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Estrogen Receptor Alpha Gene (ESR1) Facilitates Th2-immune Response and Enhances Th2 Cytokines in Experimental Atopic Dermatitis Mice

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ABSTRACT

Background: Molecular markers are involved in atopic dermatitis (AD) pathogenesis. The estrogen receptor (ESR)-1 gene, encoding ERα, is reported to express aberrantly in AD patients.

Objective: To detect the biological functions of ESR1 in 2,4 dinitrochlorobenzene (DNCB)-treated mice.

Methods: The DNCB-treated mice received a topical application of emulsion containing the 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyl ethoxy) phenol]-1H-pyrazole dihydrochloride (MPP; an ESR1-selective antagonist) to dorsal skins and ears. Then the dermatitis scores, histopathological changes, and cytokine levels were evaluated.

Results: MPP specifically downregulated ESR1 expression in DNCB-applied mice. Functionally, application of MPP abolished the DNCB-induced promotion in dermatitis score. Additionally, MPP administration protected against DNCB-induced dermatitis severity, suppressed mast cell infiltration and reduced production of immunoglobulin E (IgE) and thymus and activation-regulated chemokine (TARC). Moreover, MPP treatment inhibited DNCB-induced production of Th2 cytokines and infiltration of $CD4^+$ T cells.

Conclusion: ESR1 facilitates Th2-immune response and enhances Th2 cytokines in AD mice.

Keywords: Antagonist; Atopic Dermatitis; ESR1; Inflammatory Cytokines; Mice

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INTRODUCTION

Atopic dermatitis (AD) represents a pruritic cutaneous inflammatory disorder implicated in genetics, environmental factors, and immune abnormalities (1). As estimated, 25% of children and 2-3% of adults are affected by this disease worldwide (2). Severe pruritus, erythema, dryness, skin hypersensitivity, rash, and edema are clinically manifested by patients with AD (3). Anxiety, stress, sleep disturbances, and school performance problems are terrible sufferings for patients with AD (4). Corticosteroids, immunosuppressants, and antihistamines are currently used to treat AD (5). However, the side effects caused by the long-term use of these agents, including skin atrophy, organ toxicity, and emotional instability, limit their clinical significance (6). Therefore, exploring effective AD therapeutics

with little adverse effect is urgent.

The AD skin lesions are pathologically featured by infiltration of fully granulated mast cells and eosinophils, lymphocytes, and monocytes/macrophages (7). Activated $CD4^+$ cells secreting T-helper 2 (Th2) chemokines including c-c motif chemokine ligand 17/thymus and activation-regulated chemokine (CCL17/TARC) and cytokines including interleukin 4 (IL-4), IL-5 and IL-13 are expressed in acute skin lesions of AD (8). IL-4, an essential Th2 cytokine, is associated with eosinophil recruitment, Th2 cell differentiation, and immunoglobulin E (IgE) production. The upregulation of IL-4 is an increased risk for AD development (9). In an experimental AD model, IL-4 upregulation in the epidermis promotes pruritis, inflammation, and bacterial infection in the skin (10). Cord blood mononuclear cells-producing IL-13 is also reported to be associated with AD development (11, 12). The upregulated IL-13 level in serum is observed in patients with AD (13). The definitive role of IL-13 that facilitates pruritic dermatitis and inflammation and increased IgE level has been suggested in skin-specific IL-13 transgenic mouse (14). IL-5 is an essential cytokine involved in the survival and proliferation of eosinophils (15). Elevated serum IL-5 level is observed in patients with AD (16). IL-5 knockout attenuates epidermal thickening and skin eosinophilia (17). Thus, the reduction of Th2-related cytokines and chemokines can be effective for the treatment of AD.

