

Topical application with conjugated linoleic acid ameliorates 2, 4-dinitrofluorobenzene-induced atopic dermatitis-like lesions in BALB/c mice

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Abstract

Atopic dermatitis (AD) is a multifactorial chronic inflammatory skin disease characterized by skin barrier dysfunction, eczematous lesions, pruritus, and abnormal immune responses. In this study, we assessed the therapeutic effect of topical applied conjugated linoleic acid (CLA) on a murine AD model that was developed by repetitive applications of 2, 4-dinitrofluorobenzene (DNFB). 2% or 5% CLA could markedly ameliorate AD-like skin lesions, scratching behaviour and skin inflammation as evidenced by the reduced inflammatory blood cells, IgE and Th2-related cytokine levels, and the infiltration of mast cells and inflammatory cells to the dermal tissues. Moreover, topical application with CLA modulated skin barrier repair including maintaining a balanced skin pH and increasing skin hydration, partially mediated by upregulating skin barrier-related protein, filaggrin (FLG). In addition, topical CLA significantly dose-dependently inhibited pro-inflammatory cytokines including interleukin (IL)-6, IL-1 β , tumour necrosis factor (TNF)- α and pro-inflammatory enzyme expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in inflamed mice skin. Its anti-inflammatory effect was associated with the inhibition of DNFB-stimulated I κ B α and NF- κ B p65 phosphorylation in mouse skin. Taken together, our results suggest that locally applied CLA exerts potentially protective effects against AD lesional skin at least in part, due to regulation of skin barrier function and inflammatory response.

KEYWORDS

atopic dermatitis, conjugated linoleic acid, filaggrin, inflammatory response, skin barrier function

1 | INTRODUCTION

Atopic dermatitis (AD), also called atopic eczema, is one of the most common chronic relapsing inflammatory skin diseases and affects 15%–20% of children and 1%–3% of adults worldwide. It is characterized by intense pruritus, dryness and eczematous lesions such as erythematous, oedema and lichenification.^{1,2} AD poses a substantial

burden on patients' quality of life, mainly due to intense pruritus that leads to sleep deprivation. Furthermore, the so-called 'atopic march', which is characterized by a typical sequence of atopic diseases in childhood preceding the development of asthma, allergic rhinitis and/or food allergies later in life, can be life-threatening.³

The pathogenesis of AD involves a complex inflammatory process and is not fully understood, but is believed to be involved in

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skin barrier defects and immune abnormalities mediated by genetic, immunologic and environmental factors. In clinic, topical emollients for barrier dysfunction, topical steroids and calcineurin inhibitors for skin inflammation and immunomodulators for immune deviation, are mainly used as treatment interventions for AD.⁴⁻⁶ However, these therapies for moderate to severe AD are non-specific and exhibit limited efficacy. Besides, long-term use of topical corticosteroids on AD causes diverse side effects, such as skin atrophy, telangiectasia and acne, which reduce patient compliance and treatment effectiveness.^{7,8} Therefore, safer strategies with fewer unexpected side effects for the prevention and treatment of AD are urgently needed.

Increased transepidermal water loss (TEWL), decreased stratum corneum water content, pH alterations, and reduced expression of skin barrier-related proteins such as filaggrin (FLG) and claudin-1 are the main indications of skin barrier dysfunction in individuals with AD. Growing evidence suggests that maintaining skin barrier integrity is possible the primary intervention to prevent AD, because impaired skin barrier facilitates penetration of contact allergens and irritants into the epidermal layer and subsequently stimulates allergic sensitization and T-helper 2 (Th2)-related inflammation in the skin.^{9,10} Besides, previous preclinical and clinical studies emphasize that anti-inflammatory therapies based on blocking NF- κ B activation may be a therapeutic target for the management of AD.¹¹⁻¹³ NF- κ B is a multifunctional transcription factor and activation of NF- κ B may exacerbate the allergic and chronic inflammation by enhancing the production of inflammatory cytokines and chemokines.¹⁴

Conjugated linoleic acid (CLA), an 18-carbon polyunsaturated fatty acid, is a mixture of positional and geometric isomer of linoleic acid characterized by conjugated double bonds.^{15,16} 28 naturally occurring CLA isomers predominantly identified from milk, dairy, and ruminant fats to date and have drawn significant attention in the last three decades for its variety of biologically beneficial properties and health effects. The *cis*-9, *trans*-11(*c9*, *t11*)-CLA is the most predominant isomer in dietary sources, representing almost 90% of total dietary CLA intake, whereas the *trans*-10, *cis*-12 (*t10*, *c12*)-CLA accounts for the remaining 1%-10% of total CLA, with negligible proportions of the other isomers. Chemically synthesized and commercial preparations of CLA refers to a mixture containing equal proportions of *c9*, *t11* and *t10*, *c12* isomers.¹⁷⁻¹⁹

Conjugated linoleic acid has been reported to possess anti-inflammatory, anti-allergic, immune-regulatory and skin moisturizing properties that are demonstrated in a wide range of inflammatory disorders including inflammatory bowel disease, diabetes, atherosclerosis and allergic diseases.²⁰⁻²² Lots of findings collectively indicate the potential value of CLA for alleviating IgE-mediated chronic allergic skin inflammation like AD.^{23,24} Moreover, CLA has been shown to inhibit NF- κ B activation and subsequent production of inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in RAW 264.7 macrophages.²⁵ Accordingly, our previous observation proved that orally administration of 100 mg/kg CLA has anti-inflammatory and immune regulation effects on allergen-induced AD mice.²⁶ Notably, recent studies have shown that repairing the skin barrier function might be the novel pharmacotherapeutic developmental strategies.^{27,28} The

protective effect of CLA in maintaining the natural barrier of skin has aroused our new attention to its topical use. CLA, similar to the structure of fatty acid secreted by natural sebum, is reported to protect skin from environmental damage by reducing moisture loss due to evaporation and rebuilding the skin barrier.²⁹ However, whether topical application of CLA works well in alleviating AD has not been extensively investigated as of yet. Herein, the present study was to investigate the therapeutic effects of topically administered CLA on 2, 4-dinitrofluorobenzene (DNFB)-induced AD in BALB/c mice and elucidate the underlying molecular mechanisms.

2 | MATERIALS AND METHODS

2.1 | Chemicals

2, 4-dinitrofluorobenzene (purity 99%) was purchased from Alfa Aesar (Heysham, UK). Olive oil was obtained from Aladdin (Shanghai, China). DEX (purity 98%) was supplied by Saen Chemical Technology Co. Ltd. (Shanghai, China). CLA (50:50 mixture of *c9*, *t11*, and *t10*, *c12*-CLA isomers, purity 80%) was supplied by Macklin Biochemical Co. Ltd (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits were produced by Meimian industrial Co., Ltd. (Jiangsu, China). Antibodies against filaggrin (FLG), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were obtained from Bioss Biotechnology Co. Ltd. (Beijing, China). NF- κ B p65, phospho (p)-NF- κ B p65, I κ B α , p-I κ B α and β -actin antibodies were acquired from Cell Signaling Technology, Inc (Beverly, MA, USA), whereas goat anti-rabbit IgG and goat anti-mouse IgG conjugated to horseradish peroxidase were the products of Jackson (Lancaster, China). All other reagents and chemicals were of analytical grade.

