Ethnic and Regional Medicine

Pseudolaric acid B extracted from the Chinese medicinal herb *Cortex Pseudolaricis* ameliorates DNFB-induced atopic dermatitis-like skin lesions in BALB/c mice

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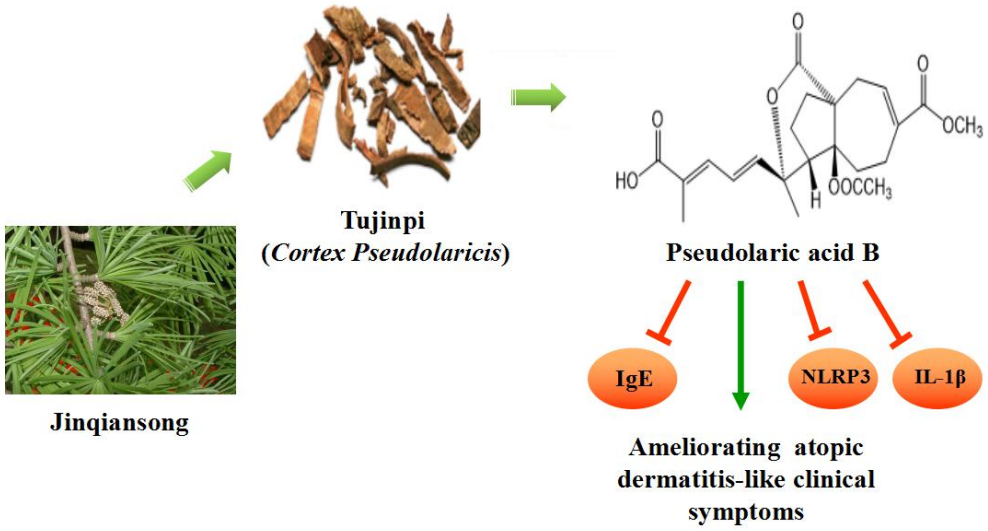
Highlights

Pseudolaric acid B significantly ameliorated the development of atopic dermatitis-like clinical symptoms and effectively suppressed the infiltration of inflammatory cells by inhibiting the expression of the NLRP3 inflammasome and IL-1β in skin lesions, and downregulating serum IgE levels.

Editor's Summary

Tujinpi (*Cortex Pseudolaricis*) is obtained from the root bark of the pine plant Jinqiansong, a rare tree that is only found in China. It was first recorded in *Bencaogangmushiyi* published in 1765 A.D. (Qing Dynasty of China).



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Abstract

Objective: Pseudolaric acid B (PB) is a newly identified diterpenoid isolated from Tujinpi (*Cortex Pseudolaricis*). In the present study, we aimed to explore the anti-inflammatory effects of PB on atopic dermatitis (AD), as well as the molecular mechanisms underlying its effects. **Methods:** BALB/c mice treated with 2,4-dinitrofluorobenzene were orally administered with PB (10 $mg \cdot kg^{-1} \cdot d^{-1}$). After evaluating the AD score, serum levels of IgE and the mRNA expression of NLRP3 inflammasome and IL-1β were measured by ELISA and qRT-PCR respectively. **Results:** The results showed that PB treatment significantly ameliorated the development of AD-like clinical symptoms and effectively suppressed the infiltration of inflammatory cells. Furthermore, PB inhibited the expression of NLRP3 inflammasome and IL-1β in skin lesions, and downregulated serum IgE levels. **Conclusion:** The anti-inflammatory properties of PB were demonstrated using the 2,4-dinitrofluorobenzene-induced mouse model of AD-like skin lesions. Our study highlighted the potential use of PB as a novel therapeutic agent for the treatment of inflammation-associated skin diseases.

Keywords: Pseudolaric acid B, Atopic dermatitis, Inflammation, NLRP3 inflammasome

摘要

目的：探讨取自土槿皮中的二萜酸土槿乙酸对特应性皮炎的抗炎免疫调节作用，并分析其作用机制。

方法：以二硝基氟苯刺激诱导 BALB/c 小鼠建立特应性皮炎动物模型，同时每日给予口服土槿乙酸干预（10 mg/kg/d），计算 SCORAD 评分，分别采用 ELISA 和 qRT-PCR 的方法检测血清 IgE 含量以及 NLRP3 炎症小体的基因表达。