Currently, transcriptomic (18, 19), metabolomic (20, 21), and proteomic (22, 23) approaches are used to improve molecular AD diagnosis. The steroid hormones and their receptors mediate eukaryotic gene expression and affect cellular differentiation and proliferation in target tissues (24). The estrogen receptor (ESR)-1 gene, encoding ERα, belongs to the family of nuclear hormone receptor (25). The gene enrichment analysis has suggested that compared with that in healthy patients, ESR1 level is aberrantly expressed in patients with AD (26). Additionally, ESR1 is highly expressed in patients with wound healing, and inhibition of ESR1 is shown to stimulate human skin fibroblasts (27). However, the biological functions of ESR1 in AD pathogenesis remain uncertain.

NC-Nga mice are widely employed to explore the mechanisms underlying AD pathogenesis (28). 2,4-dinitrochlorobenzene (DNCB), an allergenic chemical, can cause skin hypersensitivity and stimulate dermatitis in NC/Nga mice (29). 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy) phenol]-1H-pyrazole dihydrochloride (MPP) can specifically downregulate ESR1 expression (30). In the current study, we proposed to detect ESR1 roles in AD initiation by downregulating the ESR1 levels using MPP in DNCB-applied NC/Nga mice. We hypothesized that ESR1 would exacerbate AD -like symptoms. We believe that the ESR1 would be a novel target for AD diagnosis and treatment.

MATERIALS AND METHODS

Animals and Treatment

All experimental protocols followed the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (2013) and were approved by the Ethical Committee of Wuhan Myhalic Biotechnology Co., Ltd (approval number: 202111036). NC/Nga mice (female, 8 weeks) obtained from Charles River (Beijing, China) were housed under controlled conditions (22±2 °C, 60%±5% of relative humidity, 12h/12h light/dark cycle) with free food and water.

Experiments began after two weeks of acclimatization. Dorsal hairs (approximately 8 cm^2) were shaved using an electronic shaver. After complete removal, the mice received an application of 200 µl of 1% DNCB (Sigma-Aldrich, St-Louis, MO, USA) dissolved in acetone/olive oil (3:1; Sigma-Aldrich) to dorsal skin and ears for 8 consecutive days. After the DNCB sensitization, the mice were subjected to 200 µl of 0.2% DNCB for 8 weeks

(3 sessions per week). On week 5, the DNCB-treated mice received a topical application of emulsion containing 20 µg/kg or 200 µg/kg of MPP (Figure 1A; Sigma-Aldrich) to dorsal skin and ears for 4 weeks (6 times per week). The MPP dosages were determined as previously described (30). The group assignment was as follows: (a) the control mice were topically applied with acetone/olive oil (3:1) for 9 weeks and emulsion without MPP for 4 weeks; (b) the DNCB mice were treated with DNCB as described above and applied with emulsion without MPP; (c) DNCB + MPP (20 µg/kg), DNCB-applied mice were applied with an emulsion containing MPP (20 µg/kg) on dorsal skin and ears; (d) DNCB + MPP (200 µg/kg), DNCB-applied mice were applied with an emulsion containing MPP (200 µg/kg) on dorsal skin and ears. Each group had 8 mice. The mice were sacrificed the next day after the last drug administration (Figure 1B).

Dermatitis Score

The dermatitis score was evaluated weekly by scoring clinical manifestations, including erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion. The dermatitis severity was examined using the scoring system as follows: 3 (severe, >60%), 2 (moderate, 20-60%), 1 (mild, <20%), and 0 (none). Three independent observers participated in the scoring, and the dermatitis score was defined by the sum of individual scores (31).

