

TRPV1 mediates itch-associated scratching and skin barrier dysfunction in DNFB-induced atopic dermatitis mice

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Funding information

None.

Abstract

In chronic pruritic diseases such as atopic dermatitis (AD), pruritus and skin lesions are exacerbated by scratching in clinical and experimental settings. TRPV1 is known to mediate itch and neurogenic inflammation, but the role of TRPV1 in itch-associated scratching in AD is poorly understood. In this study, we examined the efficacy of cutting off nails and TRPV1 antagonist, ruthenium red (RR) in a murine model of AD induced by DNFB and further investigated the underlying mechanism. Nail clipping or RR could markedly ameliorate the general AD-like symptoms as manifested by the reduced clinical severity of dermatitis, IgE and Th2-related cytokine levels, and mast cell degranulation. Moreover, scratching behaviour, the levels of pruritogenic mediators, including HIS, TSLP, IL-31 and SP, and skin pH and TEWL were all significantly decreased in nail clipping or RR-treated mice, suggesting a reduction in itch-associated scratching and skin barrier defects. Immunofluorescence staining and Western blot results revealed that antipruritic effect of nail clipping or RR in AD may be explained, at least in part, by the suppression of TRPV1 activation. In summary, these data show that TRPV1 mediates itch-associated scratching and subsequent skin barrier defects, suggesting its potential as a therapeutic target in AD.

KEYWORDS

atopic dermatitis, itch, scratching behaviour, skin barrier dysfunction TRPV1 antagonist

1 | BACKGROUND

Atopic dermatitis (AD) is a common chronic inflammatory skin disease, characterized by a T helper (Th)-2 cell-dominant inflammatory infiltrate and a defective epidermal permeability barrier.^{1,2} Itch (or pruritus) is the major symptom of AD and causes a negative impact on quality of life. Intense itching is an unpleasant sensation, which evokes a strong desire to scratch. Moreover, itch-induced scratching aggravates skin lesions, and a vicious itch-scratch circle and consequent skin barrier destruction are easily established.^{3,4} Previous studies exhibited that reducing physical stimulus to the skin, such as nail clipping, inhibits the development of AD in patients and AD-like

phenotypes in a spontaneous mouse model.^{5,6} However, the underlying mechanism through which scratching exacerbates dermatitis remains to be investigated.

Transient receptor potential vanilloid type 1 (TRPV1), a non-selective cation channel with high permeability to calcium widely expressed in the epidermal keratinocytes, can be activated by heat, capsaicin, mechanical stimulus and endogenous inflammatory mediators. Stimulation of TRPV1 is reported to trigger neurogenic inflammatory mediator and neuropeptide releases, which ultimately contributes to the development of pruritus and diverse dermatoses.^{7,8} Given the involvement of the TRPV1 receptor in the itching associated with pruritic inflammatory skin disease, we hypothesized

that TRPV1 mediated itch-evoked scratching and subsequent skin barrier disruption in the development of AD.

2 | QUESTIONS ADDRESSED

In our study, we examined the preventive efficacy of cutting off hind toenails in a murine model of AD induced by 2,4-dinitrofluorobenzene (DNFB) and attempted to clarify whether and how TRPV1 mediated scratching behaviours in this process. In addition, we employed the TRPV1 antagonist, ruthenium red (RR; a non-selective Ca^{2+} channel blocker), as a contrast to observe its effect on AD. RR has been previously described as an antagonist of TRPV1 and was found to block the capsaicin-induced increase in intracellular calcium in single DRG neurons from neonatal rats.^{9–11} The topical route was used in this work to avoid the side effects because of the extensive distribution of TRPV1 in the body.

3 | EXPERIMENTAL DESIGN

Atopic dermatitis-like symptoms were induced in female Kunming (KM) mice with 6 week old by the repeated topical application of hapten DNFB on the shaved dorsum twice a week for 2 weeks as illustrated in Figure S1. After 3 days of acclimation, KM mice were randomly divided into four groups ($n = 5$ per group): Normal group, Model group, Model +nail clipped group and Model +1 mM RR group. A total of 100 μL RR in the vehicle of distilled water: propylene glycol = 3:7 was topically applied onto the shaved back of the mice once daily from Day 0 to Day 13. Normal group, Model group and Nail clipped group were given an equal volume of vehicle. To inhibit toenail scratching, all hind toenails of each mice in the nail clipped group were cut every 3 days with a nail clipper during the experimental period. The mortality, body weight and food/water consumption were also monitored every 2 days in all animals.

On days 0, 7 and 12, we monitored the mice through video and quantified the number of scratching bouts within 30 min after DNFB application. On the 13th day after dissection, the dorsal skin of each mouse was harvested and an 8-mm punch biopsy was performed with a puncher and then measured by a micrometre calliper (0–25 mm) or an electronic analytical balance. Moreover, a part of skin sample was sliced into 5- μm -thick sections and then stained with haematoxylin and eosin (H&E) to visualize epidermal thickness and assess histological changes or Toluidine Blue (TB) to detect the infiltration of granulated and degranulated mast cells. The other part was used for total protein isolation (by RIPA lysis buffer) to examine the expression of β -actin, TRPV1 and neurokinin-1 receptor (NK-1R). Serum and skin homogenate levels of IgE, IL-4, histamine (HIS), thymic stromal lymphopoietin (TSLP), interleukin (IL)-31 and substance P (SP) were also measured by ELISA kits (Quanzhou Ruixin Biological Technology Co., Ltd) according to the manufacturer's protocol, respectively.

