PJ-001, a small-molecule proteolysis-targeting chimera, ameliorates atopic dermatitis-like inflammation in mice by inhibiting the JAK2/STAT3 pathway and repairing the skin barrier

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Abstract. Atopic dermatitis (AD) is a common allergic skin disease, and its pathogenesis involves genetic and environmental factors, as well as the immune response and skin barrier. PJ-001 is a small-molecule proteolysis-targeting chimera, which can degrade proteins related to the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway. In the present study, 0.5% 2,4-dinitrofluorobenzene was used to induce a mouse model of AD. Following treatment with PJ-001, the number of scratches and the severity of skin damage in the AD mice were recorded. Pathological changes in skin lesions were observed with hematoxylin and eosin staining. The expression levels of JAK2/STAT3, Toll-like receptor 4/nuclear factor-κB (TLR4/NF-κB), Beclin 1 and microtubule-associated protein 1 light chain 3 (LC3) were detected using western blotting. Furthermore, reverse transcription-PCR was used to detect the mRNA expression levels of filaggrin (FLG) and keratin 17, and the change in interleukin-10 levels in the splenic tissue of the mice. Compared with in the control group, the model group exhibited severe skin lesions. Following treatment with PJ-001, the AD-like inflammation in mice decreased. The expression levels of LC3 II/LC3 I and Beclin 1 were significantly reduced ($P<0.01$), and the expression levels of JAK2, STAT3, TLR4 and NF-κB were significantly downregulated ($P<0.001$). Additionally, the mRNA expression levels of FLG were significantly upregulated ($P<0.001$). These results indicated that PJ-001 may alleviate the skin condition in a mouse model of AD. The underlying mechanism may involve inhibition of the JAK/STAT signaling pathway, thereby suppressing the release of inflammatory factors, reducing excessive autophagy at the site of skin lesions, and enhancing the skin barrier function. In conclusion, PJ-001 could be considered a potential therapeutic option for AD.

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Introduction

Atopic dermatitis (AD) is a common chronic, recurrent inflammatory skin disease with a complex pathogenesis. Its main symptoms include dry skin, chronic eczematous lesions and severe itching. Patients often experience allergic rhinitis, asthma and other specific diseases, which significantly impact their quality of life (1). One of the key mechanisms involved in AD is the disruption of skin barrier function. Keratin 17 (K17) is the main structural protein and characteristic component of keratinocytes (KCs) and other epithelial cells. As a marker of cell proliferation and inflammation, K17 is rarely expressed in healthy skin, but is highly expressed in skin with abnormal differentiation and inflammatory injury (2). Loss of function and mutation of the filaggrin (FLG) gene reduce the accumulation of keratin filaments in the skin, thus affecting the levels of natural moisturizing factor, causing dry skin and creating conditions associated with the occurrence of AD (3,4). Therefore, increasing the FLG content and controlling K17 expression may be a viable strategy for the treatment of AD.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway mediates the intracellular signaling of various cytokines, including AD-related cytokines, in particular, Th2 cytokines, including interleukin (IL)-4, IL-5, IL-13 and IL-31, and thymic stromal lymphopoietin, contribute to the symptoms of chronic inflammation and pruritus in AD. Furthermore, the JAK/STAT is involved in the regulation of the epidermal barrier and the modulation of peripheral nerves related to the transduction of pruritus. The pathogenesis of AD is related to activation of the downstream JAK/STAT pathway by its receptors (5). When IL-4 and IL-13 bind to their respective receptors, the JAK family is activated and phosphorylates STAT3 (6-8). STAT3, a versatile transcription factor, promotes excessive proliferation of KCs and endothelial cells upon phosphorylation, causing increased permeability of the skin barrier to pathogens (9). In addition,

IL-31 induces STAT3 activation in KCs when it binds to its receptor, yielding the production of β-endorphin, which may contribute to peripheral pruritus in AD. IL-6, as a cytokine that activates STAT3, has been shown to regulate autophagy through inhibitory or stimulatory effects (10). Moreover, STAT3 can influence autophagy regardless of its phosphorylation status (11). Beclin 1 plays a crucial role in the regulation of the autophagic process and serves as a marker for the initiation of autophagy. Microtubule-associated protein 1 light chain 3 (LC3) is involved in the formation of autophagosomes and serves as specific protein marker of autophagosomes. The combination of Beclin 1 and LC3 enables accurate detection of changes in autophagic activity. Autophagy plays a critical role in the differentiation of keratinocytes and in their function as a skin barrier. Autophagy occurs continuously and actively in the epidermal layer. Various stresses applied to the epidermis can serve as triggers to activate autophagy, which contributes to keratinocyte differentiation. For example, mitochondrial reactive oxygen species produced during oxidative phosphorylation and released into the cytoplasm in suprabasal keratinocytes trigger autophagy, which is necessary for epidermal differentiation (12). Therefore, inhibiting cytokine, growth factor and hormone receptor signaling pathways may prove to be an effective treatment for AD.

Proteolysis-targeting chimera (PROTAC) technology is a novel approach in small-molecule drug development. Over the past two decades, targeted protein degradation using PROTAC molecules to harness the ubiquitin proteasome system has transitioned from research to industry (13,14). Several companies have now disclosed programs at the preclinical and early clinical development stages. With clinical proof-of-concept demonstrated against two well-known cancer targets (oestrogen receptor and androgen receptor), research is now exploring previously considered 'undruggable' targets (14). PJ-001 is a cereblon-based degrader of JAK2 synthesized by our laboratory using PROTAC technology. In our previous experiment, PJ-001 was used to induce degradation of JAK protein in Jeko-1 cells and it was observed that the half-maximal degradation concentration (DC_{50}) was <100 nM (unpublished data). The present study evaluated the therapeutic effect of PJ-001 using the 2,4-dinitrofluorobenzene (DNFB)-induced mouse model of AD, providing experimental and theoretical evidence for the potential application of small-molecule protein digesters in the treatment of AD.

