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The novel house dust mite allergen Der p 39 exacerbates atopic dermatitis-like inflammation in mice by inducing skin barrier dysfunction

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ABSTRACT

Background: House dust mite (HDM) allergens can induce or exacerbate allergic inflammation, including atopic dermatitis (AD). Substances that damage the epithelial barrier can trigger or worsen AD. The mechanism by which the novel HDM allergen Der p 39 induces allergic inflammation remains unclear. Our aim was to investigate the effects of Der p 39 on AD-like inflammation and associated mechanisms.

Methods: Dinitrochlorobenzene (DNCB) and Der p 39 were utilized to establish AD model mice. Inflammation severity was evaluated with physiological and morphological assays. The effects of Der p 39 on inflammatory cytokine release and skin barrier protein expression were examined in HaCaT cells (human epidermal keratinocytes). Mitogen-activated protein kinase (MAPK) activation was examined by western blots. MAPK inhibitors were employed to assess MAPK involvement in filaggrin expression.

Results: Der p 39 worsened allergic inflammation (tissue thickness) in murine ears pretreated with 1% DNCB. Compared to controls, Der p 39-sensitized tissues showed epidermal and dermal thickening with elevated numbers of mast cells and eosinophils in inflammatory lesions. Der p 39 increased transcription and production of pro-inflammatory interleukins (ILs), down-regulated expression of the skin barrier proteins filaggrin and loricrin, and upregulated phosphorylation of ERK, JNK and p38 in HaCaT cells. Inhibition of MAPK signaling rescued filaggrin expression in Der p 39-treated cells.

Conclusions: The HDM allergen Der p 39 enhances allergic inflammation and promotes MAPK pathway-mediated epidermal barrier dysfunction, suggesting that Der p 39 may possess pathogenic and clinically relevant immunomodulatory potential.

Keywords: House dust mite, Der p 39, Skin barrier, Atopic dermatitis, Skin inflammation

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INTRODUCTION

House dust mites (HDMs), including *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farina*, constitute a pervasive source of major inhalatory allergens.[1–4] The identification and naming of HDM allergens are clinically significant for diagnosing and treating HDM-induced allergic diseases. In our previous work identifying the troponin C-like protein Der p 39 as a minor HDM allergen, Der p 39 was demonstrated to have an immunoglobulin E (IgE) binding epitope in its C-terminal region.[5] The mechanism mediating Der p 39-induced allergic inflammation needs to be further elucidated.

According to the epithelial barrier hypothesis,[6] the onset and exacerbation of chronic inflammatory diseases can be attributed to persistent epithelial inflammation triggered by exposure to diverse epithelial barrier-damaging substances, including environmental allergens.[7,8] Consistent with this hypothesis, HDM allergens, such as Der p 1 and Der f 1, activate immune cells in the underlying tissues, and initiate a cascade of Th2-polarized responses, leading to the development of allergic inflammation.[9] HDM-derived proteases have been shown to degrade the outermost cuticle on the skin, resulting in impaired barrier functionality.[7,10] These proteases have also been shown to delay skin barrier recovery through a mechanism involving PAR2 receptor activation.[11–13] Additionally, HDM allergens have been shown to induce inflammation by releasing proinflammatory cytokines and chemokines in epithelial tissues and in keratinocytes.[3,10,14] Possible effects of Der p 39 on regulating skin barrier function have yet to be addressed.

Atopic dermatitis (AD) is a widespread inflammatory disease associated with immune dysfunction and epidermal barrier issues. Recent clinical studies have demonstrated that the onset of AD is linked to skin barrier damage.[15] More specifically, the production of cytokines and chemokines by keratinocytes that have been activated by skin barrier defects have been reported to lead to a Th2-biased immune response characteristic of AD pathogenesis.[16] Furthermore, exposing mice to HDM extract has been shown to cause inflammation and signs of AD consistent with AD in human patients.[11] Many patients diagnosed with AD have high levels of HDM allergen-specific IgEs, and HDM avoidance can be effective for alleviating allergic symptoms in patients.[17]

The effects of Der p 39 on AD allergic reactivity are unclear. Accordingly, the present study aims to examine whether Der p 39 exacerbates inflammation severity in AD model mice and to explore whether Der p 39 may contribute to AD pathogenesis by way of regulatory influences on skin barrier function. Pro-inflammatory effects of Der p 39 were investigated in AD-like model mice. Additionally, because decreased levels of filaggrin or loricrin protein have been shown to be positively associated with skin barrier disruption in the development of AD,[18] potential effects of Der p 39 on the expression of skin barrier-related functional proteins filaggrin or loricrin were investigated in a line of human epidermal keratinocytes, namely HaCaT cells. To further investigate the regulatory mechanism of Der p 39 on barrier-related protein expression, we examined MAPK (mitogen-activated protein kinase) signaling activity in treated HaCaT cells, including ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase), and p38 signaling.

MATERIALS AND METHODS

Reagents

2,4-Dinitrochlorobenzene (DNCB) was sourced from Tokyo Chemical Industry (Tokyo, Japan). The information of antibodies in this study is listed in the Supplementary Data Table S1.

