



Polyozellin alleviates atopic dermatitis-like inflammatory and pruritic responses in activated keratinocytes and mast cells

Na-Hee Jeong^a, Soyoung Lee^b, Jin Kyeong Choi^c, Young-Ae Choi^a, Min-Jong Kim^a,
Hyun-Shik Lee^d, Tae-Yong Shin^e, Yong Hyun Jang^{f,*}, Kyung-Sik Song^{g,*}, Sang-Hyun Kim^{a,*}

^a Cell & Matrix Research Institute, Department of Pharmacology, School of Medicine, Kyungpook National University, Daegu, Republic of Korea

^b Immunoregulatory Materials Research Center, Korea Research Institute of Bioscience and Biotechnology, Jeongseup, Republic of Korea

^c Molecular Immunology Section, Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, MD, USA

^d School of Life Sciences, College of Natural Sciences, Kyungpook National University, Daegu, Republic of Korea

^e College of Pharmacy, Woosuk University, Jeonju, Republic of Korea

^f Department of Dermatology, School of Medicine, Kyungpook National University, Daegu, Republic of Korea

^g GHAM BioPharm Co. Ltd., College of Pharmacy, Kyungpook National University, Daegu, Republic of Korea



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ABSTRACT

Polyozellus multiplex is an edible mushroom that offers beneficial pharmacological effects against intestinal inflammation and cancer. Previous studies have demonstrated that polyozellin, a major component of *P. multiplex*, has therapeutic activities against inflammation, cancer, and oxidative stress-related disorders. This study aimed to determine the pharmacological effects of polyozellin on inflammatory and pruritic responses, the major symptoms of atopic dermatitis (AD), and to define its underlying mechanism of action. Our results showed that polyozellin inhibited the expression of inflammatory cytokines and chemokines through blockade of signal transducer and activator of transcription 1 and nuclear factor- κ B in activated keratinocytes, the major cells involved in AD progression. Based on the histological and immunological analyses, oral treatment with polyozellin attenuated the *Dermatophagoides farinae* extract (DFE)/2,4-dinitrochlorobenzene (DNCB)-induced atopic inflammatory symptoms in the skin. Pruritus is an unpleasant sensation for AD patients that causes scratching behavior and ultimately exacerbates the severity of AD. To find a possible explanation for the anti-pruritic effects of polyozellin, we investigated its effects on mast cells and mast cell-derived histamines. Oral treatment with polyozellin reduced the DFE/DNCB-induced tissue infiltration of mast cells, the serum histamine levels, and the histaminergic scratching behaviors. Additionally, polyozellin decreased the immunoglobulin E-stimulated degranulation of mast cells. Taken together, the findings of this study provide us with novel insights into the potential pharmacological targets of polyozellin for treating AD by inhibiting the inflammatory and pruritic responses.

1. Introduction

Atopic dermatitis (AD) is a chronic, inflammatory, and highly pruritic skin disease characterized by the infiltration of immune cells, including mast cells and eosinophils [1]. AD has become a major public health problem which affects up to 25% of all children and 3% of adults worldwide [2]. Additionally, AD can eventually lead to asthma and allergic rhinitis in the majority of patients [3]. A variety of mechanisms are considered to be involved in AD including genetic backgrounds, environmental factors, destruction of the epidermal structure, and abnormal immune responses; however, the pathogenesis of AD is still not completely understood [4].

Studies investigating the mechanisms of AD have focused on the imbalance in the different types of T helper (Th) cells during immune response [5]. AD is initially associated with Th2 cells and the skin lesions are associated with the activation of Th1, Th2, and Th17 cells [6]. Acute skin lesions are characterized by intensely pruritic erythematous papules, hyperkeratosis, increased immunoglobulin (Ig) E levels, and Th2-mediated abnormal inflammatory responses. The chronic phases of AD are characterized by lichenification, parakeratosis, and a predominantly Th1 response [5]. T cell imbalances are prominent in the skin of patients with AD and keratinocytes represent more than 95% of the skin cells [7]. Moreover, the keratinocytes form a functional skin barrier and provide the first line of defense against the invasion of

* Corresponding authors.

E-mail addresses: yhjang@knu.ac.kr (Y.H. Jang), kssong@knu.ac.kr (K.-S. Song), shkim72@knu.ac.kr (S.-H. Kim).

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pathogen and allergens by releasing inflammatory cytokines [8]. T cell-related cytokines and chemokines affect inflammatory responses; therefore, their inhibition can aid in the development of a useful therapeutic strategy for AD [6].

Pruritus is one of the most important symptoms of inflammatory skin diseases and allergic disorders, specifically AD. However, the mediators of the pruritic symptoms in AD patients have not yet been identified [9]. Numerous studies have shown that IgE-mediated mast cell activation and the mast cell-derived inflammatory mediators contribute to the pathophysiology of itching [10,11]. In addition, increased mast cells have been found to penetrate the epidermis and dermis of skin lesions [11]. During the pathogenesis of AD, mast cells are the major effector cells and express high-affinity receptors for IgE, FcεRI [1]. In response to IgE, the mast cells release preformed and *de novo* synthesized inflammatory mediators (e.g., histamine, tryptase, and cytokines) and trigger the allergic response [11]. In particular, histamine from mast cells is a potent inducer of pruritus in AD and histamine-derived itching is considered to be essential for the diagnosis of AD [10,12]. Overall, these findings demonstrate the importance of histamine as a marker of mast cell degranulation and pruritus induction during the generation or deterioration of itching in AD [12]. Although antihistamines are frequently used, they are not sufficient to improve the itch-related allergic symptoms [13].

Aeroallergens, including house dust mite (HDM), are recognized as the most common cause of allergies [14,15]. *Dermatophagoides farinae* extract (DFE) is a main species of HDM, and its body and feces are known environmental allergens. Various studies have reported that DFE causes atopic skin inflammation [14,16]. Previously, we have demonstrated that the application of DFE/2,4-dinitrochlorobenzene (DNCB) induced atopic skin inflammation in BALB/c mice [17] and this model was used in the presented study.

Until recently, corticosteroids, calcineurin inhibitors, as well as antihistamines, have been widely used for the treatment of AD, but their continued use can cause adverse effects such as skin thinning, osteoporosis, and emotional lability [18]. Therefore, further studies are required to develop safer and more effective AD therapies [19]. *Polyozellus multiplex* (Thelephoraceae) is an edible wild mushroom species, which is usually harvested as a medicinal mushroom in North America and Asia [20]. The chemical components of *P. multiplex*, including polyozellin (6,12-diacetoxy-2,3,8,9-tetrahydroxybenzo[1,2-b;4,5-b']bis-benzofuran), thelephoric acid, polyozellic acid, and kynapcin-9, -12, -13, -24, and -28 have been isolated and characterized [21,22]. Polyozellin has been shown to exert beneficial effects towards the prevention of various conditions such as inflammation, cancer, and oxidative stress-related disorders [20,23,24]. This study used the DFE/DNCB mouse model to determine whether polyozellin is effective against atopic skin inflammation caused by DFE and focused on its inhibitory effects against the inflammatory and pruritic responses in AD.