Materials and methods

Reagents and instruments. DNFB was obtained from MilliporeSigma. Total RNA Extractor (Trizol), Tween-20, TBS buffer, Premixed Powder (1X) and TBE Buffer, Premixed Powder (1X) were purchased from Shanghai Shenggong Biology Engineering Technology Service, Ltd. Bradford protein concentration determination kit (detergent-compatible), bovine serum albumin (BSA) and RIPA buffer (cat. no. 89900) were purchased from Thermo Fisher Scientific Inc. BeyoRT™ III cDNA first-strand synthesis premix (5X), Easy-Load™ PCR Master Mix (Green), BeyoECL Moon, agarose, nucleic acid green, antibodies and primers were purchased from Beyotime Institute of Biotechnology. Crisaborole ointment was purchased from Pfizer, Inc. Compound dexamethasone ointment was purchased from China Resources Sanjiu Medical & Pharmaceutical Co., Ltd.

Drug formulation. A 0.5% DNFB solution was prepared by adding 51 μl DNFB solution (purity: 98%) to 9.949 ml acetone/olive oil solution (4:1 ratio by volume). The mixture was uniformly suspended by vortex shaking, resulting in a final volume of 10 ml (15). For 4% PJ-001 solution preparation, the aqueous phase was prepared by weighing 4 g PJ-001 sample and adding to the following reagents: 15 g 70% ethanol, 10 g propylene glycol, 10 g glycerol, 33.9 g water and 0.3 g nipagin. The oil phase consisted of 8 g Vaseline, 2.5 g monoglyceride, 2.5 g peregal, 5.5 g octadecyl alcohol and 8 g liquid paraffin. The aqueous and oil phases were heated and melted, mixed at 80˚C and stirred well. The mixture was then cooled to 30-40˚C to produce a cream (100 g). According to the preparation method of crisaborole ointment (2% concentration), 2% PJ-001 was also prepared (16).

Experimental animals. A total of 42 specific pathogen-free grade BABL/c male mice (age, 6 weeks; weight, 18-22 g) were purchased from Charles River Laboratories, Inc. The mice were housed in individually ventilated cages maintained at constant temperature (20-26˚C) and humidity (40-60%). The animals were housed in a 12 h light/12 h dark cycle, with free access to water and food. Both the feed and water provided to the mice were sterilized. The animal license number was SYXK (Su) 2016-0045. At the end of the experiment, the mice were euthanized with CO_2 (17). The mice were placed in a container, and the concentration of CO_2 was gradually increased using a flow rate of 5.5 l/min to achieve a CO_2 displacement rate of 30% (18) until their breathing stopped (3.0-3.5 min). Subsequently, bilateral pneumothorax chest opening was performed on the mice to prevent their recovery from asphyxia. It is crucial to allow an adequate interval between euthanasia procedures, 1-2 min, to ensure the dissipation of CO_2 from the container. This precautionary measure guarantees that subsequent groups of animals are not suddenly exposed to high levels of CO_2 gas. At the end of euthanasia, mouse spleens are collected and weighed. The skin was collected from the lesion site for further experiments.

Establishment of a mouse model of AD. All animal experiments were approved by the Ethics Committee of Experimental Animal Management and Animal Welfare of Jiangnan University [approval no. JN. No20210930b0801218(353)]; Wuxi, China). After 1 week of adaptive feeding, the mice acclimated to the unfamiliar surroundings and were than randomly assigned to the following six groups based on their body mass: Control group, model group, dexamethasone group, crisaborole group, 4% PJ-001 group and 2% PJ-001 group (n=7 mice/group). The weight deviation of each mouse was consistently around 20% of the average body weight. The experimental process is shown in Fig. 1. On the first and second days of the experiment, 100 μl acetone/olive oil (4:1) solution without DNFB was applied to the back of the control group mice once a day. In all mice, with the exception of the control group, 100 μl acetone/olive oil (4:1) solution containing 0.5% DNFB was applied to the back once a day. On days 3-6 of the experiment, the intervention was suspended. On days