Preparation of recombinant Der p 39 protein

A codon-optimized sequence encoding Der p 39 (GenBank accession MZ643463.1) was synthetically generated and subcloned into the prokaryotic expression vector pET-His (Miaoling Biotech, Wuhan, China). The recombinant Der p 39 protein was expressed as a soluble protein in E.coli ClearColi® BL21(DE3) (Lucigen, USA) and purified using Ni-NTA affinity chromatography (GE Healthcare, USA) as described in our previous publication.[5] Subsequently, endotoxin was removed using a Toxin Eraser endotoxin removal kit (GenScript, USA). Endotoxin level was assessed using a Limulus

Amebocyte Lysate (LAL) endotoxin detection kit (GenScript, USA).

Animals

Female BALB/c mice (17–20 g, 5–6 weeks old) were obtained from the Guangdong Medical Experimental Animal Center (Guangdong, China). All mice were housed in specific pathogen-free rooms, with 6 mice per cage, at a constant temperature of 24 ± 1 °C and a humidity level of 50 ± 10%. They were maintained on a 12-h light and dark cycle.

Induction of AD-like lesions in mouse ears

DNCB treatment is an established method for generating murine AD models.[19] BALB/c mice were randomly divided into 5 treatment groups (n = 6 per group): (1) Control, (2) Der p 39 (8 mg/kg), (3) 1% DNCB, (4) Combination (1% DNCB plus 8 mg/kg Der p 39 treatment), and (5) 2.5% DNCB. Treatment injections were administered into mouse ears every 48 h for 28 d. On day 28, ear thickness was measured with a digital micrometer, ear lesions were photographed, and then treated ears were removed for histological analysis.

Scoring of AD-like symptoms

Dermatitis severity of mouse ears was scored once a week according to a previously developed dermatitis score (DS) system.[20] The Total Dermatological Score (DS) was calculated as the sum of 4 symptom subscores: Dryness, Erythema and redness, Edema, and Excoriation/crust formation. Subscores range from 0 to 3, representing the severity of the respective symptom as none, mild, moderate, or severe.

Histology

The ear tissue samples were fixed in a 4% formaldehyde solution, embedded in paraffin wax, and subsequently cut into a series of 5-μm thick tissue sections. The tissue sections were stained with hematoxylin and eosin (H&E) to prepare the assessment of the epidermal and dermal thicknesses, as well as eosinophil infiltration. Additionally, the sections were stained with toluidine blue (TB) to evaluate the level of mast cell infiltration. The stained tissue sections were mounted and examined under a light microscope (Olympus Corporation, Tokyo, Japan).

Cell culture

HaCaT cells (BaNa Culture Collection, Henan, China) were cultured at 37°C in a humidified atmosphere containing 5% CO_2 with Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (Gibco, NY, USA) and 1% penicillin-streptomycin (Hyclone, UT, USA).

Cell viability analysis

The viability of HaCaT cells was assessed using the Cell Counting Kit-8 assays (CCK-8) (MedChem Express, Monmouth Junction, NJ, USA). The cells were exposed to specified concentrations of Der p 39 for 24 h, after which the CCK-8 reagent was added. After 2 h, a multi-well plate reader (Bio-Rad, Hercules, CA, USA) was employed for detecting the absorbance value of each well at 450 nm.

Quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR)

HaCaT cells were treated with Der p 39 (100 μg/mL) for specified durations and then collected for RNA extraction. Total RNA was extracted using an RNA Extraction Kit (Accurate Biotech, Changsha, China) and subjected to generate cDNA using HiScript III RT SuperMix (Vazyme, Nanjing, China). Gene expression levels were analyzed via real-time RT-qPCR with TB Green Premix Ex Taq™ (Takara, Tokyo, Japan) in a qTOWER 2.2 system (Analytik Jena, Upland, CA, USA), normalized to *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) mRNA levels employing the $2^{-\Delta\Delta Ct}$ method. The primers used are listed in the Supplementary Data Table S2.

Enzyme-linked immunosorbent assay (ELISA)

HaCaT cells were stimulated with different ranges of doses Der p 39 for experimentally specified durations. Supernatants were collected for estimation of interleukin-6 (IL-6) and IL-8 concentrations with human IL-6 and IL-8 ELISA kits (Elabscience, Wuhan, China) according to the manufacturer's guidelines. Absorbance measurements were performed at 450 nm using a multi-well plate reader (Bio-Rad, Hercules, CA, USA).

Western blot analysis

HaCaT cell lysate was extracted with RIPA buffer (Beyotime, Beijing, China) and quantified with a BCA assay kit (Beyotime, Beijing, China). Lysate samples were separated by electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were incubated at 4C overnight with primary antibodies targeting loricrin, filaggrin, GAPDH, ERK, JNK, and p38, as well primary antibodies targeting the phosphorylated (p-) proteins p-ERK, p-JNK, and p-p38, respectively. Membranes were reacted with anti-rabbit or anti-mouse IgG-HRP secondary antibody for 1 h at room temperature. The immunoreactive protein bands were visualized using ChemiDoc imaging system (Bio-Rad, Hercules, CA, USA), and their quantification was conducted with software ImageJ 1.80v (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The data are reported as mean ± standard deviation (SD). Statistical differences between groups were analyzed using an unpaired Student's t-test for 2 groups or one-way analysis of variance (ANOVA) with Tukey's post - hoc test for comparisons of multiple groups. All analyses were conducted by software Prism 8 (GraphPad, La Jolla, CA, USA). Differences were considered statistically to be significant at $p < 0.05$.