2. Materials and methods

2.1. Extraction, isolation, and identification

Polyozellin was isolated from *P. multiplex* as previously described and identified by Professor KS Song, College of Pharmacy, Kyungpook National University, Daegu, Republic of Korea [22,25]. Briefly, the fresh fruiting body of the mushroom (3 kg) was refluxed in methanol for 3 h at 70 °C. The extract was dried and evaporated, and the residue was successively portioned with benzene, chloroform, and ethyl acetate. The soluble ethyl acetate fraction was suspended in methanol, and the suspension was then centrifuged at 3 kg for 5 min. Next, the supernatant was enriched *in vacuo*. HPLC on the Senshu Pak ODS column (methanol:water = 65:10 containing 1% acetic acid) provided polyozellin as a dark green powder (243 mg). The yield of dried extract from starting crude materials was about 0.0081%. The purity of the isolated polyozellin was more than 95% according to HPLC analysis using UV

254 nm [Kintex C18 (5 u, 250 × 4.6 mm), 0–100% methanol containing 1% acetic acid for 30 min, 1 ml/min].

2.2. Reagents and cell culture

HaCaT and RBL-2H3 were maintained at 37 °C in 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin G, 100 µg/ml streptomycin (Gibco, Grand Island, NY). Recombinant tumor necrosis factor (TNF)-α and interferon (IFN)-γ were purchased from R&D systems (Minneapolis, MN). Anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), and o-phthalaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Cell passages 2–7 were used in the study. For the induction of atopic skin inflammation, DFE (Greer Laboratories, Lenoir, NC) and DNCB were used as an antigen and hapten, respectively. All other reagents were purchased from Sigma-Aldrich unless otherwise noted. DFE containing 0.5% Tween 20 was dissolved in phosphate buffered saline (PBS). DNCB (1%) was freshly dissolved in a 1:3 v/v mixture of acetone/olive oil solution.

2.3. Quantitative PCR (qPCR)

Total RNA was isolated from the HaCaT and mouse ear tissues using the RNAiso Plus kit (Takara Bio, Shiga, Japan). HaCaT (2 × 10⁵ cells/well in a 24-well plate) were pretreated with or without polyozellin (0.1, 1, or 10 µM) or dexamethasone (Dexa, 10 µM) for 1 h before being stimulated with TNF-α and IFN-γ at the same concentration (10 ng/ml) for 6 h. The mouse ears (total n = 30) were prepared at the end of the *in vivo* experimental period and homogenized using an RNAiso Plus kit. Subsequently, first-strand cDNA was obtained using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) and qPCR was carried out using the Thermal Cycler Dice TP850 and SYBR Premix Ex Taq (Takara Bio). The conditions for PCR were similar to the ones described in previous research [17]. The mRNA expression levels were normalized to the GAPDH levels *in vitro* and β-actin levels *in vivo* of each sample.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The IL-6 and CCL17 levels in HaCaT were quantified by a specific ELISA kit (R&D Systems). HaCaT (2 × 10⁵ cells/well in a 24-well plate) were pretreated with or without polyozellin (0.1, 1, or 10 µM) or Dexa (10 µM) for 1 h, and then stimulated with TNF-α and IFN-γ at the same concentration (10 ng/ml) for 15 h. These cultured cells were collected and centrifuged at 400g for 5 min. The cytokine levels were analyzed according to the manufacturer's protocol and the standard curves were constructed using the standard samples.

2.5. Western blot

HaCaT (1 × 10⁶ cells/well in a 6-well plate) were pretreated with or without polyozellin or Dexa at the same concentration (10 µM) for 1 h and then treated with TNF-α and IFN-γ at the same concentration (10 ng/ml) for 20 min to activate signal transducer and activator of transcription 1 (STAT1) and nuclear factor (NF)-κB [26]. Equal volumes of the whole-cell lysates were separated on 10% SDS-PAGE gel electrophoresis and then analyzed with the following primary antibodies: STAT1, phospho-STAT1 (Cell Signaling, Beverly, MA), IκBα, nucleus p65 NF-κB, β-actin, and lamin B1 antibodies (Santa Cruz, CA). The immunoreactive bands were visualized using an enhanced chemiluminescent substrate (Thermo Fisher Scientific).

2.6. Animals

Female BALB/c mice (18–20 g, 5 weeks of age) were purchased from Dae-Han Experimental Animal Center (Daejeon, Korea) and

housed under conditions of controlled temperature $22 \pm 2^\circ\text{C}$, humidity $55 \pm 5\%$, and a 12 h light/dark cycle throughout the experiment and maintained for one week before starting the experiment. All mice procedures were performed in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals, and the study was approved by the Institutional Animal Care and Use Committee at Kyungpook National University (IRB # 2017-0007).

2.7. Induction of atopic skin inflammation

The DFE/DNCB-induced AD model was established by repetitively painting the ears of the mice with DFE and DNCB as described previously [17]. The BALB/c mice were randomized into six groups ($n = 5/\text{group}$, total 30 mice): vehicle (control), DFE/DNCB plus vehicle (AD), DFE/DNCB plus polyozellin (0.1, 1, or 10 mg/kg) or Dexamethasone (1 mg/kg). The surfaces of both ear lobes were stripped five times with surgical tape (Nichiban, Tokyo, Japan). After stripping, each ear was painted with 20 μl of DFE (10 mg/ml) or 1% DNCB repeated once a week for 4 weeks. Vehicle (PBS), polyozellin or Dexamethasone were orally administered until the end of the 4 weeks induction period (6 times/week). Ear swelling was measured 24 h after DFE or DNCB application using a micrometer (Mitutoyo, Co., Tokyo, Japan). We counted scratching for 5 min on the last day of AD induction. Scratching behavior tests were defined as hind limb scratches directed to the ears. After sacrifice, the blood samples were collected from the celiac arteries of mice. Ear tissues of the mice were removed and subjected to histopathological analysis and RNA extraction.