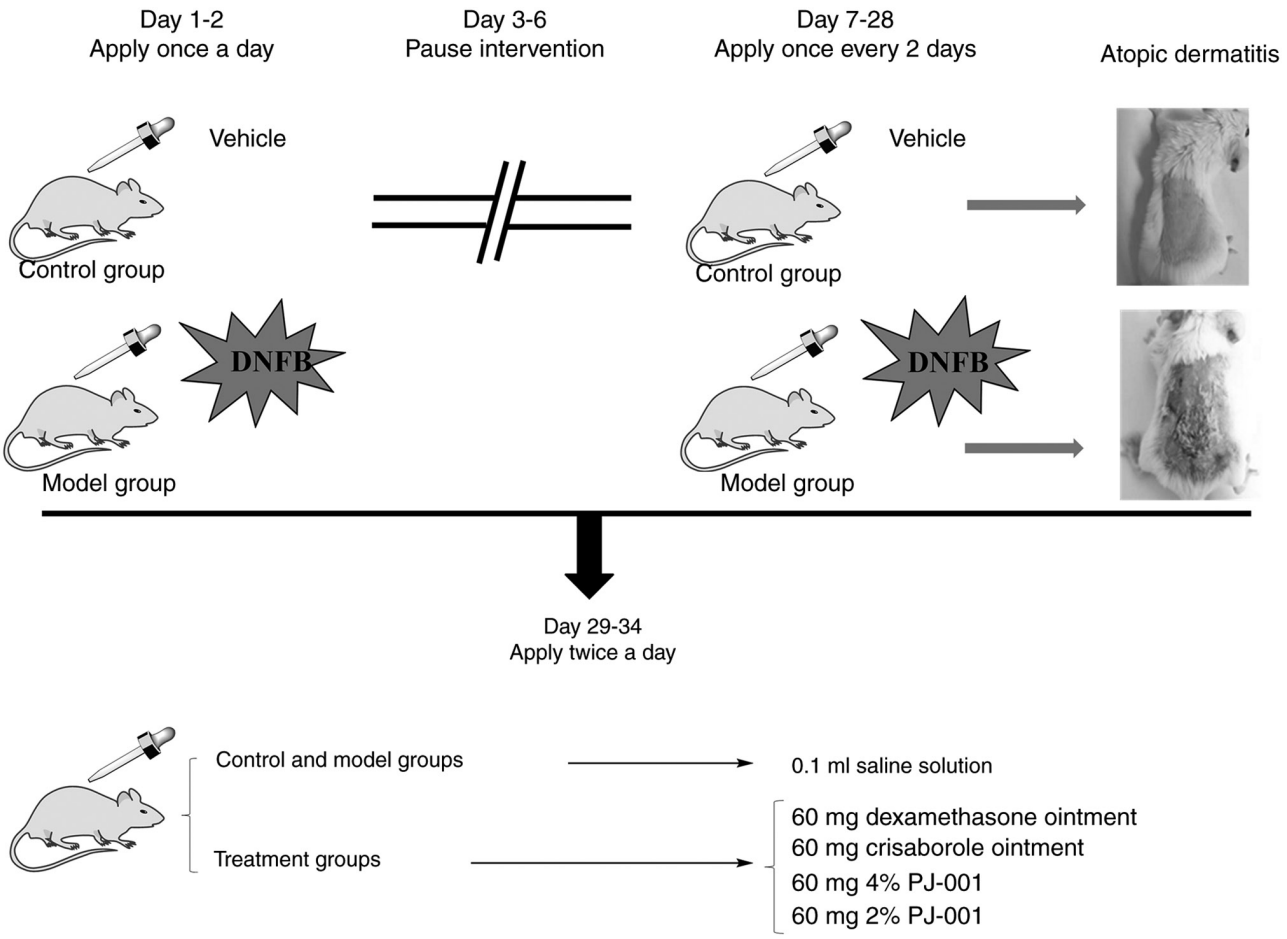

Figure 1. Atopic dermatitis mouse modeling flowchart. DNFB, 2,4-dinitrofluorobenzene.

7-28 of the experiment, the control group was administered the vehicle [acetone/olive oil (4:1) solution without DNFB], and the remaining mice were coated with 70 μl acetone olive oil (4:1) solution containing 0.5% DNFB every 2 days (15,19). At the end of the modeling process, 2 non-compliant mice (with skin broken during shaving or being over/underweight) were excluded from each group, and the remaining mice were subjected to 6 days of topical administration. Both the control and model groups were administered 100 μl saline, twice a day. The other groups were treated with 60 mg compound dexamethasone ointment, crisaborole ointment (19), 4% PJ-001 or 2% PJ-001, twice a day.

Changes in mouse skin lesions and scratching frequency. On days 0, 2, 4 and 6 of administration, changes in the specific dermatitis lesions were observed and the severity of mouse skin lesions was scored using the scoring standard provided in Table I (20). At 1 h after the last application administration, scratching frequency in mice was recorded over a duration of 10 min; for all the mice, scratching the back skin once was counted as one effective scratching incident, and continuous scratching for >3 sec was counted as two incidents.

Hematoxylin and eosin (H&E) staining. Following euthanasia, skin from the back of the mice was collected and immersed in 10% (v/v) neutral formalin at 4˚C for 24 h; then, the tissues were embedded in paraffin and sliced into 5-μm slices. The samples were then placed in xylene for 5 min at room temperature for dewaxing. The xylene was then replaced with fresh xylene, followed by another 5 min of dewaxing. The samples were subsequently cleaned sequentially with different gradient concentrations of ethanol and distilled water to rehydrate them. Next, the sections were stained with hematoxylin solution for 5 min at room temperature. The excess staining solution was washed off by soaking the samples in water for ~10 min, followed by another wash in distilled water. The samples were then stained with eosin staining solution for 2 min and washed twice with water. Dehydration and permeabilization were performed using absolute ethanol and xylene sequentially, and the slices were sealed with neutral gum. Finally, the slides were placed under an optical microscope for image collection and analysis (15).

Western blotting. Mouse skin tissue was cut into small pieces and mixed with precooled RIPA lysis buffer. The mixture was then centrifuged at 4˚C and 9,500 x g for 10 min. The resulting supernatant was collected as the protein lysate. Protein quantification was performed using the Bradford protein concentration assay kit, and the proteins were separated on 10% gels using SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membranes were then blocked with 5% BSA at room temperature for 1 h and incubated with the following primary antibodies: Toll-like receptor 4 (TLR4; 1:1,000; cat. no. AF8187), nuclear factor-κB (NF-κB; 1:1,000; cat. no. AF1234), JAK2 (1:1,000;

Table I. Lesion severity score.

Observation index	Rating/Score
Skin level with healthy skin	0
Skin slightly elevated above normal skin surface	1
Skin is moderately raised, and the edges of the plaques are circular or sloping	2
Skin hypertrophy with obvious protrusions	3
Skin is highly thickened and has extremely prominent protrusions	4

Table II. Primer sequences used for PCR.

Primer	Sequence (5'-3')	Molecular weight, bp
IL-10	F: AGGCGCTGTCATCGATTTCT	489
	R: AGGAAGAACCCCTCCCATCA	
FLG	F: AAAAGATGTCCGCTCTCCTGG	252
	R: TTGCCAGCTTTAGCACCAGT	
K17	F: GACCACCCGTTAAGGACTCA	161
	R: AGGCCACAGTTCACTTCAGGT	
GAPDH	F: ATCAGCAATGCCTCCTGCAC	242
	R: TTCCCGTTCAGCTCAGGGAT	

FLG, filaggrin; K17, keratin 17; IL-10, interleukin-10.

cat. no. AF1489), STAT3 (1:1,000; cat. no. AF1492), Beclin 1 (1:1,000; cat. no. AF5123), LC3 (1:1,000; cat. no. AL221) and GAPDH (1:10,000; cat. no. AF1186) at 4˚C overnight. Subsequently, the membrane was incubated with secondary antibodies (1:10,000; cat. no. A0208) at room temperature for 1 h. The aforementioned primary antibodies were all anti-rabbit polyclonal/monoclonal antibodies from Beyotime Institute of Biotechnology. A 5% BSA solution and antibodies were prepared using TBS buffer containing 1% Tween-20. Beyo-ECL Moon Kit was used to develop the membrane and a Gel Imaging System (Tanon Science and Technology Co., Ltd.) was used to capture images. The gray value was analyzed using ImageJ (1.46r) software (National Institutes of Health) (21). The following formula was used for semi-quantification: Protein expression (% of control)=(GA-target/GA-GAPDH)/(GC-target/GC-GAPDH) x100. G indicates grayscale value; A-target indicates target protein of the model and treatment group; A-GAPDH indicates GAPDH protein of the model and treatment group; C-target indicates target protein of the control group; C-GAPDH indicates GAPDH protein of the control group.