2.2 | Experimental protocol and AD model

Male BALB/c mice with 6-week-old were purchased from the Animal Center of China Three Gorges University (Yichang, China). The animals were housed in controlled room at 22 $\pm$ 2 C with a 12-h dark/light cycle and fed a regular unrestricted diet. After 1 week of acclimation, they were randomly divided into 5 groups with 5 mice each as follows: (1) Normal group; (2) Model group; (3) Model + 2% CLA (w/v; g/mL) group; (4) Model + 5% CLA (w/v; g/mL) group; (4) Model + 0.05% DEX (w/v; g/mL) group. 0.05% dexamethasone (DEX) was used as a positive control in the present study, equivalent doses of corticosteroid compound contained in Piyanping ointment, available on the market.

A series of experimental models have been developed in order to better clarify the pathogenesis of AD or to evaluate efficacy of candidate therapies. However, there are no perfect mouse models for AD because genetically or chemically induced AD in mice only recapitulates part of the features observed in AD patients but never all. In our study, a contact sensitizing hapten, DNFB was epicutaneously applied to the dorsal skin of BALB/c mice to induce AD-like symptoms as previously described with some

modifications.³⁰ As illustrated in Figure S1, 1 day after complete dorsal hair removal (approximately 2×3 cm), 100 μ l of 1.0% DNFB (w/v) solution (dissolved in acetone: olive = 3:1) was applied on the dorsal skin on day 1. In the secondary induction, 100 μ l of 0.5% DNFB was applied onto the dorsal skin on days 3, 7, 9 and 13. DNFB-treated BALB/c mice were painted with a total of 100 μ l of 2%, 5% CLA or 0.05% DEX diluted in a vehicle of propylene glycol daily from the first day to the thirteenth day. Normal group and Model group were treated with an equal volume of the vehicle propylene glycol. The mortality, body weight and food consumption were carefully monitored for each mouse every third day during the experimental period. Experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, and the experimental protocol used in this study was approved by the Welfare and Ethical Committee for Experimental Animal Care of Wuhan University (Wuhan, China).

2.3 | Measurement of AD severity score and scratching behaviour episodes

FT3008 multifunctional skin analyzer (USA) was used to observe detailed changes of the skin surface on day 14. Clinical skin scores were monitored macroscopically on days 0, 8 and 14, which is a standard method for evaluation of AD.³¹ Total skin severity score (maximum score: 12) was defined as the sum of individual scores (0, no symptoms; 1, mild; 2, moderate; 3, severe) for four different manifestations: erythema/haemorrhage, oedema, excoriation/erosion and scaling/dryness. Assessments were performed by two investigators who were blind to the grouping of the animals.

To investigate AD-like behavioural changes, we measured and recorded the frequencies of mouse rubbing of dorsal skin with hind paws for 10 min period immediately after DNFB sensitization on days 0, 8 and 14.

2.4 | Analysis of organ indexes and haematological parameters

On the 14th day after dissection, heart, liver, lung, kidney, spleen and thymus were harvested and weighed with an electronic analytical balance to calculate their respective organ indexes (organ weight/body weight, $100 \times \text{g/g}$). Blood sample was collected from each subject in an EDTA-containing tube. We performed blood routine examination for determination of haematological parameters white blood cells (WBC), lymphocytes, monocytes, eosinophils, basophils and neutrophils with a XN-350 (Sysmex, Japan) fully automatic haematology analyzer.

2.5 | Histopathologic evaluation

The dorsal skin of each mouse was obtained on day 14 and fixed in fixed fully in 4% paraformaldehyde to make paraffin-embedded skin

sections. Skin tissues were sliced into 5- μ m-thick sections and then stained with haematoxylin and eosin (H&E) to visualize epidermal thickness and assess histological changes or toluidine blue to detect mast cells. Mouse epidermal thickness and the total numbers of mast cells including granulated and degranulated forms in the dermis were calculated from 5 randomly selected area in 3 sections by using Image-Pro software.

2.6 | Measurements of skin hydration and pH

A Skin-pH-Meter PHSJ-4F (Leici, Shanghai) and a Skin-moisture-meter KS-109-3 (KAKUSAN, China) were used to evaluate shaved dorsal skins. Skin hydration and pH level of the dorsal skin areas were measured at days 0, 7 and 13 under standard conditions ($25 \pm 5^\circ\text{C}$, $55 \pm 5\%$ RH).

2.7 | Measurements of IgE and inflammatory cytokines

After the experimental mice were sacrificed, the whole blood was obtained from the eyeball and were centrifuged at 1500 g for 15 min at 4°C to collect serum samples. At the same time, the dorsal skin and spleen tissues of mice were harvested and homogenized with ninefold pre-cooling saline solution. Afterwards, all the homogenates were centrifuged to gather the supernatants for further biochemical detection. The total amount of IgE in the serum and spleen were measured using a commercially available mouse ELISA kit according to the manufacturer's instruction. Serum and skin homogenates levels of IL-4, IL-13, IL-6, IL-1 β and TNF- α were also measured using ELISA kits.

2.8 | Immunofluorescence microscopy

Briefly, paraffin-embedded tissue sections at 4.5 μ m thicknesses were de-paraffinized with xylene and immediately rehydrated via gradually diminishing concentrations of ethanol. After dewaxed, antigen repair was done with EDTA antigen repair buffer (pH 9.0, Solarbio) in microwave oven. Slides were then rehydrated in PBS and blocked with 5% BSA in PBS for 0.5 h at 37°C , followed by overnight incubation in a 4°C humidified chamber with anti-FLG antibody (1:200). They were then washed with PBS three times and incubated with a fluorescein isothiocyanate (FITC)-labelled second antibody for 1 h at room temperature. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, USA), and the images were captured by inverted fluorescence microscope (Olympus, Japan).

2.9 | Western blotting

Dorsal skin tissues were homogenized in RIPA lysis buffer containing 1% phenyl-methane sulfonyl fluoride (PMSF) and then centrifuged at

4°C and 24 000 g for 10 min to gather the supernatant. Following centrifugation, amounts of protein in the lysates were quantitated using a BCA kit. The supernatant fraction was subjected to immunoblot analysis as we previously described. The following antibodies were used: anti-FLG (1:1000), anti-NF-κB/p65 (1:500), anti-phospho-NF-κB/p65 (1:500), anti-Iκα (1:500), anti-phospho-IκBα (1:500), anti-COX-2 (1:1000), anti-iNOS (1:1000) and anti-β-actin (1:50 000). Data analysis was performed by a computerized densitometry software ImageJ.