4 | RESULTS

4.1 | Nail clipping or RR ameliorates the general AD-like symptoms

On experimental day 13, the clinical manifestations of skin lesions were observed and the skin severity scores were also recorded based on the sum of individual scores (0, no symptoms; 1, mild; 2, moderate; and 3, severe) for each of the following four signs and symptoms: erythema/haemorrhage, oedema, excoriation/erosion and scaling/dryness. As indicated in Figure 1A,B, dorsal skin of mice treated with repeated DNFB produced severe erythema, oedema, haemorrhaging and scarring with dermatitis score of 7.40 ± 0.55 ($p < 0.01$). In contrast, mice in the nail clipped or RR-treated groups statistically decreased dermatitis score of DNFB-induced skin lesions to 4.50 ± 0.53 and 4.00 ± 1.52 ($p < 0.01$ or $p < 0.05$). DNFB-induced skin swelling, presented by the difference in the thickness/weight of the dorsal skin with identical size, was also significantly reduced by nail clipped or RR treatment (Figure S2).

Scratching behaviour of each mouse was monitored and measured by video at different time points (Days 0, 7 and 12) during the experimental periods. In line with the development of skin lesions, repeated application of DNFB onto the shaved skin significantly increased scratching episodes. Compared with the model group, nail clipping or RR did not significantly reduced the number of scratching behaviour on Day 7. While on the 12th day, nail clipping or RR significantly markedly reduced the scratching bouts in DNFB-sensitized mice (Figure 1C). Our results strongly support the idea that itch provokes a strong desire to scratch the affected skin and scratching aggravates the AD-like skin lesions and makes the dermatitis worse, thus leading to further itching.⁵ Histological observation also revealed that clipped nails or RR topical treatment ameliorated epidermal hyperplasia and the infiltration of inflammatory cells into the dermis (Figure 1D,F). Activation of mast cells by various stimulants provokes the release of inflammatory cytokines and granular mediators correlated with the AD-like skin lesion development and intense pruritus.^{12–14} On examination using toluidine blue (TB) staining, larger number of degranulated mast cells increased along with bouts of scratching of DNFB-applied mice and these effects were reversed by treatment with nail clipping or RR (Figure 1E,G,H).

Theoretically, the core immune mechanisms of AD are the overproduction of IgE and the dominant Th2 type immune response.^{15,16} In addition, skin barrier defects in AD are driven by Th2-related cytokines, such as IL-4, IL-5 and IL-13.^{17,18} We observed a significant increase in serum levels of IgE and IL-4 in the model group as compared to normal group with p value < 0.05 (19.25 ± 1.51 vs. 11.37 ± 0.46 ng/mL and 129.95 ± 14.81 vs. 99.71 ± 13.06 pg/mL). Likewise, sensitization and repeated topical application of DNFB significantly elevated IL-4 release in lesional skin of AD mice. However, AD mice with clipped nails produced less IgE and IL-4 in the serum or skin lesions than those whose nails were not clipped (Figure 1I). Significant reduction of IgE and IL-4 levels was also observed in AD mice treatment with RR.

4.2 | Nail clipping or RR ameliorates itch-associated scratching and skin barrier defects

Itching and subsequent repeated scratching behaviours play a critical role in AD pathogenesis, promoting the transformation from the acute phase of AD to the chronic phase.^{19,20} We also attempted to determine the participation of pruritogenic mediators, including HIS, TSLP, SP and IL-31, that were reported to participate in AD itching process.^{21–23} HIS, as an activation marker of mast cell degranulation, has been identified as one of the major itch mediators in AD. However, the limited efficacy of antihistamines suggests that HIS is not the only mediator involved in AD-related pruritus.^{3,4} Overproduction of TSLP and IL-31 triggers pruritus and Th2 immune response in

AD.^{24,25} Furthermore, substance P, one of the most potent endogenous pruritogenic peptides, is also speculated to be a key mediator of itching in AD through a histamine-independent mechanism.²³ As illustrated in Figure 2A, DNFB treatment caused 1.4-, 1.1-, 1.2 and 1.1-fold increases of HIS, TSLP, SP and IL-31 in serum as compared with the normal group, and similar escalating trends have also been observed in the skin homogenates. Nail clipping or RR markedly inhibited the examined pruritogenic mediators in serum or dorsal skin, whereas the TSLP level was not significantly affected by RR.

