

ORIGINAL ARTICLE

***Lactococcus lactis* KR-050L extract suppresses house dust mite induced-atopic skin inflammation through inhibition of keratinocyte and mast cell activation**M. Jin¹, S. Lee², Y.-A. Choi¹, H.-J. Jang², S.W. Lee², P.-H. Park³, T.-Y. Shin⁴, M.-C. Rho², Y.H. Jang⁵ and S.-H. Kim¹ ¹ CMRI, Department of Pharmacology, School of Medicine, Kyungpook National University, Daegu, Korea² Immunoregulatory Materials Research Center, Korea Research Institute of Bioscience and Biotechnology, Jeongseup, Korea³ College of Pharmacy, Yeungnam University, Gyeongsan, Korea⁴ College of Pharmacy, Woosuk University, Jeonju, Korea⁵ Department of Dermatology, School of Medicine, Kyungpook National University, Daegu, Korea**Keywords**atopic dermatitis, house dust mite, keratinocytes, mast cells, *Lactococcus lactis*.**Correspondence**

Yong Hyun Jang, Department of Dermatology, School of Medicine, Kyungpook National University, 2-101 Dongin-dong, Jung-gu, Daegu 41944, Korea.

E-mail: yhjjang@knu.ac.kr

Sang-Hyun Kim, CMRI, Department of Pharmacology, School of Medicine, Kyungpook National University, 2-101 Dongin-dong, Jung-gu, Daegu 41944, Korea.

E-mail: shkim72@knu.ac.kr

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Abstract

Aims: Atopic dermatitis (AD) is a chronic inflammatory skin disease, with a steadily increasing prevalence. Lactic acid bacteria (LAB) have been widely used in the food industry and are an attractive option for preventing and treating allergic skin diseases. We previously isolated new LABs including *Lactococcus lactis* KR-050L from Gajuknamu kimchi, and showed the anti-inflammatory effects of extract of *L. lactis* KR-050L culture broth (LLK). In this study, we investigated the effects of LLK on AD.

Methods and Results: For the *in vitro* study, we used human keratinocytes (HaCaT) and mast cells (RBL-2H3). *In vivo* study, we investigated the effects of LLK on *Dermatophagoides farinae* extract (DFE) and 2,4-dinitrochlorobenzene (DNCB)-induced atopic skin inflammation in mice. LLK suppressed expression of pro-inflammatory cytokines and chemokines by down-regulation of p38 MAPK, STAT1 and nuclear translocation of NF- κ B in keratinocytes. Topical application of LLK suppressed AD symptoms based on reduction in ear thickness, serum IgE levels and immune cell infiltration. Furthermore, LLK inhibited serum histamine levels and mast cells infiltration *in vivo*, and reduced mast cells activation *in vitro*.

Conclusions: These results suggest that LLK inhibits AD symptoms through inhibition of keratinocytes and mast cells activation.

Significance and Impact of the Study: LLK is a potential therapeutic candidate for AD treatment.

Introduction

Atopic dermatitis (AD) is one of the most common inflammatory skin diseases characterized by epidermal barrier defects and immune system dysregulation (Bending and Ono 2017). AD pathogenesis involves interactions between genetic and environmental factors. Aeroallergens, including house dust mite (HDM) allergens, have been implicated in AD pathogenesis (Dai *et al.* 2011). *Dermatophagoides farinae* extract (DFE) is one of the most common HDM allergen mainly associated with AD (Karuppagounder *et al.* 2015). AD is a biphasic

T-cell-mediated disease, with T helper (Th) 2 cells being predominantly detected in the acute phase of the disease. AD begins with an acute phase characterized by excessive Th2 and Th17 cell activation. Transition to the chronic phase is marked by Th1 cell activation and continued Th2 and Th17 cell activation (Guttman-Yassky *et al.* 2017). Various types of cells including basophils, eosinophils and mast cells are involved in Th2 immune response by producing pro-inflammatory mediators (Gittler *et al.* 2012; Leung 2016). Th17 cells produce pro-inflammatory cytokines and chemokines that regulate the release of anti-microbial peptides in keratinocytes (Moy

et al. 2015). Keratinocytes activation is a hallmark of AD pathogenesis. Activated keratinocytes produce pro-inflammatory factors that promote chronic, self-amplifying immune activation. In patients with AD, keratinocytes are important for promoting and maintaining inflammation (Albanesi *et al.* 2005; Werfel 2009; Jin *et al.* 2016).

At present, topical application of corticosteroids and immunosuppressants has been used to promote rapid improvement of AD. Immunosuppressants such as cyclosporine A (CsA) and tacrolimus (FK506), which interfere with calcineurin are highly effective treatment for severe AD (Trautmann *et al.* 2001). However, long-term use of those drugs exerts serious adverse effects (Karuppagounder *et al.* 2015). The beneficial role of probiotics in clinical and developmental immunology, especially the beneficial role of *Lactobacillus* strains in AD, has gained significant interest in recent years (Yeom *et al.* 2015; Choi *et al.* 2016). Lactobacillales or lactic acid bacteria (LAB) produce lactic acid as a metabolic end-product, and are known to prevent AD symptoms (Masuda *et al.* 2012; Kim and Park 2014; Yeom *et al.* 2015). It has been reported that kimchi, a traditional Korean fermented food, contains different LABs such as *Leuconostoc* and *Lactobacillus* (Jung *et al.* 2011). Previously, we showed that the extract of culture broth (cells + supernatant) of *Lactococcus lactis* KR-050L (LLK), a newly isolated LAB from Gajuknamu kimchi, exerts anti-inflammatory effects by the reduction in interleukin (IL)-6/signal transducer and activator of transcription (STAT)3 pathway, which is also known to play an important role in AD (Hwang *et al.* 2017; Ryu *et al.* 2018). On the basis of this, we elucidate the effect of LLK on atopic skin inflammation, and determine the underlying mechanisms of action.

Materials and methods

Reagents

LLK was prepared as described previously (Hwang *et al.* 2017). DFE (Greer Laboratories, Lenoir, NC) was used as an antigen. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. DFE was dissolved in phosphate-buffered saline (PBS) containing 0.5% Tween 20, and 2,4-dinitrochlorobenzene (DNCB, 1%) was dissolved in an acetone/olive oil (1 : 3) solution. Recombinant human tumour necrosis factor (TNF)- and interferon (IFN)-were purchased from R&D systems (Minneapolis, MN).

