

A novel mouse model of atopic dermatitis with epicutaneous allergen sensitization and the effect of *Lactobacillus rhamnosus*

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Abstract: Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease, and the pathogenesis is not completely understood. Although there are some mouse models of AD, it is not easy to establish model to represent the natural AD development in human. In this study, we developed an AD model based on outside-inside theory and investigated the effect of *Lactobacillus rhamnosus* (Lcr35), which have known as an immune modulator in allergic diseases. SKH-1 hairless mice underwent three 1-week exposures (separated by 2-week intervals) to an ovalbumin (OVA) or saline (control) patch at the same site to develop the mouse model of AD. Lcr35 (1×10^9 CFU) was administered orally every day from 1 week before the first sensitization until the end of the study. The AD model induced erythematous and itchy skin, increasing TEWL and increasing skin inflammation as assessed by histology in the mice. Oral Lcr35

attenuated all disease parameters previously mentioned.

OVA-specific IgE and skin expression of interleukin-4 (IL-4) and thymic stromal lymphopoietin (TSLP) increased in AD mice, but were reduced in AD mice treated with Lcr35. Moreover, Lcr35 treatment led to an increase in CD4⁺CD25⁺ Foxp3⁺ Treg cells in the mesenteric lymph nodes of AD mice. In conclusions, based on the 'outside-inside' theory, topical allergen may induce AD without skin injury. Oral application of Lcr35 prevented the development of AD in this model by suppressing production of the inflammatory cytokines, IL-4 and TSLP in the skin via a mechanism that may involve CD4⁺CD25⁺Foxp3⁺Treg cells.

Key words: atopic dermatitis – mice – probiotics – regulatory – thymic stromal lymphopoietin – T-lymphocytes

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Introduction

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease characterized by pruritus and eczematous skin lesions and is frequently found in infants and children (1,2). Characteristic features of AD include itching, rash, oedema, haemorrhage and erosion of skin, elevated immunoglobulin (Ig) E levels and infiltration of eosinophils, neutrophils, mast cells and lymphocytes into the skin (3–5).

The pathogenesis of AD is not completely understood. The elevated serum IgE levels in patients with AD naturally suggest that T helper (Th) 2 cytokines may participate in disease pathogenesis (6). Recent evidence indicates that thymic stromal lymphopoietin (TSLP) may play a role in the initiation and maintenance of allergic immune responses, and TSLP expression is increased in children and mice with AD (7–10). In addition, the TSLP receptor is preferentially expressed on T cells undergoing mitosis and expressing IL-4, suggesting that TSLP could amplify Th2 responses at sites of allergic inflammation (11).

The outside-inside theory of AD describes genetic susceptibility to disturbed barrier function, which allows penetration of allergens into the skin (12). Although an AD mouse model exists, in which mice with a null mutation in the filaggrin gene develop flaky tails (13), it is not easy to establish AD in these mice because of the high cost associated with maintaining colonies of these mice. Therefore, some studies attempted to induce disturbed skin

barrier function and inflammation in the skin using physical or chemical injury (14–17); however, these models do not explain the natural development of AD in humans.

According to the currently adopted definition by FAO/WHO, probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (18) by improving the balance of intestinal microflora. Daily intake of live *Lactobacillus rhamnosus* (Lcr35) GG can prevent the development of atopic eczema in children (19,20). Recent studies show that some strains of probiotic can prevent the development of AD in NC/Nga mice, which have a character of spontaneous development of AD in conventional environment (14,21,22). The mechanism underlying the protective effects of probiotics against allergic diseases reportedly involves immune suppressive cytokines (IL-10, TGF- β) and regulatory T (Treg) cells (21,23,24), but the protective mechanisms involved in mouse models of AD are not clear.

Here, we evaluated the effects and mechanism of action of Lcr35 on AD development using SKH-1 hairless mice. We developed an AD model involving allergic skin sensitization and showed that Lcr35 protects the skin by suppressing the production of Th2-related and promoting cytokines (IL-4 and TSLP).

Materials and methods

Animals

Female hairless mice (SKH-1/Hr, 4-weeks-old) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed under

conditions of controlled humidity (40%) and temperature ($22 \pm 2^\circ\text{C}$). The mice were cared for and used in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the Asan Medical Center and Ulsan University College of Medicine.