Histological Evaluation

The dorsal skin was resected and

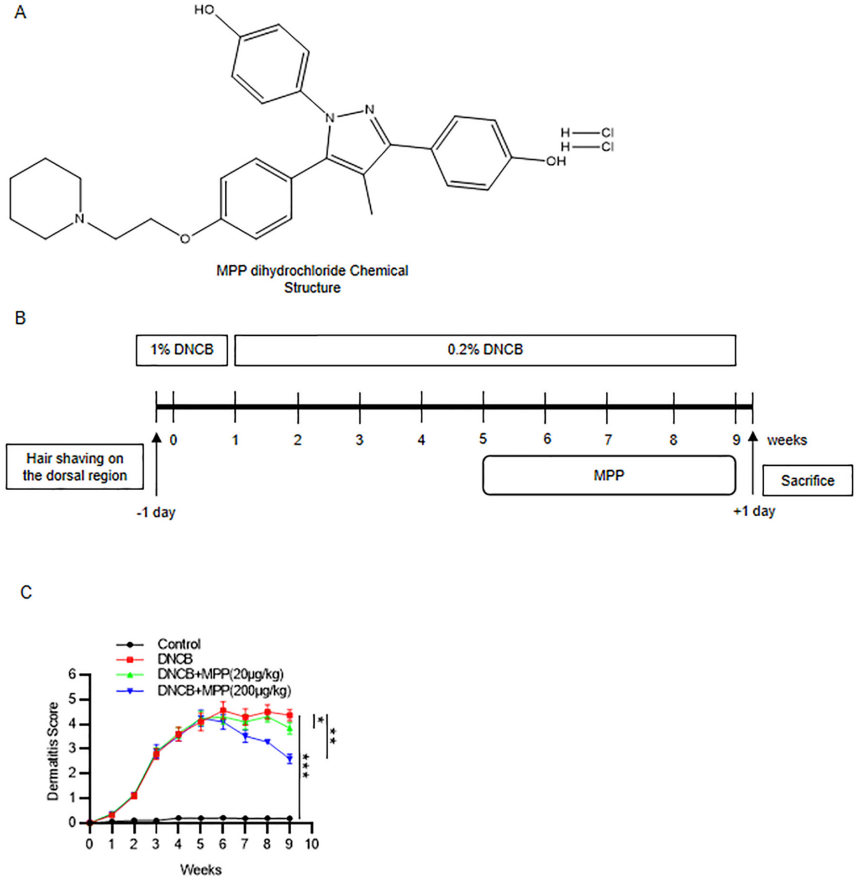

Figure 1. MPP alleviates dermatitis severity. (A) Chemical structure of ESR1 selective antagonist MPP. (B) Schematic diagram of the experimental protocol. Application of DNCB was conducted to induce AD-like symptoms. After the AD induction, an emulsion containing MPP was applied to the mice to detect the function of ESR1 inhibition in the DNCB-induced skin lesions. (C) Dermatitis scores in the control, the DNCB, and the DNCB+MPP (20 µg/kg, 200 µg/kg) groups. N=5 mice per group. *P<0.05, **P<0.01, ***P<0.001. ESR-1: Estrogen receptor -1; DNCB: 2,4-dinitrochlorobenzene

fixed in 10% neutral formalin at room temperature for 1 day, paraffin-embedded, and sectioned into 5-μm slices using a rotary microtome (Leica Biosystems, Shanghai, China) followed by deparaffinization and rehydration. Then, the tissues were stained with hematoxylin and eosin (H&E) or toluidine blue obtained from Solarbio (Beijing, China), and observed using a light microscope (Olympus, Tokyo, Japan). The dermatitis severity was assessed using the previous scoring system (32). Histological scores for individuals were defined by the sum of scores. Four randomly selected fields were used to count the number of mast cells, and the results were presented as the means of five independent numbers.

Enzyme-linked Immunosorbent Assay (ELISA)

The collection of blood samples was conducted before the mice were sacrificed, and the collected samples were subjected to 10-min centrifugation at 2000 rpm at 20 ºC, followed by the separation of the upper serum layer. To determine the concentration of the TARC and IgE in serum, commercial assays were used according to the manufacturers' instructions: a mice IgE sandwich ELISA kit (Jining Shiye, Shanghai, China; lower detection limit of 0.1 μg/ml) and a mouse TARC sandwich ELISA kit (Jining Shiye; lower detection limit of 1.0 pg/ml).