Cell culture and viability

A human keratinocytes cell line (HaCaT) and RBL-2H3 cells were incubated in Dulbecco's modified Eagle's

medium (Invitrogen, Grand Island, NY), supplemented with 10% foetal bovine serum and antibiotics (100 U ml⁻¹ penicillin G and 100 µg ml⁻¹ streptomycin), at 37°C in 5% CO₂. Cell viability was determined by performing 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. HaCaT cells were seeded in 96-well plates (1 × 10⁴ cells per well) and were treated with different concentrations of LLK for 24 h. Next, MTT solution (5 mg ml⁻¹) was added to each well containing the sample, and the cells were incubated for another 2 h at 37°C. Dimethyl sulphoxide was used to dissolve formazan crystals. The absorbance of each well was compared to that of the control sample and expressed as a percentage. To confirm the results of MTT assay, HaCaT cells (3–6 passage) were plated in 12-well plates (2 × 10⁵ cells per well) and were allowed to attach for 24 h. HaCaT cells were treated with LLK for 24 h, trypsinized, and counted using a haemocytometer.

Animals

Six-week-old female BALB/c mice were purchased from SLC Inc. (Hamamatsu, Japan). The mice were housed 5–10 mice per cage in a laminar airflow room maintained at a temperature of 22 ± 2°C and a relative humidity of 55 ± 5% throughout the study. The food and water were provided *ad libitum*, and the mice were kept on 12-h light/12-h dark cycle. The care and treatment of the mice were performed in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (KNU-2016-00422).

Development of DFE/DNCB-induced atopic skin inflammation in the mouse ear

DFE/DNCB-induced atopic skin inflammation was induced as described previously (Choi *et al.* 2014). A schematic representation of the experimental procedure is provided in Fig. S1c. Mice (total *n* = 30) were divided into the following six groups (*n* = 5 per group): vehicle, DFE/DNCB plus vehicle, DFE/DNCB plus LLK (25, 50, or 100 µg per ear), or FK506 (50 µg per ear). Surfaces of both the ear lobes were very gently stripped with surgical tape (Nichiban, Tokyo, Japan). After stripping, 20 µl of DNCB (1%) was applied on each ear, followed by the application of 20 µl DFE (10 mg ml⁻¹) after 3 days. DFE/DNCB treatment was repeated once a week for 4 weeks. Two weeks after the first induction, tail bleeding was performed to determine serum IgE level. After confirming the atopic condition based on the serum IgE level, the mouse ears were treated with LLK (25, 50, or

100 μg per ear), or FK506 (μg per ear) by painting five times per week, for 3 weeks (total 15 times). Ear thicknesses were measured 24 h after DFE or DNCB application using a dial thickness gauge (Mitutoyo, Co., Tokyo, Japan).

On day 28, mice were killed using carbon dioxide. Blood samples were collected from the celiac artery. Serum was obtained by clotting the blood at room temperature. The clot was removed, the sample was centrifuged at 2000 g and 4°C for 15 min, and supernatant was retained. After blood collection, the ears were removed and used for performing histopathological analysis and RNA extraction. Total serum IgE and IgG2a levels were measured using an ELISA kit (BD Biosciences, Oxford, UK), according to the manufacturer's instructions. For detecting DFE-specific IgE, 96-well plates (Nunc, Wiesbaden, Germany) were coated with 10 mg DFE. DFE-specific IgE level was expressed as O.D. value.

Histological observation

The mouse ears (total $n = 30$) were fixed with 10% formaldehyde and were embedded in paraffin. The ears were cut into 5- μm sections and were stained with haematoxylin and eosin (H&E). Lymphocytes infiltration, epidermal thickening and dermal fibrosis were determined using a microscope as described previously (Jin *et al.* 2016). Eosinophils were counted in 10 high-power fields for each sample at $\times 400$ magnification. Epidermal and dermal thickening were analysed using H&E-stained sections at $\times 200$ magnification. Thickening was measured in five randomly selected fields for each sample. For measuring mast cell infiltration, mouse ear sections were stained with toluidine blue and the number of mast cells was counted in five randomly selected fields for each sample at $\times 400$ magnification.

qPCR

Cytokines expression was determined by performing qPCR with Thermal Cycler Dice TP850 (Takarabio, Shiga, Japan), according to the manufacturer's protocol. At the end of the *in vivo* experimental period, the mouse ears (total $n = 30$) were excised and total RNA was isolated. HaCaT cells were pretreated with LLK for 1 h, followed by stimulation with TNF- α (10 ng ml^{-1}) and IFN- γ (10 ng ml^{-1}) for 6 h. Total cellular RNA was isolated from the cells (2×10^5 cells in 24-well plates) as previously described (Jin *et al.* 2016). First-strand complementary DNA (cDNA) was synthesized using RT Premix (iNtRON Biotech, Sungnam, Korea). Reverse transcription conditions were 45°C for 60 min and 95°C for 5 min. qPCR was performed in 25 μl reaction

mixture containing 2 μl cDNA (100 ng), 1 μl sense and antisense primer solution (0.4 $\mu\text{mol l}^{-1}$), 12.5 μl SYBR Premix Ex Taq (Takarabio), and 8.5 μl dH₂O. Conditions for qPCR were similar to those used previously (Jin *et al.* 2016), except for the primers of mouse IL-5 (F 5'-GAA GTG TGG CGA GGA GAG AC-3', R 5'-GCA CAG TTT TGT GGG GTT TT-3'). The normalization and quantification of mRNA expression were performed using TP850 software supplied by the manufacturer. The gene expression levels were normalized to β -actin, and the values of fold-changes are represented.

Western blotting

Samples for Western blotting were prepared as previously described (Jin *et al.* 2016). Briefly, cells (2×10^6 cells in six-well plates) were treated during 30 min to evaluate activation of p38 MAPK, STAT1 and NF- κB . Cells were rinsed twice with ice-cold PBS, and total cell lysates were prepared in 200 μl lysis buffer. The lysates were spun in a micro-centrifuge at 15 000 g and 4°C for 20 min, and supernatant obtained was collected. Proteins were electrophoresed by performing SDS-PAGE on 8–12% gels and were transferred to nitrocellulose membranes. The membranes were stained with reversible Ponceau S to ensure equal loading of the samples on the gel. Nucleus and cytosolic levels of p65 NF- κB and I $\kappa\text{B}\alpha$ were determined using anti-NF- κB (p65) and anti-I $\kappa\text{B}\alpha$ antibodies (Santa Cruz Biotech., Santa Cruz, CA), respectively. Phosphorylation of p38, and STAT1 were determined using anti-phospho-p38, and anti-phospho-STAT1 antibodies (Cell Signaling, Beverly, MA). Immunodetection was performed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA).

β -Hexosaminidase release

The release of β -hexosaminidase used as a marker of mast cell degranulation. Anti-dinitrophenol (DNP) IgE (100 ng ml^{-1})-sensitized RBL-2H3 cells (5×10^5 cells in 12-well plates) were pretreated with LLK for 1 h after washing three times with PBS and then stimulated with DNP-human serum albumin (HSA) (100 ng ml^{-1}) for 4 h. After incubation, the cells were taken and centrifuged to separate the supernatant and cell pellet. Cell pellets were lysed in 40 μl media culture 1% Triton X-100, and then the supernatant was transferred to 96-well plates and incubated with an equal volume of β -hexosaminidase substrate (1 mmol l^{-1} 4-nitrophenyl *N*-acetyl-beta-D-glucosaminide in 0.05 mol l^{-1} citrate buffer, pH 4.5) for 1 h at 37°C. The reaction was stopped by adding 200 μl stop solution (0.05 mol l^{-1} sodium carbonate buffer, pH 10). Optical density was measured at 405-nm

using a spectrophotometer. β -Hexosaminidase release was calculated by dividing the content in the culture media by the combined absorbance of the culture media and cell lysate.