RESULTS

Der p 39 aggravates AD-like symptoms in DNCB-induced mice

DNCB-induced AD-like model mice were generated to investigate the pro-inflammatory effects of Der p 39 (Fig. 1A). Ear lesions with skin thickening, dryness, erythema, and excoriations (Fig. 1B–D) were observed in the 2.5% DNCB treatment group. Interestingly, Der p 39 alone also induced mild ear dryness and erythema together with a slight increase in ear weight and thickness. The combination treatment of 1% DNCB and Der p 39 produced more severe AD-like inflammation in treated ears than either the 1% DNCB treatment or Der p 39 treatment alone (Fig. 1B–D). The Der p 39 group had greater DS scores than the Control group, and the Combination group had greater DS scores than either the 1% DNCB group or the Der p 39 group (Fig. 1E). These results suggest that Der p 39 exposure may aggravate AD-like inflammation in DNCB-induced mice.

Der p 39 increases skin pathological inflammation in AD-like lesions

Histopathology of ear-skin lesion tissue samples stained with H&E or TB (Fig. 2A–E) revealed that 2.5% DNCB treatment increased tissue (epidermal or dermal) thickness and infiltration of mast cells or eosinophils into ear lesions. In 1% DNCB-induced lesions, exposure to Der p 39 exacerbated tissue (epidermal or dermal) thickening and mast cell/eosinophil infiltration. Der p 39 treatment alone also caused tissue (epidermal or dermal) thickening and increased numbers of infiltrated mast cells in ear-skin lesions.

Der p 39 stimulates pro-inflammatory factors IL-6 and IL-8 in HaCaT cells

CCK-8 assays of cytotoxicity showed that Der p 39 at concentrations of ≤200 μg/mL did not impact the viability of HaCaT cells (Fig. 3A). As demonstrated by RT-PCR analysis, the mRNA levels of the pro-inflammatory cytokine IL-6 and chemokine IL-8 were found to be upregulated in a time-dependent manner following treatment with 100 μg/mL Der p 39, compare to control cells (Fig. 3B&C). In concordance, ELISAs showed that Der p 39 treatment produced time-dependent increases in IL-6 and IL-8 secretion in HaCaT cells (Fig. 3D&E). These findings indicate that pro-inflammatory effects of Der p 39 may be mediated, at least in part, by augmented expression of IL-6 and IL-8 in keratinocytes.

Der p 39 diminishes the expression of skin barrier function proteins in HaCat cells

TNF-α/IFN-γ stimulation, which served as a positive pro-inflammatory control treatment, resulted in decreased expression of the skin barrier-related proteins filaggrin or loricrin in HaCaT cells. Interestingly, Der p 39 treatment reduced expression of filaggrin or loricrin in a time- and dose-dependent manner (Fig. 4A–F), suggesting that Der p 39 may exert proinflammatory effects partly by disrupting skin barrier function.