Reverse Transcription-quantitative Polymerase Chain Reaction Analysis (RT-qPCR)

The total RNA was isolated using the RNAsio reagent (Takara, Kyoto, Japan). A Nanodrop spectrophotometer (DeNovix, Wilmington, DE, USA) was used to examine the concentration and quality of the isolated RNA. The reverse transcription into complementary DNA (cDNA) was accomplished via the PrimerScript RT Reagent kit (Think-Far Technology, Beijing, China). RT-qPCR was executed with synthesized cDNA and SYBR Premix EX Taq (Takara) into the Applied Biosystems QuantStudio 5 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR thermocycling conditions were as follows: 30-44 cycles of one minute at 94 ºC (denaturation), 1 min at 50-58 ºC (annealing), and 1 min at 72 ºC (extension). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal reference. The relative expression level was calculated using the $2^{-\triangle\triangle Ct}$ method (33). The primer sequences used in this study are listed in Table 1.

Western Blotting

The total proteins were isolated using RIPA buffer (Sigma-Aldrich) with protease inhibitor (ApexBio Technology, Shanghai, China), and the concentration was detected using an Enhanced BCA Protein assay kit

Table 1. Sequences of primers used for reverse transcription-quantitative PCR

Genes	Sequence (5'→3')
IL-4 forward (mouse)	GATTCATCGATAAGCTGCACC
IL-4 reverse (mouse)	CATGATGCTCTTTAGGCTTTCC
IL-5 forward (mouse)	AAGCAATGAGACGATGAGG
IL-5 reverse (mouse)	ATTCTTCAGTATGTCTAGCCC
IL-13 forward (mouse)	GTATGGAGTGTGGACCTGG
IL-13 reverse (mouse)	TGTTGGTCAGGGAATCCAG
ESR1 forward (mouse)	CCTCTGGCTACCATTATGGG
ESR1 reverse (mouse)	AGTCATTGTGTCCTTGAATGC
ESR2 forward (mouse)	GAATCTCTTCCCAGCAGCA
ESR2 reverse (mouse)	GCCCTTGTTACTGATGTGC
GAPDH forward (mouse)	ACTCTTCCACCTTCGATGC
GAPDH reverse (mouse)	CCGTATTCATTGTCATACCAGG

IL: Interleukin; ESR-1: estrogen receptor -1; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

(Yeasen, Shanghai, China). Proteins (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes. After being blocked using 5% skimmed milk, the membranes were incubated overnight with primary antibodies against IL-4 (#MAB404, 1 μg/ml; R&D Systems, Shanghai, China), ESR2 (sc-52494, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), IL-13 (ab79277, 1:1000; Abcam), GAPDH (ab125247, 1:5000; Abcam, Shanghai, China), ESR1 (ab32063, 1:1000; Abcam) and IL-5 (#BAF405, 0.1 μg/ml; R&D Systems) at 4 ºC, followed by an incubation with secondary antibodies for 2 h at room temperature and three times of Tris-buffered saline washing. The blots were developed using enhanced chemiluminescence (Yeasen) and imaged using the chemiluminescence detection system (Bio-Rad, Hercules, CA, USA). The intensity of the blot was quantified by Image Lab 3.0 software.

Immunohistochemistry Staining

The deparaffinized slices were subjected to a 30-min boiling in 10 mM sodium citrate buffer to retrieve antigens, and incubated with 3% H_2O_2 for 30 min to prevent endogenous peroxidase. Subsequently, the slides were blocked with 5% normal goat serum (Beyotime, Shanghai, China) and washed, followed by an incubation with an antibody against biotin anti-mouse CD4 (cat. no. 100403, 1:100; Dakewe Biotech Co, Beijing, China) overnight. Thereafter, the sections were incubated with the biotinylated goat anti-mouse IgG (cat. no. BA-9200, 1:200; Neobioscience, Shenzhen, China) for 2 h at room temperature and an Avidin/Biotinylated Enzyme Complex kit (Neobioscience) for 1 h. Finally, the slides were incubated with the 3,3-diaminobenzidine (Yeasen), counterstained by hematoxylin staining, and observed using a light microscope (Olympus).