%Degranulation

$$= \text{OD}_{\text{Cultured media}} / (\text{OD}_{\text{Cultured media}} + \text{OD}_{\text{Cell lysate}}) \times 100$$

Histamine assay

Histamine content was measured using *o*-phthaldialdehyde spectrofluorometric procedure as previously described (Jin *et al.* 2016). The blood was collected from the mice (total $n = 30$) and serum was obtained by centrifugation (400 g, 10 min, 4°C), and was used to measure histamine content. The plate was read on fluorescence spectrometer LS-50B (Perkin-Elmer, Norwalk, CT) using 355-nm excitation and with 450-nm emission filters.

Statistical analysis

Statistical analyses were performed using Prism 5 (Graph-Pad Software, San Diego, CA). Effects of LLK treatment were analysed using one-way analysis of variance, followed by Dunnett's test. A value of $P < 0.05$ was considered statistically significant differences.

Results

LLK inhibits the activation of keratinocytes

Keratinocytes play an important role in the pathogenesis of AD-like skin inflammation and promote the chronic phase of AD by producing inflammatory factors (Werfel 2009; Park *et al.* 2015). Human keratinocytes produce pro-inflammatory cytokines and chemokines such as chemokine ligand (CCL)17 and CCL22 in response to stimulation with TNF- α and IFN- γ , which are the key mediators of AD pathogenesis (Choi *et al.* 2014; Park *et al.* 2015). Prior to determining the effects of LLK on keratinocytes, we examined the cytotoxicity of LLK by the MTT assay and cell counting in a human keratinocyte cell line (HaCaT). LLK did not exert cytotoxic effects on HaCaT cells up to a concentration of 100 $\mu\text{g ml}^{-1}$ (Fig. S1a,b). Next, we investigated the effect of LLK on pro-inflammatory cytokines and chemokines in TNF- α /IFN- γ -stimulated HaCaT cells. LLK decreased TNF- α /IFN- γ -stimulated gene expression of TNF- α , IL-1 β , IL-6, CCL17, and CCL22 in keratinocytes (Fig. 1a). The effectiveness of LLK was similar to that of CsA. CsA or tacrolimus (FK506) is an immunosuppressant for the systemic therapy of severe AD. FK506 is available in form of an

ointment containing a nonsteroidal topical calcineurin inhibitor for treating AD (Zhai *et al.* 2011). In this study, CsA and FK506 ointment were used as *in vitro* and *in vivo* positive controls, respectively.

A previous study showed that NF- κ B and JAK/STAT signalling pathways facilitate the production of pro-inflammatory chemokines (CCL17 and CCL22) and cytokines (TNF- α , IL-1 β and IL-6) in TNF- α /IFN- γ -stimulated HaCaT cells (Park *et al.* 2015). In addition, TNF- α /IFN- γ activates several intracellular MAPK signalling pathways in keratinocytes (Choi *et al.* 2014). Western blot analysis revealed that LLK decreased the activation of p38 MAPK, STAT1 and NF- κ B (Fig. 1b).

LLK inhibits the ear thickening and histopathological observation

To determine the effects of LLK on AD, we used DFE/DNCB-induced atopic skin inflammation mouse model. LLK (25, 50 or 100 μg per ear) or FK506 was topically applied on the ears of BALB/c mice with DFE/DNCB five times per week for 3 weeks. Schematic representation of the experimental procedure was provided in Fig. S1c. During the induction period, we measured ear thickening after 24 h of each DFE or DNCB application. Until 2 weeks, there was no significant difference in ear thickness between each group of mice. After 14 days of AD induction, mice treated with LLK showed significant reduction in ear thickening (Fig. 2a and Fig. S1d).

Microscopic analysis showed alterations, including epidermal and dermal thickening, hyperkeratosis, large ulcers, and inflammatory cell infiltration, in mice with DFE/DNCB-induced atopic skin inflammation. From day 19 onward, mice treated with LLK (20, 50, or 100 μg per ear) showed marked reduction in epidermal and dermal thickening and inflammatory cell infiltration compared with DFE/DNCB-treated mice (Fig. 2b–e). Histological analysis showed that LLK treatment of mice with DFE/DNCB-induced atopic skin inflammation markedly decreased hyperkeratosis, large ulcers, and inflammatory cell infiltration (Fig. 2b). LLK significantly decreased epidermal and dermal thickening and eosinophil infiltration compared with that in mice with DFE/DNCB-induced atopic skin inflammation (Fig. 2c,d).

LLK suppresses the serum immunoglobulin and inflammatory cytokines

Cytokines promote immunoglobulin isotype switching, with IL-4 and IFN- γ promoting IgE and IgG2a isotype switching respectively (Brandt and Sivaprasad 2011). Our previous study showed that serum immunoglobulin and cytokine levels were increased in mice with DFE/DNCB-

