Journal of Inflammation Research

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ORIGINAL RESEARCH

Acupoint Autohemotherapy Attenuates DNCB-Induced Atopic Dermatitis and Activates Regulatory T Cells in BALB/c Mice

Shi-Hua Yan[1,2], Yong Chen[3], Zhi-Qian Huang[1], Wen-Xi Zhong[1], Xiao-Tian Wang[1], Yang-Can Tang[1], Xu-Yi Zhao[1], Yu-Shan Wu[1], Chun Zhou[4], Wei Zhu[5], Wei Xiao[1,6], Xuan Li[2], Dong-Shu Zhang[1,2]

[1]School of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong, 510515, People's Republic of China; [2]Department of Traditional Chinese Medicine, The Tenth affiliated Hospital, Southern Medical University (Dongguan People's Hospital), Dongguan, Guangdong, 523058, People's Republic of China; [3]Department of Rheumatology and Immunology, Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou, 563000, People's Republic of China; [4]School of Pharmaceutical Sciences; Guangdong Provincial Key Laboratory of Shock and Microcirculation, Southern Medical University, Guangzhou, Guangdong, 510515, People's Republic of China; [5]Guangzhou Center for Disease Control and Prevention, Guangzhou, 510440, People's Republic of China; [6]Key Laboratory of Glucolipid Metabolic Disorder, Ministry of Education, Guangdong Pharmaceutical University, Guangzhou, Guangdong, 510006, People's Republic of China

Correspondence: Dong-Shu Zhang, School of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong, 510515, People's Republic of China, Email nyzds@sina.com; Wei Xiao, Key Laboratory of Glucolipid Metabolic Disorder, Ministry of Education, Guangdong Pharmaceutical University, Guangzhou, Guangdong, 510006, People's Republic of China, Email xw7688@smu.edu.cn

Video abstract

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Purpose: Acupoint autohemotherapy (A-AHT) has been proposed as an alternative and complementary treatment for atopic dermatitis (AD), yet the exact role of its blood component in terms of therapeutic efficacy and mechanism of action is still largely unknown.

Methods: This study aimed to evaluate the therapeutic efficacies and action mechanisms of intramuscular injections of autologous whole blood (AWB) and mouse immunoglobulin G (IgG) (autologous or heterologous) at acupoints on 2,4-dinitrochlorobenzene (DNCB)-induced AD mouse models. Serum levels of total immunoglobulin E (IgE), IgG, interleukin-10 (IL-10), and interferon-gamma (IFN-γ) were measured, as well as mRNA expression levels of Forkhead box P3 (FoxP3), IL-10 and IFN-γ in dorsal skin lesions, and IL-10$^+$, IFN-γ$^+$ and FoxP3$^+$CD4$^+$T cells in murine spleen.

Results: It showed that repeated acupoint injection of AWB, autologous total IgG (purified from autologous blood in AD mice) or heterologous total IgG (purified from healthy blood in normal mice) effectively reduced the severity of AD symptoms and decreased epidermal and dermal thickness as well as mast cells in skin lesions. Additionally, AWB acupoint injection was found to upregulate FoxP3$^+$, IL-10$^+$ and IFN-γ$^+$ CD4$^+$T cells in murine spleen, suppressing the production of IgE antibodies and increasing that of IgG antibodies in the serum. Furthermore, both AWB and autologous total IgG administrations significantly elevated FoxP3 expression, mRNA levels of IL-10 and IFN-γ in dorsal skin lesions. However, acupoint injection of heterologous total IgG had no effect on regulatory T (Treg) and Th1 cells modulation.

Conclusion: These findings suggest that the therapeutic effects of A-AHT on AD are mediated by IgG-induced activation of Treg cells.

Keywords: autologous whole blood, acupoint injection, atopic dermatitis, anti-idiotypic immunomodulation, Tregs

Introduction

Atopic dermatitis (AD) is a common inflammatory skin condition characterized by eczematous lesions and intense itching. The underlying pathogenesis of AD is complex, involving compromised skin barrier dysfunction, abnormal T cell subsets, increased levels of serum IgE, and inflammatory cytokines or it may arise from other skin diseases such as

Received: 17 January 2024
Accepted: 16 April 2024
Published: 9 May 2024

scaly diseases.[1–4] Traditional treatments, such as emollients, topical corticosteroids, calcineurin inhibitors, H1 receptor antagonists, systemic corticosteroids, and immunosuppressive agents, are often ineffective.[5] Recent clinical trials have demonstrated the efficacy of monoclonal antibodies targeting IL-4 receptor alpha or IL-13 (dupilumab).[3,6] However, this approach was not effective in significant portion of patients with AD who had been treated by dupilumab.[6,7] Moreover, Janus kinase (JAK) inhibitors which are new targeted immunomodulatory are not recommended for pregnant or nursing individuals due to potential risks such as infections, blood and liver issues, high cholesterol, hypertension, and complications like tuberculosis and blood clots.[8] Given the heterogeneous nature of AD, personalized immunomodulatory therapy that reduces hypersensitivity and corrects immune dysfunction may prove to be a more effective approach.[9]

Autohemotherapy, also referred to as autologous blood injection, is a medical procedure that has been used by physicians for over a century, initially reported in 1913. This technique involves injecting a patient's own whole blood intramuscularly to treat certain diseases, such as AD and chronic urticaria.[10,11] In China, a novel approach known as acupoint autohemotherapy (A-AHT) has been developed to combine autohemotherapy with acupuncture therapy, based on TCM theory. Studies have shown that A-AHT can regulate Th1/Th2 immune responses, which is a key factor in AD progression, through the continuous stimulation of acupoints and blood components, and the selectivity of acupoints (ST 36 and LI 11) and injection (AWB compared to normal saline).[12]

This investigation aims to evaluate the immunomodulatory effects of acupoint injection of either autologous or heterologous total IgG or AWB in DNCB-induced AD mice. Our hypothesis is that the therapeutic effects of A-AHT are due to the presence of autologous total immunoglobulin in the blood, which induces anti-idiotypic immunomodulation and the activation of regulatory T cells.[13–15] Previous research has suggested that human polyclonal IgG, derived from multiple healthy blood donors, is a potent and anti-inflammatory agent for treating autoimmune and allergic diseases.[16] This study aimed to determine the efficacy of A-AHT in AD mice and its mechanism of action, primarily focusing on the anti-idiotypic immunomodulation and activation of Treg cells.