2.9.1 | Statistical analysis

Results were expressed as mean ± standard deviation (SD), and mean differences were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test using GraphPad Prism (version 5.0). A *p*-value of <0.05 or 0.01 was considered to be statistically significant.

3 | RESULTS

3.1 | Effect of CLA on DNFB-induced AD-Like clinical manifestations

We first examined whether different doses of CLA had therapeutic relevance on AD-like symptoms and responses in mice induced by repetitive application of DNFB to shaved dorsal skin. The clinical skin severity of dermatitis was scored by visually observation at days 0, 8 and 14. As indicated in Figure 1A, throughout the first week, the extent of erythema, oedema, scaling, eschar and epidermolysis varied across the Normal, Model and CLA group mice. The extent of symptoms (expressed as the total clinical score) was 7.2 ± 0.8 for AD group mice, while scores of 2%, 5% CLA and 0.05% DEX group mice were 6.2 ± 1.3 , 5.2 ± 0.8 and 6.4 ± 0.9 , respectively. In the second week, mice in the Model group exhibited severe erythema, oedema, scaling, eschar, and epidermolysis with dermatitis score of 6.2 ± 1.3 . In contrast, mice in the CLA-treated groups exhibited reduced haemorrhaging and scarring, compared with mice in the Model group. 5% CLA statistically decreased DNFB-induced increases in dermatitis score to 4.4 ± 1.0 . Notably particularly, the lesion-severity score of DEX group was 4.6 ± 0.9 and the mice in the DEX group showed significantly (*p* < 0.05) reduced haemorrhaging and epidermolysis, with most of the mice exhibiting only scaling. Detailed changes of the skin surface were observed on day 14 using a portable electronic magnifier and

topical application of CLA clearly further demonstrated its protective effects for the DNFB-induced AD-like skin lesion in mice.

3.2 | Effect of CLA on scratching behaviour

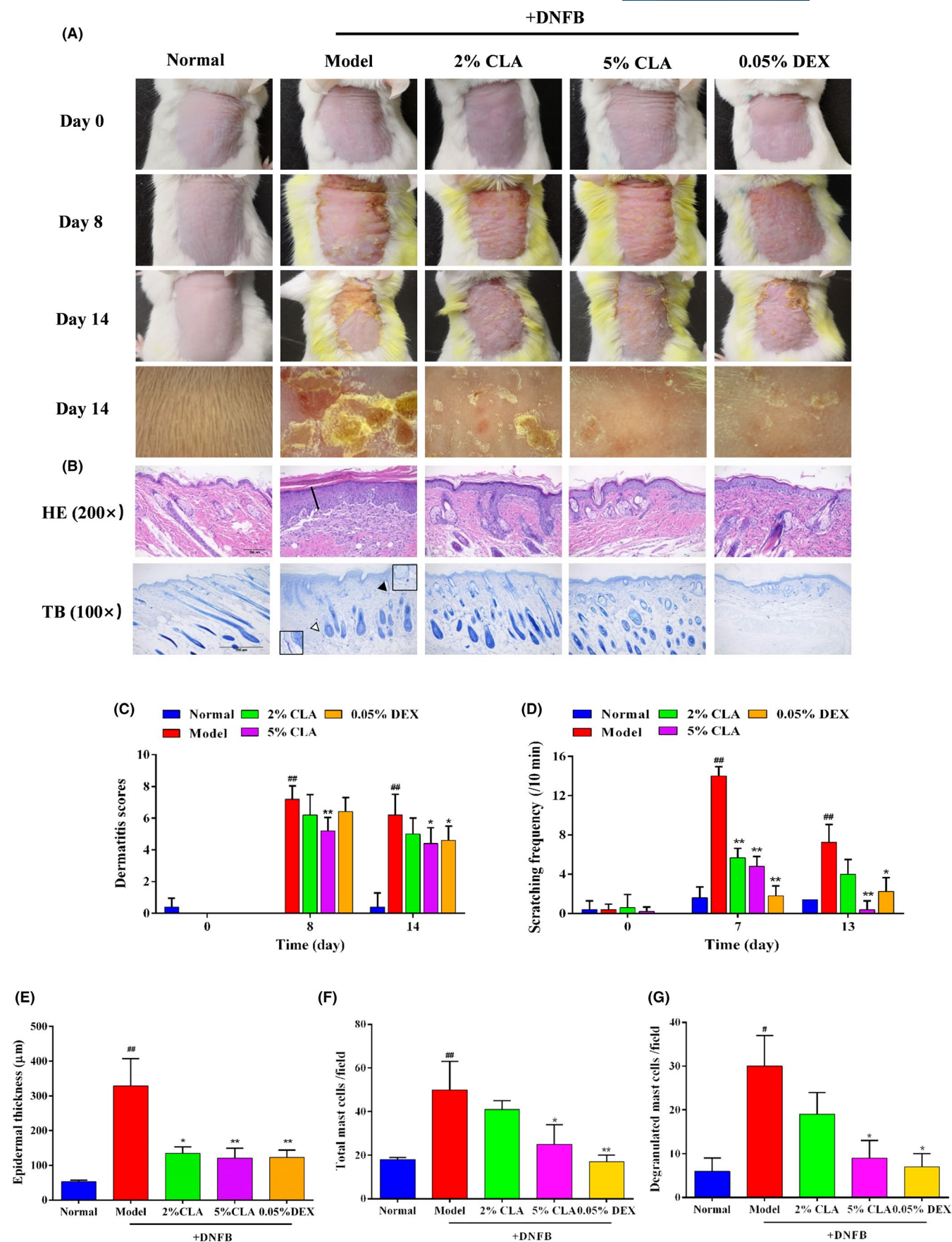
Intensive pruritus is a representative clinical manifestation of AD, and a vicious itch-scratch cycle is easily established.^{32,33} During the period, scratching behaviour was measured and recorded for 10 min after treatment with DNFB. Our experiment revealed that repeated applications of DNFB onto the dorsal skin promoted an increase in the frequencies of scratching episodes in mice corresponded to the severity of skin lesions. On day 13 after the final DNFB application, topically applied 5% CLA significantly inhibited the scratching behaviour episodes when compared with the Model group (Figure 1D).

3.3 | Effect of CLA on histopathological changes

Epidermal hyperplasia and the infiltration of inflammatory cells, including mast cells and eosinophils, are histopathological changes that occur in skin lesions of AD patients and DNFB-treated mice.^{31,34} H&E staining was performed to investigate the effect of CLA on the histopathological features of the dorsal skin in the DNFB-induced AD lesions. Histopathological analysis revealed that CLA treatment significantly markedly ameliorated epidermal thickness and infiltration of inflammatory cells in the dermis in comparison with that with vehicle treatment (Figure 1B). Quantitative analysis indicated that 2% and 5% CLA demonstrated 59.1% and 63.3% reductions in epidermal thickness in a dose-dependent manner (Figure 1E).