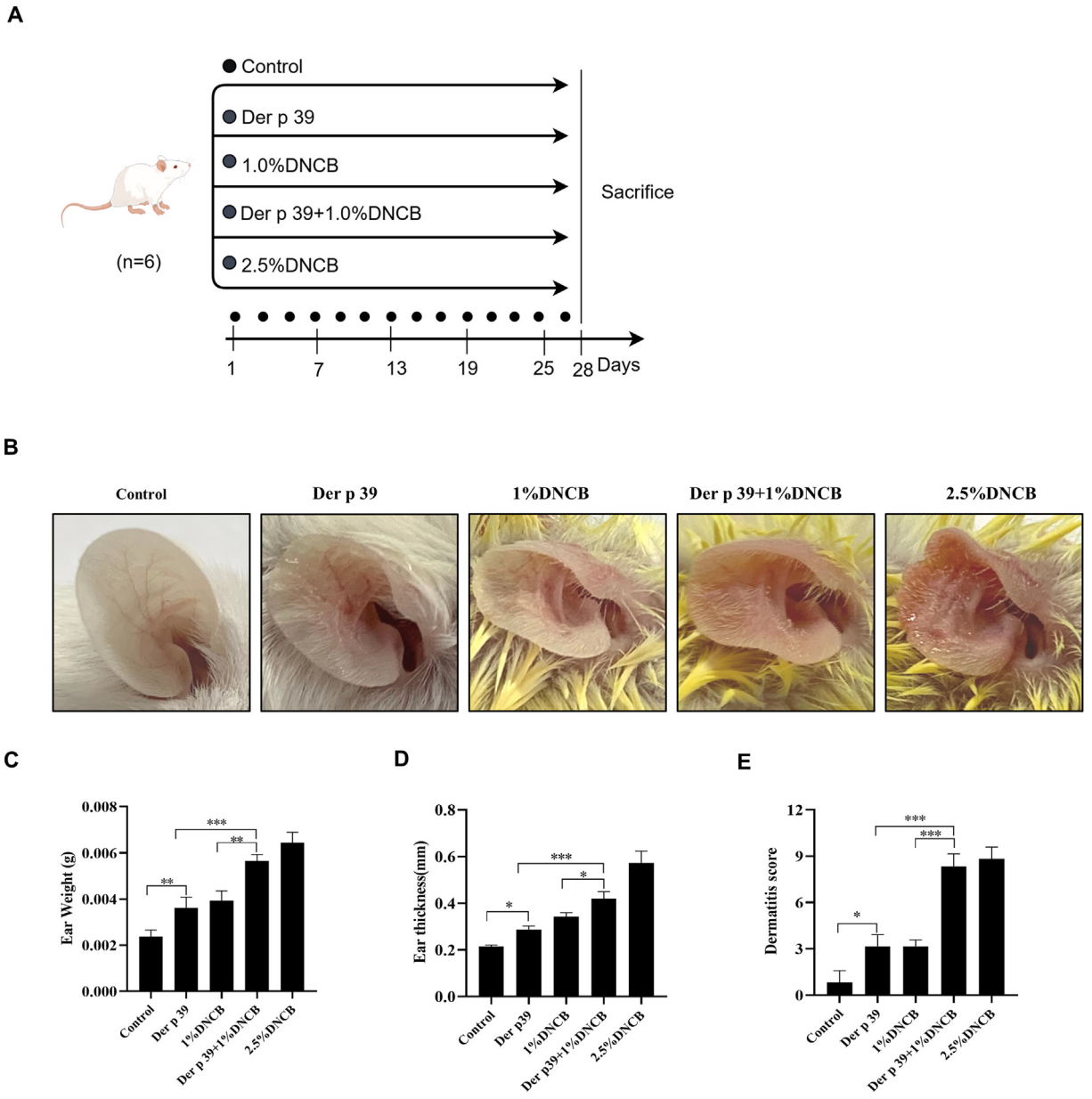

Fig. 1 Experimental design schematic and Der p 39 effects on AD-like inflammation. (A) Diagram of the study protocol (The figure was created by Figdraw). Mice were divided into 6 groups (n = 6 per group). AD-like skin inflammation lesions were induced with DNCB and/or Der p 39 as described in the Materials and Methods. (B) Representative images of tissues from each group. (C) Ears were weighed after euthanasia. (D) Changes in ear thickness were measured on the last day of the experiment. (E) DNCB/Der p 39 induced DS in mice. Data are expressed as means ± SD (n = 6). One-way ANOVA, Tukey's post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 vs. respective control

Der p 39 diminishes filaggrin expression via MAPK activation in HaCaT cells

Western blot analysis showed that the levels of *p*-ERK, p-P38, and *p*-JNK were significantly and dose-dependently elevated in HaCaT cells exposed to Der p 39 (Fig. 5A & B), indicating that Der p 39 had the effects of enhancing activation of p38, JNK, and ERK signaling pathways. The administration of various MAPK inhibitors, including the ERK inhibitor (U0126), the JNK inhibitor (SP600125) and the p38 inhibitor (SB203580), individually mitigated the