Statistics Analysis

The data were analyzed by GraphPad Prism (GraphPad Software, San Diego, CA, USA), and described as the mean±standard deviation. A One-way analysis of variance followed by Tukey's post hoc analysis and Student's *t*-test was performed to compare the differences. $P<0.05$ was considered statistically significant.

RESULTS

MPP Alleviates Dermatitis Severity

The skin conditions were evaluated for 9 weeks (twice a week). The results revealed that the DNCB application significantly increased the dermatitis scores. However, the MPP application suppressed the DNCB-induced promotion in dermatitis scores dose-dependently (Figure 1C).

MPP Attenuates the DNCB-stimulated Histopathological Changes

The results of H&E staining demonstrated that the DNCB induction promoted inflammatory changes like hyperkeratosis and epidermal hyperplasia. However, the DNCB-induced histopathological changes were attenuated by the MPP treatment dose-dependently (Figure 2A). Additionally, the DNCB group exhibited remarkably higher histological scores than the control, while the MPP abolished the DNCB-induced promotion in histological dermatitis scores dose-dependently (Figure 2B). Then, as shown by toluidine blue, mast cell infiltration enhanced in DNCB-applied mice compared with the controls, while the administration of the MPP markedly limited the impact of the DNCB dose-dependently (Figure 2C-D). Next, we detected the MPP roles in the DNCB-induced release of IgE and TARC using the ELISA kits. We observed that the DNCB application remarkably upregulated IgE and TARC levels, while the MPP abolished the enhancing effect of the DNCB at the dose of 200 μg/kg, and no significant