2.8. Histological analysis

The excised mouse ears were fixed with 10% formaldehyde, embedded in paraffin, and then sliced into 5 μm sections. The samples were stained with hematoxylin and eosin (H&E) and toluidine blue (TB). After H&E staining, infiltrated lymphocytes, epidermal thickening, and dermal fibrosis were observed by microscopy, as described previously [17]. After TB staining, the mast cell infiltration was measured. Eosinophils and mast cells were counted in a blind manner (total magnification: $\times 400$). The dermal and epidermal thickness were analyzed in the H&E-stained sections under a magnification of $\times 200$. Five randomized histological fields were considered for each section of each mouse.

2.9. Measurement of serum IgE and IgG2a levels

The blood samples were centrifuged at 400g for 15 min at 4°C and then the serum was collected. The serum collected from the whole blood was kept at -80°C until further use. Serum IgE (total IgE and DFE-specific IgE) and IgG2a levels were measured using a specific ELISA kit (BD Biosciences, San Diego, CA) according to the manufacturer's protocol. The concentrations were estimated using a standard sample from the manufacturer's set. For the detection of DFE-specific IgE, 20 $\mu\text{g}/\text{ml}$ mite extract in PBS was used as a coating solution. The DFE-specific IgE level was indicated by the O.D. value.

2.10. β -hexosaminidase assay

RBL-2H3 (5×10^5 cells/well in a 12-well plate) were primed with anti-DNP IgE (50 ng/ml) overnight [27]. The primed cells were pretreated with or without polyozellin (0.1, 1, or 10 μM) or Dexamethasone (10 μM) for 1 h, and then challenged with DNP-HSA (100 ng/ml) for 1 h. The cells were collected and then centrifuged at 150g for 5 min at 4°C . Subsequently, each supernatant was incubated with the substrate (1 mM 4-nitrophenyl-N-acetyl- β -D-glucosaminide in 0.1 M citrate buffer, pH 4.5) for 1 h. The reaction was terminated by a stop solution (0.1 M Na_2CO_3 -NaHCO₃). The absorbance was measured using a

spectrophotometer (Molecular Devices, Sunnyvale, CA) at 405 nm.

2.11. Histamine assay

The histamine levels were measured in cell culture medium and serum. RBL-2H3 (5×10^5 cells/well in a 12-well plate) primed with anti-DNP IgE (50 ng/ml) were incubated with polyozellin or Dexamethasone for 1 h and then stimulated with DNP-HSA (100 ng/ml) for 4 h. The mouse blood samples were centrifuged at 300g for 15 min at 4°C . For histamine analysis, the samples were mixed with 0.1 N hydrochloric acid (HCl) and 60% perchloric acid and then centrifuged. The supernatant was transferred and mixed with 5 M sodium chloride, 5 N sodium hydroxide, and *n*-butanol and centrifuged. HCl and *n*-heptane were added to the supernatant. In the last step, the serum histamine levels were measured using the *o*-phthalaldehyde spectrofluorometric procedure [27]. Fluorescence intensity was measured using a fluorescence plate reader (Molecular Devices) at 380 nm excitation and 440 nm emission wavelengths.

2.12. Statistical analysis

The data between groups were analyzed using Prism 5 (GraphPad Software, San Diego, CA). Statistical analysis was done using one-way analysis of variance followed by Duncan's multiple range tests. $*p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. The inhibitory effects of polyozellin on the TNF- α and IFN- γ -stimulated keratinocytes

The chemical structure of polyozellin is shown in Fig. S1A. First, the cell viability was examined by MTT assay to rule out the cytotoxic effects of polyozellin in keratinocytes and mast cells. Since polyozellin did not show cytotoxicity up to 10 μM in both HaCaT and RBL-2H3, further experiments were performed at a non-toxic range below 10 μM (Fig. S1B).

We investigated the effects of polyozellin on the levels of cytokines and chemokines in the TNF- α and IFN- γ stimulated keratinocytes by qPCR and ELISA (Fig. 1A and B). While TNF- α and IFN- γ treatment significantly increased the mRNA levels of inflammatory cytokines and chemokines relative to the normal conditions, pretreatment with polyozellin decreased the expression and secretion of TNF- α , IL-1 β , IL-6, and CCL17/TARC in the activated keratinocytes. In particular, the highest concentration of polyozellin (10 μM) showed inhibitory effects equivalent to the positive control drug, Dexamethasone (10 μM).

Since STAT1 and NF- κB are well-known transcription factors modulating the expression of various genes associated with inflammatory responses [28,29], we next tested whether polyozellin treatment affects the activation of these transcription factors. Western blot results showed that polyozellin downregulated the activation of STAT1 and p65 NF- κB , as well as the degradation of I $\kappa\text{B}\alpha$ (Fig. 1C).

3.2. The inhibitory effects of polyozellin on DFE/DNCB-induced atopic skin inflammation

To investigate the effects of polyozellin on atopic skin inflammation, we generated an experimental AD model by using DFE and DNCB. A schematic diagram of the experiment schedule is shown in Fig. S1C. Atopic skin inflammation was induced in the mouse ear with DFE/DNCB application for 4 weeks. In this study, oral treatment with polyozellin reduced the clinical symptoms and histopathological aspects, including erythema, scarring, edema, epidermal hyperplasia, and inflammatory cell infiltration (Fig. 2A and B). Histological analysis showed that the epidermal and dermal thickness, as well as the accumulation of eosinophils, were significantly decreased by the oral