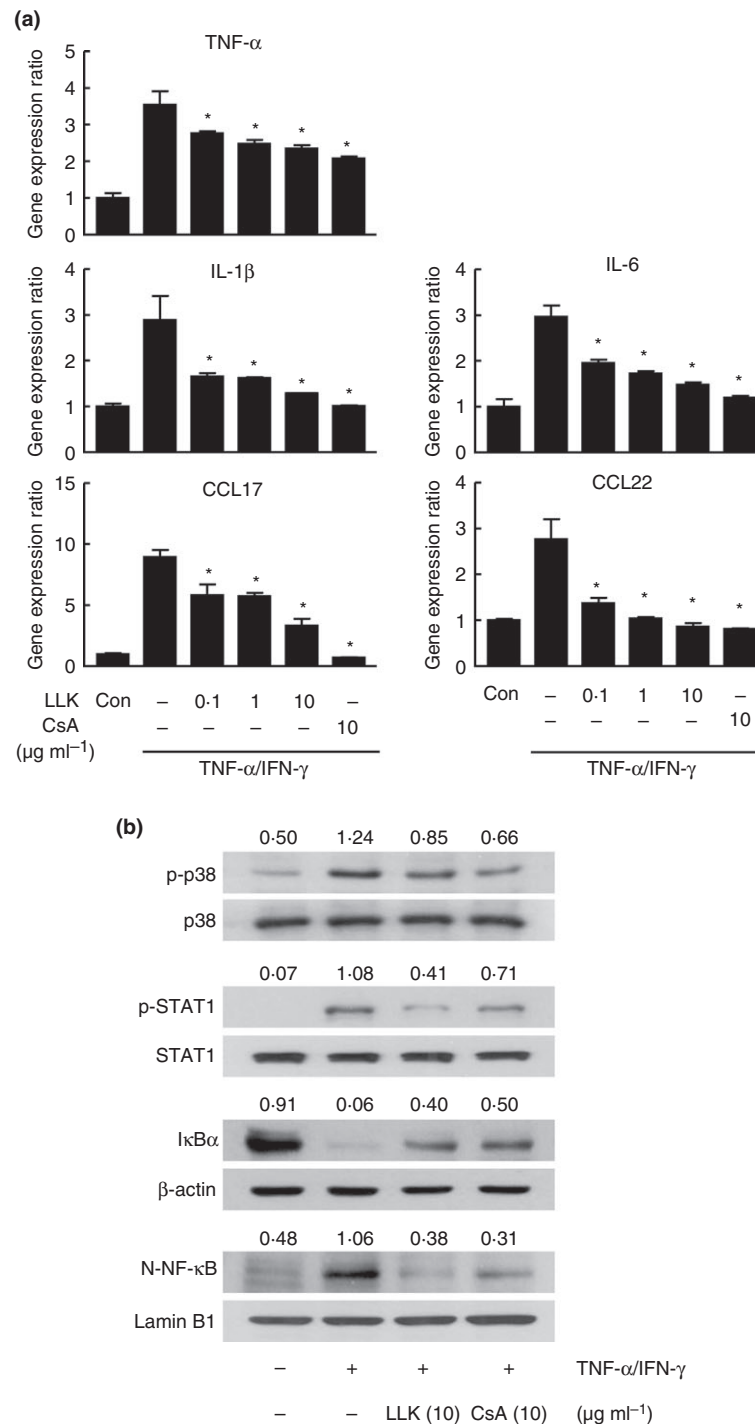


Figure 1 Effect of LLK on the Th2-mediated inflammation in TNF-α/IFN-γ-stimulated HaCaT cells. (a) The cells were pretreated with LLK (0.1, 1 or 10 $\mu\text{g ml}^{-1}$), or CsA (10 $\mu\text{g ml}^{-1}$) for 1 h followed by stimulation with TNF-α (10 ng ml^{-1}) and IFN-γ (10 ng ml^{-1}) for 6 h. Gene expression levels were determined by performing qPCR. The gene expression levels were normalized to β -actin, and the values of fold-changed are represented. Data are presented as mean $\pm$ SEM ($n = 5$); * $P < 0.05$, significantly lower than TNF-α/IFN-γ-stimulated group. (b) The cells were pretreated with LLK (10 $\mu\text{g ml}^{-1}$) or CsA (10 $\mu\text{g ml}^{-1}$) for 1 h, followed by stimulation with TNF-α (10 ng ml^{-1}) and IFN-γ (10 ng ml^{-1}) for 30 min. The activation of p38 MAPK, STAT1, and NF-κB was analysed by Western blotting. The band intensity was digitized and normalized by the relative ratio. The ratios of bands were measured using ImageJ software. β -actin and lamin B1 was used as loading controls. The experiment shown is representative of three independent experiments. N: nuclear; p: phosphorylated; Con: control; CsA: cyclosporine A.