Reverse transcription (RT)-PCR. Mouse skin tissue was cut into small pieces and precooled total RNA extractor (TRIzol) was added, according to the manufacturer's protocol. The extracted RNA sample was then placed into a MK-20 dry thermostat and heated at 70˚C for 5 min. The concentration and purity of the RNA sample were measured using a micro nucleic acid protein analyzer. RNA was then subjected to RT to obtain cDNA, and the reaction procedure was as follows: 42˚C for 60 min and 80˚C for 10 min. Then, primers were added to amplify the target gene; PCR conditions were as follows: 94.0˚C for 3 min; followed by 35 cycles at 94.0˚C for 30 sec, 56.0˚C for 30 sec and 72.0˚C for 1 min; and a final step at 72.0˚C for 1 min, finally maintained at 4˚C. Primer sequences are shown in Table II. The amplified products were subjected to 2% agarose gel electrophoresis, and the results were analyzed by ImageJ software with GAPDH as an internal control. The agarose gel was prepared using TBE buffer, then microwaved and mixed with Nucleic Acid Green.

Statistical analysis. Data were analyzed using GraphPad Prism 8.0.1 statistical software (Dotmatics), and the experimental results are presented as the mean ± standard deviation and median. Statistical analyses and comparisons among groups were performed using one-way analysis of variance followed by Tukey's post hoc test for parametric data, and the Kruskal-Wallis test followed by the Dunn-Bonferroni post hoc test for non-parametric data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of scratch frequency and skin lesion severity scores in mice. As shown in Fig. 2A, the number of scratches in the model group was significantly higher than that in the control group. However, the number of scratches in the compound dexamethasone group, crisaborole group and 2% PJ-001 group was significantly reduced compared with that in the model group ($P<0.01$). This finding indicated that 2% PJ-001 had anti-pruritic properties. The skin lesion score of the model group mice was higher than that of the control group (Fig. 2B). As expected, the crisaborole group, 4% PJ-001 group and 2% PJ-001 group exhibited a decrease in skin lesion scores.

Histopathological changes in mouse skin. As shown in Fig. 3B, compared with the control (Fig. 3A), the model group exhibited excessive and incomplete keratinization, thickening of the spinous layer, mild sponge edema, decreased hair follicles, dilated capillaries and a substantial presence of inflammatory cell infiltration. In comparison to the model group, the crisaborole group demonstrated a marked thinning of the epidermal layer and an increase in hair follicles, although incomplete keratinization and a small amount of inflammatory cell infiltration persisted (Fig. 3D). The 2% PJ-001 group exhibited a notable increase in the structure of hair follicles and a slight decrease in the thickness of the spinous layer; however, severe edema was observed (Fig. 3F). In the 4% PJ-001 group, the corneum and spinous layer became thinner, cell edema decreased and inflammatory cell infiltration decreased slightly (Fig. 3E). The compound dexamethasone group showed no marked difference compared with the control group, with only a slightly thicker epidermal layer and minimal infiltration of inflammatory cells. Cellular edema could still be observed (Fig. 3C).

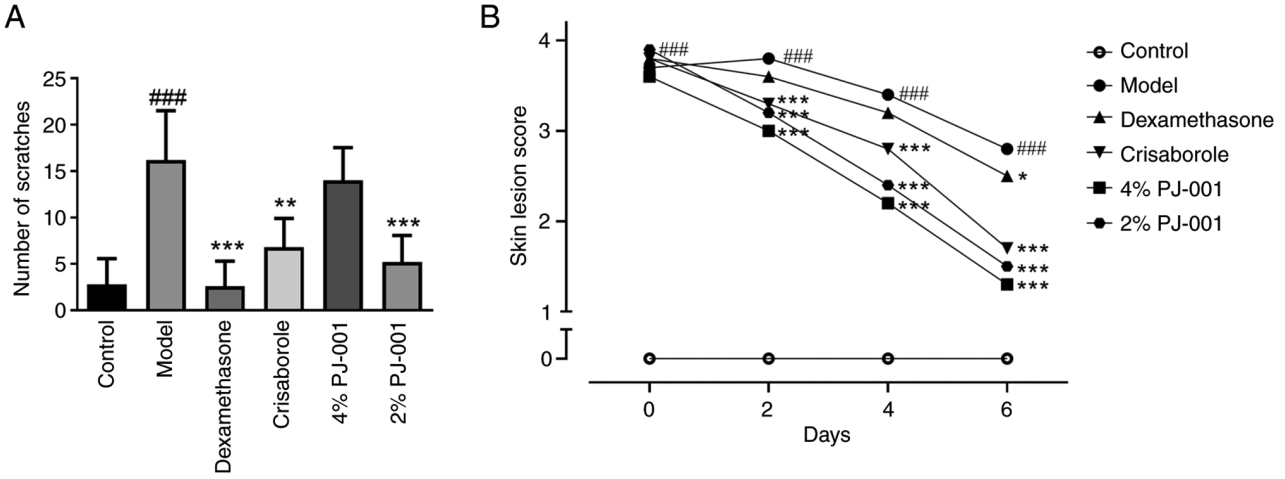

Figure 2. Number of scratches and the severity of skin lesions in mice. (A) Data were collected within 10 min of the last application of the ointment (day 34) and are presented as the mean ± standard deviation (n=5). ###P<0.001 vs. Control; **P<0.01, ***P<0.001 vs. Model. (B) Before each administration, changes in specific dermatitis lesions were observed, and the severity of mice lesions was scored. Data are presented as median values. ###P<0.001 vs. control; *P<0.05 and ***P<0.001 vs. model.