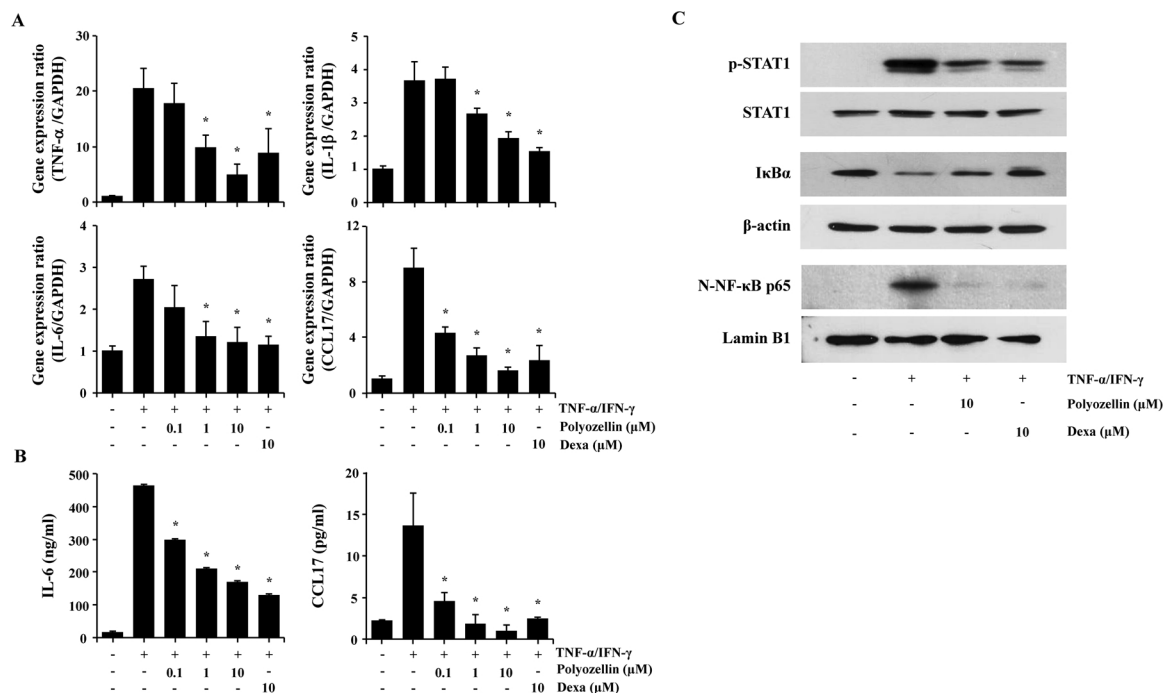


Fig. 1. The effects of polyozellin on the expression and secretion of cytokines and chemokines, as well as the inflammatory signaling pathway, in HaCaT. The expression and secretion of cytokines and chemokines in TNF- α and IFN- γ -stimulated HaCaT were determined using qPCR and ELISA. (A) Cells pretreated with polyozellin or Dexa for 1 h were stimulated with TNF- α and IFN- γ for 6 h. mRNA expression of TNF- α , IL-1 β , IL-6, and CCL17 was determined by qPCR in the activated keratinocytes. (B) Cells were pretreated with polyozellin or Dexa at different concentrations (0.1–10 μ M) for 1 h and stimulated with TNF- α and IFN- γ for 15 h. The collected cells were centrifuged at 400 g for 5 min. The protein levels were measured by ELISA. Data are presented as mean $\pm$ SEM ($n = 3$). * $p < 0.05$, significantly lower than the TNF- α and IFN- γ -stimulated group. (C) Cells were pretreated with polyozellin or Dexa for 1 h and then activated with TNF- α and IFN- γ . The activation of STAT1 and p65 NF- κ B and the degradation of I κ B α were analyzed by Western blot. β -actin and lamin B1 were used to confirm equal sample loading. The bands are representative of two independent experiments. N-p65 NF- κ B, nuclear p65 NF- κ B. Dexa: dexamethasone.

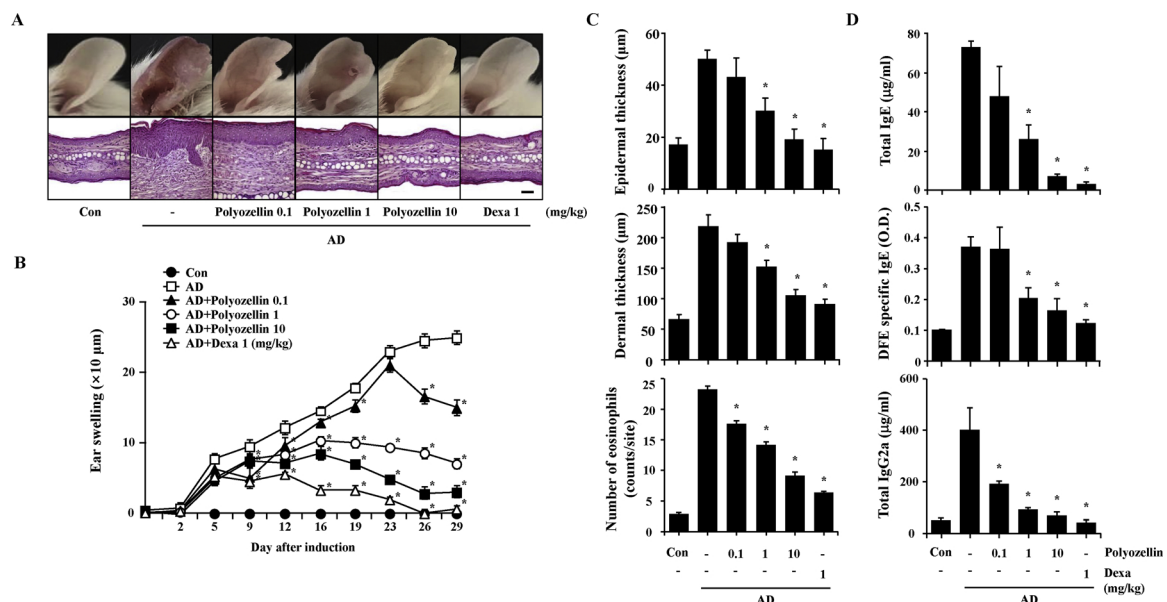


Fig. 2. The effects of polyozellin on the clinical severity of DFE/DNCB-induced atopic skin inflammation. DFE/DNCB was repeatedly applied on the ears of BALB/c mice for 4 weeks to induce AD. (A) Representative ear skin photographs of the different groups of experimental mice (first panel) and histological analyses using H&E staining (second panel; magnification of $\times 400$). The ear tissue was fixed with 10% formaldehyde and embedded in paraffin. The paraffin blocks were cut into 5 μ m sections and stained with H&E. (B) Ear swelling during the course of AD. Ear swelling ($n = 5$ /group) was measured 24 h after DFE/DNCB treatment with a micrometer. (C) The thickness of the epidermal and dermal skin was determined using microphotographs of H&E staining. The numbers of infiltrated eosinophils were counted in the H&E-stained skin sections at $\times 400$ magnification. The number of cells is presented as the mean of the number of cells at five random sites of each animal. Scale bars: 100 μ m. (D) The concentration of total IgE levels and IgG2a levels and the optical density of DFE-specific IgE levels in the serum were quantified by ELISA.

