

# Improvement of atopic dermatitis-like skin lesions by IL-4 inhibition of P14 protein isolated from *Lactobacillus casei* in NC/Nga mice

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**Abstract** Atopic dermatitis (AD) is a chronic inflammatory skin disease, with a complex etiology encompassing immunologic responses. AD is frequently associated with elevated serum immunoglobulin (Ig) E levels and is exacerbated by a variety of environmental factors, which contribute to its pathogenesis. However, the etiology of AD remains unknown. Recently, reports have documented the role of lactic acid bacteria (LAB) in the treatment and prevention of AD in humans and mice. The LAB, *Lactobacillus casei* (LC), is frequently used in the treatment of AD. To identify the active component of LC, we screened fractions obtained from the ion exchange chromatography of LC extracts. Using this approach, we identified the candidate protein, P14. We examined whether the P14 protein has anti-atopic properties, using both in vitro and in vivo models. Our results showed that the P14 protein selectively downregulated serum IgE and interleukin-4 cytokine levels, as well as the AD index and scratching score in AD-like NC/Nga mice. In addition, histological examination was also effective in mice. These results suggest that the P14 protein has potential therapeutic effects and that it may also serve as an effective immunomodulatory agent for treating patients with AD.

**Keywords** Atopic dermatitis · P14 protein · Immunoglobulin E · Interleukin-4 · NC/Nga mouse

## Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease, characterized by pruritic and eczematous skin lesions (Leung and Bieber 2003). AD is a multifactorial condition, including genetic and environmental components, such as infectious agents, food allergens, and aeroallergens (Morar et al. 2006; Tanaka et al. 2007). In recent years, the prevalence of AD has increased substantially, particularly in industrialized countries. Worldwide, AD affects up to 20 % of children and 1 to 3 % of adults (Fujiwara et al. 2004). The early onset of AD in infancy is recognized as the most prevalent chronic skin disorder in childhood, affecting individuals throughout their lifetime through its persistence or aggravation following the onset of serious problems in adulthood (Leung and Bieber 2003). The majority of AD patients show elevated serum IgE levels against specific environmental allergens or microbial proteins (Sicherer and Leung 2009). Pruritus, a sensation that urges individuals to scratch, is an important clinical feature of AD; it destroys the skin barrier, aggravating the dermatitis, resulting in typical skin lesions (Wahlgren 1999). Although the pathogenesis of AD has not yet been fully defined, it has been reported that clinical signs and symptoms are associated with underlying immunological changes. Allergic diseases such as rhinitis, asthma, and atopic eczema are characterized by the hypersensitivity of the T helper 2 (Th2) cells to a specific allergen. This leads to an immune response leading to IgE production and the accumulation of eosinophils, significant contributors to the pathogenesis of AD (Uehara et al. 1990; Woodfolk 2007). The breakdown in the balance between Th1 and Th2 cytokines has been reported to be central to the pathology of AD (Abbas et al. 1996; Umetsu and DeKruyff 1997). Specifically, it is generally believed that the

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immune response in AD is skewed toward a Th2 response, thus resembling a Th1 deficiency. Studies of patients with AD have shown that Th2 cytokines such as IL-4, IL-5, and IL-13 are overproduced (Parronchi et al. 1991). Until recently, steroid therapy was widely used in the treatment of AD. However, this treatment causes severe side effects, including atrophy, acne, cataracts, growth retardation, and red burning skin, prohibiting use over long periods of time (Hengge et al. 2006; Lyu et al. 2003; Rapaport and Lebwohl 2003; van Buul-Offers et al. 2005). More importantly, steroid treatment has a general immunosuppressive effect that occurs through a reduction in the cytokine production, associated with both Th2 and Th1 (Gupta and Chow 2003). Despite their severe side effects, steroid-based drugs are used to treat dermatological disorders in over 80 % of the world's population (Mainardi et al. 2009). To circumvent the toxicity of steroid-based drugs, several groups have conducted clinical trials of complementary or alternative medicines. However, despite numerous trials evaluating the effects of complementary or alternative medicines on allergic diseases, including AD, their efficacy and safety, as well as the therapeutic mechanisms, remain unclear (Donsky and Clarke 2007; Klovekorn et al. 2007).

Lactic acid bacteria (LAB) are noninvasive, nonpathogenic Gram-positive commensal bacteria, which are recognized for promoting human health (Mercenier et al. 2003). LAB are facultative anaerobic or microaerophilic, nonspore-forming bacteria that produce lactate as the major end product from the fermentation of carbohydrates, found in the normal gut microflora of mammals, including humans. Strains of the LAB *Lactobacillus* have probiotic properties, available in many dairy products, which exert a number of health benefits to the host by regulating the immune system (Reid et al. 2003). Recent reports have documented the role of strain-specific LAB in the prevention of allergic disorders in mice and humans. To explain this effect, several authors have reported LAB to reduce some allergic manifestations in mice and humans (Fujiwara et al. 2004; Hengge et al. 2006; Kalliomaki and Isolauri 2003; Yesilova et al. 2012). Research into the use of LAB for the treatment of allergic disorders is increasing, especially on the use of *Lactobacillus casei* (LC) in the treatment of AD (Chapat et al. 2004; Klewicka et al. 2011; Ogawa et al. 2006).

NC/Nga mice originated from Japanese fancy mice at Nagoya University (Japan) in 1957, and these mice develop AD-like skin lesions and IgE hyperproduction under conventional conditions with itching, erythema, and hemorrhage, followed by edematous superficial erosion, deep excoriation, scaling, dryness of the skin, and retarded growth (Matsuda et al. 1997; Vestergaard et al. 2000). These pathophysiological observations for AD in NC/Nga mice highly resemble those in human AD. This strain of mouse is therefore considered to be a useful animal model for the evaluation of pathologic mechanisms of human AD (Suto et al. 1999).

In this study, we attempted to identify the active protein form LC cell extracts that alleviated the symptoms of AD in NC/Nga mice. Screening studies using LC protein fractions obtained from ion exchange chromatography helped identify a 14-kDa protein active against AD. This protein was named P14, based on its molecular size. We examined whether the P14 protein exerted anti-atopic properties both in vitro and in vivo. The toxicity and effects of the P14 protein in vitro and ex vivo were also examined. Significantly, our results demonstrate that the P14 protein selectively downregulated the levels of IgE and IL-4 cytokines in AD-like NC/Nga mice. These results suggest that the P14 protein alleviates the symptoms of AD and may serve as an effective immunomodulatory agent for patients with AD.