Probiotic

The *Lcr35* used in this study was obtained from Lyocentre® laboratory (Aurillac, France) and prepared according to the manufacturer's directions.

Development of AD and oral administration of *Lcr35* in mice

To develop a mouse model of AD using skin sensitization, each mouse had a total of three 1-week exposures to ovalbumin (OVA) patch separated from each other by 2-week intervals. OVA (100 µg, grade V, Sigma Inc., St Louis, MO, USA) was applied using a gauze (1×1 cm) attached to the dorsum skin with Tegaderm™ (3M Health Care, St Paul, MN, USA). The OVA patch was replaced once per week. The mice were euthanized on the final day of the schedule after the third sensitization. The negative control group ($n = 5$) received sterile saline (100 µl) applied using the same procedure. *Lcr35* cells were suspended in saline, and the mice in the treatment group ($n = 5$) received oral *Lcr35* (1×10^9 CFU/600 µl/mouse/day) daily from 1 week before (5-weeks-old) the primary sensitization up until the study endpoint.

Clinical scores

The dorsum lesions were scored for erythema and scratching behaviour was observed for 20 minutes after each sensitization. The scores for each parameter were graded as 0 (no symptoms), 1 (mild), 2 (moderate), and 3 (severe). Erythema and scratching were assessed after each sensitization by the same investigator throughout the study.

Assessment of epidermal permeability barrier function

To determine whether epidermal permeability barrier function was altered in OVA-induced AD, we measured transepidermal water loss (TEWL) using a Vapometer®SWL-3 (Delfin Technologies Ltd., Kuopio, Finland). We assessed baseline TEWL at the beginning of the experiment followed by three more assessments after each sensitization.

Quantitation of serum levels of immunoglobulin

Sera were obtained from blood taken during exsanguination of the mice after airway measurement on the final day of the schedule. For the detection of total IgE, 96-well plates were first coated overnight with purified anti-mouse IgE (0.5 µg in 100 µl carbonate-bicarbonate buffer, PharMingen, San Diego, CA, USA). For the detection of OVA-Ig, the plates were coated overnight with OVA (100 µg in 1 ml carbonate-bicarbonate buffer; Sigma Chemical Co.). Remaining binding sites were blocked, and plates were incubated with 100 µl of diluted serum (1/10 dilution in carbonate-bicarbonate buffer) per well. After washing, following substances were sequentially added, incubated and washed: peroxidase-labelled rat anti-mouse OVA-IgE (20 ng/100 µl, Acris Antibodies, Herford, Germany), and 100 µl of TMB solution (Sigma Chemical Co.) were added to per well. Then, optical density was measured at 450 nm.

Histology and immunohistopathology

The dorsal skins of the experimental mice were removed on the final day of the schedule and fixed in 10% phosphate-buffered formalin and embedded in paraffin. Serial paraffin sections (4.5-mm

thick) were stained with haematoxylin and eosin (H&E) for evaluation of oedema.

Paraffin-embedded section was cut into 5 µm and deparaffinized in xylene and rehydrated with sequential treatment of graded ethanol solution. After blocking the non-specific antibody binding by incubation with a serum-free protein (Dako, Carpinteria, CA, USA) for 15 min at room temperature, sections were incubated with primary antibodies (1:100 dilution) for 30 min at room temperature. Sections were then drenched with a horseradish peroxidase (HRP)-conjugated secondary antibody for 30 min at room temperature and washed with tap water. Stained sections were visualized by microscopy equipped with digital camera. Primary antibodies for mouse goat-polyclonal anti-mouse IL-4 and rabbit-polyclonal anti-mouse TSLP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies, anti-goat IgG-HRP and anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology).

Flow cytometry

Mouse Treg cells were collected from mesenteric lymph nodes (MLNs). Mouse regulatory T-cell staining kits with FITC-labelled anti-CD4, PE-labelled anti-CD25, APC-labelled anti-Foxp3 and each isotype control (eBioscience, San Diego, CA, USA) were used for analysis of CD4, CD25 and Foxp3 expression according to the manufacturer's directions. Staining was analysed by flow cytometry on a FACS Calibur with CellQuest software (BD Biosciences, Mountain View, CA, USA).

