Lactobacillus acidophilus Strain L-92 Induces CD4⁺CD25⁺Foxp3⁺ Regulatory T Cells and Suppresses Allergic Contact Dermatitis

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The anti-allergic mechanism of heat-killed *Lactobacillus acidophilus* strain L-92 has not been fully investigated. Recent studies have reported that CD4⁺CD25⁺Foxp3⁺ (forkhead box P3) T regulatory (Treg) cells play important roles in controlling allergic diseases. Hence, we examined the effect of orally administered L-92 on CD4⁺CD25⁺Foxp3⁺ cell populations. BALB/c mice were supplemented daily with L-92 by gavage for 5 weeks. 2,4-Dinitrofluorobenzene (DNFB) was used to induce allergic contact dermatitis (ACD) in mice. Fluorescent-activated cell sorter (FACS) analysis was used to determine CD4⁺CD25⁺Foxp3⁺ T cell populations in spleen and cervical lymph nodes (CLN). Interleukin-10 (IL-10), transforming growth factor- β (TGF- β), and Foxp3 mRNA expressions in mouse ear skin were investigated by real-time reverse transcription-polymerase chain reaction (RT-PCR). The percentage of CD4⁺CD25⁺Foxp3⁺ T cell populations were significantly increased in both spleen and CLN of L-92-fed group than vehicle and control. In addition, L-92 produced higher levels of Foxp3, IL-10 and TGF- β compared to control mice. These results suggest that L-92 can up-regulate the number of Treg cells to suppress the progression of DNFB-induced contact dermatitis in mice.

Key words contact dermatitis; *Lactobacillus acidophilus* strain L-92; regulatory T cell; forkhead box P3

Commensal bacteria of the gut flora affect host physiology through diverse mechanisms, including modulation of the host immune system and attenuation of gastrointestinal (GI) diseases. Some clinical and experimental animal studies reported that certain commensal bacteria may also be able to regulate immune responses outside of the GI tract. Among them, species of lactobacilli and bifidobacteria are prominent commensal bacteria with anti-allergic properties. For example, a double-blind placebo-control study, *Lactobacillus* (*L.*) *rhamnosus* GG¹⁾ and *L. fermentum* VRI-003 PCC²⁾ reduced the development of eczema in young children. Within the experimental allergy animal models, several lactic acid bacterial (LAB) strains, including *L. casei* strain Shirota,³⁾ and *L. brevis* strain SBC8803⁴⁾ inhibits antigen specific immunoglobulin E (IgE) production, and *L. paracasei* strain KW3110⁵⁾ was found to suppress atopic dermatitis (AD)-like skin lesions. We also previously demonstrated that heat-killed *L. acidophilus* strain L-92 (L-92) can suppress both 2,4-dinitrofluorobenzene (DNFB) and mite antigen-induced AD-like skin lesions in mice.⁶⁾ Despite some pre-clinical studies on animal models as well as clinical trials have highlighted the beneficial roles of these bacteria, the exact mechanisms behind the anti-allergic effects still remain obscure.

Allergic contact dermatitis (ACD) is a delayed-type hypersensitivity caused by an allergic reaction to some chemical (hapten) in contact with the skin. The allergic condition causes itching and inflammation of the skin manifested by varying degrees of erythema, edema, and vesiculation. Knowledge of the mechanism of ACD was derived mainly from animal mod-

els in which allergic reactions were induced by hapten-painting of the skin. As ACD is sometimes referred to as contact hypersensitivity (CHS), both ACD and CHS are considered as synonymous and defined a hapten-specific T cell-mediated delayed-type hypersensitivity reaction.⁷⁾ Pathophysiology of ACD consists classically of two distinct phases, *i.e.*, the sensitization or afferent phase and the elicitation or efferent phase. Hapten is taken up by skin dendritic cells (DCs) that migrate to the draining lymph nodes (LN), where they present haptenated peptides on major histocompatibility complex (MHC) class I and II molecules resulting in the induction of hapten-specific CD8⁺ and CD4⁺ T cells, respectively.⁷⁾ The elicitation phase starts upon subsequent contacts of the skin with the hapten; effector T cells are recruited and activated in the dermis and trigger the inflammatory process responsible for the cutaneous lesions. DNFB is a well-known hapten for its ability to cause CHS, which was reported to elevate Th1 response but after repeated exposure can shift from the Th1 to the Th2 response or mixed response.⁸⁾

