

Development of a new eczema-like reconstructed skin equivalent for testing child atopic dermatitis-relieving cosmetics

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Abstract

Objective: Atopic dermatitis (AD) is the most common chronic inflammatory skin disease, and it has serious effects on children's and families' quality of life. We aimed to screen and evaluate the efficacy of different formulas on relieving of atopic dermatitis clinical symptoms by developing an eczema-like reconstructed human skin equivalent in vitro.

Method: Some research has reported that thymic stromal lymphopoietin (TSLP) may be a potential therapeutic target for the treatment of AD. We developed an eczema-like in vitro skin equivalent by coculturing the cocktails polyinosinic-polycytidylic acid sodium salt (poly(I:C)) and lipopolysaccharides (LPS). The eczema-like skin equivalent was characterized by overexpression of TSLP and impaired skin barrier function. Three cosmetic formulas with the potential of anti-inflammation and skin barrier promotion were topically applied onto the eczema-like skin equivalent, mimicking in vivo application. The inhibitory effect on TSLP was examined by ELISA. Effects on tissue viability and skin barrier function were determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) method.

Conclusion: The results show that eczema-like skin equivalent induced by cocktails of poly(I:C) and LPS can mimic the skin characters of the atopic dermatitis. The cocktails can induce high TSLP expression, impaired cell viability, and skin barrier function. The cosmetic formulas with the potential of anti-inflammation and skin barrier promotion were evaluated to be helpful to decrease and relieve the impact of AD with the decreased TSLP and the higher tissue viability than the eczema-like skin equivalent without any cosmetic application. The eczema-like skin equivalent can be used to screen and evaluate formulas on AD relieving.

KEYWORDS

anti-inflammation, atopic dermatitis, eczema, reconstructed skin equivalent, skin barrier recovery, thymic stromal lymphopoietin

1 | INTRODUCTION

Atopic dermatitis (AD), also known as eczema, is a common chronic, relapsing, inflammatory skin disease. As one typical type of allergic skin disease, AD has been widely reported to develop in childhood, with a characteristic age-dependent distribution.¹ It generally develops in early childhood, and distributes more often on facial, scalp, and extensor involvement in infants and young children, and predominant flexural involvement in older children and adults. Understanding of the pathogenesis of AD is still poor, although it is clear that no single defect accounts for the array of clinical features associated with AD. Main pathophysiology reported refers to the following factors.

Genetic factor was estimated to be related to approximately 80% of AD.²⁻⁴ Many susceptibility loci enriched within the AD population are within or near genes critical to skin barrier function, innate immunity, and Th2-mediated inflammation, highlighting the importance of these pathways in AD pathogenesis.^{5,6} Damaging mutation in genes critical to normal barrier function of the skin has been shown to strongly segregate with early and severe forms of AD and ichthyosis. Mutations in filaggrin (FLG), desmoglein-1 (DSG1), corneodesmosin (CDSN), and peroxisome proliferator-activated receptors (PPARs), resulting in impaired barrier function, are always indicated by an increased transepidermal water loss (TEWL) and decreased water-binding capacity. Furthermore, dysregulation of genes encoding proteases or anti-proteases involved in the process of cornification, innate immune receptors, chemotactic factors, and antimicrobial proteins has been reported in AD skin.⁶

Imbalances in epidermal innate immunity may render AD skin more susceptible for bacterial and viral infections and may explain the higher rate of skin infections in AD skin compared with psoriasis.^{7,8} The high levels of IgE in the blood and the infiltration of eosinophils into the inflammatory skin tissue are the major pathophysiological characteristics for AD patients and are mainly attributable to Th2-type inflammatory events with a high secretion of Th2-type cytokines such as TSLP, IL-4, and IL-6.^{8,9} Genetic predispositions including "genetic immunology type" such as TSLP¹⁰ and IL-4 and "genetic barrier type" such as filaggrin along with environmental factors play roles in the development and resolving of AD.¹¹

A number of mechanistically distinct anti-AD therapies are available, including UVB, corticosteroids, and calcineurin inhibitors. Most of these drugs target the adaptive immune system rather than the epidermal keratinocytes or the interaction between them. To further study the pathological features and cosmetic or pharmacologic intervention of AD, there is a need for developing of skin models that mimic AD.