Materials and Methods

Reagents

DNCB was purchased from Sigma (Cat. No. 237329-10G, Missouri, USA). Olive oil was purchased from Pythonbio (Guangzhou, China). HITRAP PROTEIN G HP 1×5 ML was purchased from Cytiva (Cat. No. 17040501, Shanghai, China). Foxp3 antibody was purchased from Affinity Biosciences (Cat. No. AF6544, Jiangsu, China). Red blood cell lysis buffer was purchased from Servicebio (Cat. No. G2015-500ML, Wuhan, China). Cell Activation Cocktail (with Brefeldin A) (Cat. No. 423303), FITC anti-mouse CD4 (Cat. No.130308), PE anti-mouse IFN-γ (Cat. No.505808), APC anti-mouse IL-10 (Cat. No.505009), PE anti-mouse FoxP3 (Cat. No.126404) were obtained from Biolegend (San Diego, USA). Mouse IgG (Cat. No. EMC116.96), IgE (Cat. No. EMC117.96), IL-10 (Cat. No. EMC005.96) and IFN-γ (Cat. No. EMC101g.96) enzyme-linked immunosorbent assay (ELISA) kits were all purchased from Neobioscience Biotechnology Company (Shenzhen, China). The Diaminobenzidine (DAB) chromogenic agent kit (Cat. No. G1211) was procured from Servicebio in Wuhan, China. TRIzol reagent was purchased from Thermo Scientific (Cat. No. 15596018, MA, United States). ReverTra Ace qPCR RT Kit (Cat. No. FSQ-101) and SYBR Green Master Mix (Cat. No. QPK-201C) were purchased from Toyobo (Shanghai, China).

Animals and Grouping

The animal experiments conducted in this study adhered to the Health Guide for the Care and Use of Laboratory Animals[17] and were sanctioned by the Medical Ethics Committee of the Guangzhou Center for Disease Control and Prevention (project identification code: GZCDC-ECAR-2021P0011). 26 SPF-grade female BALB/c mice which were used in "Preparation of Autologous Immunoglobulin" and 30 SPF-grade female BALB/c mice which were used in "Treatment" aged 6 weeks and weighing 18–21g were procured from the Experimental Animal Center of Southern Medical University, Guangzhou, China (Certificate No. SCXK 2016–0041) and housed indoors in SPF conditions. The mice were kept under a controlled environment of a 12-hour light/dark cycle, relative humidity ranging from 40%±10%, and a temperature of 22±1 °C. After 1 week of acclimatization, 30 mice which were used in "Treatment" were divided

into 5 groups by a random number table as follows: normal control (NC), AD model (AD), acupoint injection of autologous whole blood (AWB), acupoint injection of total IgG purified from whole blood of DNCB-induced AD mice (AD-IgG), and acupoint injection of total IgG purified from whole blood of in normal mice (N-IgG). Six mice in each group.

Establishment of AD Mice Model

Figure 1A depicts the AD model establishment process and treatment timing. On day 0, the mice's backs (2.5cm*1.5cm) were shaved, followed by challenging them with 200 μL of 1% DNCB dissolved in acetone/olive oil (3:1) four times (day 1-day 4, once a day). Seven days after the first induction, 200 μL of 0.5% DNCB was applied onto the mice's backs every other day for 28 days (day 8–day 35) to foster further AD development.[18]

Preparation of Autologous Immunoglobulin

Twenty-six BALB/c mice were randomly divided into two groups: the AD model donor group (AD-D) and the normal donor group (N-D, n=13 per group). In the AD-D group, the establishment of the AD model was carried out as described above. In the N-D group, only the backs of the mice were shaved. Thirty-five days after the modeling process was completed, the mice were humanely euthanized by administering a dose of 100 mg/kg of 1% sodium pentobarbital through intraperitoneal injection, followed by cervical dislocation. Subsequently, 1 to 1.8mL of whole blood were extracted from the inferior vena cava of each mouse. After centrifugation at 3000 rpm for 15 minutes, a serum concentration ranging from 20% to 40% was obtained.

The total IgG from the mouse serum was purified using the AKTA Pure 25 System (GE Healthcare Life Sciences, Germany).[19] The tubes of the system were cleaned successively with 20% ethanol, deionized water, 1M HCl, deionized water, and 1M NaOH. The HITRAP PROTEIN G HP column was then installed and cleaned successively with 0.1 M glycine, deionized water, CH_3COOH (power of hydrogen, pH 2.5), deionized water, and Binding Buffer (pH7, 100mL is composed of 0.358g $Na_2HPO_4 \cdot 12H2O$ plus 0.877g NaCl plus 0.372g EDTA·2Na dissolved in water to 100mL, and then adjust the pH value to 7 with NaOH solution) in turn. Once complete, the serum samples were added and passed through the column. After all the serum had entered the tube, the proteins specifically bound to the column were eluted

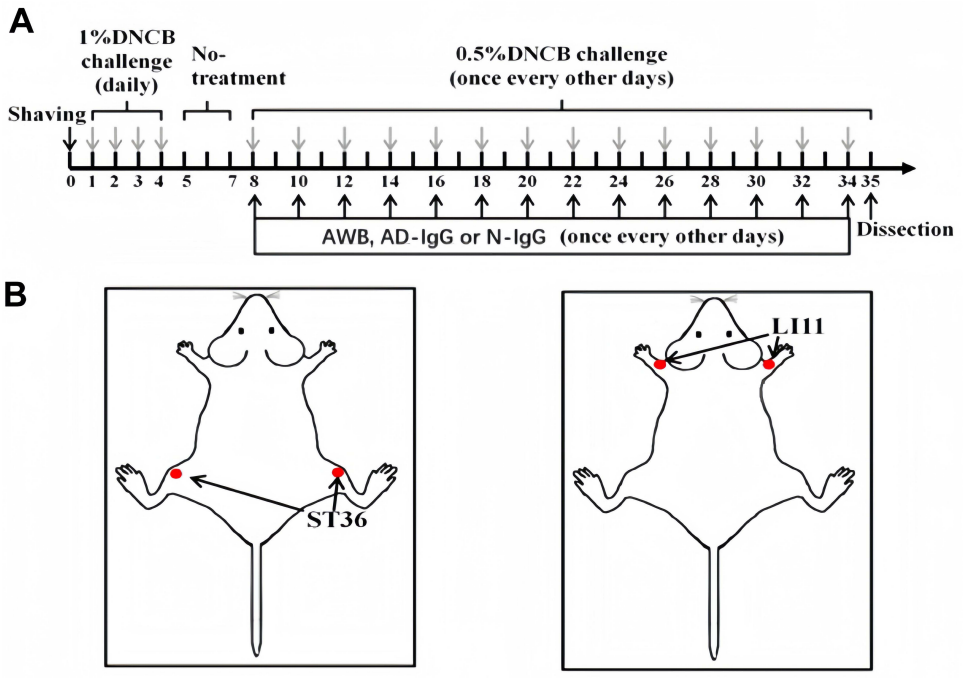

Figure 1 Time Line of Establishment of Mouse Model and Treatment. (**A**) The experimental procedure. (**B**) The location of ST36 and IL11.
Notes: ST 36: Zusanli, IL 11: Quchi.