## Materials and methods

### Preparation of *L. casei*

*L. casei* (LC) KCTC 12398BP was obtained from the culture collection maintained at Cell biotech Co., Ltd., Gimpo, Korea. LC KCTC 12398BP was cultured in MRS broth (Difco, MI, USA) at 37 °C for 18–24 h. The bacterial cells were then harvested using centrifugation and washed twice with sterile phosphate-buffered saline to remove any metabolic substances associated with it.

### Detection of IL-4 cytokine levels in vitro and ex vivo

The mouse macrophage cell line RAW264.7 was seeded into 24-well plates at a cell density of  $2 \times 10^6$  cells/mL and stimulated with 10 ng/mL of lipopolysaccharide (LPS) (Sigma-Aldrich, MO, USA). Following LPS stimulation, the RAW264.7 cells were treated with 1, 10, and 100 µg/mL of P14 for the indicated period of time. After incubation at 37 °C in an atmosphere of 5 % CO<sub>2</sub> for 24 h, supernatants were collected, and the levels of IL-4 determined using ELISA kits (BD Biosciences, CA, USA).

Splenocytes were obtained from Balb/c mouse (6 weeks of age), and the cytokine levels in isolated splenocytes were then examined (Kim et al. 2011). Spleens isolated from Balb/c mice were homogenized using a cell strainer (BD Biosciences). The splenocytes were then resuspended in culture medium, RPMI-1640 containing 10 % fetal bovine serum, and 50 µM of β-mercaptoethanol. To examine the cytokine levels following P14 treatment, the isolated splenocytes were seeded into 24-well plates at a cell density of  $2 \times 10^6$  cells/mL and then stimulated with 1 µg/mL of concanavalin A (Con A) (Sigma-Aldrich). Following Con A treatment, the splenocytes were treated with 0.1, 0.2, 1, 5, and 10 µg/mL of P14 and incubated at 37 °C in an atmosphere of 5 % CO<sub>2</sub> for 24 h. After incubation, the supernatants were collected, and the

levels of IL-4 were determined using ELISA kits (BD Biosciences). The dosage of P14 used in these experiments was the minimal concentration required for maximal activity, based on preliminary dose-response experiments.

#### Screening proteins decreasing IL-4 cytokine levels in vitro from LC extracts

LC cells were harvested by centrifuging at  $1000\times g$  for 10 min. The pelleted cells were washed twice in PBS containing 0.1 % Triton X-100. The LC pellet was then lysed using a high-speed bench top homogenizer (FastPrep24 homogenizer, MP Biomedicals, CA, USA) at 6.5 m/s, and the lysate was centrifuged at  $10,000\times g$  for 15 min to remove the cell debris. Crude proteins were obtained from dialysis of the lysate against 20 mM Tris buffer (pH 8.0) for 24 h at 4 °C. Purification was carried out by performing ion exchange chromatography (IEC). The dialyzed protein was applied to a HiPrep DEAE-FF 16/10 (GE Healthcare Bio-Sciences, Uppsala, Sweden) and eluted using a linear gradient of NaCl in buffer A. The proteins were eluted at a NaCl concentration of 0–500 mmol/100 mL at pH 8.0. Each fraction was applied at a flow rate of 5 mL/min. Fractions with high decreasing IL-4 activity were pooled and concentrated. The purification of the fraction with the high/low IL-4 activity was conducted using AKTA FPLC (GE Healthcare Bio-Sciences). The molecular weight of the purified protein was determined by performing 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in mini-protein cell (Bio-Rad Laboratories, CA, USA). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories). Protein concentrations were determined using the Bio-Rad Protein Assay Kit.

To determine the N-terminal sequence of the identified protein, the band was excised from the SDS gel and transferred to polyvinylidene difluoride (PVDF) (Bio-Rad Laboratories). The excised PVDF membrane strips containing 10–50 pmol of protein were analyzed using an ABI492 protein sequencer (Applied Biosystems, CA, USA) to determine the N-terminal sequence.

#### Cloning and expression of the P14 protein in *E. coli*

Genomic DNA from LC KCTC 12398BP was used as a template for PCR to amplify the gene encoding the P14 protein. The primers, the coding region of the *p14* gene, were amplified by performing PCR using the primers p14F, 5'-ATGGATCCATGCGGGGTTCTCATCATC-3' (Bam HI), and p14R, 5'-ATCTCGAGCTAGAAGACCTGTTCGTC-3' (Xho I), each containing restriction sites for cloning into the pRSET-A vector (Invitrogen, NY, USA). Ligation mixtures were transformed into DH5 $\alpha$  (Enzynomics, Daejeon, Korea) by heat shock. Expression of recombinants was induced using

1 mM IPTG at 37 °C. Recombinant proteins were purified by Ni-NTA agarose (Qiagen, Maryland, USA), according to the manufacturer's instructions. Purified fusion protein was digested using Enterokinase (EK) (Sigma-Aldrich) to remove the 6 $\times$  His tag at the N-terminus. The EK was then eliminated using an EK removal kit (Sigma-Aldrich). Purified His-tag P14 and P14 protein were then separated by SDS-PAGE. The P14 protein band was removed from the gel and subjected to in-gel trypsin digestion (Shevchenko and Shevchenko 2001). Tryptic peptides were extracted from the gel slices and prepared for mass spectrometry (MS) analysis. Time-of-flight (TOF) MS and TOF MS/MS analysis of peptides was performed using nano-ESI on a Q-TOF2 mass spectrometer (AB Sciex Instruments, CA, USA). A potential of 1 kV was applied to the pre-coated borosilicate nanoelectrospray needles (EconoTip™, New Objective, USA) in the ion source, combined with a nitrogen back-pressure of 0–5 psi to produce a stable flow rate (10–30 nL/min). The quadrupole analyzer was used to select precursor ions for fragmentation in the hexapole collision cell. Product ions were analyzed using an orthogonal TOF analyzer, fitted with a reflector, a micro-channel plate detector, and a time-to-digital converter. The data was processed using a peptide sequence system.