A study model for eczema was described by Engelhart,¹² in which lymphocytes were added into reconstructed human skin. It was reported that TNF- α and Th2 cytokines induced atopic dermatitis-like features on epidermal differentiation proteins and

stratum corneum lipids in human skin equivalents.¹³ In vitro reconstructed human skin equivalent was useful for biological studies and evaluation of novel therapeutic agents.¹⁴⁻¹⁸ Murine models are useful for understanding the molecular mechanisms and pathogenesis of AD, although the pathogenesis of inflammation is not truly identical to that of humans.^{19,20} Another major default of animal model is the ethical limitations for the use of animals in human research.

In this study, we used reconstructed human skin equivalents with addition of proinflammatory cytokines to screen effective actives and formulas targeting the epidermal keratinocytes and the interaction between the keratinocytes and the immune system, especially focusing on skin barrier function and specific inflammation cascade of AD.

2 | MATERIALS AND METHODS

2.1 | Culture of keratinocytes

Normal human keratinocytes were harvested from the foreskin of healthy donors (>12 years old) undergoing medical surgery. The skin tissue was collected with informed consent form and institutional approval. Primary cells were isolated as previously described by Rheinwald and Green.²¹ The primary keratinocyte culture medium was KSFM (Gibco/BRL). Cultures were incubated in a humidified, 5% CO₂ atmosphere at 37°C, and the medium was changed every 2 days. Keratinocytes were digested with trypsin-EDTA when reaching over 70% confluence and subcultured to passage 7 for the following preparation of reconstructed skin equivalents.

2.2 | Preparation of eczema-like skin equivalents

Keratinocytes were seeded into the culture insert ($\varnothing$ = 9 mm, NUNC) with a density of 3.2×10^5 per tissue, followed by culture in KSFM for 48 hours. The inserts were then elevated to the air-liquid interface with differentiation medium, which contained DMEM with hydrocortisone (0.4 μ g/mL), insulin (5 μ g/mL), ascorbic acid 2-phosphate (1 mmol/L), bovine pituitary gland extracts (BPE, 25 μ g/mL), and antibiotics. On the 10th day of air-liquid interface culture, the inflammatory cocktail, including 10 μ g/mL of poly(I:C) and 5 μ g/mL of LPS (Sigma), was supplemented into the culture medium, and the skin models were followed by culture for another 24 h to make eczema-like skin equivalents.

2.3 | Active application with eczema-like skin equivalents

At present, the clinical treatment of eczema is mainly achieved through anti-inflammation and skin barrier enhancement. Therefore, in order to verify the applicability of eczema-like skin equivalents and screen actives thereof, anti-inflammatory and skin barrier

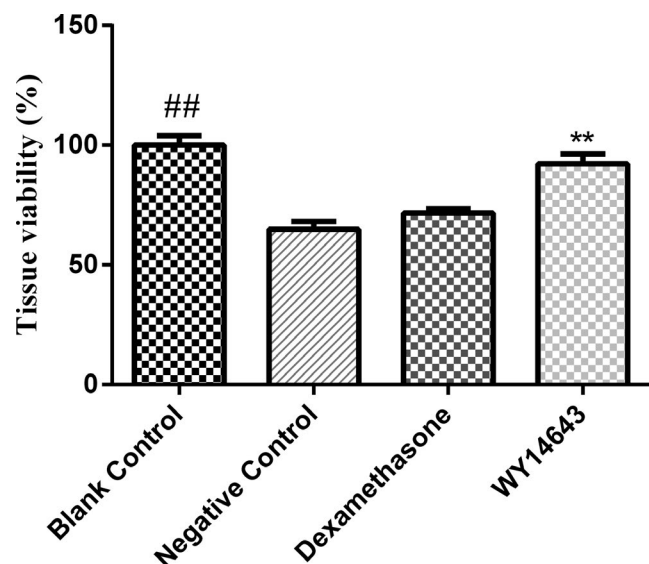


FIGURE 1 Verification of eczema-like reconstructed skin equivalents by tissue viability (## $P < 0.01$, compared with blank control; ** $P < 0.01$, compared with negative control)

promotive actives were selected, and their anti-inflammation and skin barrier promotion effects were evaluated *in vitro*. Actives were applied topically or systemically for 24 hours before the addition of poly(I:C) and LPS. Finally, skin equivalents and the underlying medium of different groups, including blank control, negative control (poly(I:C) & LPS only), and active groups (actives and poly(I:C)&LPS), were harvested to test tissue viability, ET_{50} , histology, and proinflammatory cytokines.