Epidermal barrier dysfunction manifested by dry, itchy and alkaline skin tends to be exacerbated by a mechanical injury inflicted by frequent scratching.^{26,27} Besides, Lee et al²⁸ exhibited that alkalinization of the skin contributed to corneocyte

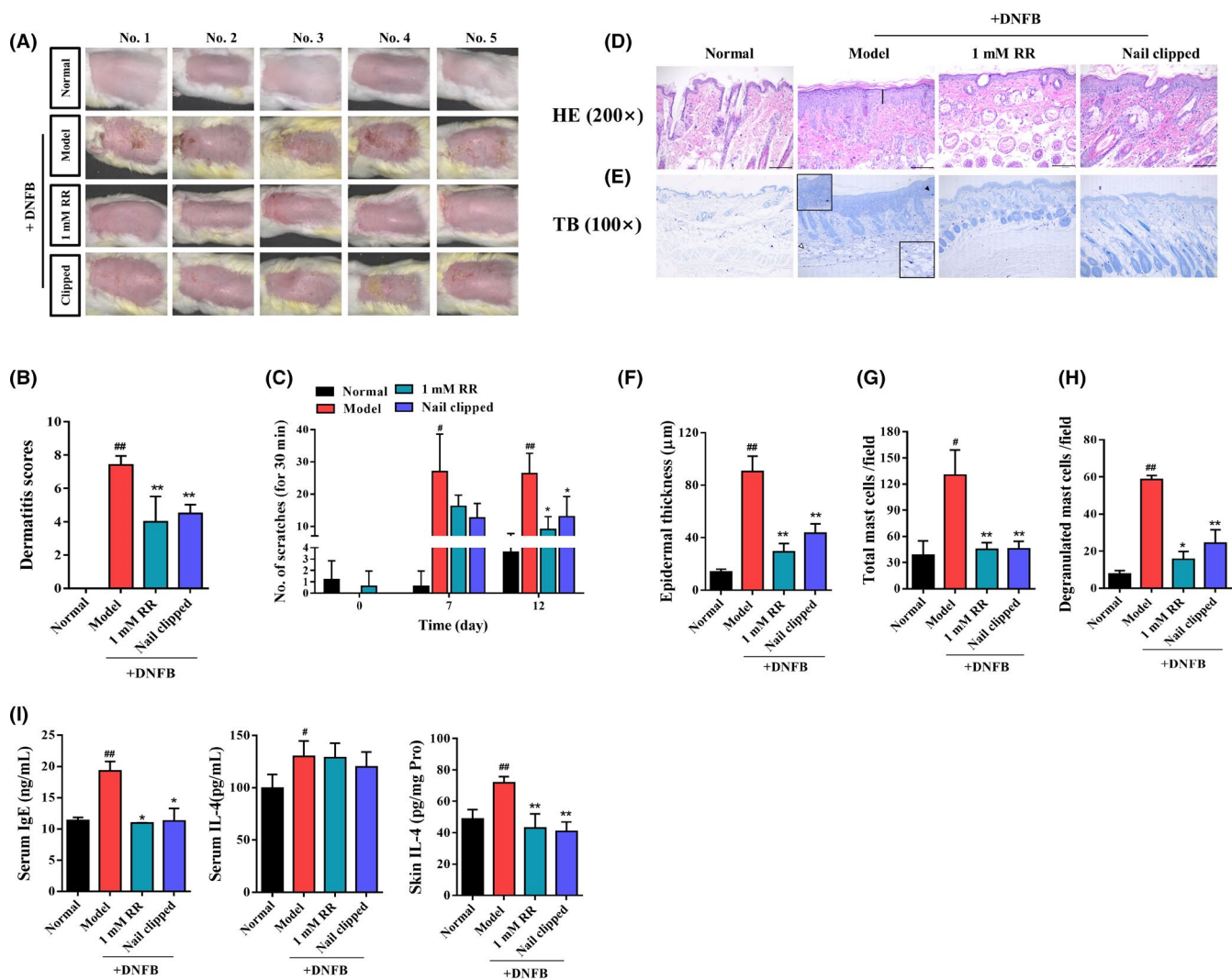


FIGURE 1 Nail clipping or RR improved DNFB-induced AD-like lesions in mice. (A) Representative photograph of dorsal skin on day 13. (B) Clinical skin severity scores for each group on day 13. (C) Quantification of scratching bouts during 30 min on Days 0, 7 and 12. (D) Representative H&E-stained dorsal skin sections (scale bar =100 μm), straight line indicates the epidermal thickness. (E) Representative Toluidine blue (TB)-stained dorsal skin sections (scale bar =200 μm). (F) Epidermal thickness was quantified in randomly selected 5 fields in each dorsal skin section. Infiltration of (G) total mast cells and (H) degranulated mast cells was quantified in randomly selected 5 fields in each dorsal skin section. (I) Quantification of IgE and IL-4 levels in AD mice by ELISA kits. Values are expressed as mean ±SD (n = 3–5). # Indicates $p < 0.05$ as compared to Normal group, ## Indicates $p < 0.01$ as compared to Normal group; *Indicates $p < 0.05$ as compared to Model group, **Indicates $p < 0.01$ as compared to Model group