Regulatory T (Treg) cells play crucial roles in the induction of peripheral tolerance to self and foreign antigens. Most frequently is the naturally-occurring population of CD4⁺CD25⁺Foxp3⁺ Treg (nTreg) cells that develop in the thymus, but also been induced in the periphery, in an antigen-specific, transforming growth factor- β (TGF- β)-dependent fashion.⁹⁾ These cells are important in the control of a wide range of immune-mediated pathologies, including autoimmune disorders, infectious, allergic and chronic inflammatory diseases.¹⁰⁾ Recent reports suggested that the forkhead box P3 (Foxp3), a transcriptional regulator which is essential for the development and function of CD25⁺ Treg cells.^{11,12)} It has been shown that Foxp3-deficient patients with immunodys-

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regulation, enteropathy, polyendocrinopathy, and X-linked syndrome (IPEX) have atopic diseases. Previous studies have shown that the treatment with commensal bacteria can regulate the allergic airway response in adult animals through the increased T cell expression of Foxp3.¹³⁾ Although few studies had performed to investigate the mechanisms underlying the effects of commensal bacteria on Treg cells but there is no study was reported whether heat-killed commensal bacteria can induce Treg cells to suppress allergic contact dermatitis (ACD). Therefore, we examined the potential role of Treg cells in mediating L-92-induced attenuation of ACD.

MATERIALS AND METHODS

Animals Female BALB/c mice (5 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Groups of 5 mice were randomly assigned to acetone-treated vehicle, DNFB-treated control, and DNFB-treated L-92-fed groups. The animal experiments were conducted according to the guidelines of the Committee on Animal Experiment of Gifu University Graduate School of Medicine.

Bacterial Cell Preparation and Oral Administration Heat-killed, lyophilized *L. acidophilus* strain L-92 (L-92) was obtained from the R&D Center, Calpis Co., Ltd., Kanagawa, Japan, and suspended in distilled water before oral administration. L-92 suspension for 30 mg/kg was prepared and administered daily before 1 week of the experiment start until to the day of sacrifice. Vehicle and control groups of mice received distilled water instead of L-92 suspension. DNFB (Sigma-Aldrich, Tokyo, Japan) was dissolved in acetone at a concentration of 0.15%. DNFB was topically applied to the ear, 2 times a week for 4 weeks, total 9 times from day 0 to day 28 to induce contact dermatitis in mice, and vehicle mice were treated with acetone in a similar manner to draw the base line. A total of 100 μ L (25 μ L for each surface of both ear lobes) antigen solution was applied topically to the mouse ear lobe. After treatments, animals were killed and both cervical lymph nodes and spleens were removed and processed for fluorescent-activated cell sorter (FACS) analysis. At the same time, ears were separated for analyzing mRNA expression by real-time reverse transcription-polymerase chain reaction (RT-PCR).

FACS Analysis Single cells from spleen and lymph nodes were resuspended at 1×10^6 cells/mL and extracellular CD4 and CD25 and intracellular Foxp3 were stained with specific antibodies using fluorescein isothiocyanate (FITC)-labeled anti-CD4 (RM4-5), allophycocyanin (APC)-labeled anti-CD25 (PC61.5), and phycoerythrin (PE)-labeled anti-Foxp3 (FJK-16s) antibodies (eBioscience, San Diego, CA, U.S.A.). Via-Probe was purchased from BD Bioscience Pharmingen (San Jose, CA, U.S.A.) to discriminate viable from non-viable cells. For Foxp3 expression, cells were stained for surface markers and then fixed, permeabilized, and stained for Foxp3 according to the manufacturer instruction. Absolute numbers of cells were calculated by multiplying the percentage of positive staining cells in the total acquired events by the total number of cells isolated from the tissue analyzed (whole spleen and cervical lymph nodes). Immunofluorescence was analysed with CellQuest software with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