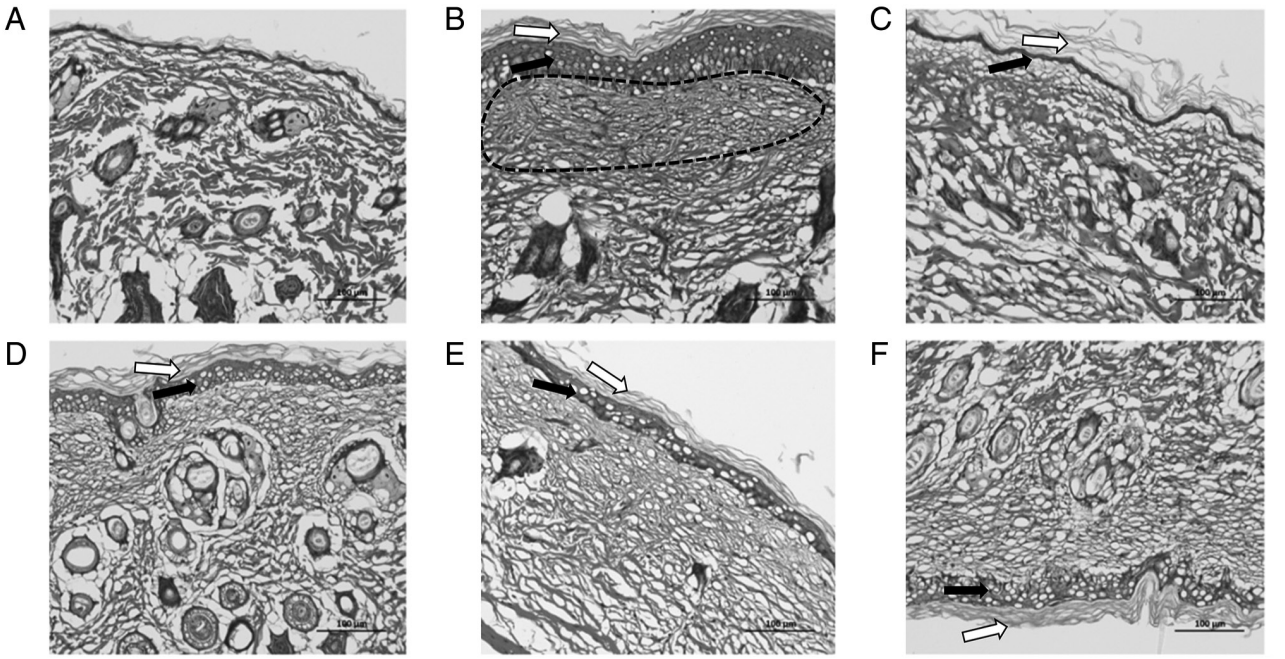

Figure 3. Mouse skin pathology was assessed by hematoxylin and eosin staining. (A) Control, (B) model, (C) dexamethasone, (D) crisaborole, (E) 4% PJ-001 and (F) 2% PJ-001 groups. White arrows indicate the stratum corneum, black arrows indicate the stratum spinosum, and the dotted area indicates the inflammatory infiltrate. All of the treatment groups had a certain degree of remission. Magnification, x5; scale bar, 100 μm.

Effects of PJ-001 on the JAK2/STAT3 pathway in mouse skin tissue. As shown in Fig. 4A-C, the protein expression levels of JAK2 and STAT3 in the AD model group were significantly increased compared with those in the control group (P<0.001). However, in the compound dexamethasone group, crisaborole group, 4% PJ-001 group and 2% PJ-001 group, the expression levels of JAK2 were significantly decreased compared with those in the model group (P<0.001). The expression levels of JAK2 in the 4 and 2% PJ-001 groups decreased by 65.2 and 61.3%, respectively. Moreover, PJ-001 also inhibited STAT3 expression. The expression levels of STAT3 in the 4 and 2% PJ-001 groups were significantly decreased by 53.4 and 29.8%, respectively (P<0.001).

Effects of PJ-001 on TLR4/NF-κB in mouse skin tissue. As shown in Fig. 4A, D and E, the protein expression levels of TLR4 and NF-κB were significantly higher in the AD model group than those in the control group (P<0.001). Compared with those in the model group, the expression levels of TLR4 were significantly downregulated in the compound dexamethasone group, crisaborole group and PJ-001 groups (P<0.01). Specifically, the protein expression levels of TLR4 in the 4 and 2% PJ-001 groups were decreased by 41.1 and 34.7%, respectively. In addition, the protein expression levels of NF-κB were significantly decreased in the compound dexamethasone group, crisaborole group, 4% PJ-001 group and 2% PJ-001 group (P<0.001). Specifically, the protein expression levels of NF-κB were downregulated