Mast cells are the major initiations of allergic inflammatory reactions, which are stimulated by an antigen bounding to the FcεRI receptor expressed on the surface of mast cell.³⁵ For investigation of whether the number of mast cells in the skin changed after treatment with 2% or 5% CLA, numbers of mast cells (both granulated and degranulated forms) in the skin dermis layers stained with toluidine blue were examined by optical microscopy (×100) (Figure 1B). Figure 1F exhibited that total mast cell counts were significantly increased after sensitization by DNFB when compared to normal mice with *p* value < 0.01 (50.0 ± 13.0 vs. 18.0 ± 1.0). However, total mast cell counts tended to increase less in the CLA-treated groups, especially the 5% CLA group, than in the propylene glycol-treated (Model) group. The degranulated forms of mast cells were also counted separately, and degranulated mast cells increased significantly after

FIGURE 1 Topical CLA alleviates AD-like lesions and histopathological changes. (A) Representative macroscopic images of each treatment group showing comparison of AD-like skin lesions at days 0, 8 and 14. (B) Haematoxylin and eosin (H&E) and toluidine blue (TB) staining. H&E staining (magnification 200×; scale bar = 100 μm), straight line indicates thickness of the epidermis. Granulated (◀) and degranulated (◁) mast cells in the skin stained with TB (magnification 100×; scale bar = 200 μm). (C) Total lesion-severity score was defined as the sum of the individual scores for each of the following four signs: erythema/haemorrhage, oedema, excoriation/erosion and dryness/scaling, the degree of each sign was scored as 0 (no symptom), 1 (mild), 2 (moderate) and 3 (severe). (D) The number of scratching behaviour within 10 min was counted at days 0, 7 and 13, respectively. (E) Epidermal thickness was quantified as means in randomly selected 5 fields per section. Infiltration of (F) total mast cells and (G) degranulated mast cells were quantified as means in randomly selected 5 fields per section. All the data are expressed as mean ± SD (*n* = 5). #*p* < 0.05, and ##*p* < 0.01 vs. Normal group; **p* < 0.05, and ***p* < 0.01 vs. Model group



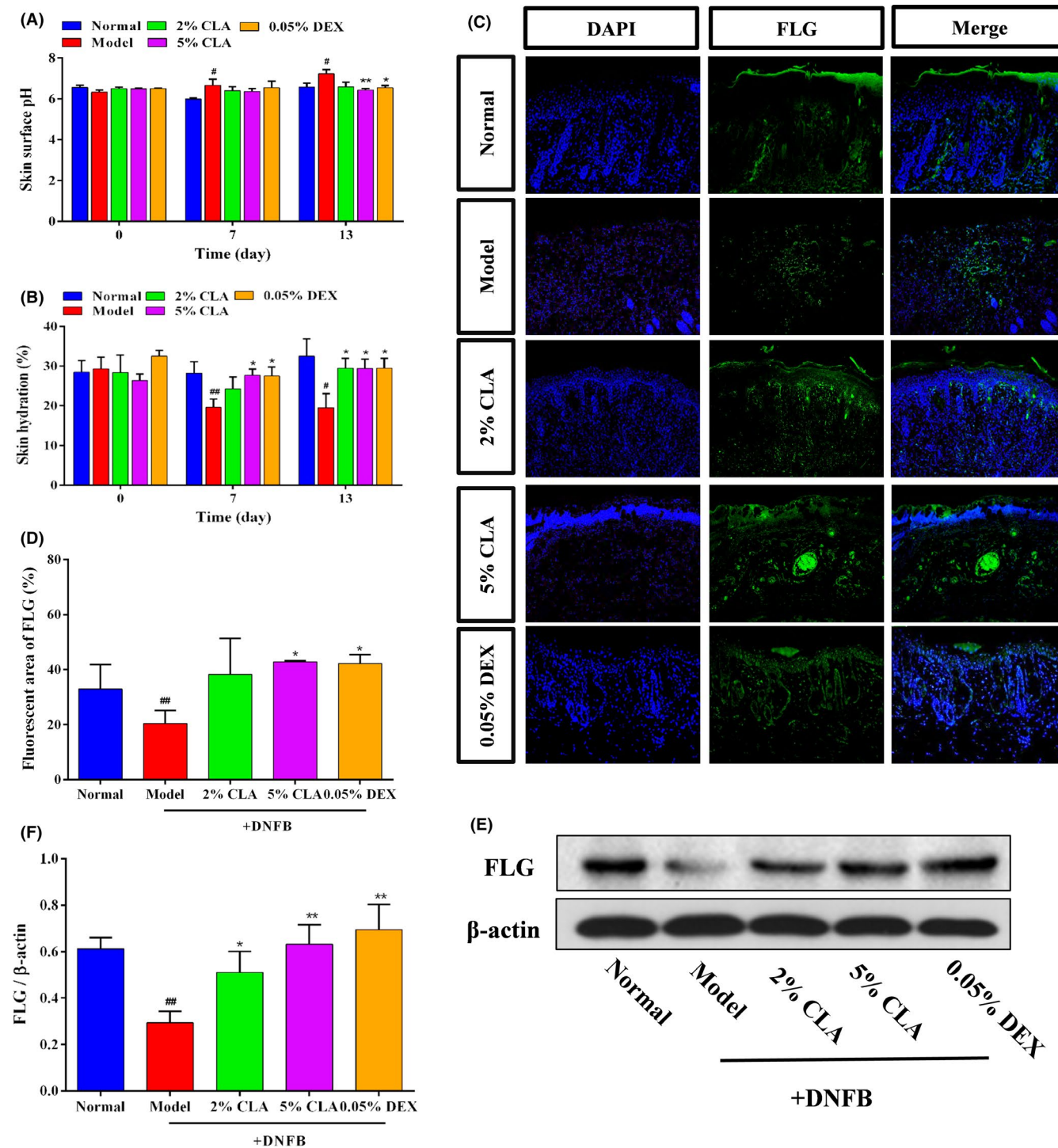


FIGURE 2 Topical CLA recovers the impaired skin barrier. (A) Measurement of pH at days 0, 7 and 13. (B) Measurement of Skin hydration (%) at days 0, 7 and 13. (C) Immunofluorescence staining of FLG protein (labelled with FITC, green) in skin biopsies from AD mice. Cells were counterstained with DAPI (blue) (magnification 100×). (D) Quantitative analysis of FLG expression was performed by the ratio of fluorescent area of FLG (green) in relation to total area of DAPI (blue) from randomly selected 3 sections in each group. (E) Western blot analysis for FLG in skin biopsies. (F) Protein relative level of FLG was normalized to β-actin. All the data are expressed as mean ± SD ($n = 3-5$). # $p < 0.05$ and ## $p < 0.01$ vs. Normal group; * $p < 0.05$, and ** $p < 0.01$ vs. Model group

AD induction ($p < 0.05$, Normal [6.0 ± 3.0] vs. Model [30.0 ± 7.0]). The topical use of 2% and 5% CLA on the AD-like skin lesions for 14 days was effective in reducing numbers of degranulated mast cells even though they did not reach the numbers in the normal group (Figure 1G).