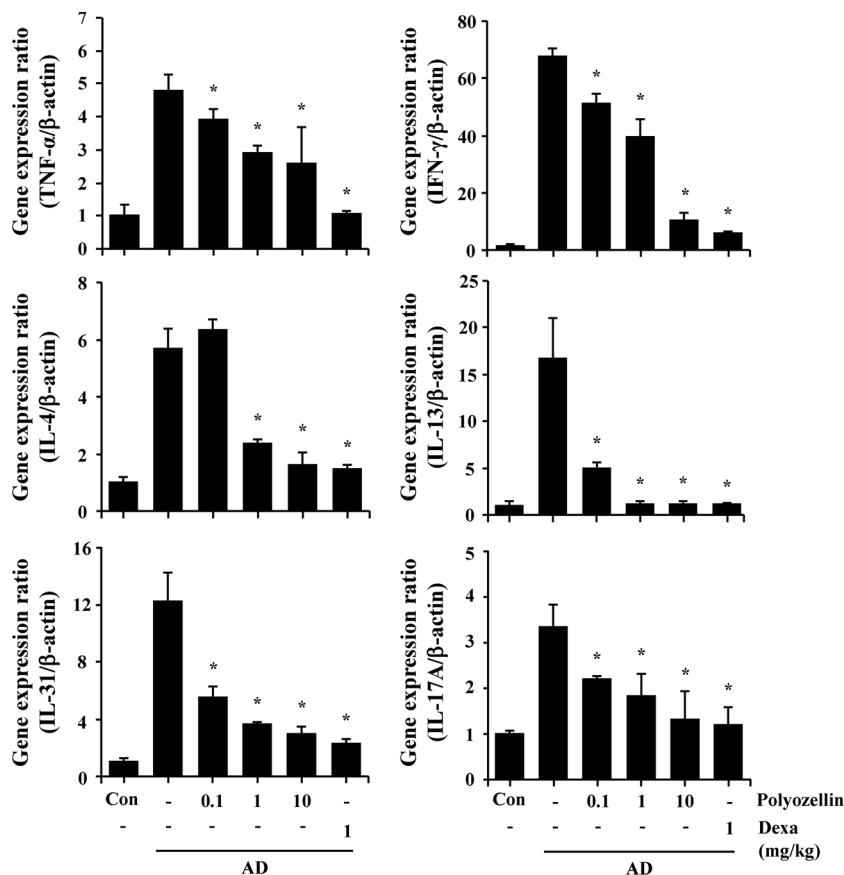


Fig. 3. The effects of polyozellin on the expression of inflammatory cytokines in mice. At the end of the experiments, the ear tissues were collected and homogenized for total RNA extraction. The mRNA expression levels of cytokines in the ear tissues were evaluated by qPCR. Data are presented as mean $\pm$ SEM ($n = 5$). * $p < 0.05$, significantly lower than those of AD mice. Dexa: dexamethasone.

administration of polyozellin (Fig. 2C). To confirm the effects of polyozellin on the Th1 and Th2 responses, we assessed the serum immunoglobulin levels of IgE and IgG2a, which are associated with hypersensitivity to allergens and chronic inflammation, respectively [1,30]. Relative to the healthy mice, the DFE/DNCB-induced mice had higher serum levels of IgE (total IgE and DFE-specific IgE) and IgG2a. In contrast, those increases were suppressed by polyozellin in a dose-dependent manner (Fig. 2D). The body weights of the mice were measured once a week to assess their general health and immunological status (Fig. S1D).

To evaluate the inhibitory effects of polyozellin on the DFE/DNCB-induced inflammatory cytokines, the mRNA levels of Th1-, Th2-, and Th17-related cytokines were measured in the ear tissues. As shown in Fig. 3, the expression of inflammatory cytokines in the DFE/DNCB-induced mice was significantly upregulated compared to the healthy mice. However, the polyozellin-treated AD mice showed marked decreases in the gene expression levels of TNF- α , IFN- γ , IL-4, IL-13, IL-31, and IL-17A in a dose-dependent manner compared to the DFE/DNCB-induced mice.

3.3. The inhibitory effects of polyozellin on the pruritic responses and mast cell degranulation

TB staining of the ear tissue showed higher accumulation of mast cells in the skin of DFE/DNCB-induced mice compared to the healthy mice, whereas the infiltration of mast cells was reduced by polyozellin treatment (Fig. 4A and B). The activated mast cells release inflammatory mediators, specifically histamine [1]. To assess the effect of polyozellin on the anti-pruritic responses, we examined the serum histamine levels, which are a major pruritogen in patients with AD and considered to be directly associated with scratching behavior [12]. The repeated application of DFE/DNCB increased the serum histamine levels and the scratching numbers, which were reduced by polyozellin

(Fig. 4C and D).

To confirm the inhibitory effects of polyozellin on mast cell activation and mast cell-derived pruritic responses, we performed β -hexosaminidase and histamine assay. Polyozellin was added at different concentrations to the RBL-2H3. As shown in Fig. 4E, pretreatment with polyozellin reduced the release of β -hexosaminidase and histamine, which are markers for mast cell degranulation, in a concentration-dependent manner.

4. Discussion

The successful prevention and treatment of AD require a multi-pronged approach, including inflammatory and pruritus treatment, allergen removal, immunotherapy, and skin care [2]. Several therapies including topical corticosteroids, calcineurin inhibitors, and immunosuppressants have been used to control the exacerbation of AD. Despite the immediate reduction of AD symptoms, they might be inappropriate for long-term use due to their adverse effects [18]. Thus, it is imperative to use alternative therapeutic agents that are less toxic [2]. Polyozellin, a naturally derived product, is known to have an inhibitory effect on inflammation, oxidative stress, and cancer [20,24,25]. These effects led us to hypothesize that polyozellin may have immunomodulatory properties that can be applied for the treatment of inflammatory skin disease, specifically AD. In the present study, we focused on the multifunctional effects of polyozellin as a therapeutic agent for AD.

AD is a chronic inflammatory skin disorder associated with many types of cells and factors, including keratinocytes, T cells, cytokines, and allergens [6,7]. Keratinocytes provide an important window to the environment by forming a skin barrier against environmental damage in the body. In response to various stimuli, the activated keratinocytes generate inflammatory cytokines as a cellular source of the danger signal [8]. Various cytokines and chemokines secreted from the