with an elution buffer (pH 2.7, 100mL is composed of 0.751g Glycine dissolved in water to 100mL, and then adjust the pH value to 2.7 with HCl) and collected. The pH of the eluted solution was adjusted to 7.0 with 1M Tris (pH 9.0). This resulted in a solution of total mouse serum IgG. The operation was carried out on ice, and all reagents were filtered through a 0.2-micron filter membrane.

To obtain the purified IgG solution, it is placed in a dialysis membrane that is sealed with a buoy and immersed in phosphate buffer. The solution is then refrigerated while being dialyzed for three days at a specific speed. It is recommended to change the PBS solution daily during the dialysis process. After obtaining the IgG solution, it is freeze-dried using a freeze dryer (Alpha 2–4/LD-Plus, Christ, Germany) to obtain IgG lyophilized powder. The concentration of IgG solution was determined using ELISA method by adding lyophilized powder to normal saline. The final step involved preparing the IgG solution (4mg/mL) to match the concentration found in the AD donor serum.

Treatment

The mice were subjected to anesthesia using a 1%, 50 mg/kg sodium pentobarbital injection. Blood was collected via the facial vein using a 1.0 mL syringe and 5# needle per treatment. For the AWB group, 0.05 mL of AWB in each acupoint was administered bilaterally to the Zusanli (ST 36) and Quchi (IL 11) acupoints.[20,21] The entire procedure was completed within 1–2 minutes. For the AD-IgG and N-IgG group, 0.05 mL of total IgG solution (4mg/mL[22]) purified from DNCB-induced AD model donor group (AD-D) or the normal donor group (N-D) was injected into bilateral ST 36 and IL 11. ST 36 is located on the posterolateral side of the knee joint, approximately 5 mm below the capitula fibula. IL 11 is located on the lateral depression of the radial proximal elbow's anterior aspect (Figure 1B).[23] During the course of the treatment, the AD group mice were subjected to blood withdrawal of 0.2 mL every other day, without the use of intramuscular injections. On the other hand, the mice in the NC group were exempted from this practice and were instead, kept stress-free by being held in the hand for a minute. All groups of mice, with the exception for the NC and AD groups, received comparable interventions beginning on day 8 of the study. This was done consistently across the various groups. On the 35th day, all mice were anesthetized using sodium pentobarbital (1%, 100 mg/kg, i.p.), and the required blood, skin tissues, and spleen samples were collected for further investigation.

Dermatitis Severity Evaluation

The severity of dermatitis on the dorsal skin of each mouse was assessed using the Severity Scoring of Atopic Dermatitis (SCORAD) scale,[24] which ranges from 0 to 18. Symptom scores were gathered each morning, within a 30-minute window after DNCB application, between day 0 and day 35. The presence and extent of erythema, papule/oedema, oozing/crust, exfoliation, dryness, and lichenification were assigned scores of 0 (none), 1 (mild, <20%), 2 (moderate, 20–60%), and 3 (severe, >60%). The cumulative score was derived by adding up the scores obtained in each category.

Histopathological Assessment

The dorsal skin tissue of the mouse was subjected to fixation in a 4% neutral buffered formalin solution for 24 hours and then embedded in paraffin, sliced into sections with a thickness of 4 μm, and subjected to staining using hematoxylin and eosin (H&E) as well as toluidine blue (TB) respectively to assess the thickness of the epidermis and dermis, as well as the quantity of mast cells. For quantitative analysis, all sections were meticulously examined using a light microscope (Nikon, Eclipse Ci-L, Japan).

Immunohistochemistry Analysis

For antigen retrieval, sections of dorsal skin which were from each group were incubated in 0.01 mol/L, pH 6.0 citrate buffer for 30 minutes in a water bath held at 100°C. Next, the tissue samples were immersed in a solution of 3% hydrogen peroxide for 25 minutes at room temperature, ensuring darkness during the process. Following three washes in phosphate buffered saline (PBS), a 3% blocking buffer (BSA) was introduced for 30 minutes at room temperature to prevent binding to non-specific sites. The FoxP3 antibody, diluted in a ratio of 1:200 with PBS (pH 7.4), was applied to the sections. The sections were then incubated overnight at 4 °C in a humidified chamber. Subsequently, staining was performed using a DAB chromogenic agent kit, afterwards, the sample was treated with a newly made diaminobenzidine

solution and then stained with hematoxylin for contrast. The sections were dried using a progressive sequence of alcohol, solidified with dimethyl benzene, and then coated with neutral gum. The relevant parts of the sample were analyzed using a Nikon E100 microscope from Japan with a magnification of 200×. The assessment of FoxP3 immunohistochemistry staining was conducted through semi-quantitative analysis utilizing Image J software from The National Institutes of Health in Bethesda, MD, USA. The results were presented in terms of average optical density (AOD).

Flow Cytometry Analysis

To obtain single-cell suspensions, mice spleen tissues were minced and filtered through a 70 m sieve.[25] In the single-cell suspension, red blood cell lysis buffer was added for 10 minutes at 4°C. Following centrifugation and resuspension, the cell population was adjusted to a density of 1×10^6 cells/mL. Next, brefeldin A was introduced into the culture, and the cells were incubated for an additional 4 hours to gather cytokine internally as explained before. IL-10 and IFN-γ were collected and stained with anti-CD4 fluorescein isothiocyanate (FITC), anti-IL-10 allophycocyanin (APC) and anti-IFN-γ P-phycoerythrin (PE). Treg cells were stained with anti-CD4 FITC and anti-FoxP3 PE. Samples were analyzed using FlowJo analytical software (TreeStar) on an LSRFortessa flow cytometer (BD Bioscience, CA, USA). Flow cytometry gating strategies are presented in Supplementary Figure 1.