结果：土槿乙酸能明显减轻特应性皮炎模型小鼠的临床症状，抑制皮损处炎症细胞浸润。此外，土槿乙酸还能显著下调皮损组织中 NLRP3 和 IL-1β mRNA 水平及血清 IgE 含量。

结论：土槿乙酸通过抗炎免疫调节作用抑制二硝基氟苯诱导的特应性皮炎，提示土槿乙酸可能成为治疗炎症相关疾病的新型候选化合物。

关键词：土槿乙酸；特应性皮炎；炎症反应；NLRP3 炎症小体

Abbreviations: TJP, Tujinpi (*Cortex Pseudolaricis*); PB, Pseudolaric acid B; AD, Atopic dermatitis; DNFB, 2,4-Dinitrofluorobenzene; IgE, Immunoglobulin E; IL, Interleukin; NLRP, Nod-like receptor family pyrin domains-containing protein.

Competing interests: Authors declare that they have no competing interests.

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Background

Atopic dermatitis (AD) is a chronic and relapsing skin disease that is histopathologically characterized by the infiltration of inflammatory cells. Skin inflammation in AD is partly caused by the complex interactions of altered keratinocytes with activated helper T cells, mast cells, and other immune cells, which ultimately lead to chronic inflammation and disruption of protective epidermal function and activities of antimicrobial proteins [1, 2]. To date, topical corticosteroids with strong anti-inflammatory properties are one of the most widely used therapies for AD treatment. However, efficacies of these therapeutic agents are limited because of long-term side effects, rebound phenomena, and intermittent recurrences [3, 4].

Tujinpi (*Cortex Pseudolaricis,* TJP), also called Shuisongpi and Jinshupi, is obtained from the root bark of the pine plant Jinqiansong, a rare tree that is only found in China. According to the records of Zhaoxuemin in *Bencaogangmushiyi* published in 1765 A.D. (Qing Dynasty of China), TJP is acrid taste, warm in nature and has toxicity. TJP relieves itching and eliminates insects, and has been used in traditional Chinese medicine for the treatment of eczema and fungal skin infections for centuries. Pseudolaric acid B (PB) is a bioactive ingredient isolated from the root bark of *Pseudolarix kaempferi Gordon* (Figure 1). Further analytical studies showed that PB exerted a wide range of pharmacological effects, including anti-inflammatory, anti-carcinogenesis, and anti-angiogenesis effects [5-7], suggesting the potential use of PB for the treatment of inflammatory skin disorders. However, further studies are required to determine whether PB can suppress the development of AD.

Several reports have shown that the BALB/c AD mouse model subjected to repeated epicutaneous administration with 2,4-dinitrofluorobenzene (DNFB) exhibited the symptoms of AD-like skin inflammation, such as epidermal thickening, dermal fibrosis, and accumulation of inflammatory cells [8, 9]. Therefore, in the present study, we aimed to explore the inhibitory effects of PB on the development of AD-like skin lesions using DNFB-induced BALB/c AD mouse model. Our findings provide useful information for the development of a new therapeutic target for AD.

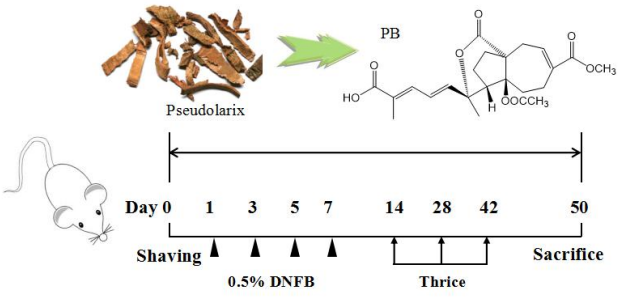

Figure 1 Treatment schedule of the atopic dermatitis model mice

Materials

PB, which was provided by Professor Chen, has the purity of more than 98% as determined by HPLC analysis. Prior to use, a stock solution of PB was prepared by dissolving in DMSO, after which the resulting solution was diluted with saline media (DMSO ≤ 0.5%) to obtain the indicated final concentrations. DNFB was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hematoxylin and eosin were purchased from Beyotime.