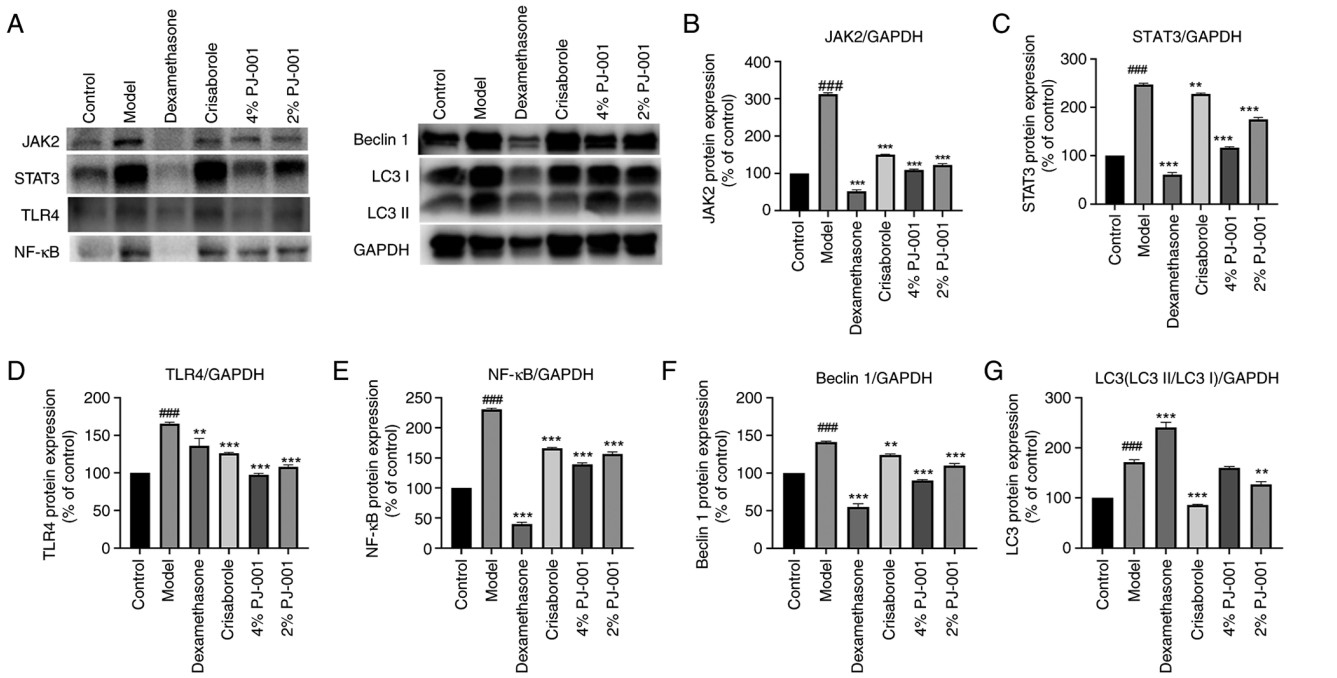

Figure 4. Effects of PJ-001 on protein expression in the skin tissue of mice. (A) Western blotting of each protein. PJ-001 effectively inhibited the JAK/STAT pathway, mediated downstream TLR4/NF-KB pathway downregulation and reduced excessive autophagy. Protein expression levels of (B) JAK2, (C) STAT3, (D) TLR4, (E) NF-κB, (F) Beclin 1 and (G) LC3 were semi-quantified. GAPDH was used as a loading control. Data are presented as a percentage of the control. A minimum of three independent experiments were carried out, and data are presented as the mean ± SD. $^{###}$P<0.001 vs. Control; **P<0.01, ***P<0.001 vs. Model. JAK2, Janus kinase 2; LC3, microtubule-associated protein 1 light chain 3; NF-κB, nuclear factor-κB; STAT3, signal transducer and activator of transcription 3; TLF4, Toll-like receptor 4.

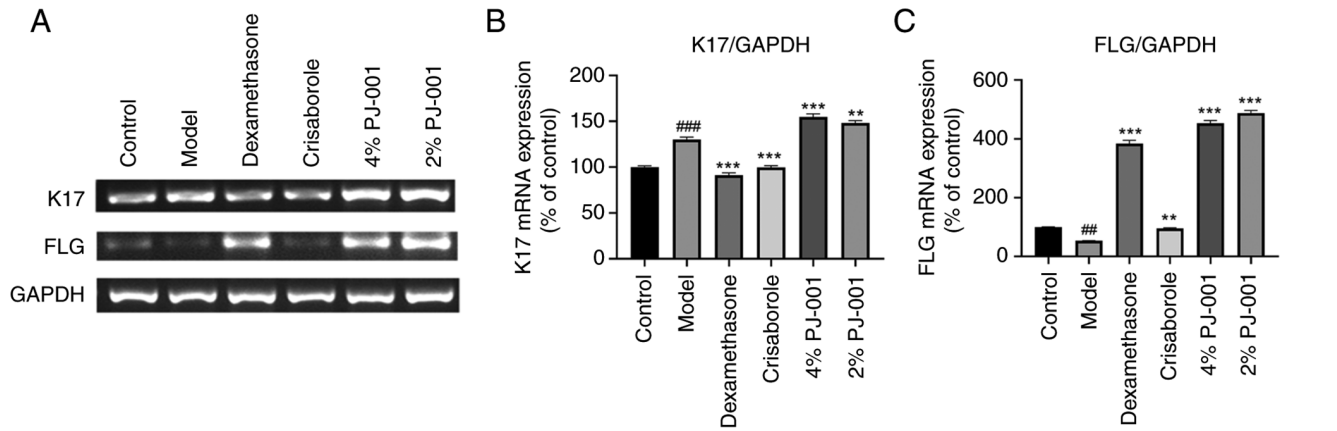

Figure 5. Effect of PJ-001 on the mRNA expression levels of K17 and FLG in skin tissue of mice. (A) Reverse transcription-PCR was performed to detect K17 and FLG mRNA. (B) K17 and (C) FLG expression levels were semi-quantified. Data are presented as a percentage of the control. GAPDH was used as a loading control. A minimum of three independent experiments were carried out, and data are presented as the mean ± SD. $^{##}$P<0.01, $^{###}$P<0.001 vs. Control; **P<0.01, ***P<0.001 vs. Model. FLG, filaggrin; K17, keratin 17.

by 46.6% in the 4% PJ-001 group and 39.9% in the 2% PJ-001 group. These findings indicated that the inflammatory pathway involving TLR4/NF-κB was inhibited by PJ-001.