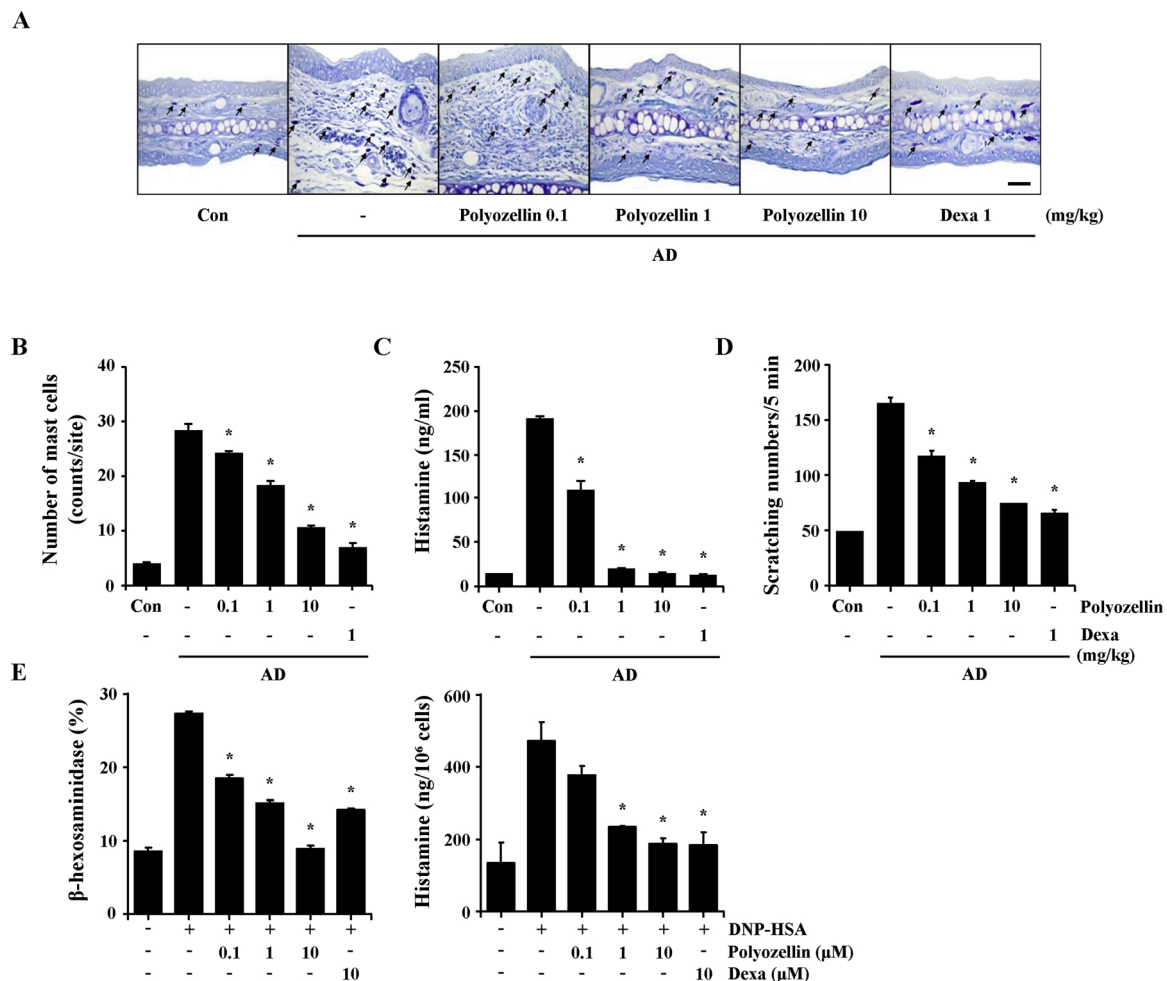


Fig. 4. The effects of polyozellin on pruritic responses *in vivo* and mast cell degranulation *in vitro*. (A) Representative images of histologic analysis using TB staining; magnification of $\times 400$. Mast cells are indicated by arrows. Scale bars: 100 μ m. (B) The number of infiltrated mast cells was counted at five random fields in each TB-stained slide. (C) Serum histamine levels were measured using the *o*-phthalaldehyde spectrofluorometric procedure. (D) The day before sacrifice, the frequency of scratching behavior was counted for 5 min. (E) RBL-2H3 were pretreated with polyozellin or Dexa for 1 h and stimulated with DNP-HSA. β -hexosaminidase levels were detected using a spectrophotometer for the measurement of mast cell degranulation. Histamine levels in the RBL-2H3 were detected using a fluorescence plate reader. Data are presented as mean $\pm$ SEM ($n = 3$). * $p < 0.05$, significantly lower than the DNP-HSA-stimulated group. Dexa: dexamethasone.

keratinocytes are involved in the initiation or pathogenesis of AD inflammatory responses [7]. For example, Th1-associated cytokines, such as TNF- α and IL-1 β , activate the inflammatory processes and accelerate chronic inflammation by inducing cytokine secretion [31,32]. IL-6 acts as a proinflammatory cytokine and induces the proliferation and differentiation of keratinocytes [8]. Additionally, inflammatory chemokines such as CCL17/TARC are directly involved in the Th2-dominant immune responses and attract immune cells into the skin lesions [33]. IL-17A, a Th17-associated cytokine, contributes to the immune dysregulation [6]. Notably, the production of these inflammatory cytokines and chemokines is tightly regulated by transcriptional regulators such as STAT1 and NF- κ B [33] and should be balanced for the treatment of AD [2,6]. In our study, we found that polyozellin suppressed the production of inflammatory cytokines and chemokines by inhibiting the activation of STAT1 and NF- κ B in keratinocytes.

Among the various allergens known to cause AD, house dust mites are a major risk factor for allergic diseases, such as AD and asthma. Among them, *D. farinae* allergens frequently induce allergic diseases and are involved in the pathogenesis of human AD; therefore, a DFE-induced AD animal model is more effective in mimicking human AD [14]. Additionally, the repeated application of DFE/DNCB to the ears of mice resulted in a process similar to the natural progression of human AD and resulted in an immune response towards Th2 [17]. Thus, we

induced atopic skin inflammation in mice by the repeated epicutaneous application of DFE with DNCB as an allergen and hapten, respectively. DFE/DNCB treatment increased the serum immunoglobulin levels of IgE and IgG2a associated with the acute and chronic phases of AD, respectively [1,30]. Especially, in the acute phase of allergic disease, IgE binds the high-affinity IgE receptor, Fc ϵ RI on mast cells, resulting in the release of inflammatory mediators [1].

Treatment with polyozellin reduced the clinical symptoms and histopathological aspects, as well as eosinophil infiltration, in the DFE/DNCB-induced AD mice. Moreover, polyozellin reduced the expression of inflammatory cytokines in the ear tissue. According to these results, polyozellin has an inhibitory effect on the acute and chronic immune responses and alleviates the clinical symptoms by downregulating the inflammatory cytokine expression (e.g., TNF- α , IL-4, and IL-31) in the DFE/DNCB-induced AD mice. Among them, IL-31 plays a role in the occurrence of pruritus by affecting the physical and antimicrobial skin barrier or by promoting the growth of sensory nerves [34].