2.4 | Tissue viability

Tissue viability of each skin equivalent was assessed by the MTT test, according to a validated test protocol. Briefly, skin equivalents were placed in 0.3 mL of MTT solution at 1 mg/mL for 180 ± 10 minutes at standard culture conditions. During the culture time, the vital dye MTT can be reduced into a blue MTT formazan precipitate by the viable cells. The precipitated blue MTT formazan from each skin equivalent was extracted with 2 mL of isopropanol for 2 hours. By measuring changes in absorbance reading of formazan accumulation in the living reconstructed skin equivalents, tissue viability can be estimated. The tissue viability of the blank control served as the reference, of which was set to 100%.

2.5 | Determination of ET_{50}

After the final incubation, Triton X-100 with a concentration of 1% (V/V) was slowly added onto the skin equivalents surface, followed by exposure of 0 hour, 1 hour, and 3 hours in a humidified, 5% CO_2 atmosphere at $37^\circ C$. Then, tissue viability of each skin equivalent was assessed by the MTT test. The tissue viability result was profiled to exposure time, and the ET_{50} was calculated as the exposure time at which reduced the tissue viability to 50%.

2.6 | Histological analysis

Skin equivalents were fixed in 4% (v/v) formalin solution, dehydrated, and then embedded in paraffin. Six-micrometer-thick sections were cut and stained with hematoxylin and eosin (H&E) following the conventional staining procedure. At least three random photographs were taken of each skin equivalent and compared for histological change.

2.7 | Proinflammatory cytokine assay

Underlying medium of each skin equivalent was collected and stored at $-20^\circ C$ until test. Concentration of TSLP and IL-8 was quantified using human TSLP quantitative ELISA Kit (R&D) and human IL-8 quantitative ELISA Kit (R&D), respectively, according to the manufacturer's instructions.

2.8 | Statistical analysis

Results are expressed as mean $\pm$ SD. Statistical comparison was performed by one-way analysis of variance (ANOVA) with repeated measures. Difference was considered as statistically significant at $P < 0.05$.

3 | RESULTS

3.1 | Verification of eczema-like skin equivalents

In order to verify the applicability of the eczema-like skin equivalents, anti-inflammatory and skin barrier promotive actives were selected, and their anti-inflammation and skin barrier promotion effects were tested on the eczema-like skin equivalents *in vitro*. Tissue viability results revealed that skin equivalents insulted by Poly(I:C) & LPS resulted in significant decrease of tissue viability as compared to the normal skin equivalents without Poly(I:C) & LPS application (Figure 1). However, the tissue viability of skin model treated with anti-inflammatory active dexamethasone and skin barrier promotive active WY14643 was increased significantly compared with the negative control (Figure 1).

3.2 | Application of cosmetic formulas improved the tissue viability and histology of eczema-like skin equivalents

To evaluate the efficacy of eczema-relieving products, we prepared the eczema-like skin equivalents by coculture with Poly(I:C) & LPS mixture for 24 hours, starting from the 10th day of normal air-liquid interface culture. After induced by Poly(I:C)&LPS for 24 hours, cosmetic formulas were separately applied onto the surface of skin equivalents. The dosage of each formula was 25- μ L per tissue, and the dosing time was maintained to be 24 hours. Results in Figure 2 showed that each formula significantly improved the tissue viability