Effects of PJ-001 on Beclin 1 and LC3 in mouse skin tissue. As shown in Fig. 4A, F and G, compared with those in the control group, the expression levels of Beclin 1 and LC3 (LC3II/LC3I) were significantly increased in the model group (P<0.001). The protein expression levels of Beclin 1 in the compound dexamethasone group, crisaborole group, 4% PJ-001 group and 2% PJ-001 group were significantly lower than those in the model group (P<0.01). Specifically, the expression levels of Beclin 1 were decreased by 36.2% in the 4% PJ-001 group compared with that in the model group, whereas they were decreased by 22.0% in the 2% PJ-001 group. Furthermore, the expression levels of LC3 were significantly decreased in the crisaborole group and 2% PJ-001 group compared with those in the model group (P<0.01).

Effects of PJ-001 on the mRNA expression levels of K17 and FLG in mouse skin tissue. As shown in Fig. 5A and B, the mRNA expression levels of K17 in the AD model group

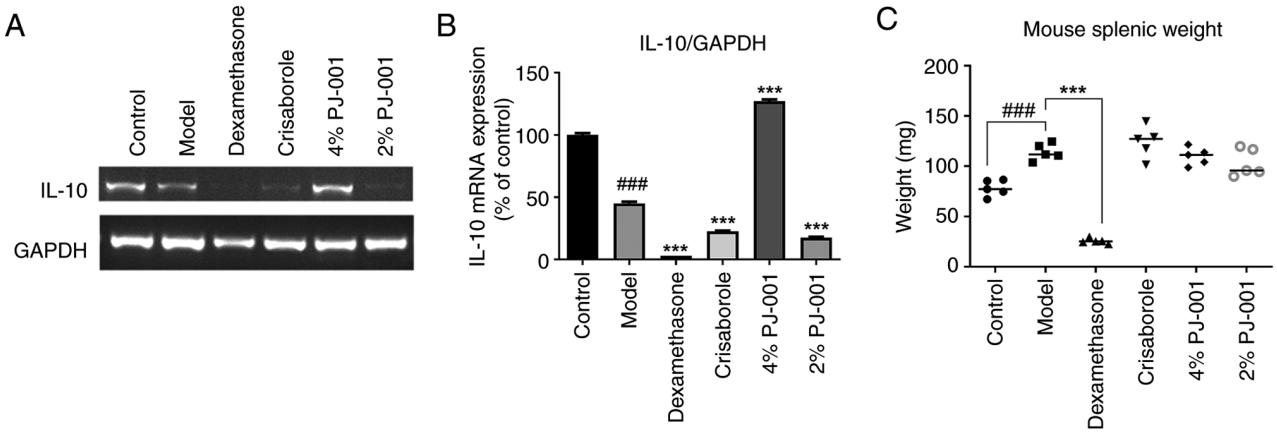

Figure 6. Effect of PJ-001 on the mRNA expression levels of IL-10 in mouse spleen tissue. (A) Reverse transcription-PCR was performed to detect IL-10 mRNA expression. (B) Semi-quantification of PCR data. GAPDH was used as a loading control. A minimum of three independent experiments were carried out, and data are presented as the mean ± SD. (C) Weight of mouse spleens (n=5). ###P<0.001 vs. Control; ***P<0.001 vs. Model. IL-10, interleukin-10.

exhibited a significant increase (P<0.001) compared with those in the control group. The positive controls, dexamethasone and crisaborole, demonstrated a notable suppressive effect on K17 mRNA (P<0.001). However, the PJ-001 treatment was ineffective in suppressing the expression and transcription of K17 (Figs. 4H and 5B). The mRNA expression levels of FLG in the AD model group were significantly reduced by PJ-001 group (P<0.001; Fig. 5A and C). By contrast, the groups treated with compound dexamethasone, 4% PJ-001 and 2% PJ-001 showed a significant increase in the expression levels of FLG (P<0.001). Specifically, the 4% PJ-001 group exhibited an 847% increase, whereas the 2% PJ-001 group showed a 911% increase (Fig. 5C).

Mouse splenic weight and IL-10 mRNA expression levels. As shown in Fig. 6C, splenic weight was significantly increased in the model group compared with that in the control group (P<0.001). However, the compound dexamethasone group exhibited a significant decrease in splenic weight (P<0.001). By contrast, there was no notable difference observed in the crisaborole group, 4% PJ-001 group and 2% PJ-001 group relative to the model group. Furthermore, the mRNA expression levels of IL-10 in the splenic tissues of mice in the model group were significantly lower than those in the control group (P<0.001; Fig. 6A and B). In the 4% PJ-001 group, the mRNA expression levels of IL-10 were significantly increased by 282.2% (P<0.001) compared with those in the model group. The compound dexamethasone group, crisaborole group and the 2% PJ-001 did not succeed in inducing IL-10 expression.

Discussion

The pathogenesis of AD is a complex process that involves various factors, including the external environment, genetic susceptibility, skin barrier dysfunction and immune response (4). Currently, there are several drugs available for treating AD, primarily systemic drugs such as topical corticosteroids and oral antihistamines; however, these drugs have limited efficacy and notable side effects. Compound dexamethasone ointment, a long-term glucocorticoid, has the ability to suppress the synthesis of inflammatory factors, reduce exudation, relieve itching, and exhibit anti-inflammatory and anti-allergic effects (22). However, as a hormone-based drug, prolonged usage can lead to skin atrophy, capillary dilation, pigmentation and secondary infections (23). Crisaborole ointment is a new small-molecule drug used to treat AD. Its main component, crisaborole, functions as a phosphodiesterase 4 inhibitor. Although this ointment has demonstrated high efficacy and safety, without causing skin atrophy, it exhibits a slow onset and does not provide immediate relief from itching, which may reduce medication compliance among patients with AD (24). To enhance treatment options for patients with AD, it is crucial to continue the development of drugs that provide faster onset and effective relief from itching, with fewer side effects.