Quantitative Real-Time PCR Analysis

RNA was isolated from mice dorsal skin tissues using TRIzol reagent and purified using chloroform. cDNA was synthesized through reverse transcription using the ReverTra Ace qPCR RT Kit. The RT-qPCR program was performed on a 7500 Real-Time PCR System (Applied Biosystems, MA, USA) using SYBR Green Master Mix and specific primers.[26] To normalize and quantify the target gene expression levels, 18S ribosomal RNA was used in the comparative CT method. Table 1 contains all primers necessary for RT-qPCR.

ELISA Analysis

The serum was collected by centrifuging blood samples from the inferior vena cava at a speed of 3000 revolutions per minute for 10 minutes, and then stored at a temperature of −80 degrees Celsius. The mouse ELISA kit was used to measure levels of total serum IgE, IgG, IFN-γ, and IL-10, in accordance with the manufacturer's instructions.

Statistics Analysis

The data were presented in the form of mean values accompanied by their respective standard deviations ($\bar{x} \pm s$). For multiple comparisons, one-way analysis of variance (ANOVA) was used, followed by least significant difference post hoc analysis (SPSS, Version 26.0, IBM Corporation, Armonk, NY, USA). A P value<0.05 indicated a statistically significant difference.

Results

Effects of AWB on AD Mice's Dorsal Skin Dermatitis Severity

Figure 2A illustrates representative images of dorsal skin from each group. Figure 2B depicts that Erythema, papules/oedema, oozing/crust, exfoliation, dryness and lichenification of the mice's skin were monitored every other day from day 0 to day 35. These findings demonstrate that DNCB can cause significant skin dysfunction over time, and the three treatments provided significant alleviation of these symptoms. SCORAD scores in the AWB group were

Table 1 Primers for RT-qPCR

Gene	Forward Primer (5`→3`)	Reverse Primer (5`→3`)
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
Foxp3	AGTGCCTGTGTCCTCAATGGTC	AGGGCCAGCATAGGTGCAAG
IFN-γ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
IL-10	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGG

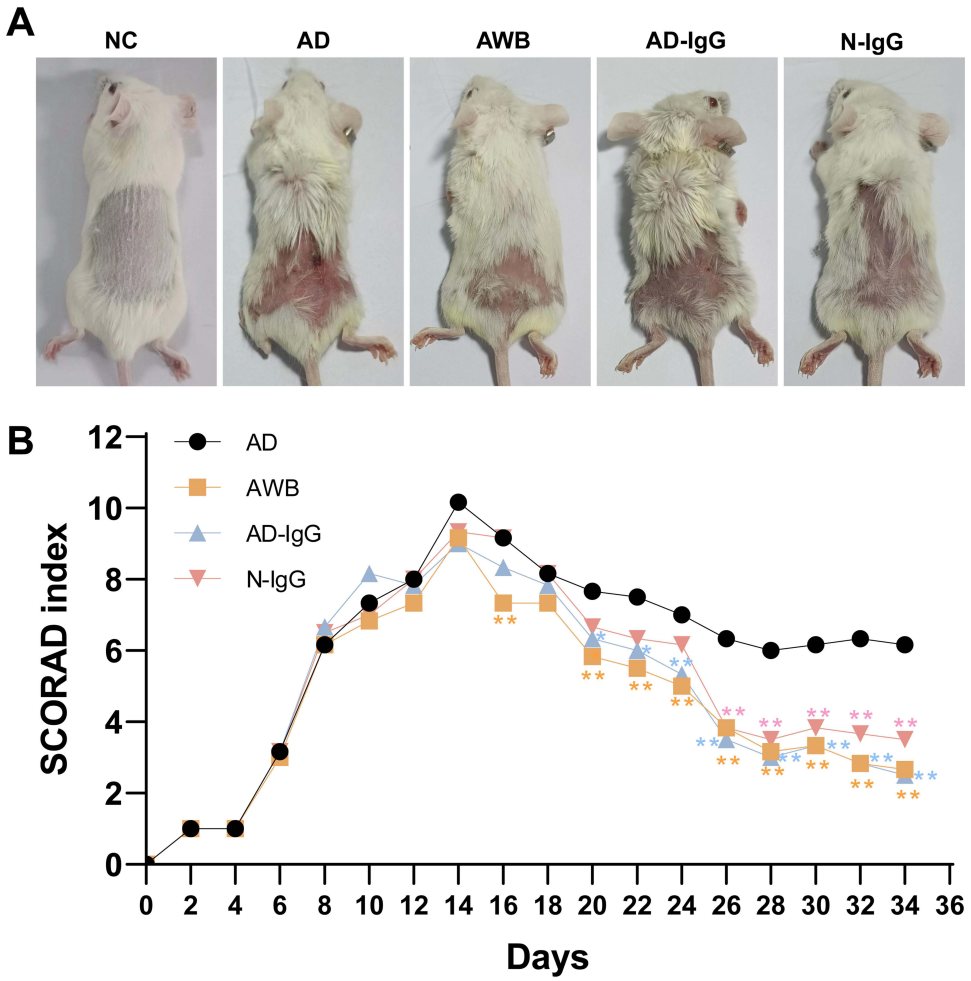

Figure 2 Effects of AWB acupoint injection on AD Mice's Dorsal Skin Dermatitis Severity ($\bar{x} \pm s$, n=6). (**A**) Clinical features of AD-like skin lesions. (**B**) SCORAD index was measured once every other day. SCORAD index were summed according to the six signs (erythema, papule/oedema, oozing/crust, exfoliation, dryness and lichenification). Data were expressed as means ± SEM (n = 6 in each group). In (**B**) *p < 0.05, **p < 0.01, vs AD group.
Abbreviations: AD, AD model; AWB, acupoint injection of autologous whole blood; AD-IgG, acupoint injection of total IgG purified from DNCB-induced AD mice; N-IgG, acupoint injection of total IgG purified from healthy blood in normal mice; SCORAD, Severity Scoring of Atopic Dermatitis.

significantly lower than the AD group on day 16. Furthermore, SCORAD scores in the AWB group and AD-IgG group were significantly lower from day 20 to 35 (P<0.01), while the N-IgG group had significantly lower scores from day 26 to day 35 (P<0.01) when compared to the AD group.