#### Toxicity of P14

The effect of P14 on cell viability was examined by performing a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST) assay. Briefly, NIH 3T3 and normal mouse cells were seeded into 96-well plates at a density of  $1\times 10^4$  cells/well and then incubated at 37 °C for 24 h. The cells were then treated with either 0.1, 0.5, 1, 5, 10, or 100  $\mu$ g/mL of P14 or 0.064 %  $\beta$ -ME. The cells were then incubated for an additional hour following the addition of 10  $\mu$ L/well of Cell Counting Kit-8 Reagent (Dojindo Laboratories, Kumamoto, Japan). The absorbance was then recorded at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Model 680; Bio-Rad Laboratories). All the experiments were performed in triplicate.

#### Animals

Male NC/Nga mice were purchased from the Shizuoka Laboratory Animal Center (Tokyo, Japan). Six-week-old male mice were randomized and housed in stainless steel cages in a controlled environment with a temperature of  $22\pm 2$  °C and 40–60 % humidity under a 12:12-h light-dark cycle. They were fed standard laboratory food and water prior to all the experiments. All the experimental procedures were carried out in accordance with The Animal Use and Care Protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Cell Biotech Co., Ltd, Gimpo, Korea.

## Murine AD model

To induce AD-like symptoms in NC/Nga mice, we used Biostir AD cream (Biostir Inc., Kobe, Japan). Biostir AD cream consists of *Dermatophagoides farinae* body (Dfb) extracts with an ointment base. The cream prepared from house dust mites and aa crude extract allergen of *D. farinae* as described previously (Yamamoto et al. 2007) was applied two times a week. Briefly, dorsal hairs were removed using an electronic clipper and hair removal cream 1 day before application. Biostir AD cream (100 mg) was then applied directly to the shaved dorsal surface. To disrupt the skin barrier, 150  $\mu$ L of 4 % SDS was topically applied on the surface, 3 h before Dfb application (Fig. 1). To investigate the inhibitory effects of P14, five experimental groups were prepared, and seven mice were assigned to each group: Negative control (NC) ( $n=7$ ), positive control (PC) ( $n=7$ ), P14 0.2 mg/head ( $n=7$ ), P14 2 mg/head ( $n=7$ ), and 0.064 % of beta-methasone ( $\beta$ -ME) ( $n=7$ ). The treatment was carried out for 4 weeks, and the mice were then sacrificed for examination.

## Evaluation of the severity of the AD-like skin lesions and scratching behavior

The severity of dermatitis in each mouse at 10–14 weeks of age was assessed by macroscopic observation before each blood sampling and scored according to the severity grade (Matsuda et al. 1997). The severity scores were expressed as a sum of the individual score and graded on a scale of 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of the following five signs and symptoms: itching, erythema/hemorrhage, oedema, excoriation/erosion, and scaling/dryness.

Individual mice were placed in a clear plastic cage and allowed to acclimatize for 15 min. Behavior was then observed for 10 min. Scratching of the nose, ears, and dorsal skin with the hind paws was recorded, while licking of the belly and dorsal skin during grooming was not recorded. Scratching behavior within the treatment area was evaluated according to the scratching score. All the evaluations were

performed blindly. Each incidence of scratching of the head, neck, dorsal skin, ears, and nose was recorded and given a score to obtain the maximum score.

## Measurement of serum IgE and IL-4 cytokines

The immunological response following Biostir AD cream-induced AD was monitored by measuring the serum levels of IgE and IL-4 (BD Biosciences). Whole blood was collected from 15-week-old mice from each of the treatment groups. The serum was then collected by centrifugation for 20 min at 12,000 rpm and stored at  $-80^{\circ}\text{C}$ , until further use. The Ig levels were determined by ELISA using detection kits for mouse IgE (BD Biosciences). Briefly, monoclonal anti-mouse Ig antibodies diluted in phosphate-buffered saline (PBS) (1:250) were placed in an immunoplate (Nunc, Roskilde, Denmark), which was incubated at  $4^{\circ}\text{C}$  overnight. After washing three times with washing buffer [PBS containing 0.05 % Tween-20 (Usb, Cleveland, U.S.A.)], 200  $\mu$ L of PBS containing 10 % fetal bovine serum (FBS) (HyClone, UT, USA) was added to each well. The samples were then incubated for 1 h at room temperature (RT), and the plates were then washed three times with washing buffer. Serum samples (100  $\mu$ L) were then added to the wells, and the plates were incubated for 2 h at RT. The samples were first diluted with PBS containing 10 % FBS for each type of Ig and IL-4. The plates were then washed three times, and 100  $\mu$ L of streptavidin-horseradish peroxidase-conjugated detection antibody (SAv-HRP; BD Biosciences) was added. The plates were then incubated for 1 h at RT and washed five times, and the enzymatic reaction was initiated by the addition of 100  $\mu$ L of substrate solution (0.1 M citric acid, 0.2 M  $\text{Na}_2\text{HPO}_4$ , *o*-phenylene diamine, and  $\text{H}_2\text{O}_2$ ; BD biosciences). The reactions were allowed to proceed for 20 min at RT and terminated by the addition of stop solution (50  $\mu$ L of 2 N  $\text{H}_2\text{SO}_4$ ; BD Biosciences). The absorbance of each well was then immediately measured at 450 and 590 nm using an ELISA reader. Samples that generate absorbance values

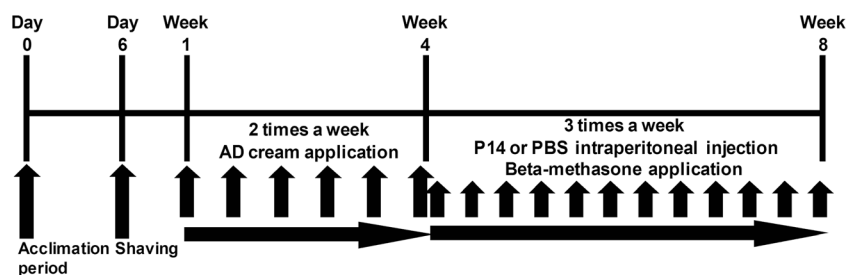


**Fig. 1** Establishment of an AD cream-induced mouse model of atopic dermatitis (AD)-like skin lesions and intraperitoneal injection of P14 in NC/Nga mice. Biostir AD cream (100 mg) was applied to the dorsal skin of mice, six times over 3 weeks, to induce AD-like skin lesions. Prior to applying the AD cream, the dorsal hair was removed. P14 was injected intraperitoneally following the last application of AD cream on week 4.