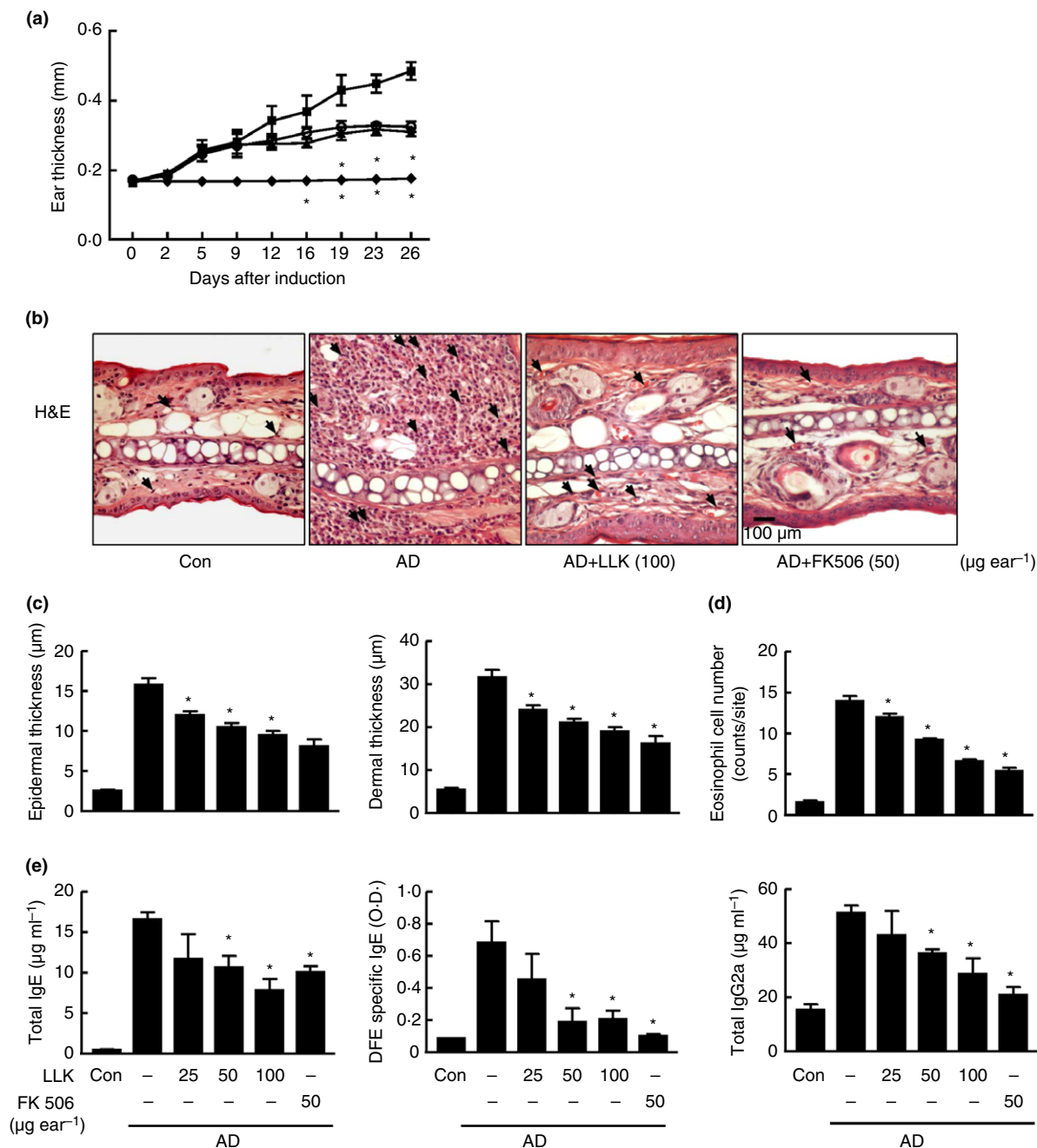


Figure 2 Ear thickening, histological observation, and serum levels in AD mice. (a) Ear thickness in mice with DFE/DNCB-induced atopic skin inflammation. The mouse ears were treated with LLK (25, 50 or 100 μ g per ear) or FK506 (50 μ g per ear) five times per week from day 8 after the first induction. Ear thickening was measured 24 h after DFE or DNCB application using a dial thickness gauge. ($\blacklozenge$) Con; ($\blacksquare$) AD; ($\bullet$) AD+LLK (100 μ g per ear); ($\blacktriangle$) AD+FK506 (50 μ g per ear). (b) Histological analysis in mice with DFE/DNCB-induced atopic skin inflammation. Representative photomicrographs of ear sections stained with H&E (original magnification $\times 400$); scale bars represent 100 μ m. Arrows indicate the bright eosinophilic granular bodies. (c) Epidermal and dermal thickening were analysed using H&E-stained sections at $\times 200$ magnification. (d) Eosinophils in H&E-stained sections were counted in 10 high-power fields at $\times 400$ magnification. (e) Serum levels in mice with DFE/DNCB-induced atopic skin inflammation. Total serum IgE levels, DFE-specific IgE levels, and serum IgG2a levels were measured by ELISA. The mice were divided into the following six groups, with five mice per group: vehicle, DFE/DNCB plus vehicle, DFE/DNCB plus LLK (25, 50 or 100 μ g per ear) or FK506 (50 μ g per ear). The experiment shown is representative of two independent experiments. Data are presented as mean $\pm$ SEM ($n = 5$ per group); $*P < 0.05$, significantly lower than DFE/DNCB-stimulated group. Con: control; FK506, tacrolimus. [Colour figure can be viewed at wileyonlinelibrary.com]

induced atopic skin inflammation (Choi *et al.* 2014). To investigate the effects of LLK on Th1 or Th2 cell response, we examined serum levels of IgE (total and DFE specific) and IgG2a. LLK decreased total IgE, DFE-specific IgE and IgG2a compared with those mice with atopic skin inflammation (Fig. 2e). Highest dose of LLK (100 μg per ear) showed similar inhibition as FK506.

AD pathogenesis is characterized by biphasic T cell polarization, with dominant Th2 cell infiltration being observed in the acute phase and Th1 phenotype switching being observed in the chronic phase. Th17 cells are essential for AD pathophysiology (Auriemma *et al.* 2013). To analyse the effect of LLK on Th1 (TNF- α and IFN- γ), Th2 (IL-4, IL-5, IL-13 and IL-31) and Th17 (IL-17A) cell immune responses, we measured inflammatory cytokines

in ear tissues using qPCR. The gene expression of Th1-associated cytokines (TNF- α and IFN- γ), Th2-associated cytokines (IL-4, IL-5, IL-13 and IL-31), and Th17-associated cytokines (IL-17A) were markedly increased in mice with DFE/DNCB-induced atopic skin inflammation; however, it was reduced by the LLK administration (TNF- α , IL-5, and IL-13 at 25, 50 and 100 μg per ear; IFN- γ , IL-4, IL-31 and IL-17A at 50 and 100 μg per ear) (Fig. 3).

LLK suppresses the degranulation of mast cells

Th2-mediated immune response in AD is mediated with multiple cell types including keratinocytes, eosinophils and mast cells. Mast cells are tissue immune cells derived

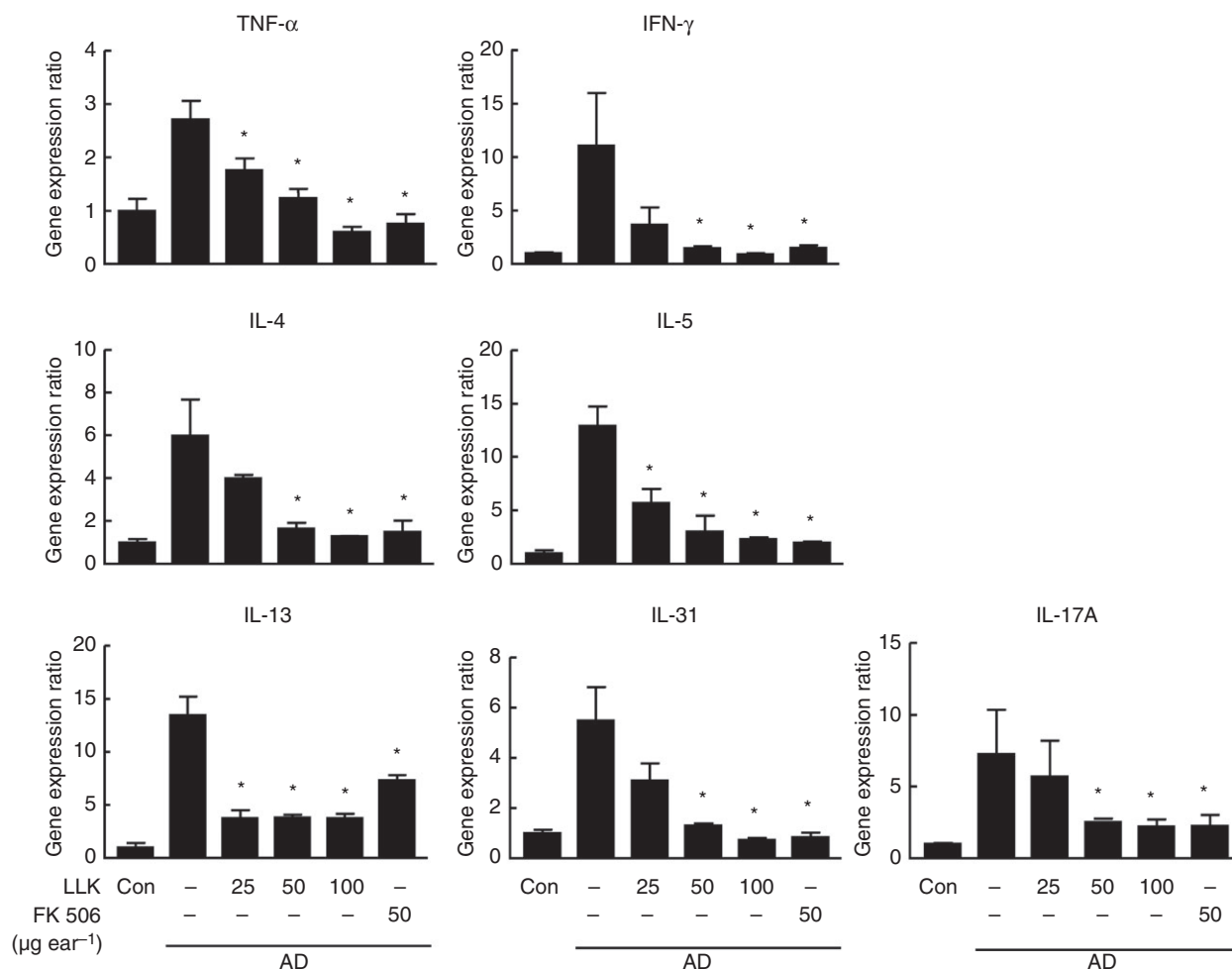


Figure 3 Expression of cytokines in the ears of mice with DFE/DNCB-induced atopic skin inflammation. The gene expression levels were normalized to β -actin, and the values of fold-changed are represented. The mice were divided into the following six groups, with five mice per group: vehicle, DFE/DNCB plus vehicle, DFE/DNCB plus LLK (25, 50, or 100 μg per ear) or FK506 (50 μg per ear). The experiment shown is representative of two independent experiments. Data are presented as mean $\pm$ SEM ($n = 5$ per group); * $P < 0.05$, significantly lower than DFE/DNCB-stimulated group. Con: control; FK506: tacrolimus.