Effects of AWB on Epidermal Hyperplasia, Dermal Thickness, and Infiltration of Mast Cells in AD Mice Dorsal Skin Tissue

H&E and TB staining were utilized to evaluate the histopathological changes caused by DNCB. Results from H&E staining revealed a significant increase in epidermal and dermal thickness in the atopic dermatitis (AD) group when compared to the normal control (NC) group (all P<0.01, Figure 3A–C). Following 28 days of treatment, mice in all three treatment groups displayed a significant reduction in both epidermal and dermal thickness when compared to the AD group (P<0.05 or P<0.01, Figure 3B and C). TB staining results also revealed a considerable increase in mast cell infiltration in the AD group compared to the NC group (P<0.01), while all three therapeutic groups demonstrated a significant inhibitory effect on mast cell infiltration (P<0.01), with the AWB group exhibiting a superior effectiveness in reducing the infiltration of mast cells than the N-IgG group (P<0.05, Figure 3D and E).

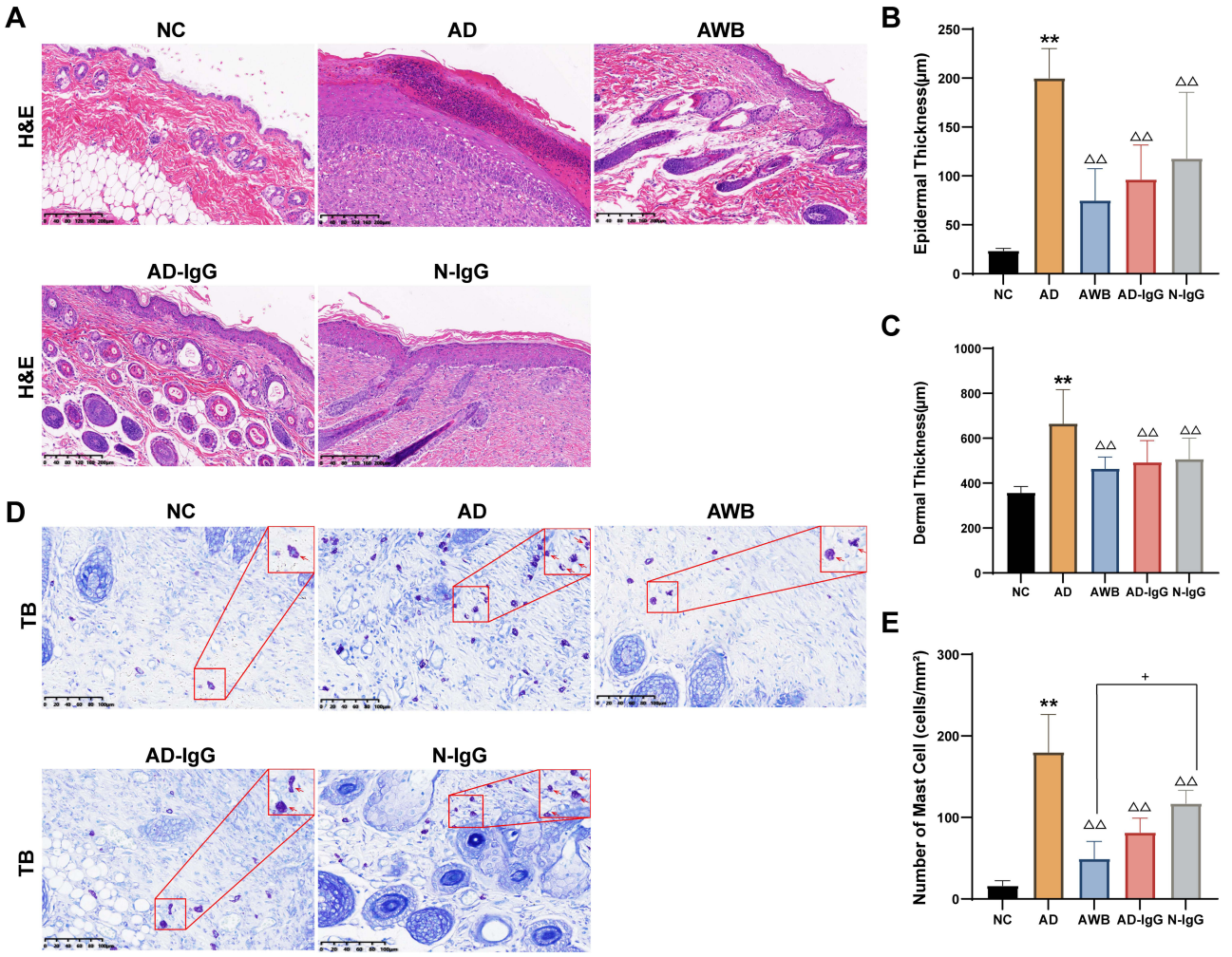

Figure 3 Effects of AWB acupoint injection on Histopathological Features in AD Mice Dorsal Skin Tissue ($\bar{x} \pm s$, n=6). (**A**) The representative image of hematoxylin and eosin (H&E). Scale bars=200μm. (**B**) Measurement of epidermal thickness and (**C**) dermal thickness. (**D**) Representative images of blue-stained toluidine mast cells in dorsal skin. Scale bars=100μm. Typical mast cell infiltrations are indicated by arrows. (**E**) number of mast cells in three fields of each section from all experimental mice. The Data were expressed as means ± SEM (n = 6 in each group). In (**B, C** and **E**) $^{**}p < 0.01$ vs NC group; $^{\Delta\Delta}P<0.01$ vs AD group. $^{+}P<0.05$ AWB vs N-IgG group.
Abbreviations: H&E, hematoxylin and eosin; TB, toluidine blue.

Effects of AWB Acupoint Injection on FoxP3-, IL-10- and IFN-γ- Producing T Cells in Spleen of AD Mice

Figure 4 showed that the AD group had a marked decrease in the proportion of $CD4^+$ T cells expressing intracellular FoxP3, IL-10, and IFN-γ when compared to the NC group (P<0.01). In contrast, FoxP3-expressing cells within the $CD4^+$ T cell population were increased in all three treatment groups (P<0.01). Furthermore, both AWB and autologous total IgG demonstrated greater efficacy in activating these cells compared to heterologous total IgG (*P*<0.05). Additionally, the AWB group had a significant rise in IL-10- and IFN-γ-producing cells within the $CD4^+$ T cell population compared to the AD group (*P*<0.01) and the AD-IgG group (*P*<0.05).