Animals

Male and female BALB/c mice aged 5-6 weeks and weighing between 18 g and 22 g were purchased from Vital River Laboratories Animal Center (Beijing, China). All experiments were approved by the Institutional Animal Care and Use Committee of Logistics University of the Chinese People's Armed Police Force and conducted in accordance to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Induction and assessment of AD

AD-like skin lesions were induced by topical application of DNFB [10]. One day before the experiment, depilatory creams were applied on the backs of 24 BALB/c mice with an area of about 4 cm^2. Mice were then randomly divided into the following three groups according to body weight (n = 8 mice per group): vehicle group, AD model group, and PB group. On day 1, the shaved dorsal skins of mice were topically sensitized with 200 μL of 0.5% DNFB dissolved in acetone/olive oil (4:1). On days 3, 5, and 7, mice were challenged with 0.5% DNFB on the same area of the dorsal skin. Finally, mice were treated with 0.5% DNFB on the shaved dorsal skins on day 14. The above procedure was repeated thrice until day 50 to generate the complete mouse model. The treatment group was orally administered with PB (10 $mg \cdot kg^{-1} \cdot d^{-1}$) during the experiment. On the other hand, mice in the control group were treated with an equal volume of vehicle (Figure 1).

Evaluation of AD-like skin lesions

Dermatitis was assessed macroscopically based on the following scoring procedure. The development of (i) dryness, (ii) erythema, (iii) edema, and (iv) excoriation was scored as either 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The total symptom severity score was defined as the sum of the individual scores.

Histological analysis

By the end of the study period, mice were sacrificed, and all dorsal skin samples were collected and subjected to histological analysis. Samples were fixed with 4% formaldehyde, embedded in paraffin, and cut into 4-μm sections. After dewaxing and dehydration, sections were stained with H&E and examined under a Motic digital microscope (OLYMPUS BX41). A minimum of three sections per experimental animal was examined for the presence and degree of inflammation in the epidermis using Image-Pro Plus V 5.0 software (Evolution VF,

Media Cybernetics). The total number of inflammatory cells was counted by a single observer unaware of the sample assignment of the individual sections as previously described [11].

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected, and plasma was obtained by centrifugation at 3000 rpm for 15 min. Serum levels of IgE were determined via sandwich ELISA using a commercially available kit according to the manufacturer's instructions (Shibukawa Co., Japan).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from skin tissues using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The purity of RNA samples was assessed by inspecting the 28S and 18S bands after 1.5% agarose gel electrophoresis; the 260/280 absorbance ratios ranged from 1.9 and 2.0. A total of 1 μg of RNA was transcribed into cDNA using a reverse transcription system (Promega, Madison, WI, USA). Amplification was performed using the following primers: NLRP3 sense, 5'-CCCTTGGAGACACAGGACTC-3' and antisense, 5'-GAGGCTGCAGTTGTCTAATTCC-3', β-actin sense, 5'-CTAAGGCCAACCGTGAAAAG-3' and antisense, 5'-ACCAGAGGCATACAGGGACA-3'. qRT-PCR with SYBR green detection was conducted using a QuantiFast SYBR® Green PCR Kit (Roche) on an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). The relative expression of target genes was normalized against β-actin. The fold change of each sample was calculated using the following formula: 2 - [Ct (target gene) - Ct (endogenous reference gene) of sample] - [Ct (target gene) - Ct (endogenous reference gene) of calibrate].

Statistical analysis

Data are presented as mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA). All analyses were performed using GraphPad Prism version 6.0 software (San Diego, CA, USA). Statistical significance was considered at $P < 0.05$.

Results

Evaluation of AD-like skin lesions

In this study, human AD-like symptoms were induced in BALB/c mice by repeated topical application of DNFB. After 4 days of sensitization, BALB/c mice began to show AD-like symptoms, including edema-like changes and skin hardening and thickening. Symptoms gradually worsened over time, resulting in increased exfoliation and erosion along with other more severe histopathological changes. On day 29, invasive erythema, rashes, erosion, and moss-like skin covered a larger area of the backs of the mice. Finally, festers found on the dorsal skin developed into scabs. Oral administration of 10 mg/kg PB significantly reduced the severity of AD-like lesions. Meanwhile, dermatitis severity was estimated based on the dermatitis score. The results showed that the AD scores in the AD model mice were considerably higher than those in the control mice. These were found to be significantly reduced by PB treatment (Figure 2A). Furthermore, we measured the serum levels of IgE, which were previously reported to be closely associated with clinical severity of AD. In the present study, repeated application of DNFB induced a significant elevation in serum IgE levels in the AD mice, but was evidently inhibited by treatment with PB (Figure 2B). No adverse effects associated with PB treatment were observed throughout the experimental period.