3.4 | Effect of CLA on skin barrier recovery

Patients with AD are known to have elevated skin pH and loss of skin hydration.^{36,37} Topical DNFB reduces skin barrier function as determined by skin hydration and PH testing. Our experiment revealed

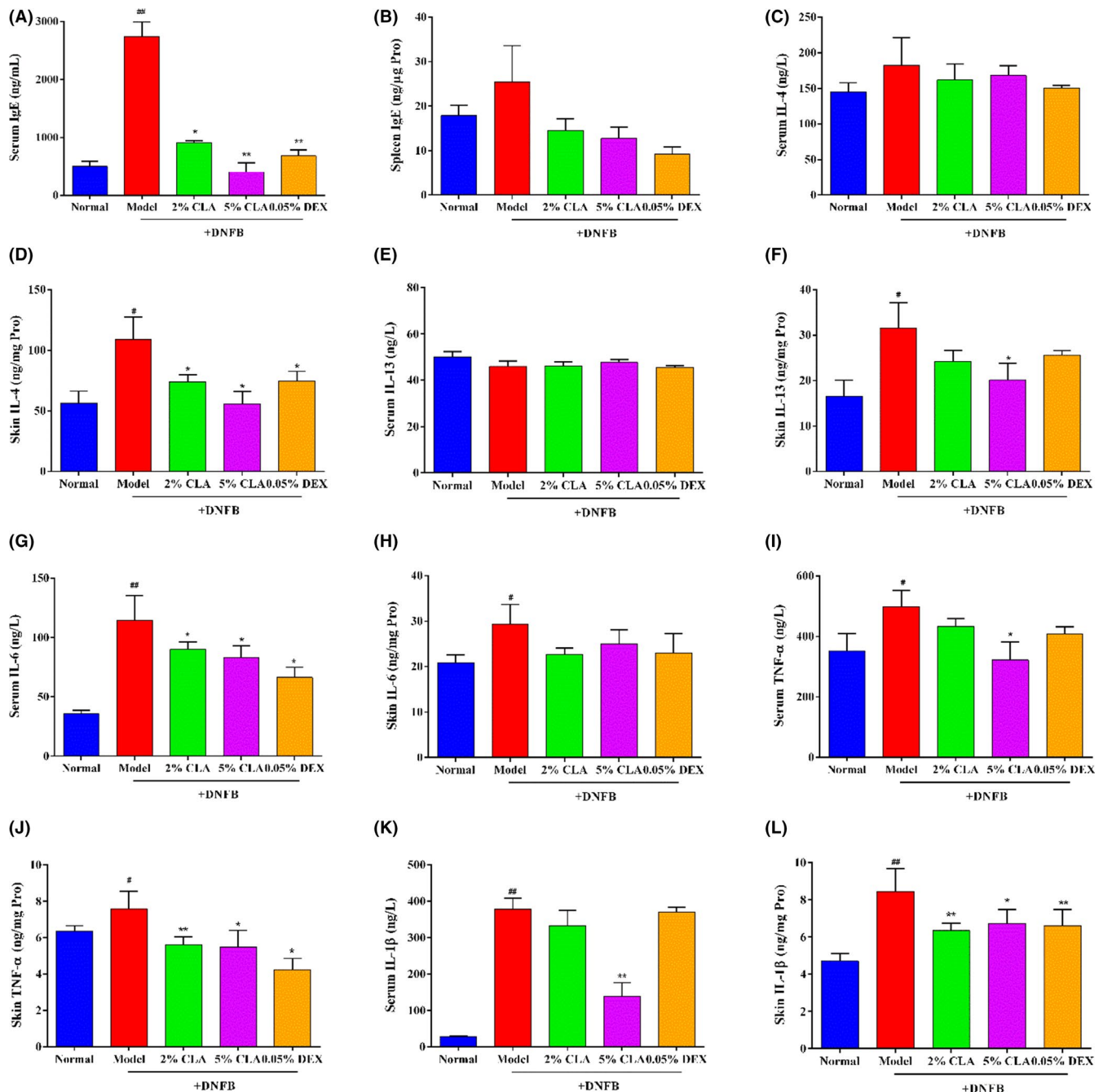


FIGURE 3 Topical CLA regulates IgE, Th2-type and pro-inflammatory cytokines levels in AD mice. Serum or spleen level (A, B) of IgE, as well as serum or skin levels of (C-L) of IL-4, IL-13, IL-6, TNF- α and IL-1 β were measured by ELISA kits were measured by ELISA kits, respectively. All the data are expressed as mean $\pm$ SD ($n = 5$). # $p < 0.05$, and ## $p < 0.01$ vs. Normal group; * $p < 0.05$, and ** $p < 0.01$ vs. Model group

that repeated application of DNFB promoted an increase in pH and a decrease in skin hydration *versus* the Normal group on days 7 and 13. After 14 days of treatment, significant differences were observed between the CLA-treated and Model groups. Co-treatment with 5% CLA reduced the decrease in pH by 11.1% and increased skin hydration by 50.8% (Figure 2A,B).

Recent studies found that the expression of FLG, a major structural protein of the stratum corneum (SC) in the skin barrier, decreased markedly in AD skin lesions.² FLG degradation products produced in the SC are components of the hygroscopic natural

moisturizing factor, which are vital for skin hydration, pH maintenance and microbial defence. Therefore, we observed the changes in FLG expression among all groups by immunofluorescence staining and Western blot. Based on the immunofluorescence analysis of skin specimens, strong positive reaction of FLG (green) was obvious in the skin of normal mice, whereas the reaction at the affected skin sites of AD mice was highly suppressed. However, it was apparently restored by topical application of 2% or 5% CLA for 2 weeks (Figure 2C,D), which was further confirmed by the result of Western blot analysis (Figure 2E,F). Overall, our results demonstrated that

TABLE 1 Effect of topical applied CLA on organ indexes (100*g per g body weight)

Group	Organ index (100*g/g body weight)					
	Heart	Liver	Kidney	Lung	Spleen	Thymus
Normal	0.59 ± 0.06	3.80 ± 0.20	1.51 ± 0.05	0.55 ± 0.05	0.28 ± 0.05	0.14 ± 0.05
Model	0.61 ± 0.08	4.45 ± 0.26 ^{##}	1.55 ± 0.06	0.56 ± 0.03	0.49 ± 0.55 ^{##}	0.11 ± 0.02
2% CLA	0.52 ± 0.05	4.28 ± 0.23	1.53 ± 0.06	0.59 ± 0.04	0.42 ± 0.10	0.11 ± 0.02
5% CLA	0.59 ± 0.08	4.59 ± 0.18	1.59 ± 0.08	0.57 ± 0.02	0.39 ± 0.03 [*]	0.10 ± 0.01
0.05% DEX	0.64 ± 0.08	4.75 ± 0.36	1.63 ± 0.08	0.56 ± 0.05	0.10 ± 0.03 ^{**}	0.10 ± 0.03

Note: All the data are expressed as mean ± SD (n = 5). [#]p < 0.05 and ^{##}p < 0.01 vs. Normal group; ^{*}p < 0.05 and ^{**}p < 0.01 vs. Model group.