Injections were administered three times a week for 4 weeks. The experimental groups were as follows: 0.2 and 2 mg/head of P14, 0.064 % of beta-methasone ( $\beta$ -ME), and PBS (100  $\mu$ L/head). On week 8, mice were sacrificed and samples were collected for immunological analyses and dorsal skin histology measurements

higher than the standard curve were diluted with standard diluent and re-assayed.

### Histological analysis

Biostir AD cream was repeatedly applied to the backs of mice and removed on the final day of the experiment (week 8). The dorsal skin was removed and fixed in 4 % paraformaldehyde (Sigma-Aldrich). The paraffin-embedded skin sections were heat immobilized, deparaffinized by immersing in xylene (Sigma-Aldrich), rehydrated using a graded series of ethanol, and washed with DW. The dorsal skins were then cut into 4- $\mu$ m sections and subjected to either hematoxylin and eosin (H&E) staining (Sigma-Aldrich) or toluidine blue (Sigma-Aldrich) staining; they were then examined by light microscopy (Leica Microsystems, Wetzlar, Germany) for histological evaluation. Mast cells were counted in three different microscopic fields for each mouse, and the number per dorsal skin surface area ( $\text{n}/\text{mm}^2$ ) was calculated.

### Statistical analysis

The data were processed using GraphPad Prism™ 4.0, and the statistical parameters, mean value, and standard deviation for each group were calculated and compared with those for the other groups. The significance was determined using ANOVA (Tukey's post test). Means were considered significantly different when  $P < 0.05$ .

### Nucleotide sequence accession number

The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL database under accession number KM454859.

## Results

### Isolation of P14 protein from *L. casei*

To find the essential protein involved in AD, LC were disrupted and the protein was purified using a DEAE-FF 16/10 IEC column. Fractions inhibiting IL-4 immune activity were selected in Raw264.7 macrophage cell lines (data not shown). The selected fraction was concentrated, and the proteins were confirmed by performing SDS-PAGE. The molecular size of the protein was 14 kDa; therefore, we named this protein P14 based on the molecular size. The proteins were transferred to a PVDF membrane, for N-terminal sequence analysis. The  $\text{NH}_2$ -terminal amino acid sequences of the P14 protein were determined as  $\text{NH}_2$ -Ala-Lys-Ser-Gln-Asp-Gln-Phe-Asn-Glu-Lys-Val-Gly-Lys-Glu. This was consistent with those of a hypothetical protein from the *L. casei* group

(Sequence ID WP\_003602441.1) in NCBI BLASTp. The protein sequence of P14 protein from this experiment was the same as the sequences of the LC protein in GenBank.

### Cloning, expression, purification, and identification of P14

The genomic DNA from LC KCTC 12398BP was used as a template for PCR to amplify the P14 gene. The P14 gene was fused with a 6 $\times$  His-tag for purification as described in "Materials and methods." The *E. coli* host strain BL21 (DE3) was transformed using the expression vector pRSET-A/His::P14. Following IPTG induction, a recombinant fusion protein of about 17 kDa was identified. The His::P14 protein was purified by Ni-NTA agarose and dialysis in PBS. To obtain a functionally intact P14 protein, the His-tag was removed following incubation with EK. A 14-kDa cleavage fragment was observed and analyzed by performing 12 % SDS-PAGE gel (Fig. 2a).

To confirm the sequence identity of P14, the band was excised from the gel and digested with trypsin for TOF MS and TOF MS/MS analysis. Three peaks from the TOF MS spectrum were selected for sequencing by MS/MS fragmentation. MS/MS fragmentation spectra of precursor ions at  $m/z$  618.82 (①),  $m/z$  634.91 (②), and  $m/z$  739.42 (③) indicated that the fragments matched the expected P14 peptide sequences (Fig. 2b).

### The effects of P14 on Raw264.7 and Balb/c splenocytes and cytotoxicity

To assess the therapeutic potential of P14, we used IL-4 as a biomarker based on its pivotal role in the murine model of AD pathogenesis. IL-4 is a major cytokine produced by Th2 cells, and increased levels of IL-4 are one of the symptoms of AD (Kim et al. 2012). To determine the effect of P14 on the inhibition of IL-4, we measured the release of IL-4 from Raw264.7 and Balb/c splenocytes treated with increasing concentrations of P14. In the Raw264.7 macrophage cell lines, the level of IL-4 in the groups treated with different concentrations of P14 was as follows: 1  $\mu\text{g}/\text{mL}$  was  $46.9 \pm 10.1$  pg/mL, 10  $\mu\text{g}/\text{mL}$  was  $53.3 \pm 6.4$  pg/mL, and 100  $\mu\text{g}/\text{mL}$  was  $91.4 \pm 11.1$  pg/mL. This decreased compared to the LPS-only treatment group, with IL-4 levels of  $105.2 \pm 10.4$  pg/mL at suppression rates of 55.2, 49.5, and 13.3 % (Fig. 3a). The release of IL-4 significantly decreased following treatment with 1 and 10  $\mu\text{g}/\text{mL}$  of P14 when compared to LPS treatment only in Raw264.7 cells. But, 100  $\mu\text{g}/\text{mL}$  of P14 was not almost reduced in vitro test. These findings imply that around 1 to 10  $\mu\text{g}/\text{mL}$  of P14 effects IL-4 inhibition in vitro. All concentrations of P14 at 0.1, 0.2, 1, 2, and 10  $\mu\text{g}/\text{mL}$  significantly reduced the amount of Th2 cytokines IL-4 when compared to only Con A treatment on Balb/c splenocytes in an ex vivo assay (Fig. 3b). Significantly, P14 treatment groups ( $92.7 \pm 1$ ,