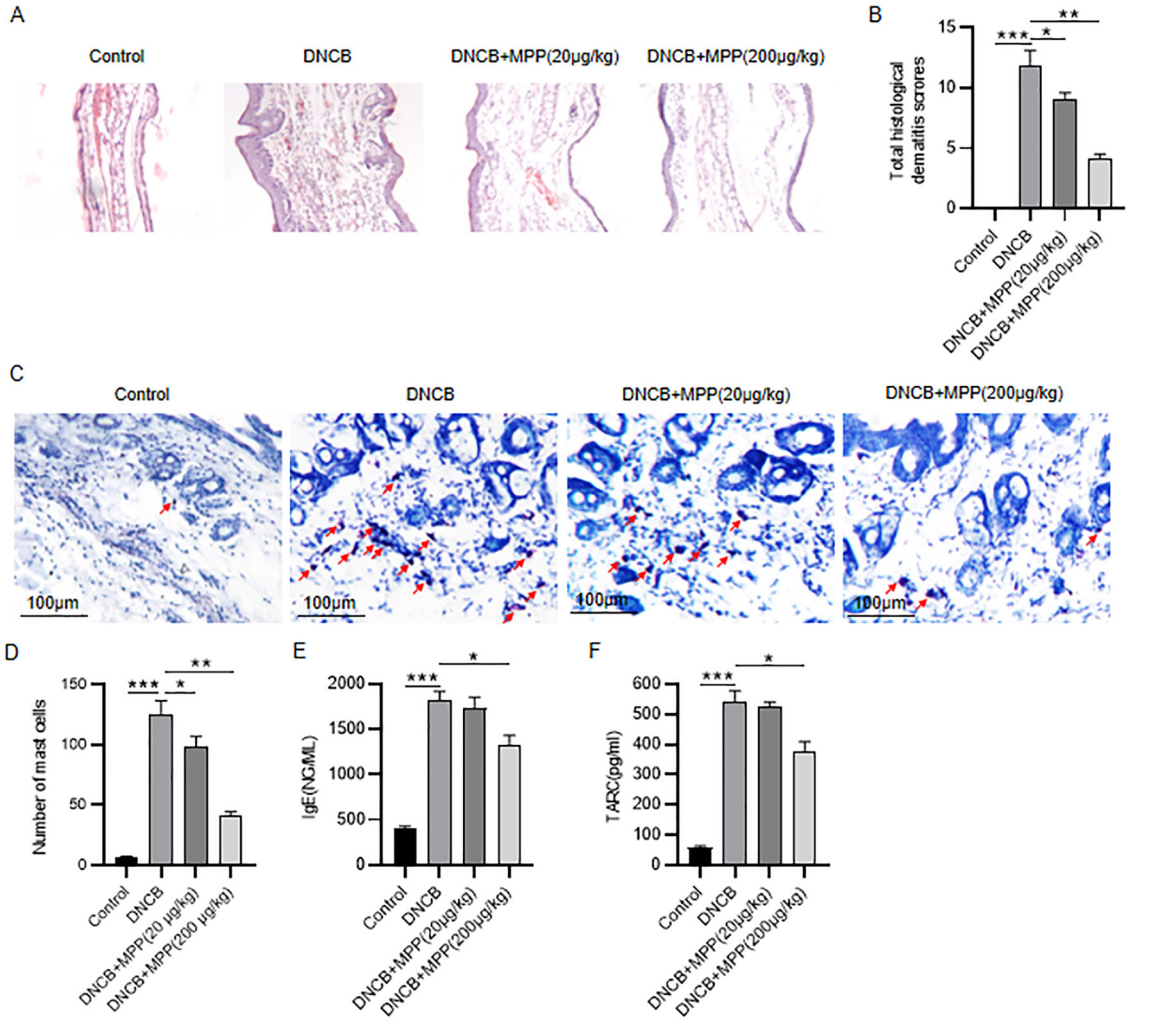

Figure 2. MPP attenuates the histopathological changes and reduces the production of IgE and TARC. (A) The dorsal skin tissues were stained with H&E for histopathological analysis. (B) The assessment of total histological AD scores. (C) Toluidine blue staining. (D) Quantification of the number of mast cells at five random sites per animal. (E) Serum level of IgE. (F) Serum level of TARC. N=5 mice per group. *P<0.05, **P<0.01, ***P<0.001. DNCB: 2,4-dinitrochlorobenzene

difference was observed following 20 μg/kg of MPP treatment (Figures 2E-F). These results suggest that the MPP treatment alleviates DNCB-induced dermatitis severity, suppresses mast cell infiltration, and reduces IgE, and TARC levels.

MPP Inhibits Th2-mediated Inflammation in DNCB-applied Mice

As RT-qPCR showed, 200 μg/kg of MPP reversed the enhancing effect of the DNCB on IL-4, IL-5, and IL-13 mRNA levels, while 20 μg/kg of MPP had no obvious effect (Figures 3A-C). The results of the western blotting confirmed the above finding that the DNCB significantly upregulated IL-4, IL-5, and IL-13 protein levels, while the MPP application had the opposite effect at the dose of 200 μg/kg (Figure 3D-E). Immunohistochemistry staining of CD4+ T cells showed that the DNCB application markedly augmented infiltration of $CD4^+$ T cells, inhibited by MPP dose-dependently (Figures 3F-G). These results show that the MPP treatment suppresses DNCB-induced Th2-mediated inflammation.

MPP Specifically Inhibits ESR1 Expression

The DNCB increased ESR1 and ESR2 mRNA levels, as shown by RT-qPCR. However, after the MPP application, the mRNA level of ESR1 decreased dose-dependently, while no obvious difference