Statistical analysis

Data represented the mean $\pm$ SEM. Statistical significance was tested by one-way analysis of variance with Dunnett's test. A probability value of <0.05 was considered significant.

Results

Assessment of clinical and epidermal permeability barrier function

Clinical assessment of AD was performed by measuring erythema and scratching behaviour. Erythema increased significantly in the OVA-treated group by 300%, 600% and 250% at sensitizations 1, 2 and 3, respectively, compared with that in the negative control group. Similarly, scratching behaviour increased by 140%, 117% and 100%. Oral administration of *Lcr35* effectively attenuated the erythema (75%, 63% and 64% reductions at sensitizations 1, 2 and 3, respectively) and scratching (48%, 42% and 55%) compared with OVA treatment only (Fig. 1a,b). Epidermal permeability barrier function was assessed by measuring TEWL. TEWL increased significantly in the OVA-treated group by 45%, 52% and 92% after sensitizations 1, 2 and 3, respectively, compared with that in the negative controls. The damage to barrier function after the third sensitization was significantly reduced by *Lcr35* (30%, $P < 0.05$; Fig. 1c) compared with that in the AD mice that received OVA treatment only. These results show that oral *Lcr35* improves the clinical signs of AD and reduces damage to epidermal barrier function in mice.

Systemic immune responses

The systemic immune response was assessed by measuring total IgE and OVA-specific IgE in the serum. Epicutaneous serial OVA treatments increased the serum concentration of total IgE (OVA: 61.20 ± 10.55 ; saline: 39.40 ± 3.68) and OVA-specific IgE (OVA: 21.23 ± 1.30 ng/ml; saline: 15.92 ± 0.59 ng/ml, $P < 0.01$) after

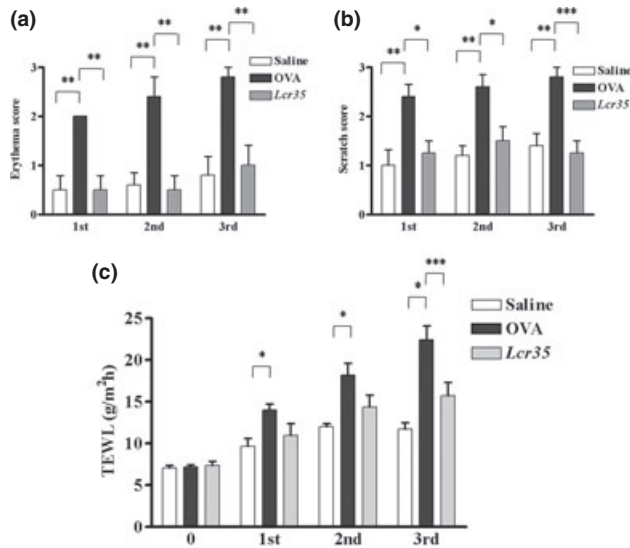


Figure 1. Effect of oral administration of *Lactobacillus rhamnosus* (*Lcr35*) on clinical signs of AD and the epidermal permeability barrier. *Lcr35* attenuated the clinical scores (a, b) and TEWL (c) in the mouse model of AD; ($n = 5$ per group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). AD, atopic dermatitis; TEWL, transepidermal water loss.

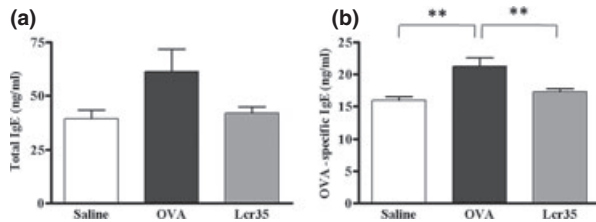


Figure 2. Effect of oral treatment with *Lcr35* on serum IgE levels in the mouse model. *Lcr35* attenuated total IgE (a) and OVA-specific IgE (b) production. OVA-specific IgE was significantly suppressed by *Lcr35* compared with that in the positive control ($n = 5$ per group; ** $P < 0.01$).

three sensitizations (Fig. 2). Oral *Lcr35* treatment significantly lowered the OVA-specific IgE levels (17.27 ± 0.43 ng/ml, $P < 0.01$, Fig. 2b). But the result of total IgE was not significant. These results suggest that oral *Lcr35* treatment attenuated the systemic immune response during AD in mice.