Effects of AWB on Levels of IgE, IgG, IL-10 and IFN-γ in Serum of AD Mice

The AD group had significantly higher serum IgE and IgG levels than the NC group (*P*<0.01). Acupoint injection of both AWB and N-IgG were found to be successful in suppressing elevated serum total IgE, while serum total IgG increased in both AWB and AD-IgG groups (*P*<0.01), with the AWB group exhibiting the greater effectiveness in elevating serum total IgG compared to the N-IgG group (P<0.05, Figure 5A and B). In addition, IL-10 and IFN-γ were decreased in

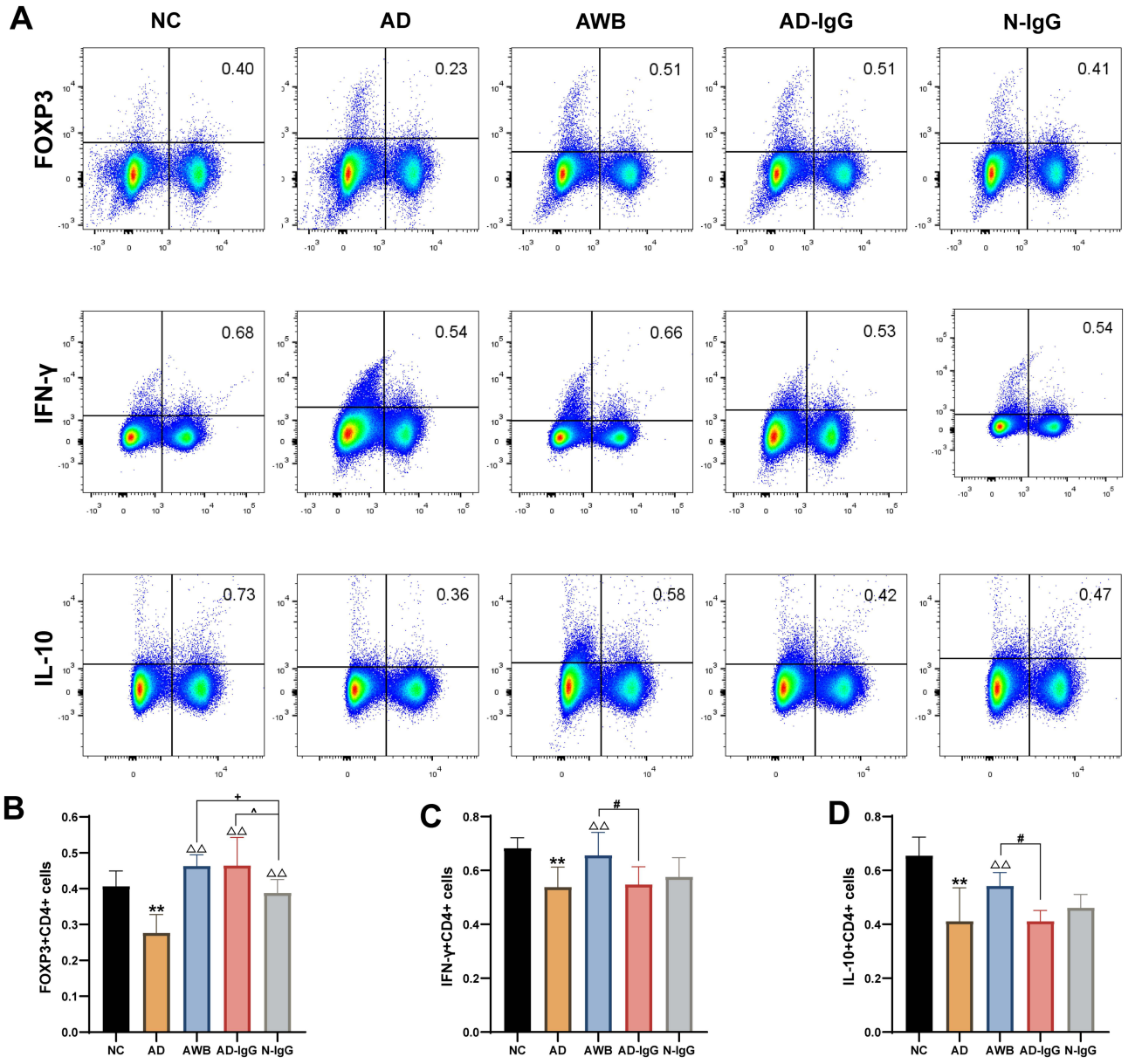

Figure 4 Effects of AWB acupoint injection on FoxP3$^+$, IL-10$^+$and IFN-γ$^+$ CD4$^+$T cells in Spleen of AD Mice ($\bar{x} \pm s$, n=6). (**A**) Flow cytometric profiles of FoxP3-, IL-10- and IFN-γ- producing CD4$^+$T cells in murine spleen. (**B**) Proportion of cells with intracellular FoxP3, (**C**) IFN-γ, and (**D**) IL-10 in CD4$^+$T cells. Data were expressed as means ± SEM (n = 6 in each group). In (B-D), $^{**}p < 0.01$ vs NC group; $^{\Delta\Delta}P<0.01$ vs AD group. $^{\#}p<0.05$ AWB vs AD-IgG group; ^ $p<0.05$ AD-IgG vs N-IgG group; $^{+}p<0.05$ AWB vs N-IgG group.

Abbreviations: FoxP3, Forkhead box P3; IFN-γ, interferon-gamma; IL-10, interleukin 10.

the AD group when compared to the NC group ($P<0.01$). Conversely, both IL-10 and IFN-γ levels were increased in the AWB group compared to the AD group ($P<0.01$), AD-IgG group and N-IgG group (P<0.01 or P<0.05, Figure 5C and D).