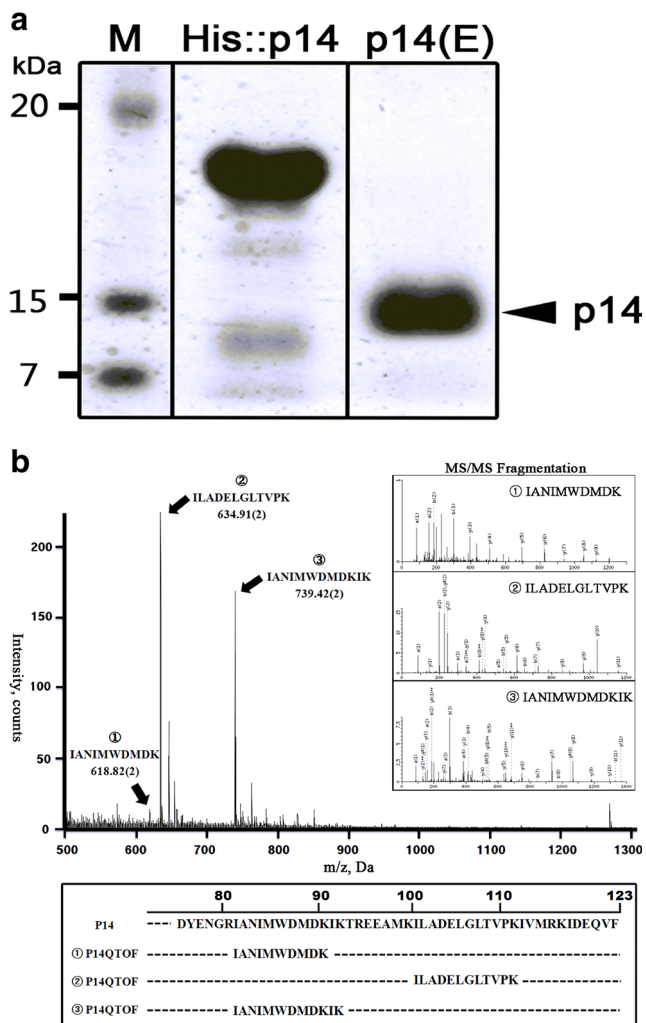


**Fig. 2** Expression, purification, and identification of P14. **a** SDS-PAGE analysis of purification N-terminal His-tagged P14. *His::P14* His-tagged P14 protein after purification using Ni-NTA agarose, *P14(E)* intact P14 protein after removal of N-terminal His-tag using Enterokinase, *M* protein molecular weight marker (kDa). The samples were separated by performing 12 % SDS-PAGE gel. **b** MS and MS/MS spectra of P14. Three tryptic peptides derived from carboxy (C)-terminal residue of P14 protein were identified by Q-TOF MS and MS/MS. Three peptide ions (m/z: 618.82 (①), 634.91 (②), 739.42 (③)) were verified by de novo sequencing of peptides using MS/MS fragmentation. The lower box shows representative annotated MS/MS fragments

75.4±4.8, 81.8±6.8, 71.2±2.6, and 79.6±3.4 pg/mL) decreased when compared to only Con A treatment group (106.9±1.3 pg/mL). P14 at 0.2 and 2 µg/mL showed a high inhibition rate of 28.6 and 32.4 %, respectively (Fig. 3b). These results indicate that P14 induces the inhibition of the Th2 cytokine IL-4 and that the optimal concentrations for maximum activity are 0.2 and 2 µg/mL.

To determine the effect of P14 on cell viability, we performed a WST assay to assess toxicity in NIH 3T3 normal mouse cells. Cells were treated with 0.1, 0.2, 1, 2,

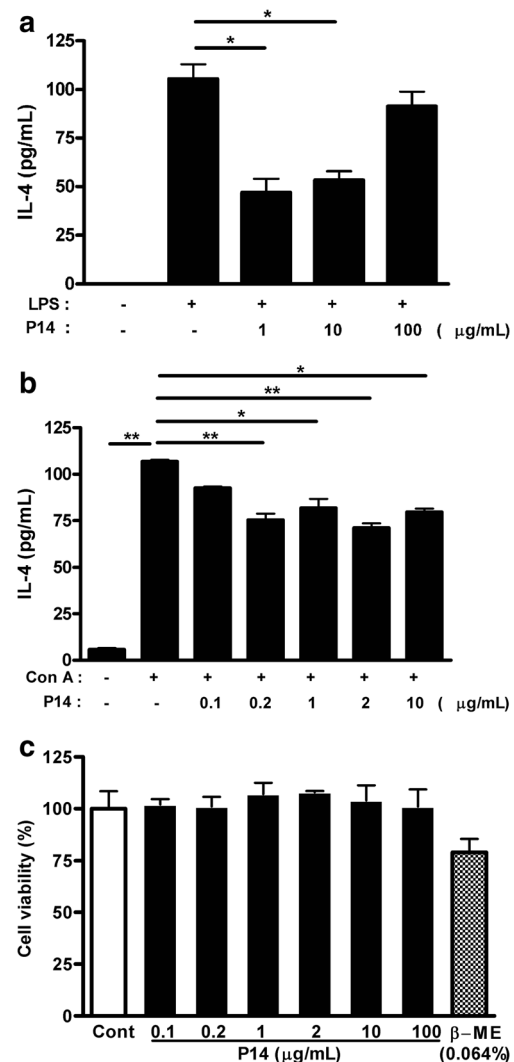


**Fig. 3** The effects of P14 in vitro. **a** Raw264.7 mouse macrophage cells were stimulated with LPS (10 ng/mL) and treated with P14 (1, 10, and 100 µg/mL). The cells were then incubated for 24 h. Secreted interleukin (IL)-4 levels were measured by enzyme-linked immunosorbent assay (ELISA). **b** Balb/c splenocytes were stimulated with concanavalin A (Con A, 1 µg/mL) and treated with P14 (0.1, 0.2, 1, 2, and 10 µg/mL). The cells were then incubated for 24 h. Secreted IL-4 levels were measured by ELISA. **c** Cytotoxicity of P14 on NIH 3T3 normal cells. Cells were treated with P14 (0.1, 0.2, 1, 2, 10, and 100 µg/mL) or β-ME (0.064 %) for 24 h, after which the cell viability was assessed by WST cell proliferation assay. Data shown represent the mean ± SD. \**P*<0.05 and \*\**P*<0.01 compared with control group (analysis of variance)

10, and 100 µg/mL of P14 or 0.064 % of β-ME. P14 treatment had no effect on cell viability; however, it decreased by approximately 79 % in cells treated with β-ME compared with the untreated cells (Fig. 3c). These results suggest that, unlike β-ME, P14 is non-cytotoxic to cells and could be used safely without the adverse effects associated with steroid therapy. Based on the above results, we determined the concentration of P14, which could be applied in vivo.