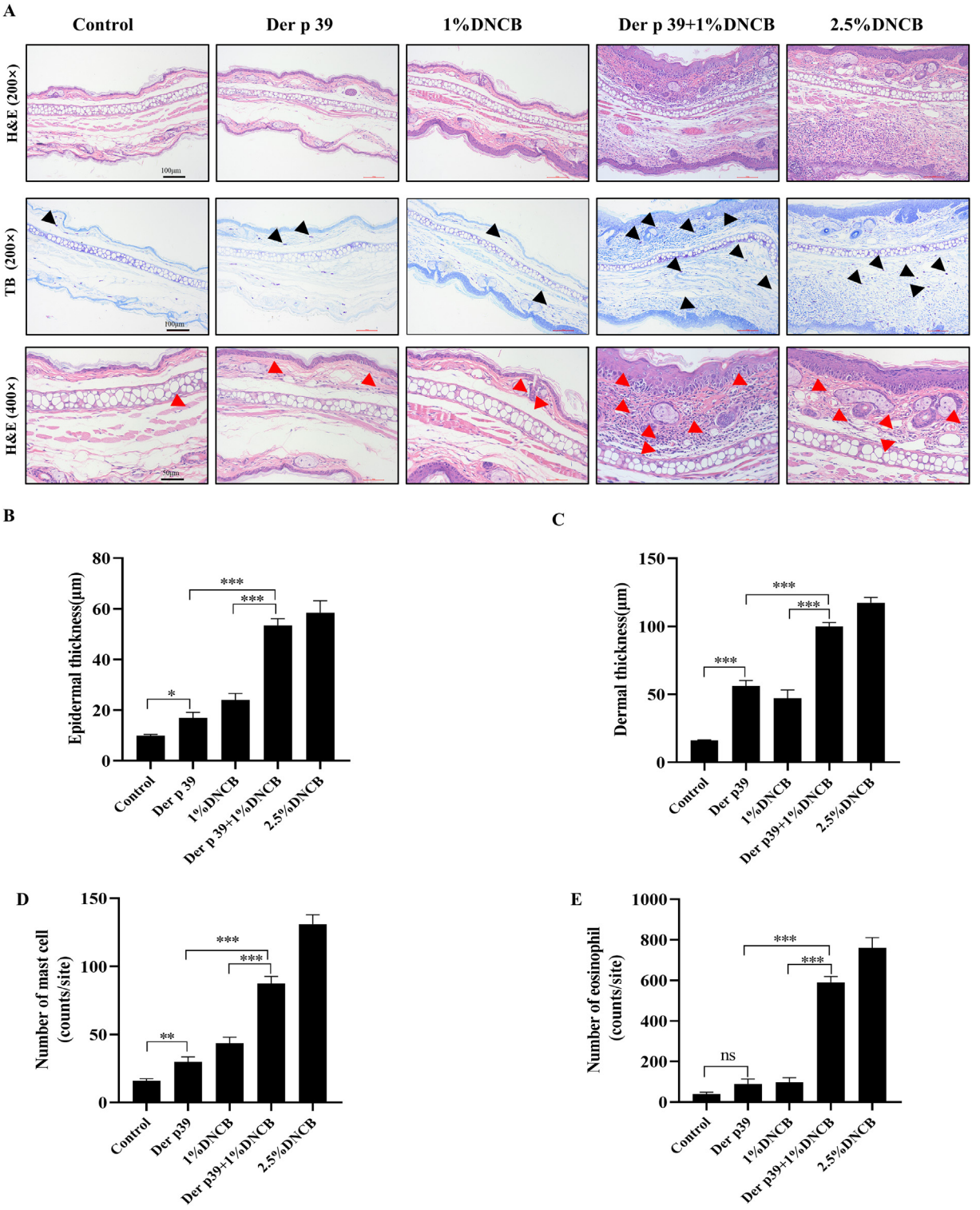

Fig. 2 Der p 39 induces histological changes and enhances leukocyte invasion in AD-like lesions. (A) Ear tissues were stained with H&E and TB. (B) Assessment of epidermal/dermal thicknesses in H&E stained sections. (C) Mast cell quantities were determined in TB-stained sections. (D) Eosinophil quantities were estimated in H&E stained sections. Data are expressed as means ± SEM (n = 6); *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Data are expressed as means ± SD (n = 6). One-way ANOVA, Tukey's post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 vs. respective control

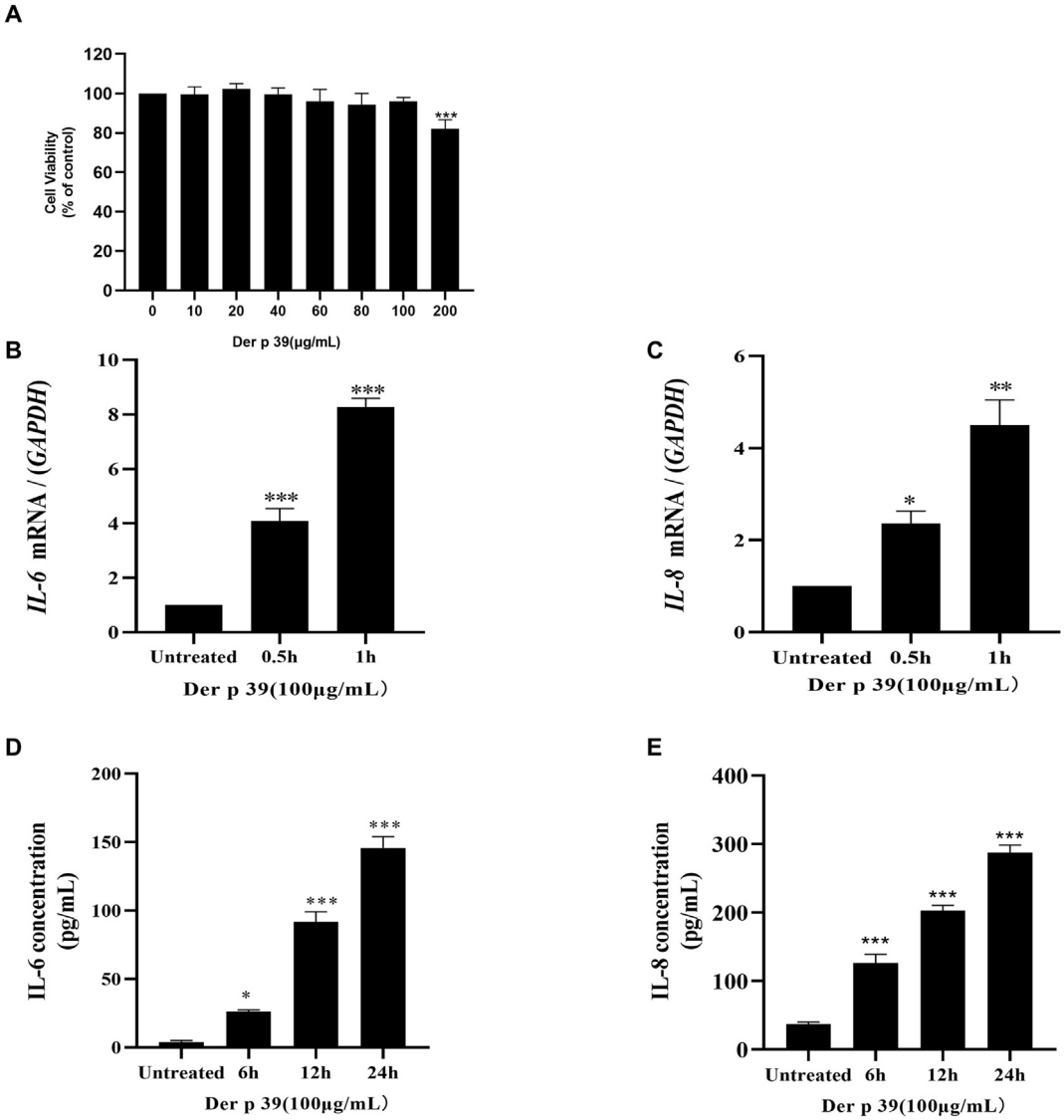

Fig. 3 Der p 39 modulates pro-inflammatory cytokine expression in HaCaT cells. CCK-8 assay analysis of HaCaT cell viability following Der p 39 treatment. (B) Following stimulation of HaCaT cells with Der p 39 for 0.5h and 1 h, expression levels of *IL-6* and *IL-8* transcripts were determined by RT-qPCR. Expression levels were normalized to GAPDH, and fold-change values are presented. (C) Secretion levels of IL-6 and IL-8 were determined by subjecting supernatants of HaCaT cells that had been stimulated with Der p 39 for 6 h, 12 h, or 24 h to ELISAs. Data are presented as means ± SD (n = 3). One-way ANOVA, Tukey's post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 vs. untreated control

aforementioned Der p 39-induced reduction in filaggrin expression (Fig. 5C–E). These results suggest that Der p 39 may regulate filaggrin expression.