Pruritus causes the patients with AD to persistently scratch their skin, resulting in skin injury, infection, and scarring, which in turn causes more itching and scratching [35]. In addition, pruritus induces the transition of a Th2-predominant acute phase to a Th1-dominant chronic phase, which is characteristic of AD symptoms [36]. Eventually, pruritus has a negative impact on the quality of life for patients

with AD. Therefore, the primary therapeutic objective should be to relieve the symptoms of pruritus, ameliorate the severity of the disease, and improve the patients' quality of life [34]. Although antihistamines are commonly approved as a treatment for itching in patients with AD, they only provide a slight relief of the symptoms [19]. As mentioned above, the mast cells are tissue-based inflammatory cells of bone marrow origin and secrete allergic mediators such as histamine and cytokines. Histamine, in particular, is an indicator of mast cell degranulation and plays a major role in allergic response through the production of an itch-scratch cycle [12]. Thus, the inhibition of histamine can be a potential therapeutic target for mitigating the AD-associated itching [12]. In the present study, polyozellin suppressed the infiltration and degranulation of mast cells, as well as the scratching numbers. These results suggest that polyozellin is effective for relieving the symptoms of AD by inhibiting pruritus.

5. Conclusion

We observed that polyozellin inhibited the development of atopic skin inflammation in a DFE/DNCB-induced AD model, as well as the activation of keratinocytes and mast cells. These results suggest that polyozellin could be a potential therapeutic agent for suppressing the inflammatory and pruritic responses of AD. Our results provide an overview of the potential pharmacological effects of polyozellin for treating AD.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2019.109743>.

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Polyozellin alleviates atopic dermatitis-like inflammatory and pruritic responses in activated keratinocytes and mast cells

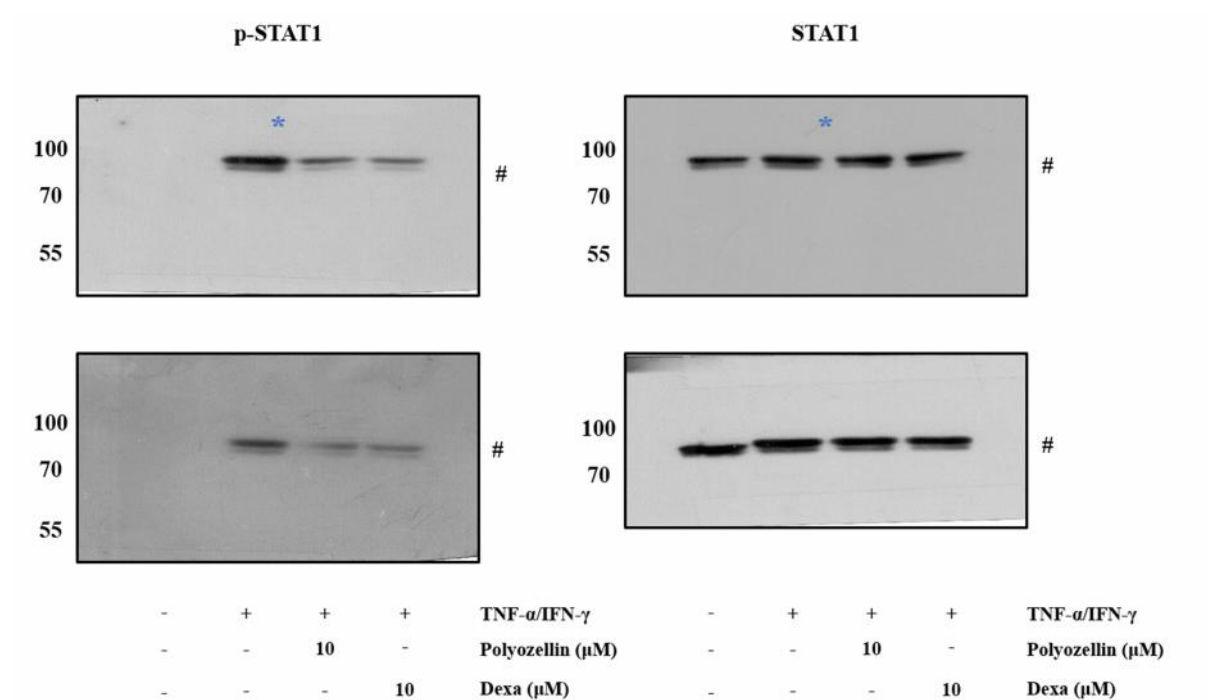
Material and method

1. Cell viability

HaCaT and RBL-2H3 viability were measured using the MTT (3-(4,5-dimethylthiazolyl-2-yl) 2,5-diphenyl tetrazolium bromide) [1]. Briefly, HaCaT (1×10^4 cells/well in a 96-well plate) and RBL-2H3 (6×10^4 cells/well in a 96-well plate) were seeded and after 24 h of treatment of polyozellin with different concentrations (0-100 μ M), 100 μ l of MTT solution (5 mg/ml) was added into each well and incubated for 2 h. After formazan crystals formed, the MTT medium was aspirated and 100 μ l of dimethyl sulfoxide was added to dissolve the formazan crystals. Absorbance on each sample compared to that of the control, was calculated and expressed as percentage.