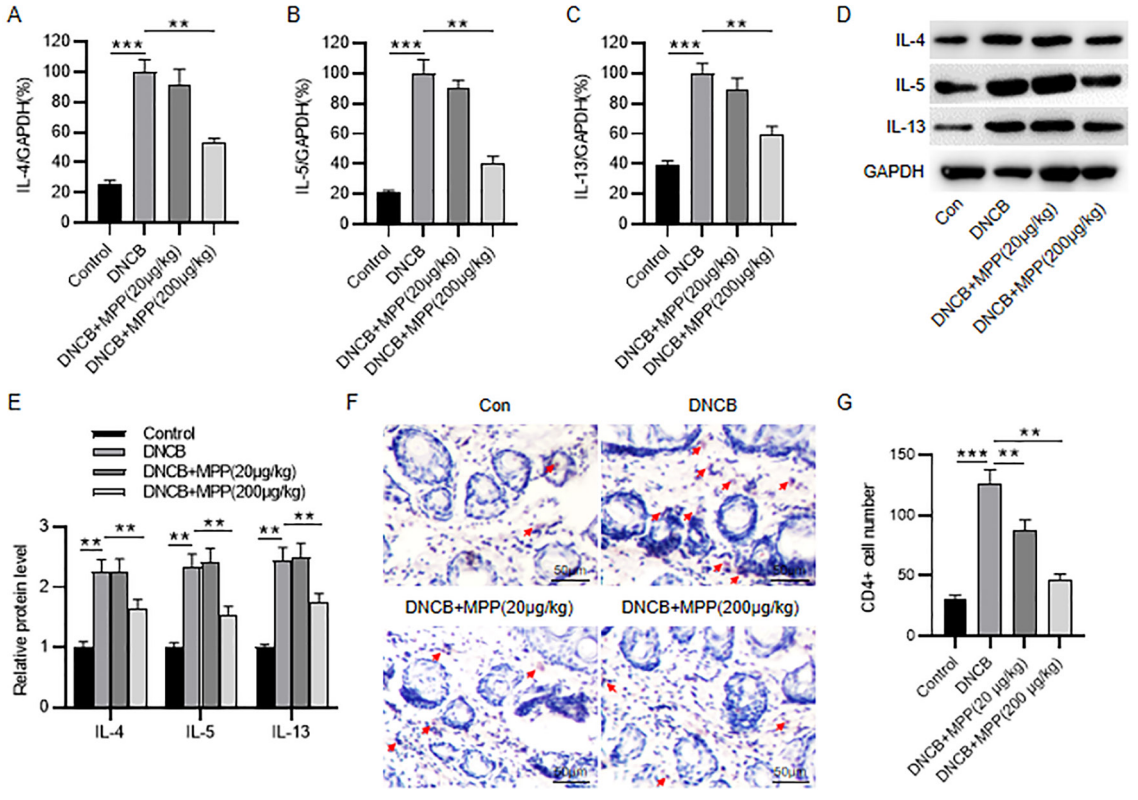

Figure 3. MPP inhibits Th2-mediated inflammation in DNCB-applied mice. (A-C) RT-qPCR of IL-4, IL-5, and IL-13 mRNA levels. (D-E) The protein levels of Th2 inflammatory cytokines were detected by western blotting. (F-G) Immunohistochemistry staining of $CD4^+$ cells. N=5 mice per group. **P<0.01, ***P<0.001. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; DNCB: 2,4-dinitrochlorobenzene; IL: Interleukin