Effects of AWB Acupoint Injection on Expressions of FoxP3, IL-10 and IFN-γ in Dorsal Skin Tissue of AD Mice

The data presented in Figure 6A showed that the NC group of mice had FoxP3 expression predominantly in the cytoplasm of their dorsal skin tissue, whereas the AD group had a significantly lower expression and mRNA levels of FoxP3 ($P<0.05$ or $P<0.01$). Both the AWB and AD-IgG groups significantly enhanced FoxP3 expression ($P<0.05$ or $P<0.01$), and these groups demonstrated greater effectiveness in elevating FoxP3 expression compared to N-IgG group

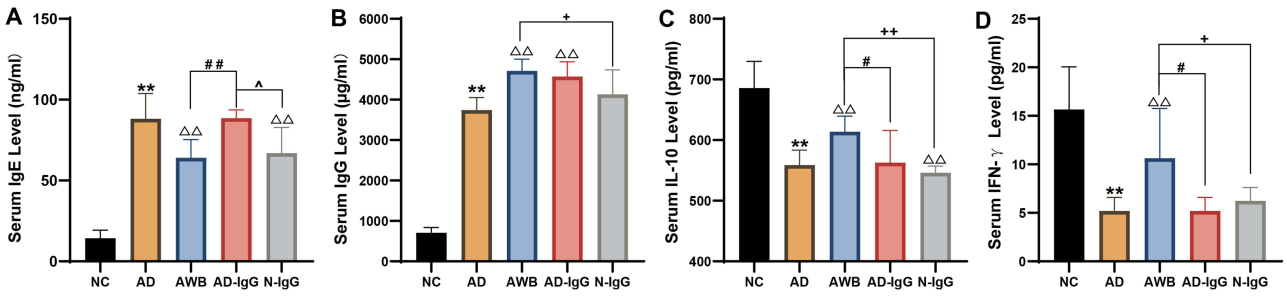

Figure 5 Effects of AWB acupoint injection on Levels of IgE, IgG, IL-10 and IFN-γ in Serum of AD Mice ($\bar{x} \pm s$, n=6). (**A**) The level of total IgE, (**B**) IgG, (**C**) IL-10 and (**D**) IFN-γ in serum. Data were expressed as means ± SEM (n = 6). In **A-D**, $^{**}p < 0.01$ vs NC group; $^{\Delta\Delta}P<0.01$ vs AD group. $^{\#}p<0.05$ AWB vs AD-IgG group; $^{\#\#}p<0.01$ AWB vs AD-IgG group. $^{\wedge}p<0.05$ AD-IgG vs N-IgG group; $^{+}p<0.05$ AWB vs N-IgG group; $^{++}p<0.01$ AWB vs N-IgG group.
Abbreviations: IgE, immunoglobulin E; IgG, immunoglobulin G.

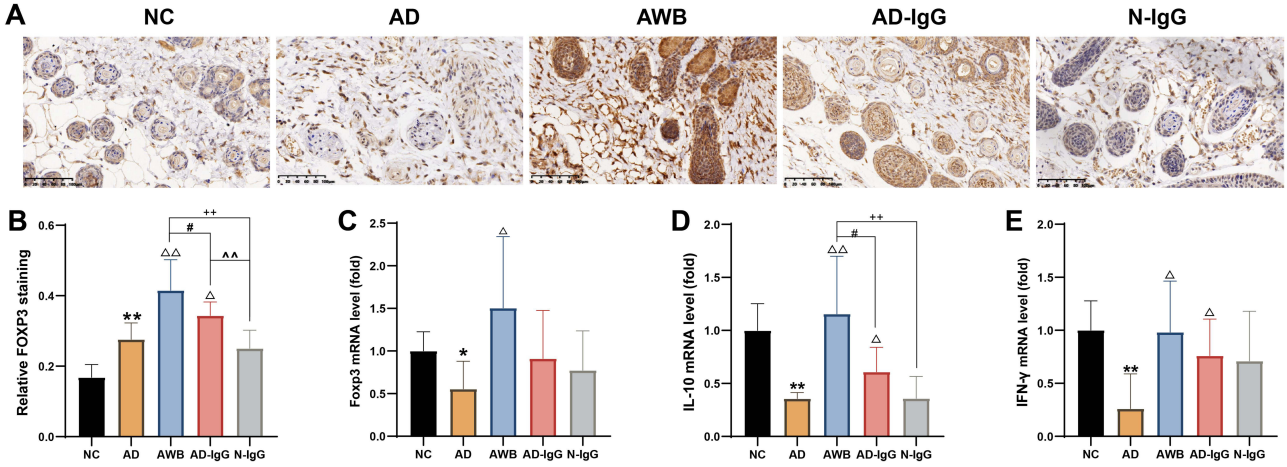

Figure 6 Effects of AWB acupoint injection on Expression of FoxP3 and mRNA levels of FoxP3, IL-10 and IFN-γ in Dorsal Skin Tissues of AD Mice ($\bar{x} \pm s$, n=6). (**A**) Representative images of FoxP3 in each group. Scale bars=100μm. (**B**) AOD analysis of FoxP3. (**C**) mRNA levels of FoxP3, (**D**) IL-10, and (**E**) IFN-γ. Data were expressed as means ± SEM (n = 6). In (**B -E**) $^{*}p< 0.05$, $^{**}p< 0.01$ vs NC group; $^{\Delta}P<0.05$, $^{\Delta\Delta}p < 0.01$ vs AD group; $^{\#}p<0.05$ AWB vs AD-IgG group; $^{\wedge\wedge}p<0.01$ AD-IgG vs N-IgG group. $^{+}$ $^{+}p<0.01$ AWB vs N-IgG group.

($P<0.05$). Additionally, AWB administration resulted in significantly increased FoxP3 mRNA levels compared to the AD group ($P<0.05$). However, acupoint injection of AD-IgG and N-IgG did not lead to a notable elevation in FoxP3 mRNA levels (Figure 6B and C). Moreover, acupoint injection of AWB and AD-IgG increased the mRNA levels of IL-10 and IFN-γ in the dorsal skin tissue of AD mice ($P<0.05$ or $P<0.01$). The N-IgG acupoint injection group did not exhibit these effects, whereas AWB administration led to a significant increase in mRNA level of IL-10 compared to N-IgG acupoint injection (Figure 6D and E).