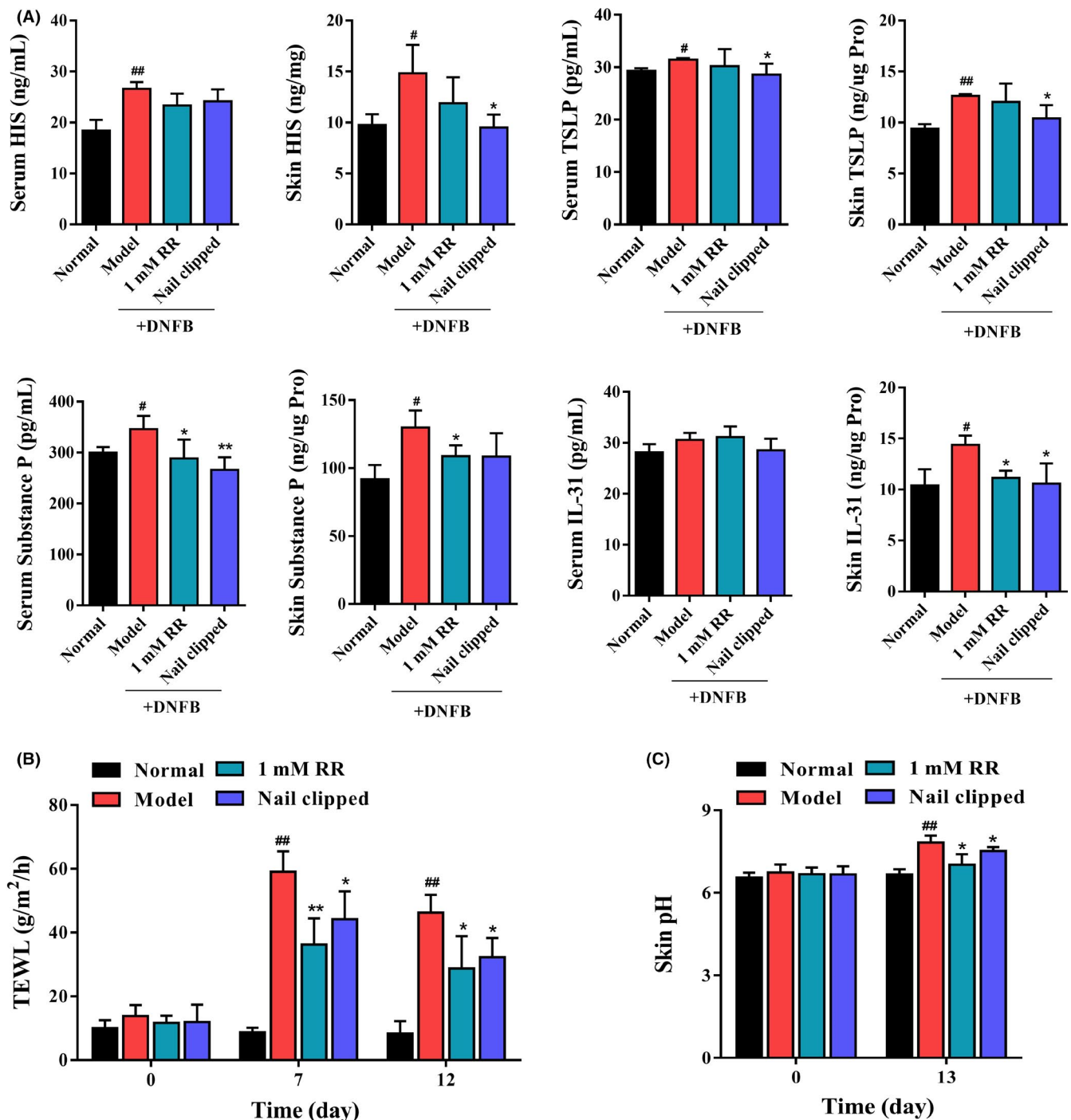
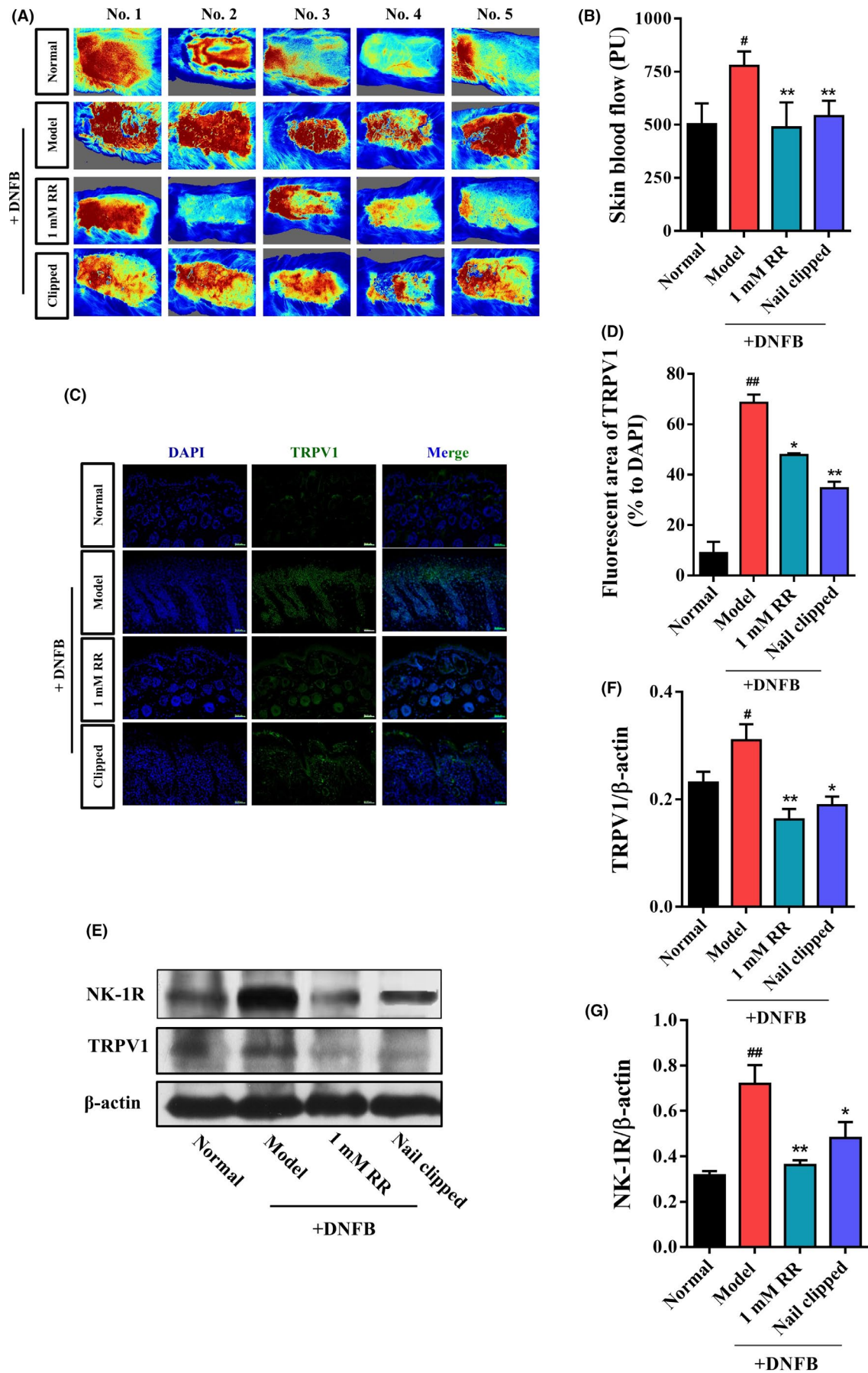