DISCUSSION

In the present study, Der p 39 was shown to worsen allergic inflammation in murine ears pretreated with 1% DNCB, and Der p 39-sensitized tissues showed epidermal and dermal thickening with increased mast cell and eosinophil infiltration into inflammatory lesions. Der p 39 increased pro-inflammatory IL production, down-regulated expression of skin barrier proteins, and upregulated phosphorylation of MAPKs in HaCaT cells. Conversely, it was shown that MAPK inhibitors could rescue filaggrin expression in Der p 39-treated cells.

HDMs are a highly prevalent indoor allergen source, causing human allergic diseases, including asthma, rhinitis and AD.[21-23] Regarding the molecular mechanisms of HDM allergenicity, Der p 1, a cysteine protease allergen, cleaves the

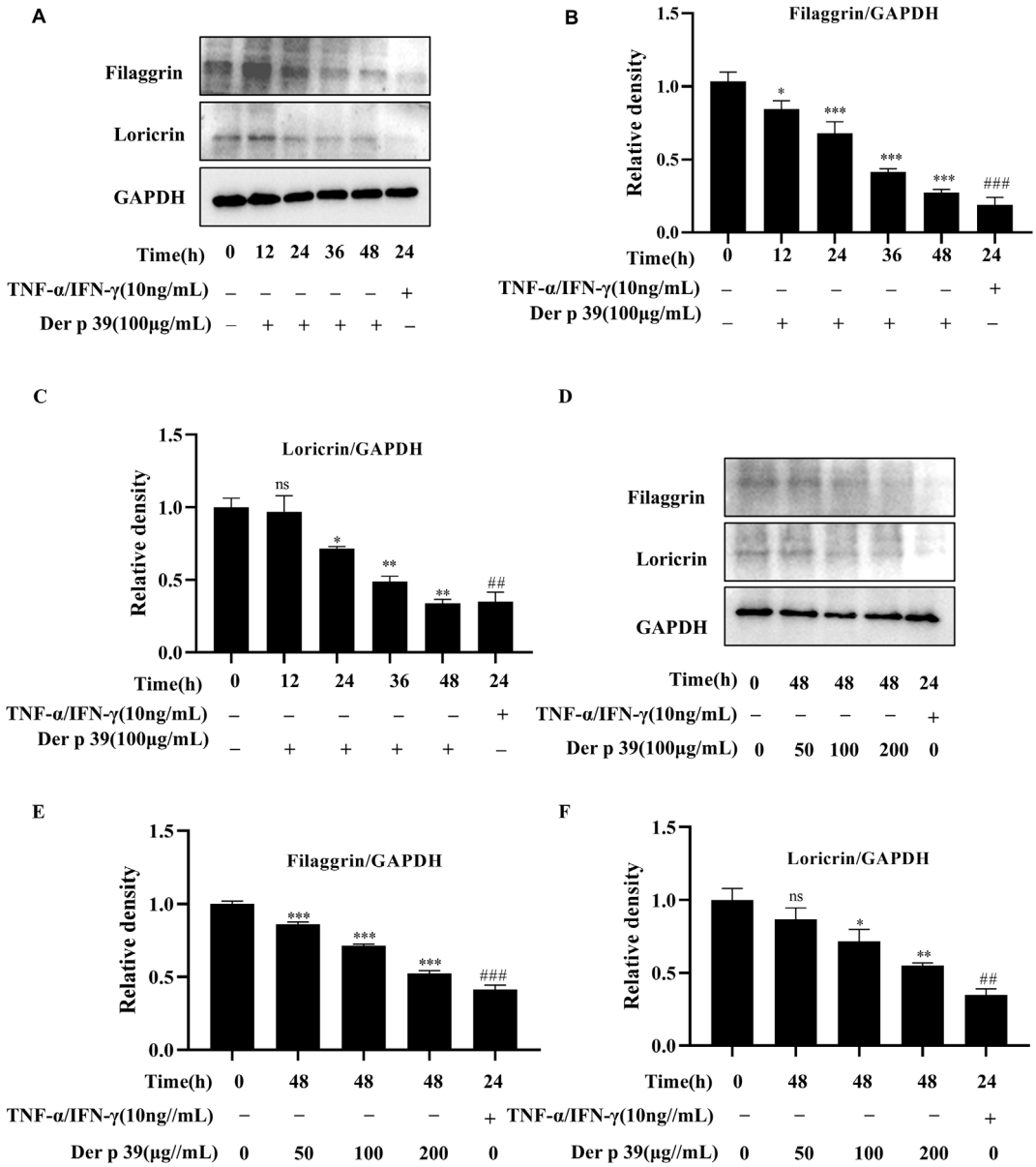

Fig. 4 Der p 39 diminishes filaggrin expression in HaCaT cells. (A) HaCaT cells were treated with 100 μg/mL Der p 39 for the indicated durations. (B, C) Quantified values of filaggrin and loricrine. (D) HaCaT cells were treated with a series of Der p 39 concentrations for 48 h. (E, F) Quantified values of filaggrin and loricrine. For both experiments, HaCaT cells were treated with TNF-α/IFN-γ (each 10 ng/mL) for 24 h as a positive control. Whole-cell lysates were used for Western blot analysis of filaggrin and loricrin levels. The results are expressed as means ± SD (n = 3). Unpaired Student' s *t*-test, ##p < 0.01, ###p < 0.001 vs. respective control. One-way ANOVA, Tukey's post-hoc test. ns, not significant, *p < 0.05, **p < 0.01, ***p < 0.001 vs. respective control