Discussion

Our research has demonstrated that the injection of AWB, either autologous or heterologous total IgG, into acupoints can alleviate AD-like symptoms and reduce the accumulation of inflammatory cells in DNCB-induced AD mice. Furthermore, we have found that AWB and autologous total IgG acupoint injection can activate Treg cells to secrete IL-10 and induce Th1 cells to secrete IFN-γ in dorsal skin tissues of AD mice. Additionally, our findings indicate that AWB acupoint injection can increase the proportion of FoxP3, IL-10, and IFN-γ in $CD4^{+}T$ cells of murine spleen, resulting in the suppression of IgE antibody production and the elevation of IgG antibody production in serum. Conversely, acupoint injection of heterologous total IgG failed to modulate Treg and Th1 cells. These results suggest that the use of A-AHT as an anti-allergic therapy may be promising, as our findings support the hypothesis that the autologous total IgG in AWB is responsible for its therapeutic efficacy. Our data suggest that the underlying mechanisms

may involve more effective activation of Treg cells and anti-idiotypic suppression of pathogenic antibodies, including IgE antibodies, compared to heterologous total IgG.

A DNCB-induced model of atopic dermatitis (AD) was employed in BALB/c mice due to its close resemblance to the signs of human AD, such as skin erosion, hemorrhage, epidermal hyperplasia, histopathological features of mast cell infiltration, increased levels of serum IgE, and infiltration of inflammatory cells with thickened epidermis and dermis.[27,28] IgE levels are key parameter that has been extensively studied in patients with atopic dermatitis. Approximately 80% of AD patients have been reported to have elevated serum IgE levels.[29] The results of this study showed that repeated acupoint injections of AWB, autologous total IgG, or heterologous total IgG effectively reduced the severity of the symptoms and skin thickening, indicating the beneficial effects of these treatments on AD. Furthermore, TB staining revealed a significant decrease in mast cell infiltration in all treatment groups, suggesting a positive effect of these methods in suppressing mast cell accumulation in AD mice. Moreover, total serum IgG was found to be significantly higher in the AWB and autologous total IgG groups, while total serum IgE was decreased in the AWB group. These results are in line with those reported in humans who have received autologous total IgG via intramuscular administration.[30]

Tregs play a crucial role in preventing autoimmune diseases by controlling and balancing immune responses, and promoting tolerance towards harmless antigens. Two main types of Tregs have been identified: natural Tregs, which are $CD4^+$ $CD25^+$ and express FoxP3, and adaptive Tregs, which can be further divided into type 1 Tregs that secrete high levels of IL-10.[31] Our study demonstrated a significant increase in both $IL\text{-}10^+CD4^+T$ and $Foxp3^+CD4^+T$ cells in the AWB group. Additionally, T-cell response was modified from pathogenic Th2 to protective Th1.[12] Furthermore, IFN-γ^+ $CD4^+T$ cells were also found to be elevated in the AWB group. These changes in Treg and Th1 cells were accompanied by alterations in systemic cytokine production in both the AWB and AD-IgG groups, with increased mRNA levels of IL-10 and IFN-γ in the dorsal skin tissue. Thus, our study provides evidence that AWB acupoint injection can activate T cells to secrete FoxP3, IL-10, and IFN-γ, thereby inhibiting IgE antibody production and enhancing IgG antibody production in AD mice. Interestingly, acupoint injection of AWB, as well as autologous or heterologous total IgG, effectively activated the $CD4^+FoxP3^+$ Treg cells in spleen of AD mice. Notably, both AWB and autologous total IgG proved to be more effective in achieving this activation compared to heterologous total IgG. Furthermore, both AWB and autologous total IgG administrations significantly elevated FoxP3 expression, mRNA levels of IL-10 and IFN-γ, whereas the N-IgG acupoint injection group failed to demonstrate these effects. These results suggest that the use of A-AHT for the treatment of AD may be clinically useful, as our findings support the hypothesis that the autologous total IgG in AWB is responsible for its therapeutic efficacy. Our data suggest that action mechanisms of A-AHT may involve activation of Treg cells and anti-idiotypic immunomodulation mediated by autologous total IgG in the AWB.

The dose and method of administration of an antigen determine the type of specific immune response it elicits.[32] When a small amount of an allergen is topically or inhaled, it can result in an allergic reaction in individuals with allergic conditions.[33] There is speculation that injections of autologous whole blood or total IgG into specific acupoints may have an immunomodulatory effect in AD mice by manipulating the dose and type of antigen-presenting cells that come into contact with the antigen.[12,30] In this way, autologous blood injections act as personalized vaccines, stimulating the immune system to recognize and respond to AD components, increasing neutralizing modulators, inducing tolerance, and restoring homeostasis.[34] The potential mechanism of A-AHT may be activation of Treg cells by anti-idiotypic immunomodulation because acupoint injections of autologous whole blood, autologous total IgG, or heterologous total IgG improved the clinical severity of AD in AD mice. Intramuscularly administered IgG might be processed by dendritic cells and activate anti-idiotypic Treg cells and improve the AD as recently suggested.[35,36] The effects of Treg and Th1 cell activation, however, were not observed with heterologous total IgG (naïve IgG) acupoint injections.

Our research into the use of intramuscular injection of autologous whole blood at specific acupuncture points (LI11 and ST36) as a potential therapy for autoimmune and allergic diseases had yielded promising results. In an AD mouse model, this approach led to clinical remission, likely due to the stimulation of dendritic cells, which activated Tregs and Th1 cells while suppressing Th2 and effector cells, as well as the downregulation of IgE antibodies. However, there are some limitations to our study, one significant constraint is the difficulty encountered in purifying IgE with extremely low serum concentrations, which precluded the detection of anti-idiotype antibodies targeting allergen-specific IgE

antibodies. Additionally, we did not monitor other inflammatory factors, such as LDH and IL-8. Furthermore, the study exclusively utilized female mice, rendering it uncertain whether the findings are applicable to male mice.

Conclusion

In conclusion, acupoint injection of AWB induced significant clinical in DNCB-induced AD mice and activation of Treg cells and Th1 cells. Further studies are necessary to clarify its mechanism of action (especially on possibility of the anti-idiotypic immune modulation by autologous IgG in the autologous blood) and to assess its potential clinical usefulness in the treatment of AD.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author DS Z upon reasonable request.

Ethics Approval and Consent to Participate

All mouse studies were performed after approval by a local animal care committee, as documented in "Materials and methods" section.

Acknowledgment

This study is supported by research grants from the Basic and Applied Basic Research Project of Guangdong-Dongguan joint fund (No. 2022A1515140106).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no competing interests in this work.

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