from haematopoietic cells, and secrete pro-inflammatory cytokines and histamine (Guttman-Yassky *et al.* 2011). Mast cell-derived histamine and other inflammatory mediators cause itching and inflammation in patients with AD (Amin 2012). Therefore, we examined serum histamine levels. LLK decreased serum histamine level compared with that in mice with DFE/DNCB-induced atopic skin inflammation (Fig. 4a). Furthermore, histopathological examinations of tissue-stained section

showed reduction in mast cell infiltration in the skin of mice treated with DFE/DNCB (Fig. 4b). Next, we examined the ability of LLK on the degranulation of mast cells by measuring the release of histamine and β -hexosaminidase in RBL-2H3 cells. DNP-HSA-challenged RBL-2H3 cells released high levels of histamine and β -hexosaminidase. LLK suppressed the histamine and β -hexosaminidase release in a concentration-dependent manner (Fig. 4c,d).

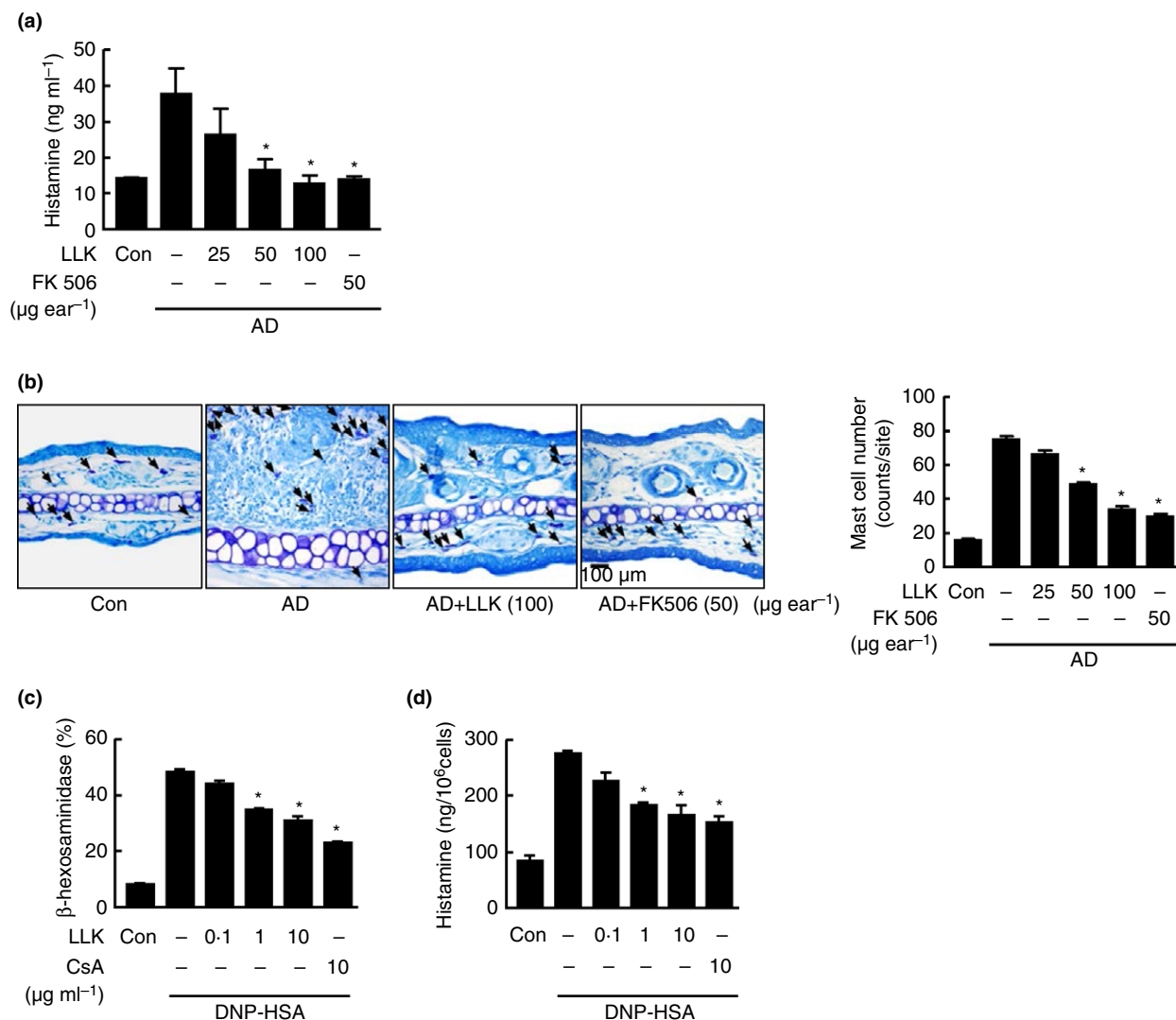


Figure 4 Effects of LLK on the degranulation of mast cells. (a) Histamine levels were measured by o-phthalaldehyde spectrofluorometric procedure (b) Mast cell (arrow) infiltration in mice ear tissue. Representative photomicrographs of ear sections stained with toluidine blue (original magnification $\times 400$); scale bars represent 100 μ m. Mast cells in toluidine blue-stained sections were counted in five fields at $\times 400$ magnification. The experiment shown is representative of two independent experiments. Data are presented as mean $\pm$ SEM ($n = 5$ per group); * $P < 0.05$, significantly lower than DFE/DNCB-stimulated group. (c and d) RBL-2H3 cells were sensitized with anti-DNP IgE (50 ng ml⁻¹). After overnight incubation, the cells were pretreated with or without drugs, including LLK (0.1, 1 or 10 μ g ml⁻¹) and CsA (10 μ g ml⁻¹) and then challenged with DNP-HSA (100 ng ml⁻¹). Histamine levels and β -hexosaminidase levels were detected using a fluorescent plate reader or a spectrophotometer, respectively. The experiment shown is representative of three independent experiments. Data are presented as mean $\pm$ SEM ($n = 5$); * $P < 0.05$, significantly lower than DNP-HSA-challenged group. Con: control; FK506: tacrolimus. [Colour figure can be viewed at wileyonlinelibrary.com]