## Alleviation of AD cream-induced AD-like skin lesions in NC/Nga mice treated with P14

To assess the therapeutic potential of P14 *in vivo*, we established a mouse model for AD-like skin lesions in NC/Nga mice. Briefly, animals were sensitized with 100 mg Biostir AD cream six times over 3 weeks and simultaneously injected intraperitoneally with PBS (100  $\mu$ L/head), P14 (0.2 and 2 mg/head), or application  $\beta$ -ME (0.064 %/head), as shown in Fig. 1. After 4 weeks, the application of AD cream was stopped, and intraperitoneal injection of P14 was started. After the cessation of AD cream treatment, the AD-like skin lesions were observed to be markedly different in the PC and P14-treated mice. Specifically, there were obvious signs of AD-like skin lesions in PC mice treated with PBS, including bleeding, severe itching, and a rash. However, in mice treated with P14, the AD-like skin lesions were milder when compared to the PC group. There were no significant differences in the AD-like skin lesions amongst mice treated with different concentrations of P14 (0.2 and 2 mg/head). NC/Nga mice have been shown to develop AD-like skin lesions following the repeated application of Biostir AD cream. The severity of the skin lesions in control increased gradually with the number of AD cream challenges. Intraperitoneal injection of P14 for 4 weeks suppressed the development of AD-like skin lesions. The severity of skin lesions in each group in week 8 is as shown in Fig. 4a. The severity of skin lesions in each group was more or less similar up to 4 weeks from the day of sensitization. However, after 4 weeks, there was radical change in the skin lesions of the PC group compared with the P14 intraperitoneal groups. The skin severity score of each group is shown in Fig. 4b. To compare the skin lesions from NC mice, we utilized the clinical severity scores proposed by Leung for human AD (Leung 1997). Figure 4b shows changes in the atopic dermatitis index. In NC mice, the dermatitis score was zero from weeks 4 to 8, but in PC mice, dermatitis appeared at day 20 and deteriorated with time. In P14 mice, the development of dermatitis was inhibited significantly when compared with PC mice. The effects of P14 at 0.2 mg/head AD index ( $5.3 \pm 0.7$ ) are lower than P14 at 2 mg/head ( $7.2 \pm 1.1$ ).  $\beta$ -ME is nearly close to the NC group ( $1.9 \pm 0.6$ ). The chronological profile of the scratching behavior seen in NC, PC, and P14 NC/Nga mice is shown in Fig. 4c. The number of spontaneous scratching episodes remained almost constant from weeks 4 to 8 in NC mice. In contrast, the number of scratching episodes significantly increased in PC and P14: 0.2 and 2 mg/head mice. However, P14 from 0.2 mg/head scratching score ( $11.9 \pm 3.9$ ) was lower than P14 of 2 mg/head ( $18.7 \pm 6.5$ ) in this case as well. The scratching behavior in mice treated with  $\beta$ -ME ( $1 \pm 0.4$ ) was almost completely inhibited during this period (Fig. 4c).

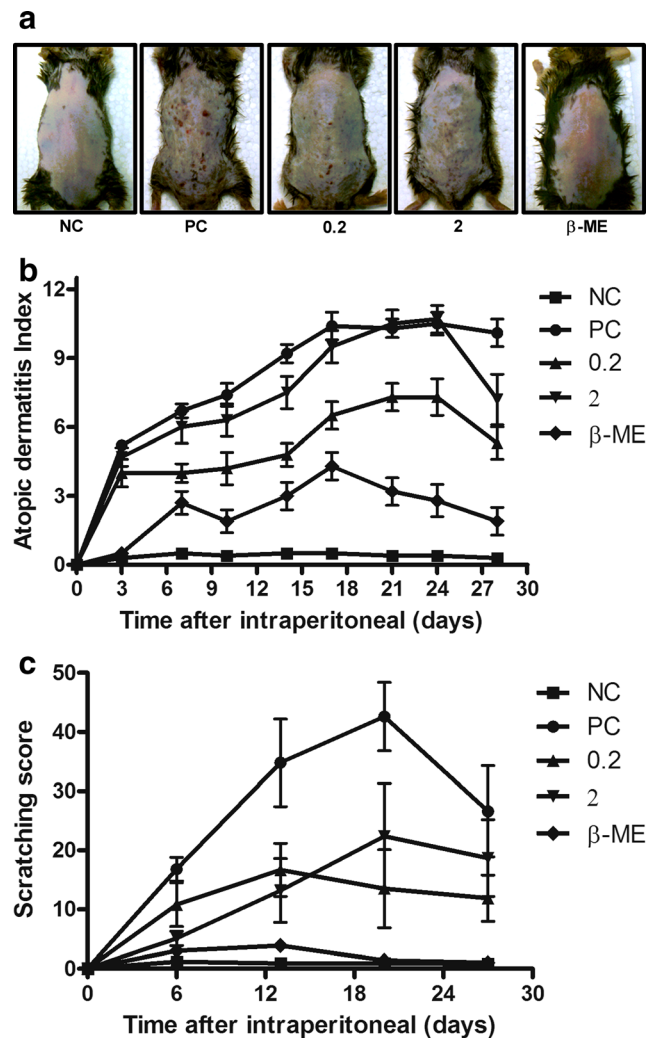


**Fig. 4** The effects of P14 on AD. **a** AD cream-induced AD-like skin lesions in NC/Nga mice. P14 is demonstrated to have an inhibitory effect on the ongoing pathogenesis of AD-like skin lesions, when compared with the control group. Intraperitoneal injection of two different doses of P14 (0.2 and 2 mg/head) and 0.064 % of  $\beta$ -ME was administered after AD cream sensitization. The photographs were obtained on week 8 before the mice were sacrificed. **b** Skin severity score. The skin severity of the mice was assessed macroscopically. The dermatitis index was evaluated weekly from week 4 to week 8. The symptoms considered were itching, erythema/hemorrhage, oedema, excoriation/erosion, and scaling/dryness. **c** The scratching behavior of each mouse was observed for 10 min after sensitization. The graph shows mean scratching behavior of P14 or  $\beta$ -ME-treated groups for 4 weeks

## Changes in serum levels of IgE and IL-4 in NC/Nga mice treated with P14

In addition to clinical features, the levels of IgE and IL-4 in the sera from NC/Nga mice were assessed to characterize the immunological response during disease progression, as increased IgE levels is a common indicator of AD. At week 8, the concentration of soluble serum IgE was significantly reduced in P14-treated mice. The IgE levels of P14 0.2 and 2 mg/head were  $33.1 \pm 18.9$  and  $43.1 \pm 27.2$   $\mu$ g/mL,

respectively, in comparison to the PC group, where the level was  $103.4 \pm 8.5$   $\mu\text{g/mL}$ , whereas the NC group of non-AD NC/Nga mice produced much lower serum IgE at  $7.7 \pm 0.9$   $\mu\text{g/mL}$  (Fig. 5a). Taken together, these results show that the increase of serum IgE correlates with the onset of AD-like skin lesions. Treatment with P14 reduces IgE production, which inhibits the symptoms of AD-like skin lesions. Thus, P14 alleviates AD-like symptoms through the concomitant downregulation of IgE.