PB suppressed the infiltration of inflammatory cells

Consistent with previous findings, histological examination of the skin lesions of BALB/c mice revealed strong inflammatory reactions at the DNFB-challenged sites [12]. The severity of skin lesions was ameliorated by PB treatment. The degree of infiltration by neutrophils and eosinophils was also significantly lower in PB-treated mice than that in vehicle-treated mice (Figure 3).

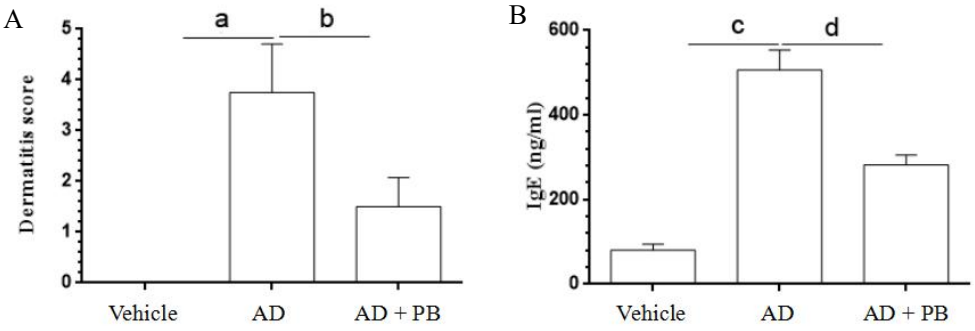

Figure 2 Effects of PB on clinical skin severity

A: Overall dermatitis score was calculated as the sum of all individual scores. B: Total serum concentrations of IgE. Data are presented as mean ± SD. A: a, AD vs. Vehicle $P < 0.001$; b, AD vs. AD + PB $P = 0.002$; B: c, AD vs. Vehicle $P < 0.001$; d, AD vs. AD + PB $P < 0.001$.