TABLE 2 Absolute numbers of WBC, lymphocyte, monocyte, neutrophil, eosinophil and basophil count in blood in all groups

Group	WBC	Neutrophil	lymphocyte	monocyte	eosinophil	basophil
	10 ⁹ /L	10 ⁹ /L	10 ⁹ /L	10 ⁹ /L	10 ⁹ /L	10 ⁹ /L
Normal	3.92 ± 0.94	0.21 ± 0.06	2.86 ± 1.07	0.04 ± 0.01	0.00 ± 0.00	0.81 ± 0.20
Model	6.54 ± 2.26 ^{##}	0.34 ± 0.03 [#]	4.06 ± 0.87	0.06 ± 0.02	0.88 ± 0.01 ^{##}	1.20 ± 0.18
2% CLA	3.96 ± 1.36 [*]	0.21 ± 0.08 [*]	1.79 ± 0.65	0.04 ± 0.02	0.67 ± 0.05	1.25 ± 0.64
5% CLA	4.43 ± 1.01 [*]	0.20 ± 0.05 [*]	2.25 ± 0.84	0.06 ± 0.04	0.61 ± 0.05 [*]	1.31 ± 0.47
0.05% DEX	3.98 ± 0.21 ^{**}	1.56 ± 0.32 [*]	1.09 ± 0.29 [*]	0.01 ± 0.01	0.12 ± 0.00 [*]	1.20 ± 0.14

Note: All the data are expressed as mean ± SD (n = 5). [#]p < 0.05 and ^{##}p < 0.01 vs. Normal group; ^{*}p < 0.05 and ^{**}p < 0.01 vs. Model group.

topical application of CLA improved skin barrier function through elevating the production of epidermal proteins, such as FLG.

3.5 | Effect of CLA on IgE levels in serum and spleen

Highly elevated IgE production has been considered as a hallmark of AD.^{1,38} Serum IgE level significantly increased about 4.03-fold in the AD group compared with normal group mice (Figure 3A), whereas this increase was inhibited by CLA in both the 2% and 5% CLA-treated groups. No significant changes were observed in spleen IgE levels (*p* > 0.05) (Figure 3B).

3.6 | Effects of CLA on organ indexes and haematological parameters

Compared to Normal or Model group, there was not any significant change in the body weight of mice treated with either 2%, 5% CLA or 0.05% DEX (Figure S2). Organ weight measurement results manifested that there were no remarkable differences in the organ coefficients among groups with the exception of the liver and the immune organ, spleen. Thus, we preliminarily assumed that there was no evident adverse effect of the topical supplementation of CLA in the mice that survived till the completion of the study. However, as the largest immune organ in the body, the spleen plays an important role in regulating the immune system and contains a range of immune cells. Also, splenic enlargement is believed to indicate abnormality of immune system function.³⁹ As shown in Table 1 and Figure S3,

DNFB-sensitized mice had enlarged spleens that were reduced to almost normal size in the CLA and DEX-treated groups.

Eosinophil, monocytes, neutrophils and lymphocytes are known to be activated in blood of AD patients. Eosinophils take part in mediating the classical hypersensitivity reaction and are directly linked with the disease severity.⁴⁰ Our data revealed significantly increased concentration of total and differential leukocyte counts in blood of sensitized mice. Compared to the Normal group, blood levels of WBCs, neutrophils and eosinophils count were elevated by 1.7-fold, 1.6-fold and 88-fold, respectively, in the Model group. On observing the WBCs, neutrophils, and eosinophils of the blood in all treated groups, remarkable reductions were seen as compared with the Model group (Table 2).

3.7 | Effect of CLA on the production of Th2 cytokine and pro-inflammatory cytokines

As AD lesions are characterized by epidermal hyperplasia associated with a dominant Th2 immune response and dermal inflammatory infiltrates, the level of Th2-related cytokines such as IL-4 and IL-13 in serum and skin was determined in our experiment. Here, our findings demonstrated that skin concentrations of IL-4 and IL-13 were significantly higher than those measured in skin obtained from normal mice (*p* < 0.05) 24 h after the final DNFB challenge (Figure 3D,F). Moreover, there was a trend towards increased serum IL-4 levels, which did not reach statistical significance (Figure 3C). Topical application of 5% CLA to the dorsal skin effectively reduced these responses, restoring normal skin levels of IL-4 and IL-13 (Figure 3D,F).

To investigate the molecular mechanisms by which CLA suppressed DNFB-induced skin inflammation, we next determined the

effects of CLA on the expression levels of pro-inflammatory cytokines in serum and mouse skin by ELISA. As illustrated in Figure 3G-L, DNFB treatment caused 13.2-, 3.2- and 1.4-fold increases of IL-1 β , IL-6 and TNF- α in serum in comparison with the Normal group. However, topical application of 2% and 5% CLA to skin effectively reduced the increased levels of IL-1 β , IL-6 and TNF- α . Similar changing trends were also found in the skin.

3.8 | Effects of CLA on DNFB-induced expressions of iNOS and COX-2 in mouse skin

iNOS is the major producer of NO when it is activated by various cytokines or bacterial lipopolysaccharides, and AD-like symptoms such as scratching can be exacerbated by iNOS-derived NO.⁴¹ COX-2 expression is increased in immune cells (macrophages, neutrophils and dendritic cells) in response to stimulation with cytokines, bacterial endotoxins and growth factors during the immune reaction.⁴² As illustrated in Figure 4A-C, DNFB treatment resulted in a dramatic accumulation of COX-2 (1.2-fold) and iNOS (1.8-fold) in mouse skin, compared to the Normal group. In contrast, topical treatment with CLA exhibited dose-dependent inhibition of COX-2 and iNOS expressions in the dorsal skin. 5% CLA evidenced 18.5% and 43.8% inhibition of COX-2 and iNOS expressions, respectively.