As P14 treatment differentially affected the decrease of IL-4 in relation to Th2 responses, we examined the effect of P14 on Th2 cytokine responses. To determine the effect of P14 on the Th2 cytokine responses, we measured the levels of IL-4, which represented Th2 immune responses in sera isolated from 14-week old NC/Nga mice. The IL-4 sera levels in the PC mice, which were determined as  $43.4 \pm 7.1$   $\text{pg/mL}$ , when treated with P14 at 0.2 and 2  $\text{mg/head}$ , decreased to  $12.4 \pm 5.1$  and  $14 \pm 9.6$   $\text{pg/mL}$ , respectively. These levels seen in P14-treated mice were similar to those of NC mice ( $10.2 \pm 0.8$   $\text{pg/mL}$ ) (Fig. 5b). These data show that treatment with P14 resulted in the reduction of both serum IgE and IL-4 levels (Fig. 5a). Overall, the *in vivo* results have demonstrated that 0.2  $\text{mg}$  rather than 2  $\text{mg}$  of P14 is more effective in reducing AD-like lesions.

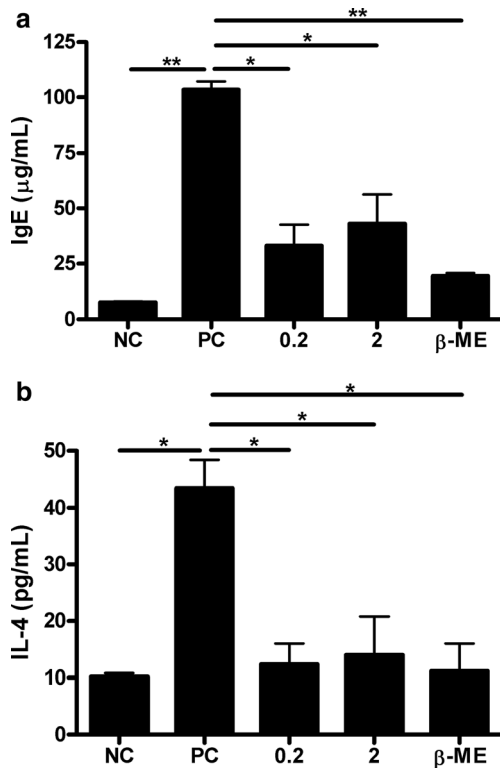


**Fig. 5** Serum IgE and IL-4 levels in AD cream-sensitized NC/Nga mice. Blood was obtained from NC/Nga mice treated with P14 or  $\beta$ -ME on week 8 for which the serum IgE levels (a) and IL-4 levels (b) were determined using ELISA. Data shown represent the mean  $\pm$  SD. \* $P < 0.05$  and \*\* $P < 0.01$  compared with PC group (analysis of variance)

## Improvements in epidermal skin lesions in P14-treated NC/Nga mice

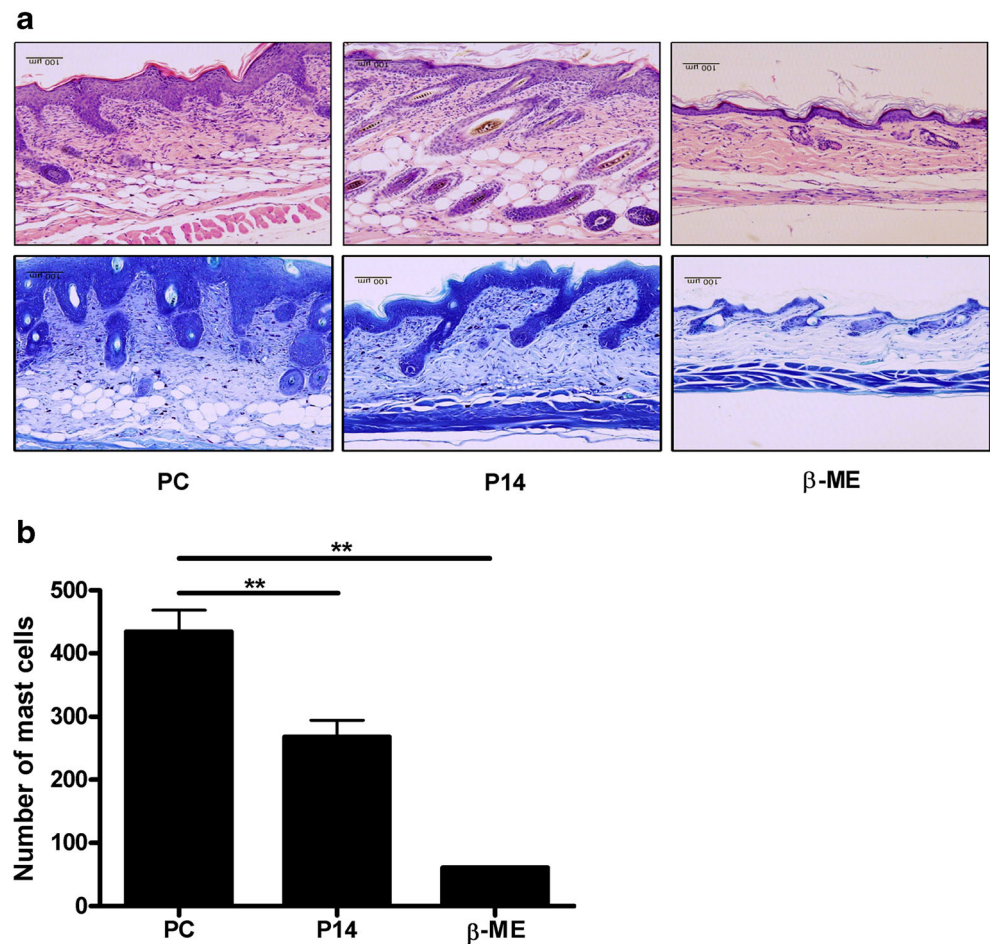
PC NC/Nga mice with fully developed AD-like skin lesions showed hypertrophy and hyperkeratosis, which are typical features exhibited by patients with AD epidermis (Fig. 6a). Dense infiltration of inflammatory cells was also observed in the epidermis in this group of mice (Fig. 6a). To analyze the effect of P14 on skin hypertrophy infiltration in AD cream-induced NC/Nga mice, the P14-treated skin was stained with hematoxylin and eosin (H&E) and then examined using an optical microscope. As shown in Fig. 6a (top), the acanthosis was clearly suppressed in P14-intraperitoneal NC/Nga mice when compared to PC mice. These results indicate that P14 changes skin hypertrophy in AD cream-induced NC/Nga mice.