FIGURE 2 Nail clipping or RR reduces the release of pruritogenic mediators and repairs the defective skin barrier. (A) Pruritic mediator levels of HIS, TSLP, SP and IL-31 from serum or lysates of mouse dorsal skin tissues. (B) Measurement of TEWL and (C) pH at different time points. Values are expressed as mean $\pm$ SD ($n = 3\sim 5$). [#] Indicates $p < 0.05$ as compared to Normal group, ^{##} Indicates $p < 0.01$ as compared to Normal group; ^{*}Indicates $p < 0.05$ as compared to Model group, ^{**}Indicates $p < 0.01$ as compared to Model group

FIGURE 3 Nail clipping or RR inhibits TRPV1 activation in the lesional skin. (A) Images of skin blood perfusion among different groups on day 13, with red areas corresponding to high perfusion and high flux areas and blue areas indicating low perfusion and low flux. (B) The comparison and analysis of skin microvascular blood flow in each group. (C) Immunofluorescence staining of TRPV1 protein (labelled with FITC, green) in skin biopsies from AD mice. (D) Quantitative analysis of TRPV1 expression was performed by the ratio of fluorescent area of TRPV1 (green) in relation to total area of DAPI (blue) from randomly selected 3 sections in each group. (E) Western blot analysis of TRPV1 and NK-1R protein in skin biopsies. Quantification of Western blot was done with ImageJ and protein relative levels of (F) TRPV1 and (G) NK-1R were normalized to β -actin, respectively. Values are expressed as mean $\pm$ SD ($n = 3\sim 5$). [#] Indicates $p < 0.05$ as compared to Normal group, ^{##} Indicates $p < 0.01$ as compared to Normal group; ^{*}Indicates $p < 0.05$ as compared to Model group, ^{**}Indicates $p < 0.01$ as compared to Model group



desquamation, dermatitis and pruritus by activating protease-activated receptor 2 (PAR-2). Higher skin hydration and lower TEWL indicate better skin barrier function. Skin barrier recovery and the interruption of itch/scratch circle are mutually beneficial in the treatment and management of AD.²⁹ In our study, nail clipping or topically applied RR effectively ameliorated impaired skin barrier, as reflected by decreased TEWL and pH values (Figure 2B,C). These findings suggest that the scratching-induced barrier damage worsened the skin lesion in AD mice, as seen in AD patients.

4.3 | Nail clipping or RR inhibits TRPV1 activation in DNFB-inflamed mouse skin

So far, our above-mentioned findings indeed confirm that nail clipping or topical TRPV1 antagonist, RR were effective in acceleration of skin barrier recovery and suppress scratching behaviours. TRPV1 is upregulated in the AD-skin lesion, and the activation of TRPV1 causes the release of pro-inflammatory and pruritic mediators, such as HIS, SP and IL-31 in lesional skin.^{30,31} To further explore the underlying mechanisms of TRPV1 involvement in the pruritus and skin barrier recovery, studies on TRPV1 activation and expression were performed. Generally, erythema, one of the main clinical signs of AD, is a good surrogate and noninvasive marker for evaluating the effects of TRPV1 activation in the skin.^{32,33} The laser Doppler blood flowmetry technique has been recently adapted to assess the dorsal skin erythema.^{32,34,35} We measured the skin blood flow using MoorFLPI-2 Laser Doppler Imager in DNFB-induced AD mice in our experiment. To our knowledge, the measurement of dorsal skin blood flow quantitatively in this AD model has been previously unreported. As shown in Figure 3A,B, the blood flow was increased 1.55-fold by topical application of DNFB for 12 days, whereas this change was significantly reversed in RR-treated group or nail clipped group. Hutter et al³⁶ reported that the activation of TRPV1 leads to the release of substance P and subsequently up-regulates neurokinin-1 receptor. In our study, TRPV1 and NK-1R (SP receptor) expressions also increased substantially in the AD-like lesion as assayed by immunofluorescence and Western blot analysis, indicating that TRPV1 was activated. In contrast, nail cutting or RR treatment significantly reduced TRPV1 and NK-1R protein expressions (Figure 3C–G). Collectively, we provide evidence that the anti-pruritic effect of nail cutting or RR in AD may be explained, at least in part, by the suppression of TRPV1 and the consequent reduction of HIS, SP and IL-31.