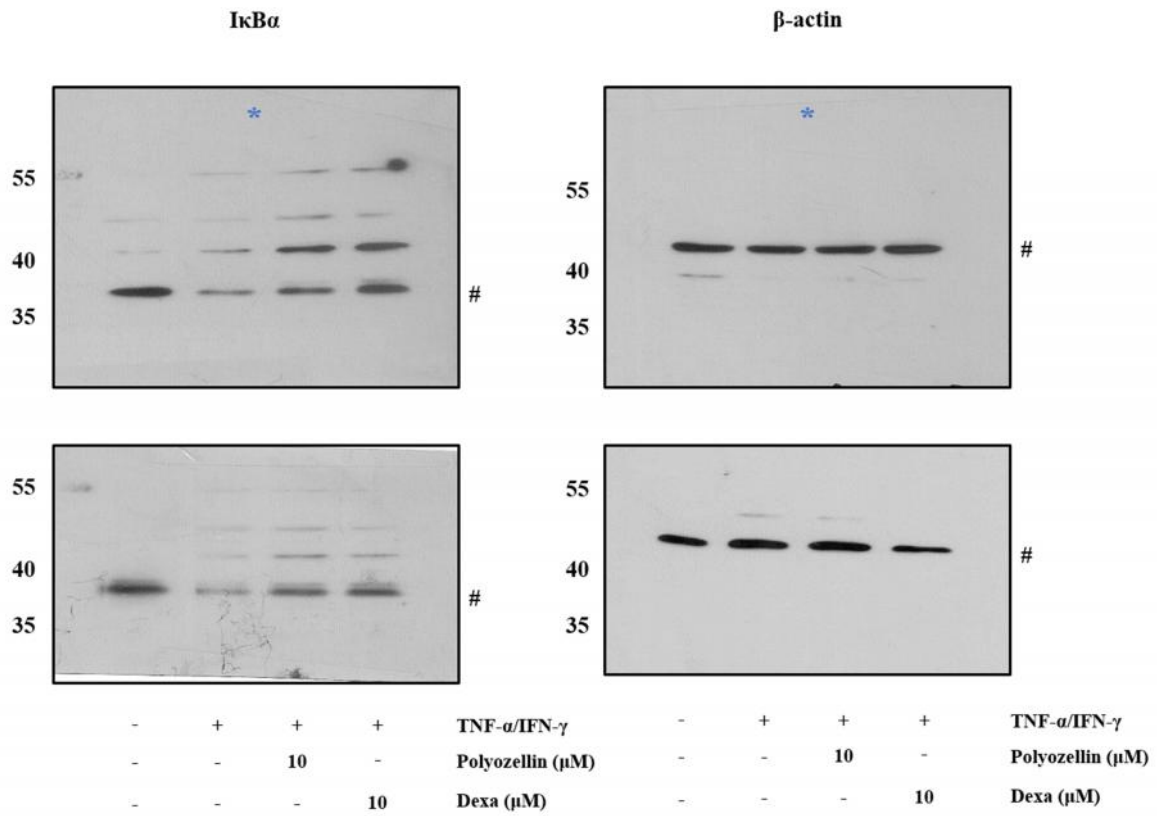
2. Experimental schedule for the induction

The surfaces of both mouse ear lobes were repeatedly stripped with surgical tape (Nichiban, Tokyo, Japan) for 4 weeks to induce atopic skin inflammation. DFE (10 mg/ml, 20 μ l/ear) or 1% DNCB (20 μ l/ear) were alternative treated to both ears once a week. After 1 week of induction, polyozellin (0.1, 1, or 10 mg/kg) was orally administered for 3 weeks. Body weight of female BALB/c mice was measured on the first day of each week for 4 weeks using a weighing scale.

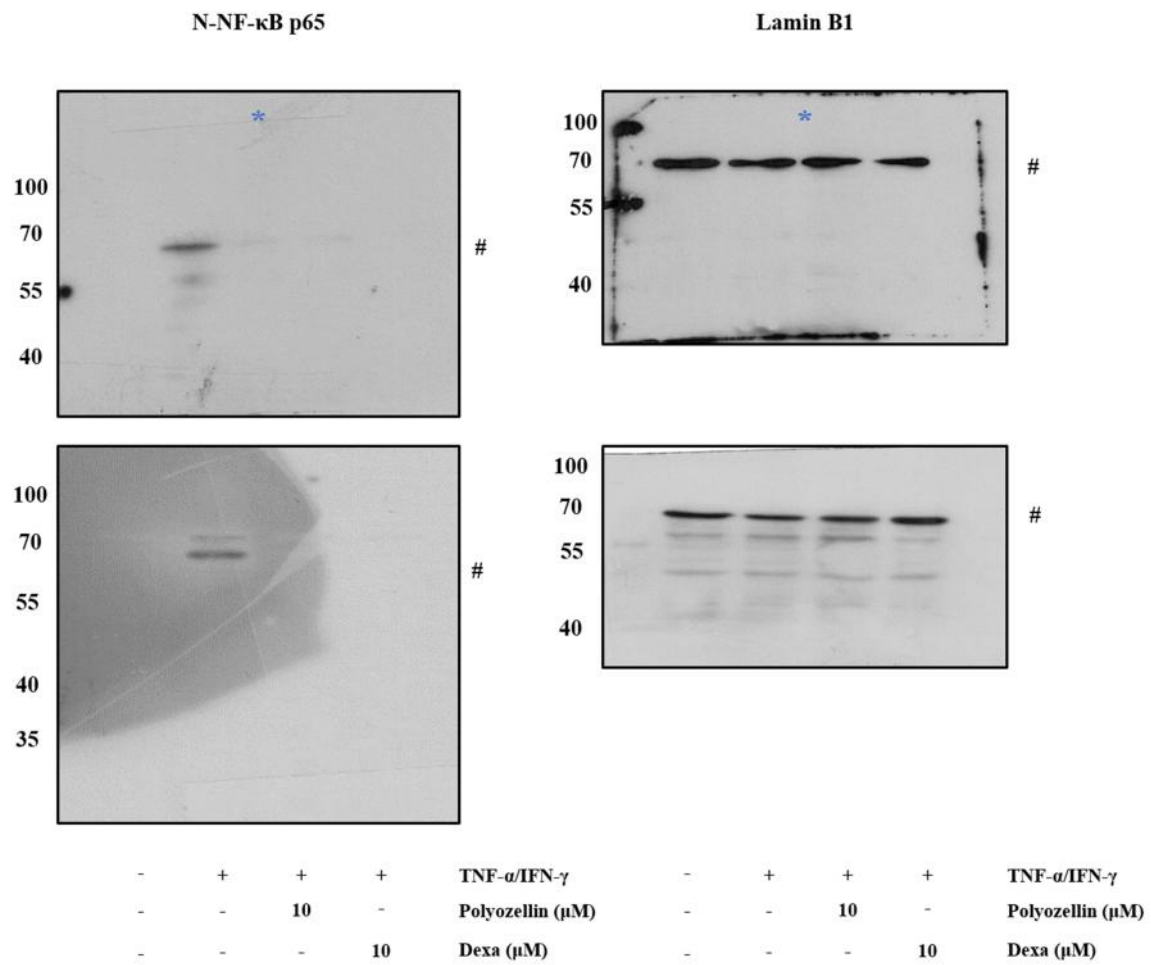


.Supplementary Figure S2. Full-length Western blot corresponding to Figure 1C.

Supplementary information of Figure 1C. Full length blot of phospho-STAT1 (84,91 kDa) and STAT1 (84,91 kDa). Hash (#) denotes target band. Blots with asterisk (blue) were used in main Figure.



Supplementary information of Figure 1C. Full length blot of IkBα (37 kDa) and β-actin (43 kDa). Hash (#) denotes target band. Blots with asterisk (blue) were used in main Figure.



Supplementary information of Figure 1C. Full length blot of nucleus NF-κB (65 kDa) and lamin B1 (67 kDa). Hash (#) denotes target band. Blots with asterisk (blue) were used in main Figure.

Reference

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