tight junction proteins occludin and claudin-1, disrupting the epithelial barrier and facilitating the transepithelial passage of allergens.[10,24,25] In addition, Der p 1 catalyzes the deactivation of α-antitrypsin, thereby exposing the lower respiratory tract to potential damage and facilitating the onset of airway inflammation and asthma.[26] Der p 2 acts as an immunomodulator by mimicking a co-receptor of Toll-like receptor 4 and can promote airway hypersensitivity.[27] Previous study showed allergen Der p 3 had the ability to stimulate store-operated calcium channels and drive mast cell migration by engaging cellular PAR4 receptors, indicating its role in triggering inflammatory responses.[28] Interestingly, here we found that Der p 39 enhanced allergic inflammation in AD models and increased keratinocyte production of the pro-inflammatory

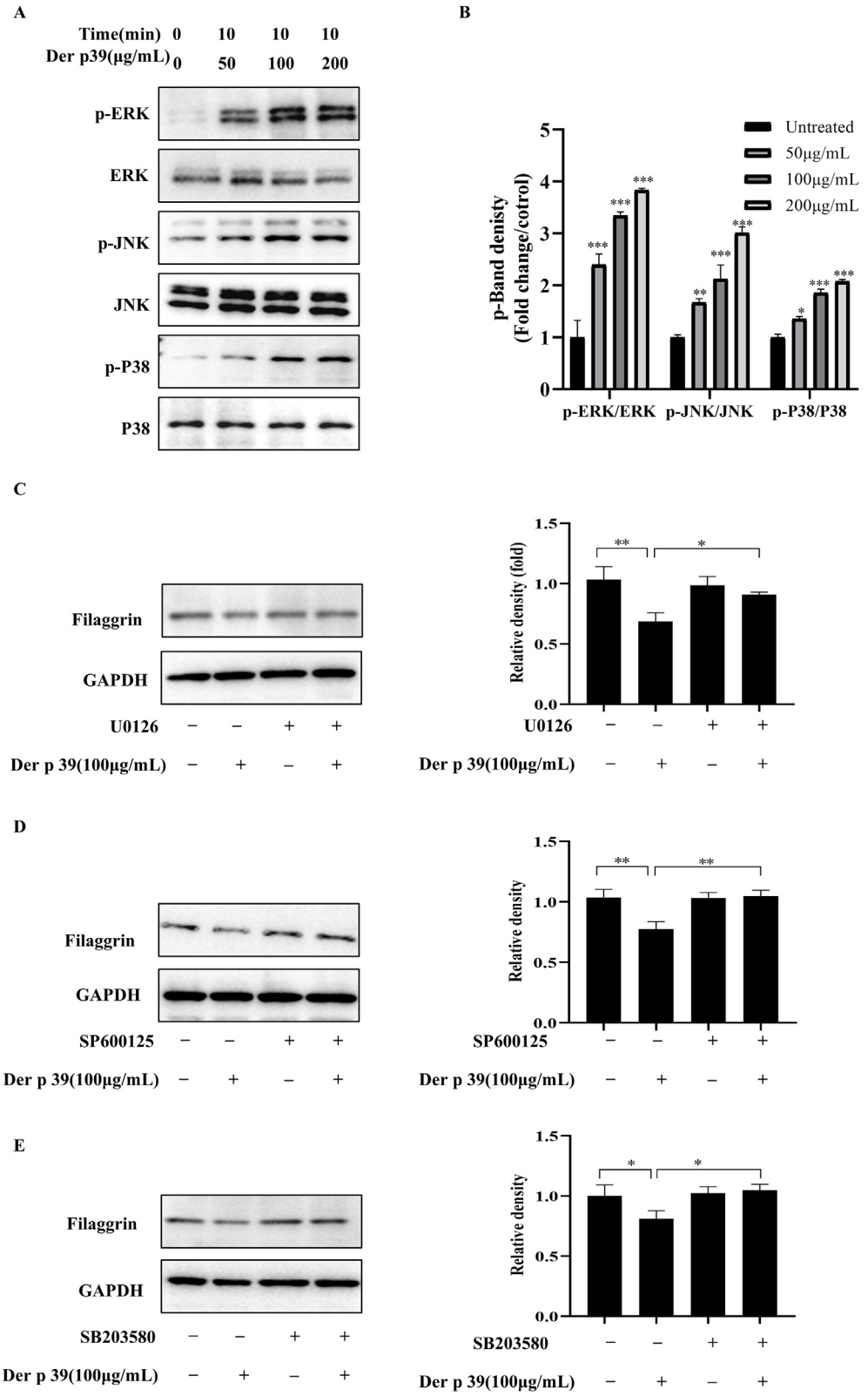

Fig. 5 Der p 39 reduced filaggrin expression in HaCaT cells by activating MAPK signaling. (A) Effects of Der p 39 on MAPK activation in HaCaT cells. HaCaT cells were stimulated with Der p 39 (50, 100, and 200 μg/mL) for 15 min, and levels of p38, ERK, and JNK proteins were analyzed in western blots. (B–E) Effects of MAPK pathway inhibition on Der p 39-suppressed filaggrin expression. After being treated with Der p 39 for 48 h, the cells were exposed to an ERK inhibitor (C), JNK inhibitor (D), or p38 inhibitor (E) for 1 h. Filaggrin levels were analyzed in western blots. The results are expressed as means ± SD (n = 3). One-way ANOVA, Tukey's post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. respective control

factors IL-6 and IL-8, which have previously been associated with AD pathogenesis.[29-32] These findings suggest that, in addition to its IgE-binding activity, Der p 39 may have immunomodulatory potential for allergic inflammation initiation and exacerbation.