5 | CONCLUSIONS AND PERSPECTIVES

Studies have shown that TRPV1 is implicated in some inflammatory skin diseases such as psoriasis, AD.^{31,37} More recently, Kemény et al³² reported that TRPV1 KO mice showed fewer changes in skin thickness and blood perfusion in comparison with WT mice following

IMQ treatment. Preclinical and clinical studies also have demonstrated that TRPV1 antagonist, PAC-14028, could suppress scratching behaviours associated with AD-like symptoms through the inhibition of TRPV1 activation.^{33,35,38,39} Here, we firstly discovered that TRPV1 inhibition by RR can suppress itch-associated scratching and the release of diverse pruritogens, and modulates skin barrier recovery. RR remarkably alleviated pruritus in murine models of AD, partially mediated by inhibiting TRPV1/SP axis and the similar mechanism was also found in reducing the physical stimulus to the skin. Our results not only further address the question of whether and how TRPV1 signalling is involved in itch-associated scratching and skin barrier dysfunction, but also provide interesting insight into how scratching exacerbates dermatitis in DNFB-induced AD mice (Figure S3).

There are several limitations to the present study. First, our current work was a preventive treatment done to cut off toenails or topically applied RR before dermatitis induction, and we were not able to fully demonstrate their potential as a therapeutic agent. Therefore, we are currently planning to examine the therapeutic effect of nail clipping or RR on scratching behaviour and skin barrier in the post-onset of AD. Second, RR has been utilized extensively as an antagonist of TRPV1 by blocking the capsaicin-induced calcium influx,^{9,11} but it suffers from poor selectivity because it was subsequently described to interact with other TRP-channels including TRPV3, TRPV4 that are also involved in itch and skin barrier.^{10,40,41} In the future, we will conduct in-depth research using TRPV1 gene knockout (KO) mice to further explore how TRPV1 mediates itch-associated scratching and skin barrier dysfunction in AD. At last, our experiments were performed in animal models, and our findings can therefore not be simply extrapolated to clinical situations. However, considering the frequent failure of conventional anti-AD therapies, we believe that breaking the vicious itch/scratch circle by cutting off nails or TRPV1 antagonists are the favourable intervention to stopping the progression of AD and TRPV1 antagonists could be used for antipruritic therapy that might satisfy the unmet medical need.

ACKNOWLEDGMENTS

None.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

HD and LT designed the experiments. LT, JG, LC and HW performed the research and contributed to analysis and interpretation of data. LT drafted the manuscript. HD and XC critically reviewed the manuscript. All authors have read and approved the final version of 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 in the online version of the article at the publisher's website.

Fig S1 Schematic representation of the experiment. Mice were sensitized on the stripped dorsal skin with hapten (100 µL of 1.0% DNFB in a 3:1 acetone-olive oil solution) on day 1, then further challenged

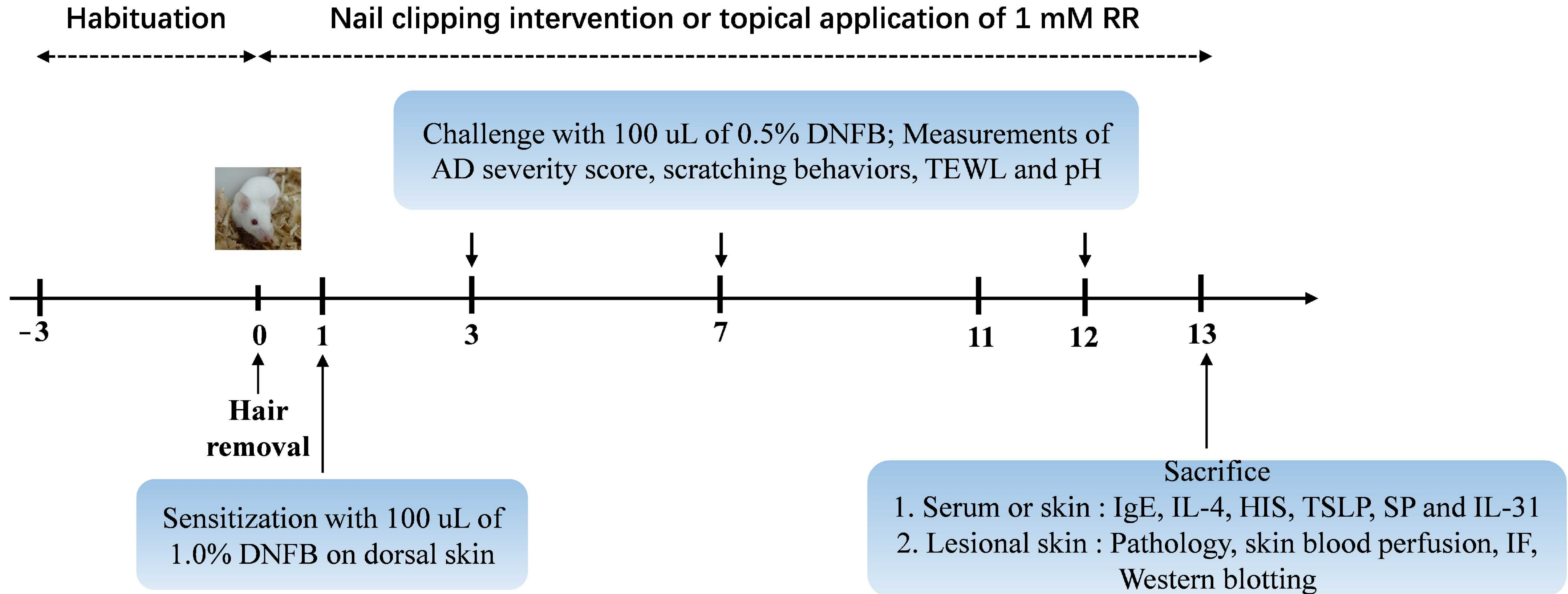
with 100 μ L of 0.5% DNFB twice weekly. 100 μ L of RR or vehicle was topically applied to the dorsal skin of each mouse once daily from Day 0 to Day 13

