed patients, it was also verified that EA intervention could restore the balance between Th1 and Th2 cytokines by increasing TNF-α and decreasing IL-4 [21]. In that study, EA increased the mRNA and protein expression of the Th1 cytokine, such as IL-2 and IFN-γ, and decreased the production of the Th2 cytokine including IL-4 and IL-10 [21]. In line with the above reports, EA treatment was demonstrated to regulate a balance between Th1 and Th2 cytokines at protein and mRNA levels in spleen T cells in a rat model of surgical trauma [22]. These studies suggest that the immunoregulatory effect of EA was closely associated with an induced immuno-shift of Th cells from a Th2 to a Th1 response. Conversely, our results indicated that EA treatment at ST36 strongly inhibited OVA-induced IFN-γ-producing $CD4^+$ T cells even though there was little change for the $CD4^+IL\text{-}4^+$ T cells (Fig. 6). Interestingly, the Th1/Th2 ratio, i.e., $CD4^+IFN\text{-}\gamma^+$ T cells/$CD4^+IL\text{-}4^+$ T cells, showed an obvious tendency to restore normal level following EA treatment, suggesting

EA intervention is a very powerful immune suppressor for Th1 polarization in DTH responses.

Differentiation of Th1 and Th2 is determined by the interactions of cytokine production as well as transcription factor expression [23, 24]. To clarify the underlying mechanisms by which EA regulated the balance of Th1/Th2, detailed studies were conducted to investigate the effect of EA treatment on the expression of the transcription factors including GATA-3 and T-bet which cross-regulate the differentiation and development of Th1 and Th2 cells, respectively. T-bet, a Th1-specific transcription factor, was reported to modulate the development of Th1 cells by inducing the synthesis of IFN-γ, and also, to negatively regulate the Th2 differentiation [25]. Our data showed that EA treatment down-regulated the mRNA expression level of T-bet and IFN-γ, but not GATA-3 and IL-4 (Fig. 7). Additionally, the expression of T-bet protein was inhibited by EA treatment despite no significant change on GATA-3, and T-bet/GATA-3 ratio showed the similar trend as Th1/Th2 ratio (Fig. 8). These findings strongly suggest that inhibiting T-bet expression led to the suppression of Th1 differentiation, which contributes to the regulating immuno-shift between Th1 and Th2 cells. Studies have reported that Th17 cells were involved in the inhibition of methylated BSA-induced DTH, which accompanies the suppression of Th1 activation [26]. In rats with experimental autoimmune encephalitis, EA treatment restored balance to Th1/Th2/Th17/Treg cell responses by stimulating the hypothalamus to increase adrenocorticotrophic hormone (ACTH) secretion [27]. Compared with these studies, EA treatment in the current study induced an immunoshift of Th1 cells to restore Th1/Th2 balance via curbing Th1 differentiation. If other T cell subpopulations, such as Th17 or Treg cells, as well as other transcription factors, such as STAT 6 and NF-κB, participated in the switch of Th1 cells in OVA-induced DTH responses, studies are needed to elucidate the cellular and immunological mechanisms.

Mechanism Exploration of EA Treatment at Acupoint ST36 Based on Traditional Meridian Theory

According to traditional meridian theory, acupoints are distributed on the skin and in deeper tissue linking a network of meridian channel inside the human body [28]. The selection and compatibility of acupoints can make an impact on the therapeutic effect for experimental or clinic application of EA treatment [29]. Studies have shown that EA stimulation on specific acupoints could induce functional immune reactions and exert different biological effect. Acupuncture at both "Shuigou" (GV26) and "Yanglingquan" (GB34) acupoints provide neuroprotection in neurodegenerative diseases and in central nervous system injuries [30]. In addition, EA treatment at the ST36 Zusanli acupoint reduced lipopolysaccharide-induced serum levels of inflammatory cytokines such as IFN-γ, TNF-α, and IL-6. The results also indicated that the anti-inflammatory potential of EA intervention was voltage-dependent and acupoint-specific, since stimulation of a non-acupuncture point did not show this effect [31]. Utilizing experimental autoimmune encephalomyelitis (EAE) model, EA stimulation at the "Zusanli" ST36 acupoints ameliorated the severity of EAE, while rats receiving non-Zusanli acupoint therapy still exhibited serious disease progression [32]. Consistent with these results, EA treatment at "Zusanli" acupoint ST36 showed the anti-inflammatory and anti-allergic effects in the present study, whereas EA stimulation at the non-Zusanli (non-acupoint) area (DTH + Sham group) exhibited the similar DTH reactions. Based on the histological analysis, a previous study indicated the differences between acupuncture points and non-acupoints. There was a large amount of dermal papillae containing capillary loops with sympathetic nerve endings, and the acupoints were innervated by superficial somatic nerves, most fibers appear to be unmyelinated [33]. In addition, another study indicated that the anti-inflammatory effect of EA was associated with release of acetylcholine by the vagus nerve as a result of transmission of message to vagal nuclei by stimulation at the ST36 acupoint, which suggests the activation of cholinergic anti-inflammatory pathways was involved in the biological effect of EA [34].

In summary, EA treatment at acupoint ST36 inhibited inflammation associated with OVA-induced DTH and induced tissue remodeling through regulation of serum IgE levels, cytokine production in footpad tissue, the percentage of IFN-γ-producing $CD4^+$ T cells, and T-bet activation. These results suggested that EA intervention might be an alternative or complementary therapeutic option for curing allergic inflammatory disease and Th1-mediated inflammation disorders.

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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.C. finished experiments and wrote the manuscript. M.L., L.Y.C., and L.W. performed partial research. Z.G.W. and N.N.Y. analyzed the data. Z.B.C. helped revise the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

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

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