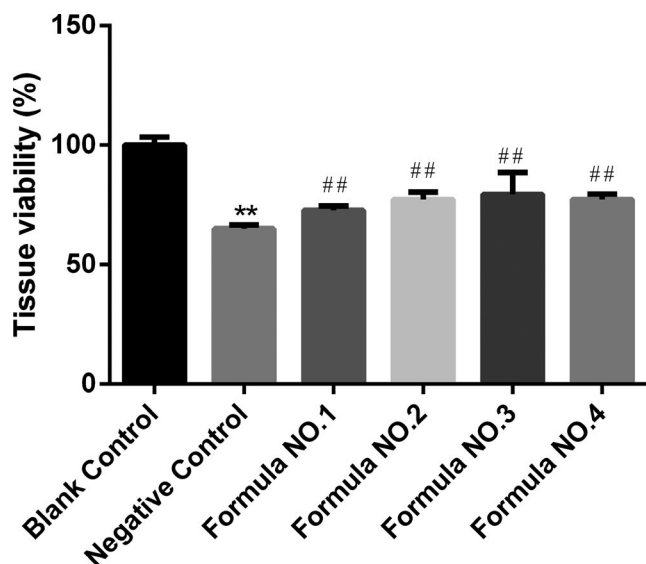


FIGURE 2 Tissue viability under different treatments: BC, blank control; NC: negative control (** $P < 0.01$, compared with blank control; # $P < 0.05$, compared with negative group; ## $P < 0.01$, compared with negative control)

as compared to the negative control. This may be explained by the adding of anti-inflammatory and skin barrier promotive actives in the formulas, which relieved the tissue damage caused by Poly(I:C) & LPS mixture. At the same time, we examined the morphology of skin equivalents of the negative control and the formulas tested. The H&E results showed that skin equivalents induced by Poly(I:C)&LPS exhibited an obviously disturbed development of the epidermal layers, and a number of impaired cells with dark cytoplasm and pyknotic nuclei were identified in the viable tissue layers (Figure 3). Compared with the negative control, application of cosmetic formulas improved tissue structure, with less impaired cells and thicker stratum corneum. The NO.3 formula presented the best epidermal

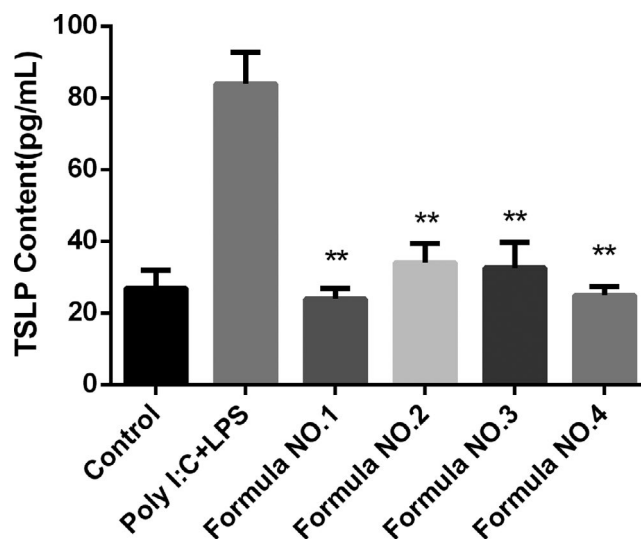


FIGURE 4 The skin barrier function promotion of different formulas under the inflammatory cocktail exposure

structure (Figure 3E), as compared to other formulas. The results of H&E staining were consistent with the results of tissue viability. In addition, formula NO.2 and NO.3 had thicker stratum corneum than the formula NO.1 and NO.4. Based on the composition of the formulas, these varied results may be relative to the addition of bisabolol and N-palmitoyl hydroxyproline cetacean wax ester in formula NO.2 and NO.3.

3.3 | Application of cosmetic formulas recovers the skin barrier function of eczema-like skin equivalents

The impaired epidermal barrier function always accompanied with the atopic dermatitis. Mutations in filaggrin (FLG), desmoglein-1 (DSG1), corneodesmosin (CDSN), and serine protease inhibitor Kazal-type 5 (SPINK5) all played important role in atopic dermatitis