Fig S2 Nail clipping or RR alleviates DNFB-induced skin swelling in AD mice. Skin swelling was evaluated by measuring the difference in their thickness (μ m) and their weight (mg) from the skin slices with equivalent area. On the day of sacrifice, the dorsal skin in each mouse was harvested and punched (holes with the diameter of 8 mm) with a puncher and then measured by a micrometre calliper (0–25 mm) or an electronic analytical balance. (A) On day 13, the skin swelling by thickness in each group (μ m). (B) On day 13, the skin swelling by weight in each group (mg). Values are expressed as mean $\pm$ SD ($n = 5$). # Indicates $p < 0.05$ as compared to Normal

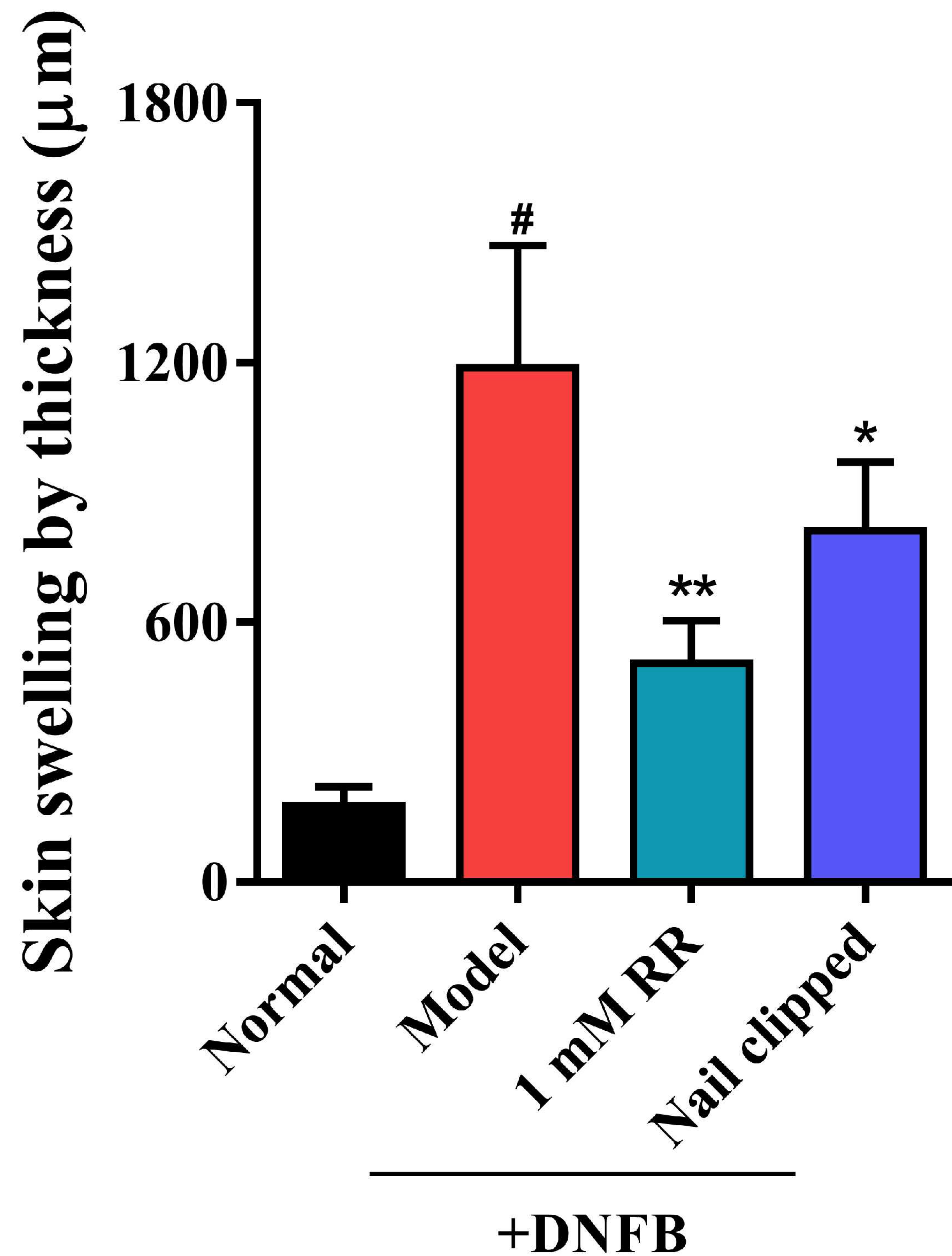
group, ## Indicates $p < 0.01$ as compared to Normal group; *Indicates $p < 0.05$ as compared to Model group, **Indicates $p < 0.01$ as compared to Model group