Analysis of mRNA Expression For evaluation of Foxp3,

interleukin-10 (IL-10) and TGF- β expression, total RNA was extracted from the ear of each mouse using Isogen (Nippon Gene, Tokyo, Japan). cDNA was synthesized using oligo(dT) primer and Superscript II reverse transcriptase (Life Technologies, Tokyo, Japan). Real-time RT-PCR was performed with a BIO-RAD Real-Time PCR System (BIO-RAD Laboratories, Tokyo, Japan) using SYBR Premix EX TaqII (Takara Bio, Otsu, Japan). The cyclic conditions were 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 60°C for 60 s. The results were normalized with the housekeeping gene mouse β -actin. Each experiment was repeated for 2–3 times.

The primer sequences used for mouse β -actin, Foxp3, IL-10, and TGF- β are listed as follows: β -actin (sense) 5-GGG ACGACATGGAGAAGA-3, (anti-sense) 5-CATACAGGGACA GCACAG-3; Foxp3 (sense) 5-CTCATGATAGTGCCTGTG TCCTCAA-3, (anti-sense) 5-AGGGCCAGCATAGGTGCA AG-3; IL-10 (sense) 5-GCAGAGAAGCATGGCCCAGAAA-3, (anti-sense) 5-GGAGAAATCGATGACAGCGCCT; TGF- β (sense) 5-GTGTGGAGCAACATGTGGAAGTCTA-3, (anti-sense) 5-TTGTTTCAGCCACTGCCGTA-3.

Statistical Analysis All results were expressed as the mean $\pm$ S.E.M. Data between two groups were compared by Student's *t*-test or Mann-Whitney's *U*-test after examining the variance using the *F*-test. $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Many investigations have demonstrated the involvement of Treg cells in controlling various aspects of allergic diseases.^{14,15)} Of the several subtypes of Treg cells that have been identified to date, the naturally-occurring CD4⁺CD25⁺ Treg (nTreg) cells have attracted great attention in recent years because of the maintenance of immunological tolerance in a variety of clinical disorders by suppressing the activation and expansion of auto-reactive T cells.¹⁶⁾ These nTreg cells produce both IL-10 and TGF- β and are known to suppress inflammation in both normal and allergic conditions.¹⁷⁾ Here, we examined the influence of L-92 on nTreg cells to clarify the immunomodulatory capacity by suppressing ACD in mice.

After topical application of DNFB on both ears of BALB/c mice showed severe spontaneous scratching, and the onset of skin inflammation was observed after 2nd painting and gradually aggravated by the repeated application of allergen up to 4 weeks, which turned into a severe phase of dermatitis with desquamation, erosion, and hemorrhage on the ear skin. Treatment with L-92 did not alter any significant inhibition of ear thickness and scratching behavior, although a trend reduction was found in comparison to the control group mice (data not shown). In contrast, no severity was observed with acetone treated vehicle group mice. FACS analysis data showed that the percentage of Foxp3 expressing CD4⁺CD25⁺ cells increased significantly in L-92-treated group compared with control in both splenocytes and cervical lymph node cells of BALB/c mice (Fig. 1), while a similar result was also observed in the vehicle group due to the normal presence or distribution of Treg (Fig. 1A). Recent studies have demonstrated that children with allergic diseases expressed fewer CD4⁺CD25⁺ T cells than normal.¹⁸⁾ Administration of L-92 also significantly increased Foxp3 mRNA expression compared with vehicle and control groups (Fig. 2A) in ACD model

Fig. 1. Effects of L-92 on Treg Cell Populations in DNFB-Induced Allergic Dermatitis Mice

BALB/c mice were given L-92 or vehicle by gavage for 5 weeks before sacrifice, then the percentage of Foxp3 expressing CD4⁺CD25⁺ cell populations in (a) spleen and (b) cervical lymph nodes were analyzed by FACS. Vehicle: non-sensitized mice, Control: sensitized mice, L-92: L-92-treated sensitized mice. Each column represents the mean $\pm$ S.E.M. for 5 mice. * $p < 0.05$ vs. control.