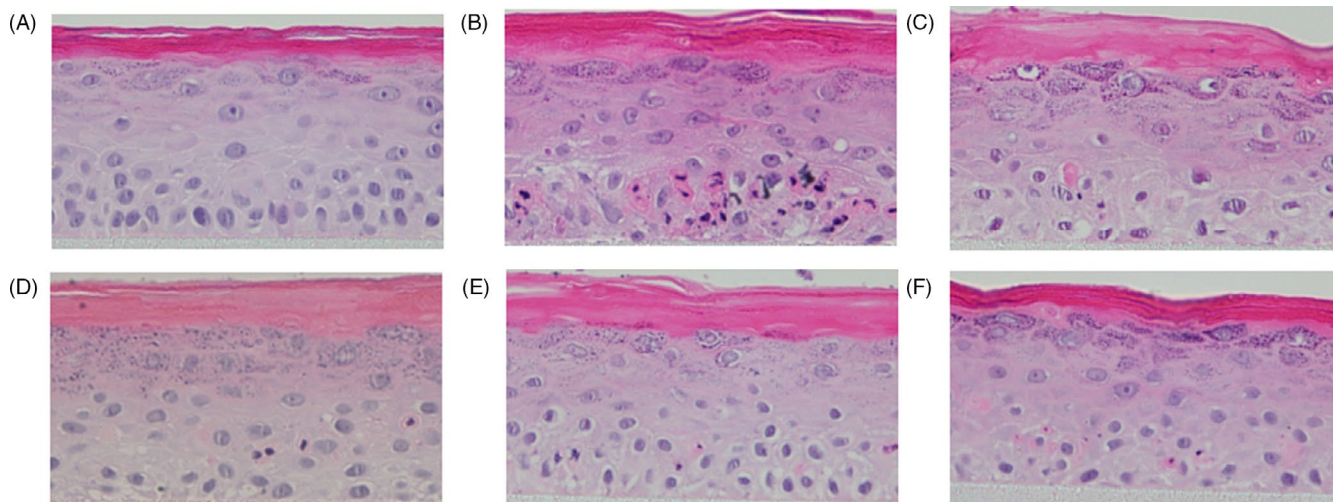


FIGURE 3 The histology of skin equivalent treated with test sample after being induced by inflammatory cocktail ($\times 20$ magnification) A, Sections of skin equivalents cultured in the absence of the inflammatory cocktail (blank control); B, sections of skin equivalents cultured in the presence of the inflammatory cocktail (negative control); C, formula NO.1; D, formula NO.2; E, formula NO.3; F, formula NO.4

pathogenesis.⁴ In this study, we evaluated the effects of different cosmetic formulas on skin barrier recovery by testing the tissue viability after exposure to 1% Triton X-100 for 0 hour, 1 hour, and 3 hours. The tissue viability result was profiled to exposure time, and the ET_{50} was calculated as the exposure time at which reduced the tissue viability to 50%. ET_{50} was positively correlated with the skin barrier function. The results shown in Figure 4 implied that the formula NO.2 can improve the skin barrier function. Other formulas did not show the efficacy of skin barrier recovery compared with the negative control. The increased ET_{50} value illustrated that actives in the formula NO.2 decreased the damage of Triton X-100 to the stratum corneum and viable tissue layers. These results could be attributed to the addition of N-palmitoyl hydroxyproline cetacean wax ester and allantoin. They were all widely used as skin conditioning agents in cosmetics. Although these two actives were also added into the formula NO.3, it did not present the same trend of ET_{50} value. This may be because of the different additional concentration. In formula NO.3, higher content of N-palmitoyl hydroxyproline cetacean wax ester was used. In formula NO.1 and formula NO.4, allantoin was the only active with the skin barrier recovery potential. So, appropriate active concentration and combinational strategy in formula design were responsible to the final efficacy.

3.4 | Application of cosmetic formulas inhibits TSLP expression of eczema-like skin equivalent

In order to evaluate the effect of cosmetic formulas application on the expression of proinflammatory factors in vitro, we analyzed TSLP content by ELISA. As shown in Figure 5, four cosmetic formulas had significant effect on the decline of TSLP secretion. The results illustrated that both bisabolol and dipotassium glycyrrhizinate can inhibit the release of TSLP by keratinocytes stimulated by Poly(I:C)&LPS. It is reported that bisabolol can reduce proinflammatory cytokine production such as TNF- α and ameliorate skin inflammation.²² The

expression of TSLP was decreased and could affect the activation of Langerhans cells and alleviate the release of Th2 cytokines.

4 | DISCUSSION

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease and affected around 15%-25% of children.^{7,23,24} It is a complex disorder and strongly associated with several comorbidities, including food, asthma, and allergic sensitization.^{25,26} It has a serious effect on children's and families' quality of life—for example, through sleep disturbance and a negative impact on schooling.^{7,23,24} To improve quality of life, the effective strategy on treatment was restoring barrier function, controlling inflammation, and using moisturizers to keep hydration. In this study, we designed cosmetic formulas target the atopic dermatitis by adding actives of anti-inflammation, hydration, and skin barrier promotion. Then, we assessed their efficacy by using eczema-like reconstructed skin equivalent in vitro.