Discussion

Probiotic therapeutics show great promise in preventing and treating clinical conditions associated with allergic responses. Probiotic bacteria can interact with the host mucosa and may beneficially modulate immune system (Choi *et al.* 2016). Previously, clinical trials involving *in vivo* animal models have reported that various types of LAB alleviate allergic symptoms (Kalliomaki *et al.* 2007; Shah *et al.* 2010). Kimchi is a rich source of LAB, and functional and biological properties of LABs isolated from kimchi (Chinese cabbage kimchi, cubed radish kimchi and young radish kimchi) have been investigated (Hwang *et al.* 2017). Previously, we screened six new strains from Gajuknamu kimchi. Among the isolated LABs, the extract of culture broth (cells + supernatant) of *L. lactis* KR-050L (LLK), exerts the most potent anti-inflammatory effects compared to the other isolated LABs (Hwang *et al.* 2017). Thus, we examined the effects of LLK on atopic skin inflammation using *in vitro* and *in vivo* models.

Patients with AD experience a 'vicious cycle of itching and scratching', and it is known that cytokines (IL-4, IL-13, and IL-31) and histamine play important role in the initiation of itching. Histamine is released by the degranulation of mast cells, and an important inflammatory mediator in patients with AD (Guttman-Yassky *et al.* 2011). Mast cells are the key effector cells in IgE-mediated immediate allergic diseases, such as asthma, allergic rhinitis, and AD (Kawakami *et al.* 2009). Mast cells are localized in many tissues, and activation of mast cell-derived factors such as pro-inflammatory cytokines and histamine contributes to itching and inflammation in patients with AD; moreover, mast cells secrete mediators that affect eosinophils. Our results showed that LLK decreased AD symptoms including mast cell and eosinophil infiltration, ear thickening, as well as dermal and epidermal thickening. LLK inhibited serum histamine levels compared with those mice with atopic skin inflammation. In addition, LLK suppressed release of histamine and β -hexosaminidase from activated mast cells. These results indicate that LLK can decrease itching and AD symptoms through inhibition of mast cell activation.

Total serum IgE and environmental allergen-specific IgE levels are elevated in patients with AD compared with those in healthy individuals (Wu and Scheerens 2014). Th1 and Th2 cells are observed in AD skin lesions, with Th2 cytokines being predominantly expressed in the acute phase of AD and Th1 cytokines being predominantly expressed in the chronic phase of AD (Spergel *et al.* 1999). IL-4 and/or IL-13 are critical for IgE production and Th2 cell differentiation, and IL-5 is critical for eosinophil recruitment (Brandt and Sivaprasad 2011). IL-31

is primarily produced by keratinocytes, Th2 cells, and mast cells and acts as a mediator of pruritus in AD (Auremma *et al.* 2013). Th1 cells produce IFN- γ that drives IgG2a production (Jin *et al.* 2009). IL-17A produced by Th17 cells is observed in both acute and chronic phase of AD skin lesions (Auremma *et al.* 2013). In addition, the number of Th17 cells are upregulated in the peripheral blood of patients with AD (Koga *et al.* 2008). Our mouse model of AD showed up-regulated Th1, Th2 and Th17 cell response after AD induction. Several studies have shown that levels of Th2 cytokines were increased in NC/Nga mouse model of AD and that a drug candidate for treating AD decreased the levels of Th2 cytokines and/or increased the levels of Th1 cytokines (Taniguchi *et al.* 2003; Kang *et al.* 2008). However, this is disputable. Many studies have also shown that drug candidates for treating AD suppressed all the Th1, Th2 and Th17 cell responses (Choi *et al.* 2014; Jin *et al.* 2016). In addition, down-regulated Th1, Th2 and Th17 cell responses are necessary for alleviating AD symptoms (Hwang *et al.* 2013). In our study, LLK significantly suppressed Th1, Th2 and Th17 cell response in the mouse ears. LLK decreased serum IgE, antigen-specific IgE and IgG2a levels. In addition, LLK also inhibited mast cells, which are known to secrete pro-inflammatory mediators, such as histamine and Th2-type cytokines. These results suggest that LLK can decrease Th2 immune response in AD through inhibition mast cell activation.

Our results also showed that treatment with LLK suppressed pro-inflammatory cytokines and chemokines in human keratinocytes. Keratinocytes are a part of the innate immune defence and contribute to inflammatory reactions in AD (Cevikbas and Steinhoff 2012). Upon allergen stimulation, keratinocytes produce pro-inflammatory cytokines and chemokines (Guttman-Yassky *et al.* 2011). Keratinocytes are a major group of epidermal cells which play key role in AD pathogenesis (Park *et al.* 2015), and are the main source of CCL17 and CCL22 (Saeki and Tamaki 2006; Qi *et al.* 2009). TNF- α /IFN- γ stimulated keratinocytes produce CCL17 and CCL22 (Jin *et al.* 2016). In this study, LLK suppressed gene expression of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) and chemokines (CCL17 and CCL22) in TNF- α /IFN- γ -stimulated keratinocytes. NF- κ B and STAT1 are important transcription factors in immune system and produce pro-inflammatory cytokines after stimulation with TNF- α /IFN- γ (Kisseleva *et al.* 2002; Nakao *et al.* 2002). Previous studies have shown that the promoters of CCL17 and CCL22 have STAT1 and NF- κ B-binding sites and suggest that these transcription factors are involved in the transcription of genes (Nakayama *et al.* 2004; Park *et al.* 2015). MAPKs signalling pathways also important in inflammatory diseases and lead to systemic

inflammatory response syndrome (Arbabi and Maier 2002). In this study, LLK inhibited p38 MAPK, STAT1 phosphorylation, as well as I κ B α degradation and NF- κ B nuclear translocation. These results demonstrate that LLK can inhibit pro-inflammatory cytokines and chemokines by down-regulation of p38 MAPKs, STAT1 and NF- κ B pathways in keratinocytes.

CsA or FK506 is a potent immunosuppressant drug prescribed for AD treatment, and FK506 ointment is used for treating moderate-to-severe AD (Trautmann *et al.* 2001; Zhai *et al.* 2011). In this study, CsA was used as a positive control *in vitro* and FK506 was used as a positive control *in vivo*. In our *in vitro* and *in vivo* models of AD, the inhibitory effect of LLK was comparable to that of FK506 or CsA.