3.9 | Effect of CLA on DNFB-induced NF- κ B activation in mouse skin

Direct evidences have reported that NF- κ B plays a critical role in regulating the expression of key pro-inflammatory biomarkers and involves in the exacerbation of AD.⁴³ Further, to investigate the mechanism by which CLA inhibited the release of pro-inflammatory cytokines and enzymes, we investigated its effect on DNFB-induced NF- κ B activation in the mouse skin. As expected, DNFB treatment remarkably increased the phosphorylation of I κ B α and NF- κ B p65, and these effects were reversed by treatment with CLA in a dose-dependent manner (Figure 4D-F). However, no changes were observed in the total levels of I κ B α and NF- κ B p65.

4 | DISCUSSION

Exploration and development of novel, safer and more effective anti-AD agents are still highly demanded due to limited therapeutic efficacy and unwanted adverse effects of the current therapies. The present study was undertaken to elucidate the pharmacological and biological effects of topical CLA on the cutaneous inflammation in a mouse model of chronic inflammatory dermatitis induced by repeated percutaneously challenge of DNFB to the skin of back. Macroscopic observation results showed that repeated application of DNFB in the Model group induced erythema and scarring on the dorsal skin of mice. However, erythema, oedema, erosion and other symptoms in the

skin of CLA-treated mice were markedly mitigated. Histopathological examinations of the CLA-treated skin area also revealed a significant reduction in the epidermal thickness and a decrease of inflammatory infiltrate, including inflammatory cells and mast cells in the dermis. In addition, the levels of total and differential leukocyte counts in blood were reduced, which is an indicator of improved inflammatory symptoms. Its curative effects for AD mice are mainly ascribed to two aspects, one is that CLA increased skin hydration and epidermal FLG expression, and reduced skin surface pH, thus accelerating skin barrier recovery. Secondly, CLA significantly suppressed the levels of inflammatory mediators in mouse serum or skin, such as TNF- α , IL-6, IL-1 β , iNOS and COX-2 through the down-regulation of NF- κ B activation.

Epidermal barrier dysfunction in AD involves dry and itchy skin, which enables the entry of antigens *via* the skin to result in the production of inflammatory cytokine and tends to be exacerbated by a mechanical injury inflicted by scratching.⁴⁴ Skin barrier repair and reduction of itch/scratch cycle are mutually beneficial in the prevention and treatment of AD. During the progression of AD, the skin barrier function collapses owing to inflammation and itching, resulting in decreased hydration of the SC and an increase in pH. FLG is a key protein involved in the epidermal barrier function. Mutations in the gene encoding FLG have been shown to be major predisposing factors for AD. It has been reported that decomposition products of FLG, a natural moisturizing factor closely related to water retention/pH reductions in SC.^{45,46} In clinic, the skin of patients with AD in whom FLG levels are decreased tends to be dry and alkaline. In our study, scratching was relatively decreased in 2%, 5% CLA and 0.05% DEX-administered mice, compared to the Model group. In addition, topical application of CLA onto dorsal skin mouse could effectively ameliorate impaired skin barrier, as evidenced by the increased FLG expression and skin hydration, and acidic pH recovery. High skin hydration facilitates skin barrier function recovery of atopic dry skin. Besides, Lee et al.⁴⁷ showed alkalization of the skin activated protease-activated receptor 2 (PAR-2), resulting in corneocyte desquamation, pruritus and dermatitis. CLA, especially 5% CLA increased the expression of FLG in skin of DNFB-induced AD mice, thereby alleviating atopic symptoms. Collectively, these results indicated that topically administered CLA may be effective in protecting the skin barrier, partially ascribing to the decrease in scratching behaviours. Apart from the decreased mechanical injury, we found that treated with CLA decreased levels of IL-4 and IL-13 in inflamed skin, which further favoured the upregulation of FLG. Th2 cytokines such as IL-4 and IL-13 have been demonstrated to play a key role in the epidermal differentiation complex that regulates epidermal barrier function. In lesional and non-lesional AD skin, FLG, loricrin (LOR) and involucrin are downregulated by IL-4 and IL-13, which also contributes to the development of a defective skin barrier.⁴⁸

Abundance of IgE and eosinophil or basophils levels in peripheral blood is key features of AD and associated with AD severity. In our study, repeated topical treatment of CLA suppressed extensively serum IgE level in DNFB-treated BALB/c mice. Furthermore, CLA treatments alleviated the expansion of WBC, eosinophils and lymphocytes, and neutrophils under the higher concentration of 5% CLA