Fig. 2. Effects of L-92 on (a) Foxp3, (b) IL-10, and (c) TGF- β Expressions in DNFB-Induced Allergic Dermatitis Mice

mRNA expression was examined by real-time RT-PCR and normalized with β -actin mRNA expression. Vehicle: non-sensitized mice, Control: sensitized mice, L-92: L-92-treated sensitized mice. Each column represents the mean $\pm$ S.E.M. for 5 mice. * $p < 0.05$, and *** $p < 0.001$ vs. control.

clearly revealed that Foxp3⁺ cells have a positive effect to suppress the allergic reaction.

Cytosine-guanosine dinucleotide (CpG) sequence motifs composed of unmethylated CpG dinucleotides are the immunostimulatory components of bacterial DNA, which can directly or indirectly activate DCs, macrophages, B cells, natural killer (NK) cells, and T cells. This action leads to the secretion of various pro-inflammatory cytokines, including IL-6, IL-12, tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) via Toll-like receptor-9 (TLR-9), and finally induces a Th1-biased immune response.^{19–21} It has also been shown that LAB contains CpG-like stimulatory oligonucleotides, which can directly ligate with TLR-2 and/or TLR-9.^{22,23} Furthermore, DCs can directly present antigens from commensal bacteria to mesenteric lymph nodes and interact with T and B cells to maintain non-inflammatory immune responses.^{24,25} Recent evidence indicates that DC is also required for the generation of Foxp3⁺ Tregs through a retinoic acid and TGF- β -dependent manner.²⁶ Pessi and colleagues reported that oral administration of *L. rhamnosus* GG can alleviate the

clinical symptoms of AD through the enhanced IL-10 production.²⁷ Subcutaneous injection of TGF- β_1 was reported to suppress the development of AD-like skin lesions in NC/Nga mice, which was accompanied by the reduction of IgE production.²⁸ Moreover, heat-killed *L. brevis* SBC8803 can suppress picryl chloride-induced AD-like skin lesions in NC/Nga mice due to the down-regulation of Th1 and Th2 response through the increased secretion of IL-10 and TGF- β_1 .⁴

In the present study, both IL-10 and TGF- β levels were found significantly higher in the L-92 group compared with vehicle and control (Fig. 2) suggested the immunomodulatory actions against allergy. Furthermore, L-92 increased IL-10 level in DNFB-induced BALB/c mice while down-regulation was observed in Torii *et al.*²⁹ due to the different antigenic stimulation. Our previous study showed that oral treatment with L-92 did not affect IL-4 production, but production of IFN- γ , IL-5, IL-13 and IgE was suppressed in DNFB-treated allergic dermatitis mice.⁶ Together with the present study, it is clear that heat-killed L-92 can exert the immunomodulatory function through increasing production of nTreg cells and

that it modulates both Th1 and Th2 responses. Although how L-92 induces nTreg was not evaluated in the present study, but the previous report suggested that oral treatment with L-92 can induce apoptosis of antigen-stimulated T cells by modulating DC function.³⁰⁾ Further studies are required to verify how L-92 induces nTreg cells, whether IL-10 and TGF- β are produced from nTreg cells or induced Treg cells, and the evaluation of Lymphocyte-activation gene 3 (LAG3) which contributes to the suppressor activity of induced Treg cells.³¹⁾

In summary, our results provide evidence that L-92-induced attenuation of the ACD response in mice is mediated by CD4⁺CD25⁺Foxp3⁺ Treg cells at least in part, although additional regulatory cell populations may play a role in mediating these effects. L-92 further supports the concept that it can exert immunomodulatory activities that are not confined to the gastrointestinal tract and may be effective for the treatment of human allergic disease progression.

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