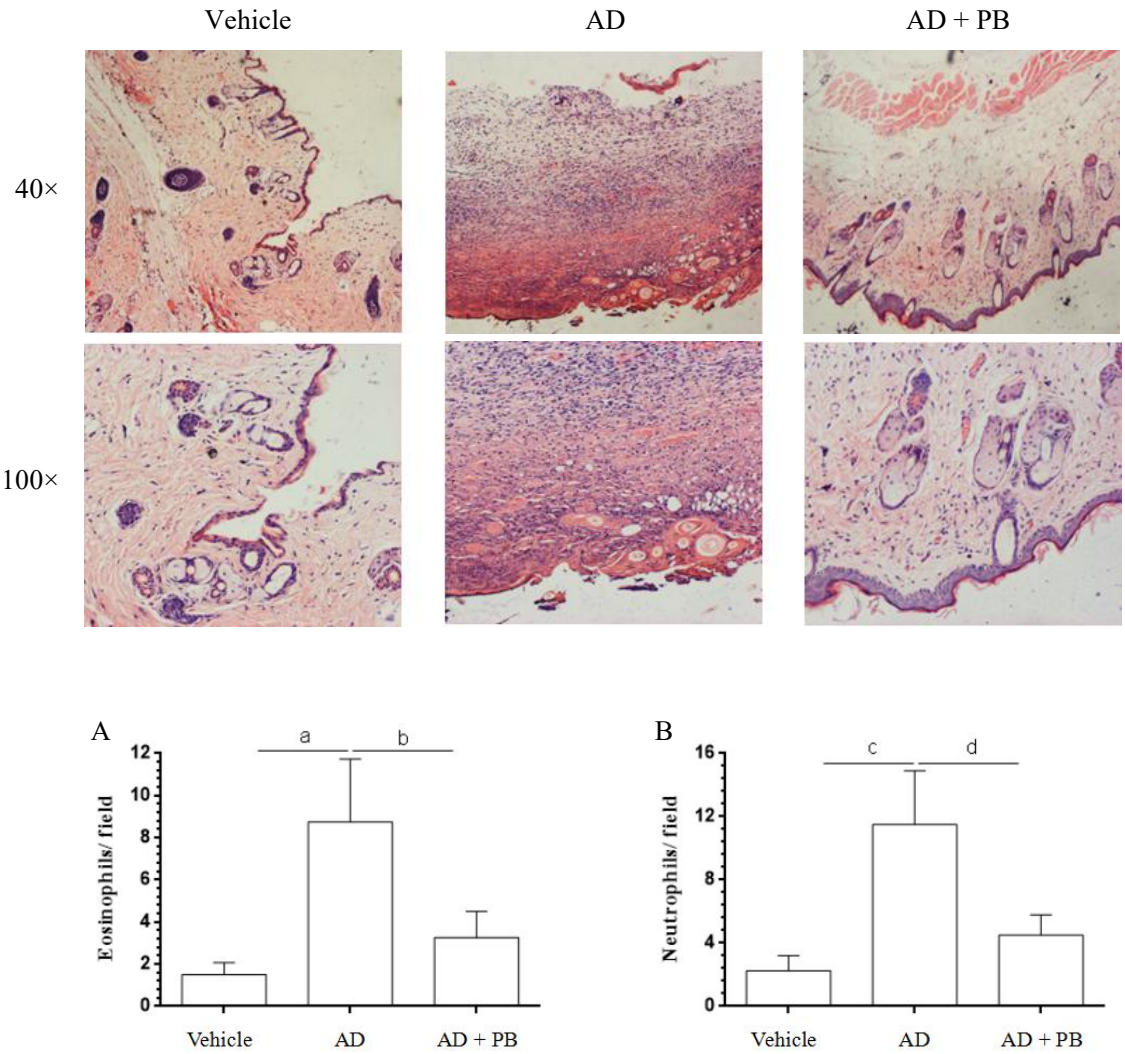

Figure 3 Inhibitory effects of PB on inflammation. Dorsal skin samples of BALB/c mice were stained with H&E to detect various inflammatory cells. Representative images showing comparison of AD-like skin lesions (40×, 100× magnification). Neutrophils are distinguished based on the presence of poly-segmented nuclei stained dark blue, whereas eosinophils have red granules that can be stained with eosin. The number of eosinophils and neutrophils were counted. Data are presented as mean ± SD. Eosinophils: a, AD vs. Vehicle $P = 0.001$; b, AD vs. AD + PB $P = 0.007$. Neutrophils: c, AD vs. Vehicle $P < 0.001$; d, AD vs. AD + PB $P = 0.004$.

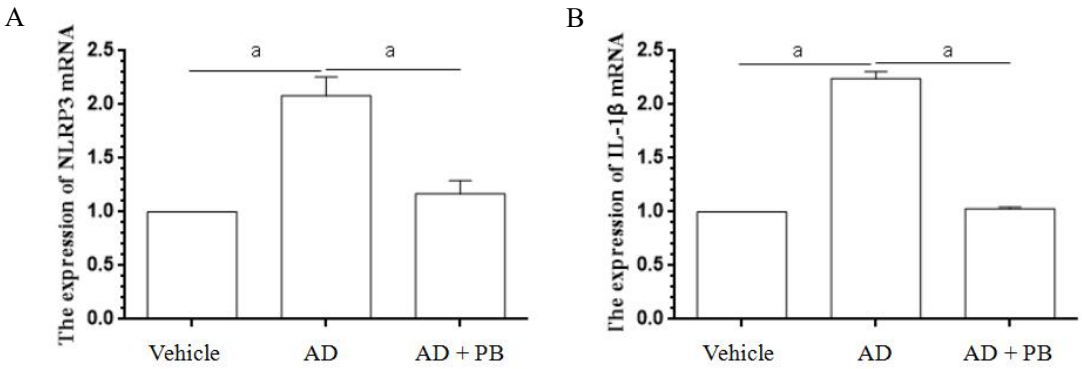

Figure 4 Effects of PB on the mRNA expression of NLRP3 inflammasome and IL-1β. Data are presented as mean ± SD. a, $P < 0.001$. NLRP3: AD vs. Vehicle $P < 0.001$; AD vs. AD + PB $P < 0.001$. IL-1β: AD vs. Vehicle $P < 0.001$; AD vs. AD + PB $P < 0.001$.

Effects of PB on the expression of NLRP3 inflammasome and IL-1β

Given that the NLRP3 inflammasome plays a critical role in mediating tissue damage during inflammation, we further analyzed the effects of PB on the NLRP3 inflammasome. The results revealed significant upregulation in the mRNA expression of NLRP3 and IL-1β in AD mice, which could be markedly reduced by PB treatment (Figure 4). The results also showed that PB treatment did not markedly alter the expression of apoptosis-associated speck-like protein and Caspase-1 (data not shown). Thus, PB could potentially inhibit the inflammatory response in AD by suppressing the expression of NLRP3 inflammasome and IL-1β.