In recent years, PROTAC drugs have advanced to the clinical research stage, representing a significant development from a conceptual standpoint. Unlike traditional small-molecule inhibitor drugs, PROTAC drugs utilize the ubiquitin-proteasome system to degrade target proteins, showing immense potential in targeting 'non-drug targets' (25). KT-474 is a potential first-in-class IRAK4 degrader that is being developed for the treatment of TLR/IL-1R-driven immune inflammatory diseases, such as AD (26). The utilization of PROTAC technology holds promise as a novel approach in drug development for the treatment of inflammatory diseases. PJ-001 is a degrader of JAK synthesized by our laboratory using PROTAC technology. The original purpose of the present study was to develop a PROTAC molecule with the potential to alleviate AD. To evaluate the effectiveness of the molecule, a comparison with clinically recommended drugs, namely dexamethasone and crisaborole, was performed. The severity of skin lesions in AD mice was assessed by comparing the number of scratches and severity scores among different groups. The results demonstrated that PJ-001 can effectively reduce the severity of skin lesions, particularly when used at a concentration of 2%. However, the results did not conform to the expected dose-effect relationship, as lower doses showed a more significant effect than higher doses. The potential explanation for this is that each drug has an upper limit, which is known as the 'hook effect' that PROTAC drugs need to overcome. When the concentration is higher than the DC_{50} value,

PROTAC molecules can saturate binding sites on either the target protein or the E3 ligase, inhibiting the formation of the required ternary complex and resulting in the generation of an unproductive binary complex. This results in loss of efficacy at higher concentrations (27). Additionally, analysis of mouse H&E pathological sections revealed that PJ-001 could reduce the thickness of the spinous layer in AD mice and alleviate excessive or incomplete epidermal keratinization. Notably, the application of 2% PJ-001 demonstrated a marked restoration of hair follicle structure, whereas 4% PJ-001 exhibited a substantial reduction in the infiltration of inflammatory cells. These findings provide direct evidence that PJ-001 effectively mitigates inflammatory infiltration, thereby improving skin itching and epidermal keratinization.

FLG, an essential component of the outer membrane of KCs, is produced during the process of KC differentiation. Its main function is to maintain epidermal homeostasis by facilitating the regular gathering of keratin filaments. Additionally, mutations or abnormal expression of FLG-related genes can result in a decrease in ceramides and antimicrobial peptides, while also causing an increase in water loss through the epidermis (3,28). Consequently, this leads to increased infiltration of environmental stimuli, allergens and microorganisms, ultimately contributing to the development of AD (29). The expression of FLG has been observed to decrease in the epidermis of patients diagnosed with AD (30). In the present study, a significant decrease in FLG mRNA expression was observed in the skin lesions of AD mice. However, following treatment with PJ-001, FLG mRNA expression was upregulated ~9 times compared with that in the model group. These findings suggested that PJ-001 has the ability to promote the expression of FLG and to enhance the resistance of the stratum corneum. As a result, it may effectively prevent the penetration and invasion of harmful substances and allergens.

The JAK/STAT pathway is a major signaling pathway regulated by cytokines; it serves a crucial role in priming innate immunity, coordinating adaptive immune mechanisms, and ultimately suppressing inflammation and immune responses (31). NF-κB is present in almost all animal cell types and participates in cell responses to various stimuli, such as stress, cytokines, free radicals, heavy metals, ultraviolet radiation, and bacterial or viral antigens. NF-κB is essential in regulating the immune response to infection (32). Both JAK/STAT and NF-κB pathways are connected through the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway. Receptors phosphorylated by JAKs recruit PI3K, which activates the PI3K/Akt pathway. This leads to the phosphorylation of inhibitor κB, enabling the activation of NF-κB. Once activated, NF-κB enters the nucleus, interacts with DNA, and initiates or inhibits the transcription of related genes (33). PJ-001 is a PROTAC molecule designed to target the JAK protein. The present study indicated that PJ-001 can effectively decrease JAK/STAT3 and TLR4/NF-κB protein expression to alleviate skin damage in AD mice. Moreover, PJ-001 induced a significant downregulation of the autophagy factors Beclin 1 and LC3, thereby inhibiting excessive autophagy in the inflammatory tissues.

IL-10, an important anti-inflammatory cytokine, has a controversial role in the pathogenesis of AD. A previous study detected high expression of IL-10 in the serum of patients with AD (34). However, Hussein *et al* (35) found no significant abnormality in the serum of IL-10 in patients with atopy. By contrast, Zhang *et al* (36) demonstrated that IL-10 levels in the supernatants of peripheral blood culture were lower in patients with severe AD compared with those in normal controls. In the present study, it was observed that 4% PJ-001 significantly increased the mRNA expression levels of IL-10 in the spleens of AD mice. We hypothesize that PJ-001 can inhibit JAK/STAT signaling, thereby modulating macrophage activation to suppress inflammatory diseases. This modulation may promote the repolarization of inflammatory (M1) macrophages into anti-inflammatory (M2) macrophages, increasing the transcription of IL-10 (37). However, evidence has suggested that IL-10 may have a dual role, as it can stimulate the immune response and potentially trigger diseases instead of suppressing them (38). In future studies, we aim to evaluate the molecular mechanism of PJ-001 in alleviating AD by inhibiting the JAK/STAT pathway using inhibitor interference. Additionally, we will utilize mature PROTACs for comparison to ensure a more comprehensive evaluation of our test compound.

In conclusion, PJ-001 can reduce the inflammatory response in a mouse model of AD by inhibiting the activation of inflammatory pathways. In addition, it may alleviate excessive autophagy in skin lesions and increase the expression of skin barrier factors. These findings suggested that targeted inhibition of the JAK2/STAT3 and TLR4/NF-κB signaling pathways represents a potential therapeutic approach for AD.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

PL and ZC drafted the final manuscript. PL, ZC and JL made substantial contributions to the conception and design of the study. PL and JL critically revised the manuscript and provided constructive feedback. YL and HS participated in animal experiments and assisted in interpreting data. PL and JL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was performed according to the guidelines laid out by the Ethics Committee for Animal Experiments of Jiangnan University (Wuxi, China) and was approved by the Ethics Committee of Experimental Animal Management

and Animal Welfare of Jiangnan University [approval no. JN. No20210930b0801218(353)].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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