Histological and immunohistopathological analysis

Epicutaneous serial OVA treatments resulted in severe infiltration of inflammatory cells, including mast cells and eosinophils, into the dermis (Fig. 3; H&E staining). Oral *Lcr35* treatment decreased both the infiltration of inflammatory cells and epidermal thickening in the OVA-treated skin (Fig. 3 H&E staining).

To assess cytokine involvement, skin sections were stained with IL-4- and TSLP-specific antibodies. Immunohistochemistry staining was evaluated by two researchers randomly with double-blind method. Five mice were included in each group. While the immunohistochemical control experiments without primary antibodies did not show any staining in observed skin, OVA-treated skin contained more IL-4- and TSLP-positive cells than negative control skin, with IL-4-positive cells mainly located in the epidermis and upper dermal layers, and TSLP-positive cells mainly located in the epidermis. The number of IL-4- and TSLP-positive cells

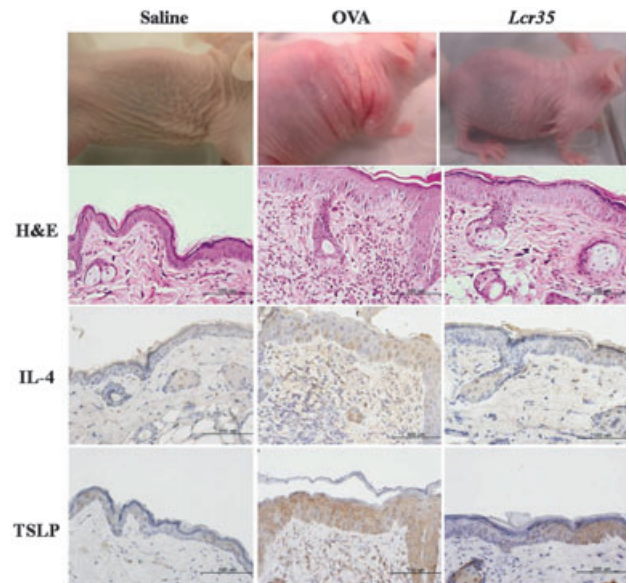


Figure 3. Effect of oral administration of *Lcr35* on skin histology and IL-4 and TSLP expression in the mouse model. AD lesions showed severe infiltration by inflammatory cells compared with the negative control (H&E). The infiltration of inflammatory cells was decreased in the *Lcr35*-treated group compared with that in the OVA-treated group. Immunohistochemical staining showed that the number of IL-4- and TSLP-positive cells was increased in the skin of the OVA-treated mice compared with that in the negative control. The number of IL-4- and TSLP-positive cells was decreased in the *Lcr35*-treated group compared with that in the OVA-treated group. AD, atopic dermatitis; IL, interleukin; TSLP, thymic stromal lymphopoietin.

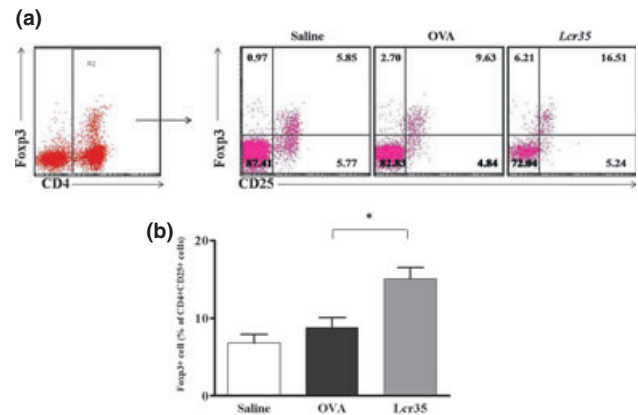


Figure 4. Effect of oral administration of *Lcr35* on the number of CD4⁺CD25⁺Foxp3⁺ Treg cells in the mouse model. (a) Flow cytometric analysis of T cells in the MLNs. Numbers inside the histograms indicate the percentage of cells within each region. (b) Relative proportions of CD4⁺CD25⁺Foxp3⁺ Treg cells among total T cells in the MLNs; Treg, regulatory T cell; MLN, mesenteric lymph node. ($n = 5$ per group; * $P < 0.05$).

was decreased significantly in the *Lcr35* treatment group (Fig. 3). These results indicate that IL-4 and TSLP expression in the skin was positively associated with AD and that their expression was inhibited by *Lcr35*.

