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Effects of *Lactobacillus rhamnosus* on allergic march model by suppressing Th2, Th17, and TSLP responses via $CD4^+CD25^+Foxp3^+$ Tregs

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Abstract Allergic march (AM) is characterized by the progression of clinical signs of atopic dermatitis (AD) to allergic asthma or rhinitis, but its pathogenesis is not completely understood. We developed mouse model of AM with three 1-week exposures (separated by 2-week interval) to an OVA or saline (control) followed by OVA challenge. The development of AM was confirmed by phenotypes of AD and allergic asthma. Increases in IL-4, IL-17, and thymic stromal lymphopoietin (TSLP) responses were associated with the progression of AM, and these responses were suppressed by treatment with *Lcr35*. Moreover, *Lcr35* treatment led to an increase in the number of $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells in the mesenteric lymph nodes (MLNs) of AM mice.
In conclusion, the oral application of *Lcr35* prevented the development of AM in this model by suppressing Th2, Th17, and TSLP responses via a mechanism that may involve $CD4^+CD25^+Foxp3^+$ Tregs in MLNs.

Abbreviations: AM, allergy march; AD, atopic dermatitis; *Lcr35*, *Lactobacillus rhamnosus*; OVA, ovalbumin; IL-4, interleukin-4; TSLP, thymic stromal lymphopoietin; Treg, regulatory T cell; MLNs, mesenteric lymph nodes; TEWL, transepidermal water loss; IHC, immunohistochemistry; IFN-γ, interferon gamma; IP-10, Interferon gamma-induced protein 10; CCL11, C-C motif chemokine 11; BAL, bronchoalveolar lavage.

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1. Introduction

Asthma is a chronic inflammatory disease of the airways, and atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease—both of these diseases are associated with Th2-related allergic responses [1,2]. Allergic march (AM) is associated with 70% of patients with a history of severe AD and characterized by the progression from AD to asthma and allergic rhinitis [2,3]. AD is considered the starting point of AM, which suggests that the development of AD may be the first step toward the progression of systemic allergic diseases. Recently, some studies have reported that thymic stromal lymphopoietin (TSLP) and IL-17A are closely related to the development of AM in experimental mice [4–6]. However, the underlying mechanism of AM is still unclear.

A few studies have attempted to develop an AM model in mice by inducing the overexpression of TSLP via transgenic skin-barrier defects or the application of OVA plus an aggravating factor of AD (e.g., TSLP) to the skin [4–6]. Previously, our group reported the development of a novel AD mouse model following the application of serial allergen sensitizations to the skin of SKH-1 hr mice [7]. Our model is a representation of the outside–inside theory of AD and can be used to describe genetic susceptibilities that affect disturbed barrier function, which allows the penetration of allergens into the skin [7,8]. Our AD model, however, is different from other models, which use physical injury (e.g., shaving, intradermal allergen injection) as a way to study damaged barriers and inflamed skin [5,6]. For this study, we developed a mouse model of AM using the AD model [7].

It has been reported that the intestinal microflora play an important role in Th1/Th2 balance, which is the key mechanism involved in allergic diseases [9]. Human epidemiological data suggest that altered microbial exposure during childhood influences the outcome of allergic sensitization [10,11]; therefore the role of probiotics in allergic disease has been highlighted recently. Probiotics are nonpathogenic microorganisms that confer a number of beneficial effects to the health of the host [12]. Several studies have characterized the abilities of probiotic strains to alter cytokine production in the gut and lymphoid tissue, demonstrating immunomodulatory effects against some allergic diseases [13–15]. We recently reported that the oral application of *Lactobacillus rhamnosus* (*Lcr35*) attenuates the features of allergic asthma and atopic dermatitis in mouse models [7,16]. Although there have been a lot of data for supporting the preventive effects of probiotics on allergic disease in animal study, their preventive effects have not been conclusively defined in human study. Therefore more well-designed studies are needed to certificate the use of probiotics on allergic diseases in clinic. Particularly, there have been no studies to investigate the effects of probiotics on AM.

Mechanisms of probiotics have known mostly as suppression of Th2-related cytokines, IL-4, IL-5, IL-13, and generation of regulatory T cells (Tregs) [16,17]. However, the results of many studies differ and cannot be fully explained by an imbalance of Th1/Th2 responses in the development of allergic diseases. Therefore, there have been disputes among researches about the mechanism of probiotics. Here, we investigate the effects of *Lcr35* on AM using a novel mouse model and explore its new mechanisms.

2. Materials and methods

2.1. Animals

Female hairless mice (SKH-1/hr; 4 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed under controlled humidity (40%) and temperature (22 ± 2 °C). All mice (n = 5 per group) were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Asan Medical Center and Ulsan University College of Medicine.

2.2. Probiotics

The *Lcr35* probiotics used in this study was obtained from Lyocentre (Aurillac, France) and prepared in accordance with the manufacturer's directions.

2.3. Study protocol

In order to develop a mouse model of AM using skin sensitization, each mouse received a total of three 1-week exposures to 100 μL OVA (grade V; Sigma, St Louis, MO) that was administered via a patch; administrations were separated by 2-week intervals. Mice received an oral dose of *Lcr35* (1×10^9 CFU/600 mL/mouse/day) starting one week before primary sensitization; administrations were continued through the endpoint of this study. The day before sacrifice, all mice inhaled 1% OVA as the airway challenge (Supp. Fig. 1).

2.4. Antibody injections

Anti-IL-17 (100 μg/mouse; R&D Systems, MN, USA), anti-TSLP (15 mg/kg; R&D Systems, MN, USA) and rat IgG_{2A} isotype-control antibodies (15 mg/kg; R&D Systems, MN, USA) were administered the day before the OVA challenge. The *Lcr35*-, anti-IL-17 Ab-, and anti-TSLP Ab-treated groups were also administered OVA sensitization and challenge. Negative controls received only saline instead of OVA during both sensitization and the airway challenge (Supp. Fig. 1).

2.5. Immunohistochemistry (IHC)

Paraffin-embedded sections were cut to 5-μm widths, deparaffinized in xylene, and rehydrated with sequential treatments in graded ethanol. Antigen retrieval was carried out by heating the tissue sections in antigen retrieval buffer (citrate buffer; pH 6.0) using microwave treatment. Tissues were incubated with a peroxidase blocking reagent (Dako, Glostrup, Denmark) for 30 min in order to block endogenous peroxidase activities. After blocking nonspecific antibody binding by incubation with a serum-free protein (Dako, Glostrup, Denmark) for 15 min at room temperature, sections were incubated overnight with primary antibodies at 4 °C. The primary antibodies used for staining and their respective dilution rates were as follows: goat-polyclonal anti-mouse IL-4 antibody (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA); rabbit-polyclonal anti-mouse IL-17 (1:500 dilution; Abcam, Cambridge, MA); rabbit-polyclonal