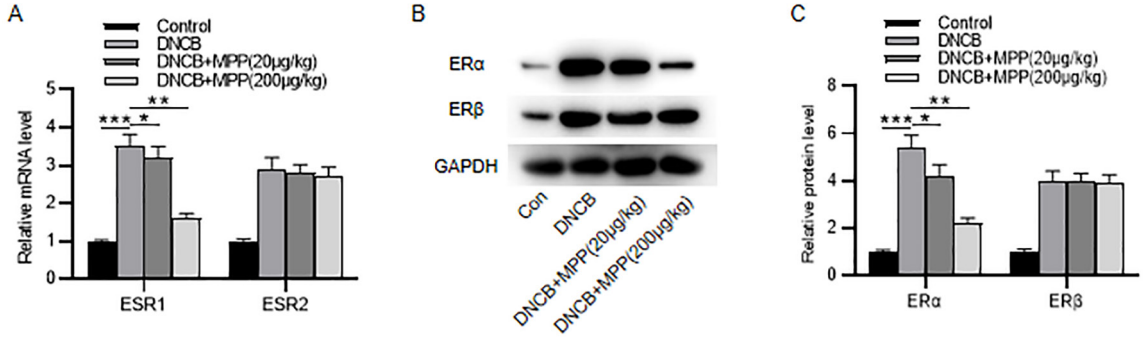

Figure 4. MPP inhibits the ESR1 expression in DNCB-applied mice. (A) The mRNA levels of ESR1 and ESR2 in the control, the DNCB, and the DNCB +MPP (20 μg/kg, 200 μg/kg) groups were detected by RT-qPCR. (B-C). Western blotting was conducted to measure the protein levels of ERα and ERβ after indicated treatment. N=5 mice per group. *P<0.05, **P<0.01, ***P<0.001. ESR-1: Estrogen receptor -1; DNCB: 2,4-dinitrochlorobenzene; ER: Estrogen receptor

was observed in the mRNA level of ESR2 (Figure 4A). As the western blotting revealed, the protein levels of ERα and ERβ were upregulated post-DNCB. However, the MPP attenuated the promoting effect of the DNCB on the protein level of ERα, while MPP exerted no function in the protein level of ERβ (Figure 4B-C).

DISCUSSION

ESR1 is aberrantly expressed in patients with AD compared with that in the controls (26, 34). Additionally, ESR1 inhibition increases human skin fibroblasts which are key cells in the cutaneous wound repair (27). However, the biological functions of

ESR1 in AD development remain uncertain. Therefore, this study first demonstrated the biological functions of ESR1 antagonist (MPP) in DNCB-induced skin lesions in vivo.

Mast cells release mediators such as histamine that exacerbate inflammation (35). In the current study, the MPP administration inhibited the thickening of the epidermis and dermis and reduced mast cell infiltration. In AD therapy, the major objective is to inhibit the inflammatory immune response (36). The imbalance between Th1 and Th2 caused by an activated Th2-type immune response is reported to initiate AD pathogenesis (37). ESR1 has been found to promote the secretion of IL-33 and airway inflammation (38). Additionally, ESR1 can regulate the anti-inflammatory activity of estrogen on human monocytes (39). In the current study, we found that post-DNCB, the number of $CD4^+$ cells and the levels of Th2 inflammatory cytokines (IL-4, IL-5, and IL-13) increased, while the application of the MPP, a selective antagonist of ESR1, limited the impact of DNCB, indicating that ESR1 inhibition suppressed the immune inflammatory activity in DNCB-applied mice.

TARC can attract Th2 cells to the site of skin lesions (40), and TARC upregulation has been found in AD-like skin lesions as previously reported (41). Mast cells secreting inflammatory cytokines are essential participants in inflammation. IgE induces the secretion of inflammatory chemokines and cytokines by binding to the surface of mast cells (42-44). An increased serum level of IgE has been observed in patients with AD (45). We herein found that ESR1 inhibition abolished the DNCB-induced promotion in the IgE and TARC release in mice.

Overall, this study demonstrated that ESR1 inhibition suppresses the Th2-associated immune inflammatory responses and DNCB-induced skin lesions in mice. However, there are limitations to this study. First, a previous study demonstrated the ESR1 downregulation in AD patients compared with the normal controls (26); thus, further evaluation of ESR1 in patients with AD is required. Second, the functions of ESR1 agonists in AD development should be further investigated. Third, the functions of ESR1 antagonists or agonists should be investigated in more species. Despite these limitations, we believe that the ESR1 would be a target for AD diagnosis and treatment.

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AUTHORS' CONTRIBUTIONS

Jianrong Niu conceived and designed the experiments. Jianrong Niu, Hui Zhou, Rong Tian, and Xudong Wang carried out the experiments. Jianrong Niu and Rong Tian analyzed the data. Jianrong Niu and Rong Tian drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

Conflict of Interest: None declared.

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