Effect of *Lcr35* treatment on the number of CD4⁺CD25⁺Foxp3⁺ Treg cells in the MLNs

To test whether the number of Treg cells increased in mice fed *Lcr35*, we determined the percentage of CD4⁺CD25⁺Foxp3⁺ Treg cells in the MLNs. *Lcr35* treatment led to a significant increase in

the percentage of CD4⁺CD25⁺ Foxp3⁺ Treg cells compared with that in OVA-treated mice (72% increase, $P < 0.01$; Fig. 4). This result indicates that *Lcr35*-induced attenuations in the allergic responses in the mouse model of AD are associated with an increase in the proportion of CD4⁺CD25⁺Foxp3⁺ Treg cells.

Discussion

This study aimed to develop an allergen-sensitization model of AD in mice and to investigate the effects of oral *Lcr35* on AD in this model. AD was induced by epicutaneous sensitization with allergen. This model could represent 'outside-inside' theory of AD. The development of AD was confirmed by observing clinical signs such as erythema and scratching, impaired epidermal skin barrier function by measuring TEWL, increased serum IgE and severe allergic inflammation on histological examination. These results suggest that outside exposure of allergen can induce localized allergic inflammation in the skin and a systemic sensitization to specific allergen. AD was significantly suppressed by daily oral *Lcr35* treatment via a mechanism involving IL-4 and the Th2-related cytokine TSLP; therefore, we conclude that *Lcr35* may inhibit allergic inflammation in this model of AD.

Previous studies have investigated mouse models of AD induced according to the 'outside-inside' theory (14–17). However, these models required skin injury induced by shaving and tape stripping, followed by repeated epicutaneous sensitization or sensitization with chemical irritants (haptens). By contrast, allergen was applied repeatedly in our model without any physical or chemical injury to the skin, that protocol in the present study is novel. In addition, probiotics prevent the development of AD in mouse models (14,21,22); however, these studies are not enough to identify the mechanism of probiotics in the skin.

Oral administration of probiotics induces regulatory dendritic cells, which, in turn, promote the generation of CD4⁺Foxp3⁺ Treg cells within mesenteric lymph nodes (23). CD4⁺ Treg cells expressing the transcription factor, Foxp3, play a critical role in preventing autoimmunity and in limiting immune-mediated inflammation (25). CD4⁺ Treg cells modulate immune responses

by regulating Th1, Th2 and Th17 responses (25). In the present study, *Lcr35* treatment led to an increase in CD4⁺CD25⁺Foxp3⁺ Treg cells in MLNs. A previous study reported that Foxp3⁺ expression in the skin was also increased by probiotics in a mouse model of AD (23).

In addition, *Lcr35* suppressed expression of IL-4 and the Th2-promoting cytokine TSLP, both of which were increased in the skin of AD mice. TSLP initiates the inflammatory cascade in AD by activating dendritic cells in the local lymph nodes and by attracting IL-4-producing Th2 cells (10). Therefore, we speculate that in our model, *Lcr35* protects against AD by activating Tregs leading to suppression of Th2-mediated inflammation in the skin. Similarly, the vaccination with *Propionibacterium acnes*, bacteria in skin flora, significantly increased CD4⁺CD25⁺Foxp3⁺ Treg cells in spleen cells and prevented clinical manifestations in the skin of AD mice (26).

Our study has some limitations. Expression of the Treg maker, Foxp3, and the immune suppressive cytokines, IL-10 and TGF- β , in the skin was not investigated directly. Therefore, we could not identify the relationship between the expansion of CD4⁺CD25⁺Foxp3⁺ Treg cells in the MLNs and suppression of skin inflammation.

This study describes the development of an AD mouse model in SKH-1 hairless mice by repeated epicutaneous allergen exposure based on the outside-inside theory of AD. The probiotic *Lcr35* suppressed allergic response in this model, with systemic suppression of OVA-specific IgE and suppression of the inflammatory cytokines IL-4 and TSLP in the skin via a mechanism that may involve CD4⁺CD25⁺Foxp3⁺ Treg cells.

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Conflict of interest

The authors have declared no conflicting interests.

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