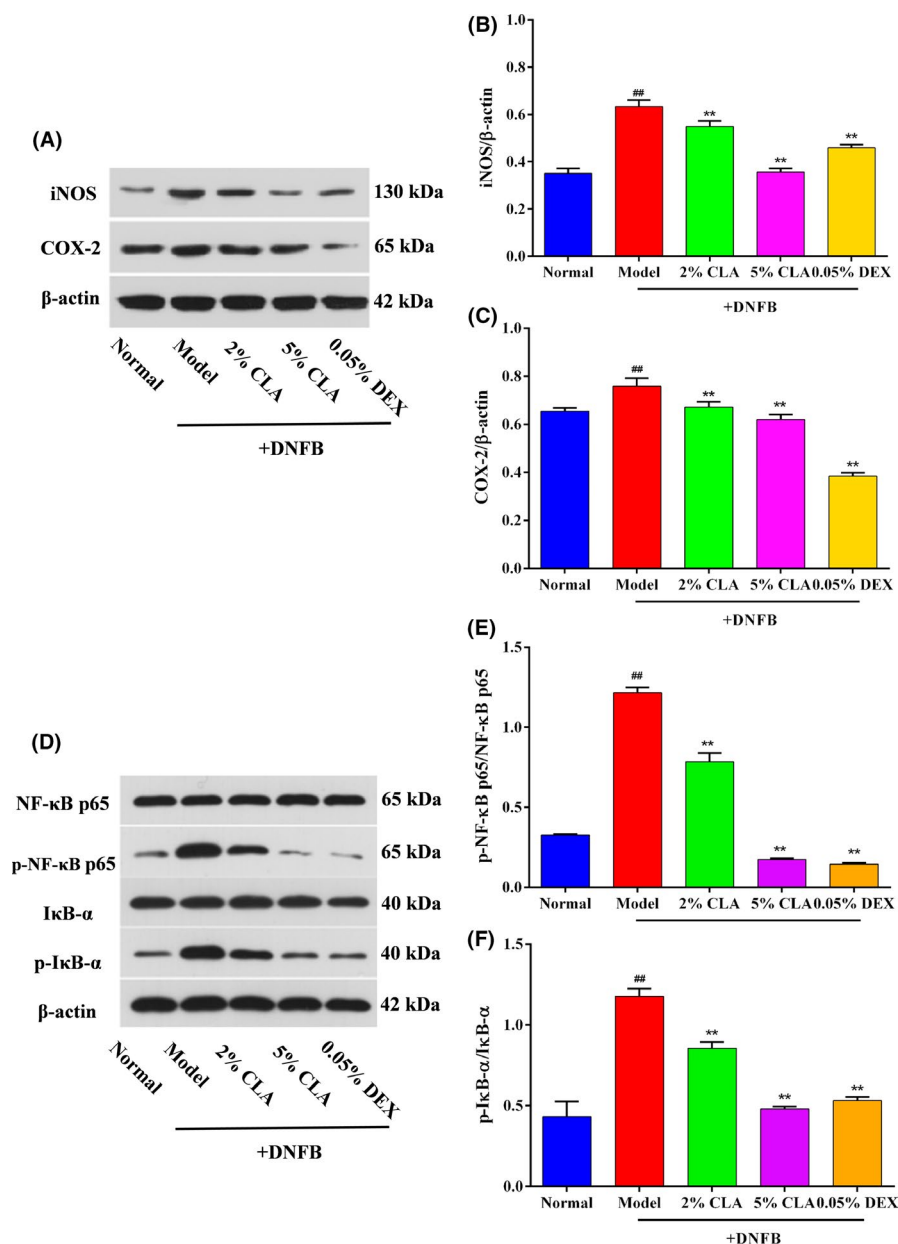


FIGURE 4 Topical CLA inhibits protein expressions of iNOS, COX-2 and DNFB-induced NF-κB activation in AD-like skin lesions. Western blot analysis for (A) iNOS and COX-2 in skin biopsies. Protein relative levels of (B) iNOS and (C) COX-2 were normalized to β-actin. Western blot analysis for (D) NF-κB p65, p-NF-κB p65, IκBα and p-IκBα proteins. Protein relative levels of (E) p-NF-κB p65 and (F) p-IκBα were normalized to NF-κB p65 and IκBα, respectively. All the data are expressed as mean ± SD ($n = 3$). # $p < 0.05$, and ## $p < 0.01$ vs. Normal group; * $p < 0.05$, and ** $p < 0.01$ vs. Model group

dosing, implicating that reduction of WBCs is an anti-inflammatory manifestation of CLA. Mast cell accumulation is another major effector in the pathogenesis of AD, closely associated with elevated levels of IgE. Indeed, mast cells are known to play a pivotal role in IgE-mediated immediate hypersensitivity reactions via the production and secretion of pro-inflammatory mediators such as histamine, chemokines and cytokines. IgE binds to the FcεRI on the mast cell surface, leading to the degranulation and expression of pro-inflammatory mediators which subsequently trigger skin lesions.⁴⁹ Topical application of CLA suppressed extensively mast cell infiltration and degranulation. Seemingly, the alleviation of skin symptoms is related to suppression of reduction of infiltration of the total degranulation mast cells in the dorsal skin.

Given the lower WBC, neutrophil and eosinophil counts in blood, we presumed that the levels of pro-inflammatory cytokines could subsequently be affected by CLA. To determine the changes of AD-relevant cytokines, we used ELISA assays to measure numerous pro-inflammatory cytokines in the lesion areas. As expected, we found that secretion DNFB-stimulated pro-inflammatory cytokines including TNF-α, IL-6, IL-1β, iNOS and COX-2 were significantly inhibited by topical treatment of CLA. NF-κB, as an important transcriptional factor is involved in the trans-activation of a number of genes, which regulates both acute-inflammatory and immune-inflammatory responses. In most cells, NF-κB exists in the cytoplasm in an inactive complex bound an inhibitory protein, an IκB-like

protein. Upon stimulation, the degradation and phosphorylation of I κ B will be activated and triggered, and NF- κ B p65 will dissociate from its inhibitory protein I κ B and also be phosphorylated, leading to the activation of NF- κ B, which will then promote the transcription of inflammatory-related genes such as IL-1 β , IL-6, iNOS and COX-2.^{50,51} In this study, after repeated stimulation with DNFB, NF- κ B was activated and the phosphorylation of I κ B α and NF- κ B p65 was increased in affected skin sites of atopic mice. And, this phenomenon was significantly reversed by topical application of CLA. Although further studies are required, the present data demonstrated that inhibition of NF- κ B activation and downstream NF- κ B dependent pro-inflammatory cytokines could be the possible mechanism for CLA against DNFB-induced local skin damage.

In the only previous experimental study of the effects of CLA on AD in mice, significant attenuation of ovalbumin-induced allergic dermatitis was achieved by topical application of milk fat enriched in c9, t11-CLA and vaccenic acid (VA) and its two major fatty acids, particularly when the two major fatty acids were administered in combination. The mechanisms responsible for the ameliorative effects displayed by enriched milk fat and c9, t11-CLA and VA individually were not clarified in the research.⁵² In our study, commercially available CLA, which contains roughly equal amounts of c9, t11 and t10, c12 isomers, was employed investigate its effect on DNFB-induced AD in BALB/c mice. Topical applied 2% or 5% CLA manifested protective effects against AD lesional skin, most likely by suppressing NF- κ B activation and increasing the expression level of skin barrier-related protein, FLG. In vitro and animal studies increasingly ascribe different biological properties to c9, t11 and t10, c12 isomers. Anti-inflammatory effects, including airway anti-inflammatory effects, are largely mediated by the c9, t11 isomer.^{22,53} Further studies are necessary to investigate and compare the therapeutic effects of t10, c12 or c9, t11 isomer alone in AD-like models.

In conclusion, topically applied 2% or 5% CLA produced a situation favourable for mitigation of AD-like clinical symptoms in mice and may represent a step towards an attractive novel strategy in the treatment of AD. To the best of our knowledge, our study firstly revealed the therapeutic effects of topical applied CLA for AD were ascribed to its ability to accelerate skin barrier repair and suppress NF- κ B activation and we believe this warrants further investigation for its potential therapeutic benefits in human clinical trials.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

HD and XC designed the experiments. LT and XL performed the experiments and data analysis. LT drafted the manuscript. HD and XC critically reviewed the manuscript.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1. Schematic diagram for the whole procedure of the in vivo experiment. BALB/c mice were evoked by repetitive painting with 100 uL of 1.0% DNFB in 3:1 (v/v) acetone/olive oil on dorsal skin once daily on day 1, then further challenge with 100uL of 0.5% DNFB to the stripped dorsal skin twice weekly. The treatment groups received topical treatment of 2%, 5% CLA or 0.05% DEX from days 1 to 14.

Figure S2. Effect of topical applied CLA on the growth of body weight. All the data are expressed as mean $\pm$ SD (n = 5). # p < 0.05, and ## p < 0.01 vs. Normal group; * p < 0.05, and ** p < 0.01 vs. Model group.

Figure S3. Representative macroscopic images of each treatment group showing morphologic features of the spleen in AD mice.

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