TSLP was produced in response to both environmental and endogenous factors, including microbes and viruses, allergen sources, cigarette smoke, cytokine, chemicals, IgE, barrier injury, and disturbed epidermal homeostasis.^{7,23} The production of TSLP was also regarded as key initiatory event. Its expression was always regulated by cytokines. Proinflammatory cytokines (TNF- α or IL-1 α) with Th2 cytokines (IL-4 or IL-13) acted synergistically to induce TSLP production in human skin explants.²⁷ And it has been reported that primary human KCs²⁸⁻³⁰ release TSLP in response to poly(I:C). We cultured reconstructed skin equivalents in vitro by adding proinflammatory factors, poly(I:C)&LPS, and the skin equivalents exhibited characteristic AD-associated features of inflammation. Firstly, treatment of skin equivalent with poly(I:C)&LPS altered the epidermal morphology similarly to observations in other in vitro AD-like models and in patients with AD.²² Secondly, keratinocytes of skin equivalents treated with poly(I:C)&LPS secreted higher TSLP level than normal skin equivalents. The TSLP is produced in large amount by keratinocytes from the lesional skin of AD patients. Therefore, our skin equivalents treated with poly(I:C)&LPS could mimic an immune response and could be used as an interesting tool for screening actives which would be helpful to decrease the incidence of AD, especially in children.

With the aid of the three-dimensional reconstructed skin equivalents, we studied the inhibition of proinflammatory factors, improvement of skin barrier function, and tissue viability of the cosmetic formulas or creams. The inhibition of the TSLP secretion of the three investigational formulas all displayed significant effect. This could be attributed to the effect of anti-inflammatory components. α -(-)-bisabolol is one kind of natural monocyclic sesquiterpene and currently used in various cosmetic formulas. It can reduce proinflammatory cytokines production such as TNF- α and ameliorate skin inflammation.²⁸ Formula NO.2 which contained higher skin lipid analogues showed best efficacy on improving the skin barrier function, from both of the tissue viability and the H&E results. Anti-inflammation of different formulas keeps in line with the skin barrier recovery. The phenomenon may be relative to the effect of formula NO.2 on restoring skin

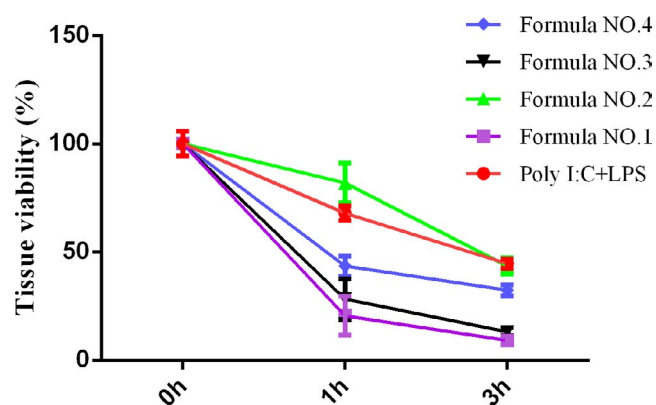


FIGURE 5 The inflammatory cocktail induces the secretion of inflammatory factors by reconstructed skin equivalent. (a) Thymic stromal lymphopoietin (TSLP) secretion by keratinocytes was assessed in normal and each experiment groups' undernatsants. Results are the mean $\pm$ SD of three different skin equivalents tested. * $P < 0.05$, ** $P < 0.01$, according to Student's t test

barrier dysfunction. Skin barrier dysfunction is a critical driving force in the initiation and exacerbation of AD.³¹ Skin barrier improvement can also inhibit TSLP secretion by enhancing ability of anti-irritant and reducing the damage exerted by insults. It can be concluded that skin equivalents treated with proinflammatory factors poly(I:C)&LPS could be used as an effective tool for screening actives which would be helpful to decrease the TSLP, a potential therapeutic target for the treatment of AD.³⁰ The formulas prepared containing anti-inflammatory and skin barrier recovery actives showed statistically significant better efficacy for the treatment of AD. This conclusion was further validated by our clinical studies.

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