Fig S3 TRPV1 mediates itch-associated scratching and skin barrier dysfunction in AD mice

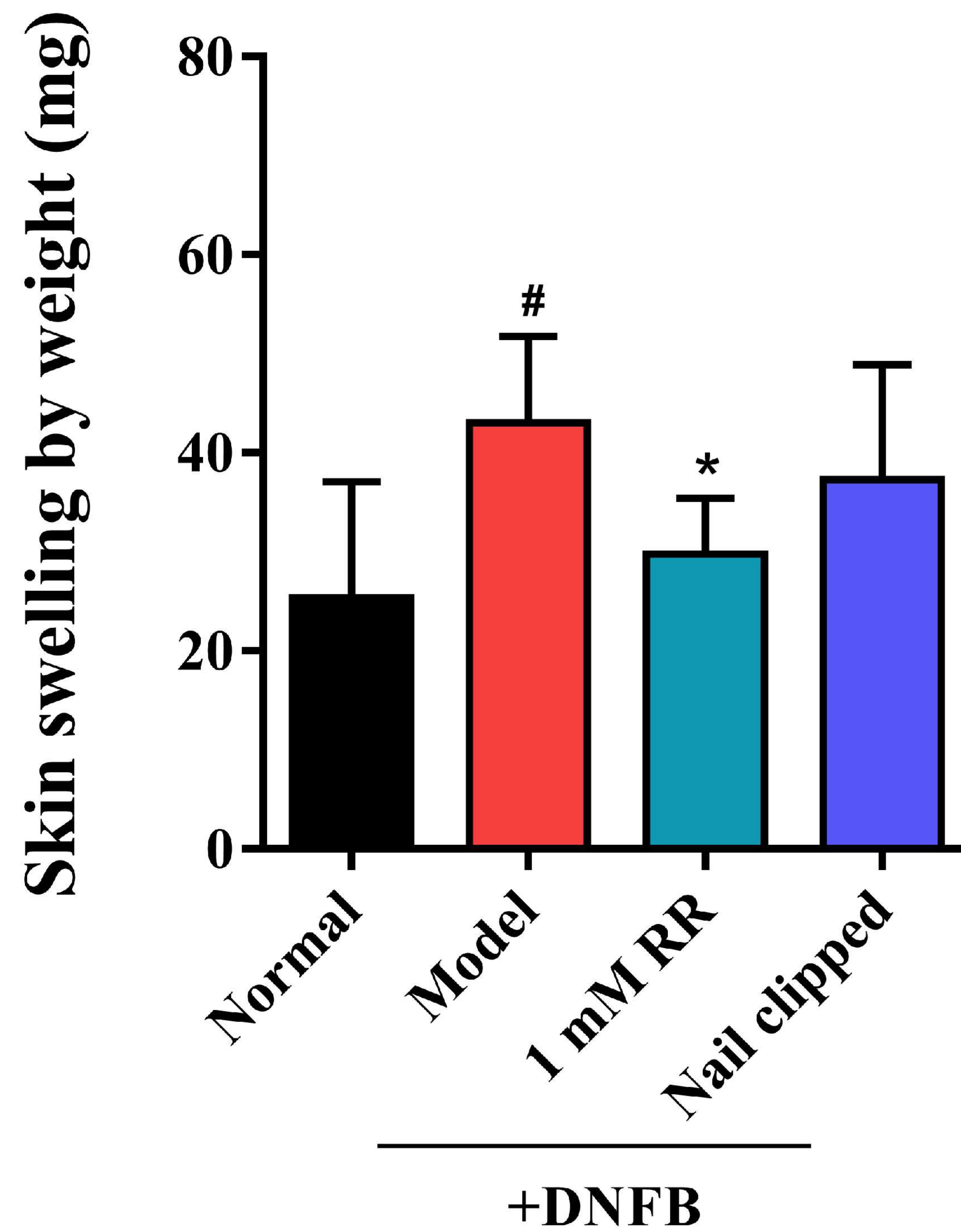
How to cite this article: Tang L, Gao J, Cao X, Chen L, Wang H, Ding H. TRPV1 mediates itch-associated scratching and skin barrier dysfunction in DNFB-induced atopic dermatitis mice. *Exp Dermatol*. 2021;00:1–8. <https://doi.org/10.1111/exd.14464>



(A)



(B)



Epidermis

Dermis

→ Promote
—| Inhibit