Toluidine blue staining of the dorsal skin sections revealed more infiltration and degranulation of mast cells in the upper dermis of the PC, in comparison to the P14-treated groups, as shown in Fig. 6a (bottom). The quantitative data on mast cell infiltration and degranulation are shown in Fig. 6a (bottom). Figure 6b shows the chronological course of number of mast cells in the lesional skin. The numbers of mast cells per millimeter square in the PC, P14, and  $\beta$ -ME were found to be  $434.4 \pm 89.9$ ,  $268.1 \pm 69.8$ , and  $60.4 \pm 3.3$ , respectively. The number of mast cells had significantly decreased by 38.3 and 86.1 % in P14 and  $\beta$ -ME mice when compared to PC mice, from week 8. These results indicate that intraperitoneal P14 suppressed AD cream-induced AD-like skin lesions in NC/Nga mice, by reducing the infiltration of mast cells.

## Discussion

Many studies have suggested that the Th1 and Th2 reaction types can reciprocally regulate one another. Thus, given the immunologic imbalance in AD, one of the goals of AD therapy is to manipulate the cytokine network and to preferentially inhibit the Th2 response. Several studies have reported inhibition of the development of AD-like skin lesions in NC/Nga mice by suppressing the Th2 response (Jeong et al. 2010; Kim et al. 2012; Lee et al. 2006). These facts led us to further investigate the distinct inhibitory effect of AD improvement on the development of AD-like skin lesions in NC/Nga mice. IL-4 is an important factor in immunoglobulin class switching to IgE and directly induces the release of IgE (Ozcan et al. 2008). Although there are few reports that IgE plays a major role in the pathogenesis of AD, it has been reported that abnormally high serum IgE levels correlate with the severity of AD (Ahmed and Nasreen 2007; Laske and Niggemann 2004). As well, the production of IL-4 cytokine in epidermal cells has been known to be the main factor for initiation of AD (Chan et al. 2001).

**Fig. 6** Comparison of the histopathological analysis of dorsal skin lesions in AD cream-treated NC/Nga mice, following repeated intraperitoneal injections of P14. **a** Images are representative examples from each group. Hematoxylin-eosin (H&E) or toluidine blue-stained sections of PC, P14-intraperitoneal NC/Nga mice, and  $\beta$ -ME-treated NC/Nga mice (original magnification  $\times 100$ ). **b** mast cells of dorsal skin lesions compared with PC, P14, and  $\beta$ -ME groups. Data shown represent the mean  $\pm$  SD.  $^{**}P < 0.01$  compared with PC group (analysis of variance)



Many studies investigating the use of probiotics for the treatment of AD have been conducted in both humans and animals, with most studies employing *Lactobacillus* and *Bifidobacterium* species (Fujiwara et al. 2004; Hengge et al. 2006; Larsen et al. 2011; van der Aa et al. 2012; Yesilova et al. 2012). Probiotic shows no side effects and stabilize the imbalance of Th1 and Th2 cytokines, reinforcing the immune function that has already been reported. Based on the success of the use of LAB in the treatment of AD, we sought to identify the active component from the well-characterized species LC. We identified the active protein P14 from LC extracts using IEC chromatography. The P14 protein was overexpressed using the *E. coli* system (Fig. 2a). Our study demonstrated that P14 decreased the levels of IL-4 in RAW264.7 and Balb/c splenocytes in vitro and ex vivo (Fig. 3a, b). In addition, P14 was confirmed to be nontoxic to NIH 3 T3 mouse normal cell lines (Fig. 3c). To examine the effective dose range and the dose dependency of P14, we performed experiments using two different doses, 0.2 and 2 mg/head. In Biostir AD cream-sensitized NC/Nga mice, P14 treatment resulted in a decrease in the AD index and skin severity score (Fig. 4b, c), as well as Th2-mediated IgE and IL-4 levels in sera (Fig. 5). These results confirm that P14 restores the impaired

Th2 responses. Macroscopic analysis showed severe hemorrhage, acanthosis, and excoriation in the control group, whereas P14 treatment prevented these skin changes (Fig. 4a). Histologically, intraperitoneal injection of P14 decreased hypertrophy, hyperkeratosis, and infiltration of inflammatory cells in the skin (Fig. 6a). Although P14 was effective at two different doses, P14 at 0.2 mg/head was slightly more effective than P14 at 2 mg/head.

Beta-methasone is a potent glucocorticoid steroid with anti-inflammatory and immunosuppressive properties. It is applied as a topical cream, ointment, foam, lotion, or gel to treat itching. Beta-methasone is sometimes prescribed as an intramuscular injection for itching from various ailments, including allergic reactions to poison ivy and similar plants. Although treatment of AD with steroids is effective, there are many unwanted adverse side effects. Therefore, the use of steroids in the treatment of AD should only be in an emergency. This has resulted in the use of LAB as a viable alternative.

In conclusion, P14 plays an important role in the inhibition of IgE and IL-4 in AD-like skin lesions of Biostir AD cream-treated NC/Nga mice, through the modulation of Th2 immune responses. P14 treatment resulted in a decrease in Th2-

mediated cytokines and was shown to improve AD in pathohistology. Therefore, these results suggest that P14 alleviates AD symptoms and represents a promising candidate for the treatment of AD. Further studies are required on aspects such as the mechanism involved in the alleviation of AD and biological role of P14 in LC. Moreover, it is necessary to conduct further research with regard to the contribution of P14 to human health.

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