Skin barrier disruption is a critical factor in AD pathogenesis.[33,34] The stratum corneum, the outermost layer of the skin, is chiefly made up of corneodesmosomes and corneocytes, functioning as a barrier to protect against water loss and the infiltration of external substances.[35] Impairment of the skin barrier can exacerbate allergen exposure and trigger inflammation through touch-induced release of cytokines.[7,33] Barrier-disrupting HDM allergen components are important targets for prevention of HDM-mediated inflammation.[11,36] HDM-derived protease components have been shown to influence epidermal barrier permeability and immune system homeostasis through activation of protease activated receptor 2 (PAR-2), including Group 1 and 3 HDM allergens[37,38] Previously, it was shown that Der p 2 peptides contribute to AD initiation and exacerbation by augmenting skin inflammation, barrier disruption, and keratinocyte hyperplasia.[39] The present results showing that Der p 39 enhanced epidermal/dermal thickness and increased mast cell infiltration into skin lesions indicate that Der p 39 can impair the skin's barrier function in a manner that contributes to AD-like inflammation.

Since keratinocytes, a main cell ingredient in the epidermis, maintain skin barrier functions, they are central effector cells in AD, wherein they interact with immune cells to generate inflammatory reaction.[33,40] Filaggrin, a significant structural protein, is responsible for the organization of cytoskeletal components and the maintenance of keratinocyte morphology. In AD, keratinocyte's filaggrin expression is diminished, consequently resulting in the abnormality of skin barrier.[41–43] Similar to Der p 38 in a previous study,[44] we found that Der p 39 diminished expression of the skin barrier protein filaggrin in HaCat cells.

Filaggrin expression is regulated by MAPK.[45] Previously, UVB-irradiation that decreases filaggrin expression and increases JNK, p38, and ERK phosphorylation in HaCaT cells has been shown to result in epidermal barrier dysfunction.[46] Similar to previous results showing that Der p 38 enhanced MAPK signaling in keratinocytes,[46] Der p 39 was found to increase MAPK activity in HaCat cells in the present study, as evidence by increased p-p38, p-JNK, and p-ERK levels. Our concordant results showing that each of several MAPK inhibitors restored filaggrin expression suggest that pharmacological MAPK inhibition may ameliorate HDM-induced epidermal barrier dysfunction and AD-like inflammation.

It can be inferred that the doses of Der p 39 used in our study were appropriate from our results showing dose- or time-dependent effects of Der p 39 on measures of inflammatory response despite having no effects on cell viability. Previous studies using similar dosages of HDM allergen proteins support this inference. For example, Jeon et al. reported that HaCat cells treated with 10 μg/mL the HDM allergen Der p 38 for 48 h showed no abnormalities.[44] Likewise, Österlund et al found that incubating BEAS-2B cells with the HDM allergen Der p 2 (180 μg/mL) did not produce cellular abnormalities.[47] Lin et al reported that the HDM allergen Der p 3, at a concentration of 60 μg/mL, activated Ca^{2+} channels in mast cells and induced cell migration through stimulating PAR4, leading to development the pathogenesis of asthma, this activation.[28] Thus, our study can reflect the effect of Der p 39 on skin barrier impairment and AD-like inflammation.

CONCLUSION

In this study, the novel HDM allergen Der p 39 was shown to enhance allergic inflammation in AD model mouse ears and to promote MAPK pathway-mediated epidermal barrier dysfunction in cultured keratinocytes. These findings suggests that Der p 39 has immunomodulatory potential for allergic inflammation initiation and exacerbation. Therefore, environmental Der p 39 should be avoided in the prevention or treatment of HDM allergy.

Abbreviations

AD, atopic dermatitis; CKK-8, cell counting kit-8; Der p, Dermatophagoides pteronyssinus; DNCB, 2,4-dinitrochlorobenzene; ELISA, enzyme linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FLG, filaggrin; HDM, house dust mite; IgE, immunoglobulin E;

JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; p-, phosphorylated.

Authors' consent for publication
I confirm that each of the authors has reviewed this paper in its submitted form and approved submission for publication of this paper to the World Allergy Organization Journal.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions
SL, ZLC and JL performed experiments and interpreted results. ZLC and SYQ contributed to the data analysis. SL, LC and WZH participated in technical discussions. JJC and KJ supervised the projects and participated in experimental design and technical discussions. SL and JJC wrote the paper. KJ and LC revised the manuscript.

Declaration of competing interest
The authors declare no competing interests.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2025.101036.

Ethics approval and consent to participate
All animal care and experimental procedures were carried out in accordance with protocols approved by the Animal Care and Use Committee of School of Medicine of Shenzhen University (No. A202300883) and were in compliance with the Guidelines on Animal Welfare of the School of Medicine of Shenzhen University.

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