This study showed that topical application of LLK suppressed AD-like skin inflammation in both *in vitro* and *in vivo* AD models. LLK inhibited the expression of pro-inflammatory cytokines and chemokines that play an important role in AD pathogenesis by decreasing p38 MAPK, STAT1 and NF- κ B activation in keratinocytes. In addition, we found that LLK treatment suppressed the activation of mast cells. It should be noted that in spite of the strong effectiveness of LLK even at low concentrations, LLK did not show clear dose-dependent inhibition of keratinocytes. This issue should be cleared in near future. Since we used LLK, an extract of culture broth of *L. lactis* KR-050L, not a single compound, the effort to find major active components of LLK is ongoing in our laboratory. However, we provide evidence that LLK could be a potential therapeutic candidate for AD treatment. Moreover, LABs isolated from Gajuknamu kimchi could be used for development and commercialization functional foods and therapeutic agents for AD.

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Author contributions

M.C.R., Y.H.J. and S.H.K. conceived and designed the experiments; M.J. and Y.A.C. performed the experiments; S.L., H.J.J. and S.W.L. analysed the data; P.H.P. and T.Y.S. contributed reagents/materials/analysis tools; M.J. wrote the paper.

Conflicts of Interest

The authors declare no conflicts of interest.

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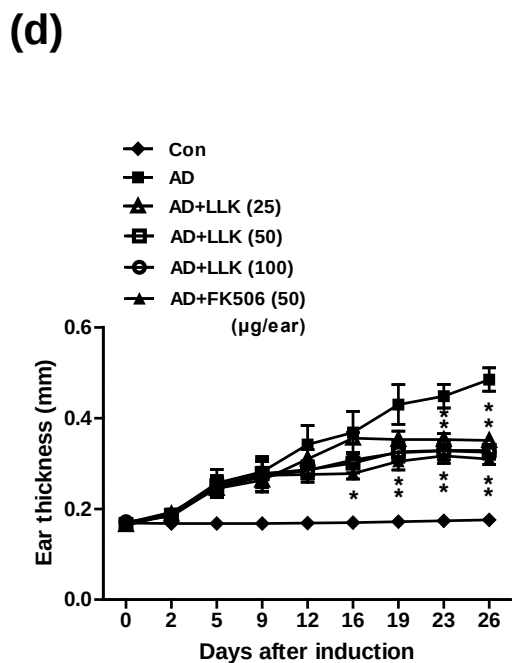
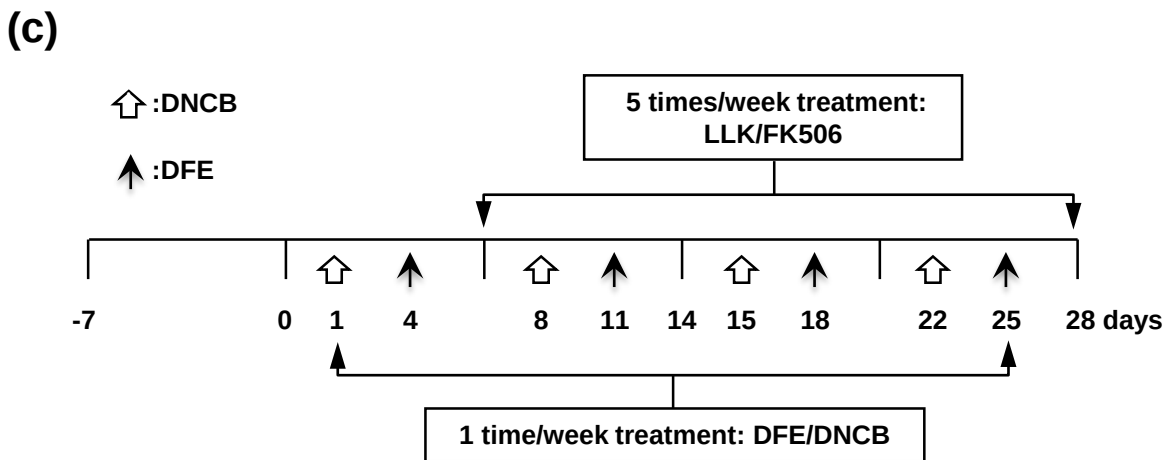
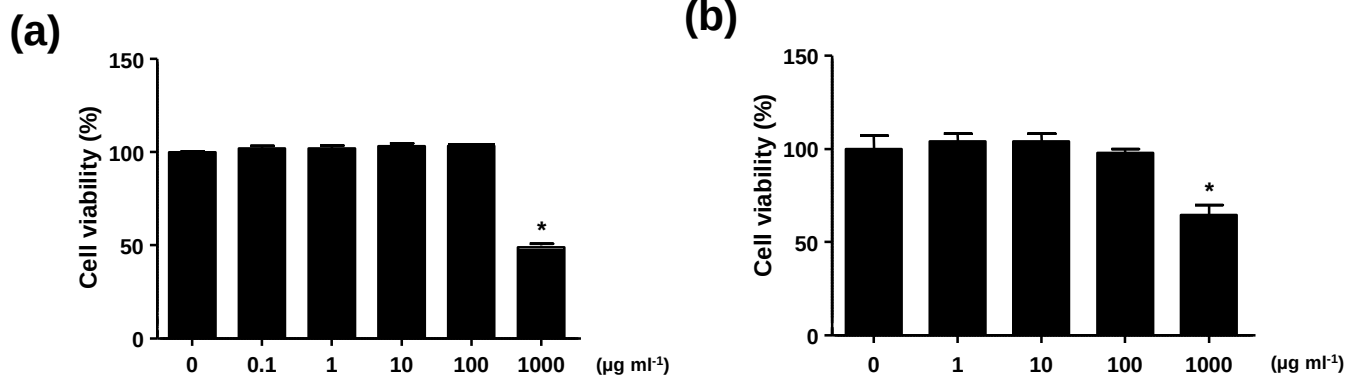
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Experimental design for establishing the mouse model of DFE/DNCB-induced atopic skin inflammation, and cell viability.

Legend for supplementary figure

Figure S1. (a and b) Cell viability of LLK on HaCaT cells. Cell viability was determined by performing the MTT assay (a), and cell number was counted using a hemocytometer (b). Data are presented as mean $\pm$ SEM ($n = 5$); $*p < 0.05$, significantly lower than control group. (c) Experimental design for establishing the mouse model of DFE/DNCB-induced atopic skin inflammation. One week after the first boost with 1 % DNCB (20 μ l/ear) or DFE (10 mg ml⁻¹, 20 μ l/ear) was alternatively applied to both ears once a week for 4 weeks. After 2 weeks of induction, the mouse ears were treated with LLK (25, 50, or 100 μ g/ear), or FK506 (50 μ g/ear) five times per week until 4 weeks after the induction (total 15 times). (d) Development of DFE/DNCB-induced atopic skin inflammation. Ear thickening was measured 24 h after DFE/DNCB application by using a dial thickness gauge. The mice were divided into the following six groups, with five per group: vehicle, DFE/DNCB plus vehicle, DFE/DNCB plus LLK (25, 50, or 100 μ g/ear), or FK506 (50 μ g/ear). Data are presented as mean $\pm$ SEM ($n = 5$ per group); $*p < 0.05$, significantly lower than DFE/DNCB-stimulated group.



Supplementary Figure S1