Discussion

AD is multifactorial disease that is attributed to both genetic and environmental factors. AD presents various clinical features, such as chronic relapsing of skin inflammation and reduced skin barrier function and is predominantly characterized by impaired immune response [13]. For many years, medical treatment of AD has involved topical or systemic administration of corticosteroids. However, these steroids often produce adverse effects, such as skin atrophy, striae distensae, and perioral dermatitis in sensitive areas. Therefore, recent efforts have focused on the identification of novel therapeutic agents for long-term control of skin inflammation without triggering adverse side effects.

Several naturally occurring drugs have been reported to be effective for the treatment of AD. Thus, the anti-inflammatory activities of natural compounds with very low risks of eliciting adverse effects can be beneficial for suppressing the development of AD lesions [14, 15]. PB is one of the compounds that have been demonstrated to exert potent anti-inflammatory, antimicrobial, and anticarcinogenic activities. PB was also shown to inhibit lipopolysaccharide-induced the production of inflammatory cytokines in murine macrophages and to effectively attenuate contact hypersensitivity in vivo [16, 17]. To expand the applications of PB for the treatment of skin inflammatory disease, DNFB-induced AD model BALB/c mice were orally administered with PB, and the therapeutic efficacy of PB in inhibiting AD-like skin lesions was evaluated. In this study, treatment of AD mice with 10 $mg \cdot kg^{-1}$ PB significantly relieved AD-like clinical symptoms and the skin infiltration of inflammatory cells.

AD is generally associated with elevated serum IgE levels and infiltration with innate immune cells, as well as adaptive immune cells. Hyperproduction of IgE is considered a hallmark of AD in human patients and mouse models with AD-like skin lesions [18]. IgE binds to FceRI on the mast cell surface and induces degranulation and expression of proinflammatory mediators, which in turn trigger the formation of skin lesions. We observed that oral treatment with PB significantly suppressed extensive inflammatory cell infiltration and downregulated serum IgE levels in DNFB-treated BALB/c mice. These results suggested that PB might be an effective therapeutic agent for the treatment of AD.

High levels of proinflammatory cytokines are well known to initiate and maintain inflammation in AD and mediate cross-talk between the innate and adaptive immune systems. The recently described cytosolic receptors of the nucleotide-binding domain and leucine-rich repeat (NLR)-containing family of proteins have been shown to play key roles in inflammatory and immune responses after exposure to various danger signals [19]. The NLR family pyrin domain-containing proteins (NLRPs) are recognized for the formation of a multiprotein complex known as the “inflammasome”, which activates the proinflammatory cytokines IL-1β, IL-18, and IL-33. Accumulating evidence has suggested the crucial involvement of inflammasomes in skin inflammation [20]. In particular, research efforts have been focused on NLRP3 (also known as NALP3), which is associated with significantly increased risk of developing AD. The NLRP3 inflammasome consists of NLRP3, apoptosis-associated speck-like protein, and Caspase-1. The NLRP3 inflammasome induces excessive secretion of IL-1β and IL-18 from macrophages, monocytes, and mast cells [21]. The majority of the clinical features of AD are a direct consequence of Th2-skewed acquired immune responses (e.g. IL-4, IL-10); however, AD patients appear to develop a biphasic Th-cell pattern that is characterized by early Th2 cytokine production during the acute phase, followed by a switch to a more Th1-like profile (e.g. IL-1β, IL-2, TNF-α) during the chronic phase. Recent studies using mouse models further indicated the importance of IL-1β in the development of AD [22]. Therefore, this study was conducted to determine whether PB treatment would effectively inhibit AD through its effects on NLRP3. Our results revealed that DNFB contributed to the development of AD by activating NLRP3 and triggering IL-1β production in the epidermis, both of which were suppressed by PB treatment.

Taken together, our findings suggested that PB can ameliorate the clinical symptoms of AD-like skin lesions, reduce serum IgE levels, and repress inflammatory cell infiltration by inhibiting NLRP3 inflammasome, thereby ultimately preventing the progression of chronic skin inflammation. These findings highlighted the potential use of PB as an alternative therapeutic agent for the treatment of AD.

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