

Anti-allergic effect of lactic acid bacteria isolated from seed mash used for brewing sake is not dependent on the total IgE levels

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Lactic acid bacteria (LAB) in fermented foods have attracted considerable attention recently as treatment options for allergic diseases, the incidence of which has been increasing worldwide. Five strains of LAB isolated from kimoto, the traditional seed mash used for brewing sake, were screened for the ability to suppress IgE-mediated hypersensitivity reaction. *Leuconostoc mesenteroides* and *Lactobacillus sakei*, the normal microflora in kimoto, significantly suppressed the reaction, but the contaminant *Lactobacillus curvatus* did not. Next, we examined the effect of *L. sakei* LK-117 on atopic dermatitis in the NC/Nga mouse model. LK-117 supplementation significantly reduced the development of atopic dermatitis-like skin lesions in a manner independent of the IgE plasma levels. In the *in vitro* intestinal model constructed using the human intestinal epithelial cell line Caco-2 and murine macrophage cell line RAW 264.7, treatment with *L. sakei* LK-117, but not *L. curvatus*, significantly upregulated TNF- α production from RAW264.7 cells. This result indicated that *L. sakei* on the apical side affected the macrophages on the basolateral side, and this organism may have the ability to improve allergy symptoms mediated by the intestinal immune system.

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The incidence of allergies, or diseases associated with an over-active immune system, is increasing worldwide. According to the hygiene hypothesis, allergies may be related to reduced exposure to environmental bacteria. Many research groups have recently been involved in the selection of lactic acid bacteria (LABs) to be used in preventing the onset of allergies such as hay fever (1,2) and atopic dermatitis (AD) (3,4), a chronic refractory skin disease (5,6). Several studies have attempted to reduce the blood levels of IgE, an indicator of allergic conditions and improve the helper type 1/helper type 2 (Th1/Th2) balance, since an imbalance in the lymphocytes due to Th2 dominance has also been associated with allergies (7,8).

In a previous study, we isolated LABs with potential anti-allergy effects from kimoto, a traditional seed mash used for brewing Japanese sake, by using IL-12 as a Th1 parameter (9), as IL-12 triggers Th1 differentiation and blocks Th2 cell production. An alternative approach to the restoration of the Th1/Th2 balance for preventing allergies is inhibition of the activation of mast cells, which play a critical role in the effector phase of IgE-dependent immediate hypersensitivity and allergy.

In this study, we used mouse ear swelling test to screen LABs that inhibit IgE-mediated mast cell activation. Next, we examined

the effect of *Lactobacillus sakei* LK-117 intake on the development of AD-like skin lesions in the NC/Nga Tnd Crlj mice, in which a simple and reproducible hapten-induced AD-like skin disease is induced via repeated application of picrylchloride (PiCl) under specific pathogen-free conditions (10,11). Finally, by using an *in vitro* intestinal model created using the human intestinal epithelial cell line Caco-2 and murine macrophage cell line RAW 264.7, we showed that LK-117 may have significant effects on macrophages.

MATERIALS AND METHODS

Bacteria The LAB strains used in this study were *Leuconostoc mesenteroides* LK-103, 3 strains of *L. sakei* (LK-117, LK-127, and LK-133), and *Lactobacillus curvatus* LK-141. All strains were isolated from seed mash during the first half of the kimoto-making process.

Reagents DeMan Rogosa and Sharpe (MRS) broth and anti-trinitrophenyl (TNP) IgE were purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). Bovine serum albumin (BSA) and PiCl were purchased from Nacalai Tesque (Kyoto, Japan). Olive oil was purchased from Nikko Chemicals Co. Ltd., (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM; with glutamine and low glucose) and recombinant murine tumor necrosis factor- α (rTNF- α) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Eagle's minimum essential medium (MEM) was purchased from Nissui Pharmaceutical Co. Ltd., (Tokyo, Japan). RPMI 1640, MEM non-essential amino acids (NEAA) solution, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). DMEM (with glutamine, high glucose) was obtained from Sigma (St. Louis, MO, USA), and fetal bovine serum (FBS) was purchased from Biological Industries (Beit, Israel).

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Animals Three-week-old female BALB/c mice were obtained from JAPAN SLC Inc. (Shizuoka, Japan), and 6-week-old male specific pathogen-free NC/Nga Tnd Crlj (NC/Nga) mice were obtained from Charles River Japan (Kanagawa, Japan).

Preparations of LAB strains LAB cultures were grown in 200 ml of MRS broth at 30°C for 2 days, harvested by centrifugation at 16,000 ×g for 15 min and washed twice with sterile water. The cells were killed by autoclaving at 121°C for 10 min and then lyophilized. To prepare LK-117 for administration to the NC/Nga mice with AD-like skin lesions, the strain was cultured in MRS broth (Merck KGaA, Darmstadt, Germany) for 26 h at 30°C, and the cells were washed twice with sterilized water. They were then autoclaved at 121°C for 10 min, and then lyophilized. Chow (CRF-1; Oriental Yeast, Co., Ltd., Tokyo, Japan) containing 1% (w/w) freeze-dried LK-117 was prepared.

IgE-mediated hypersensitivity reaction The IgE-mediated hypersensitivity reaction was evaluated according to a previous method (12,13). After preliminary breeding for 13 days, 5-week-old female BALB/c mice were divided into 6 groups. Each lyophilized LAB sample (2 mg) was suspended in 200 µl of phosphate buffered saline (PBS). The sample solution (200 µl) was orally administered using a metal stomach tube once a day for 4 days. PBS was orally administered in the same manner to the animals in the control group. Four days later, the mice were intravenously administered 100 µl of anti-TNP IgE (20 µg/ml) in 0.1% BSA-PBS in the tail vein. After 30 min, 10 µl of 0.8% PiCl solution in olive oil/acetone was applied on ventral side of the right ear. The mice were sacrificed and the ear thickness was measured using an upright dial thickness gauge (Ozaki Manufacturing Co., Tokyo, Japan). An ear swelling response was determined as the difference in the ear thickness before and after the challenge. Every measurement was made in triplicate, and the average of 3 results was used in the subsequent analysis.

Induction of AD-like skin lesions As shown Fig. 1, AD-like skin lesions were induced in the NC/Nga mice by sensitization and repeated challenge with PiCl (Tokyo Kasei Kogyo, Tokyo, Japan) as described previously (14,15). Briefly, after preliminary breeding for 13 days, NC/Nga mice were evenly divided into groups on the basis of their weights and total clinical score. The mice were fed a basic diet containing LAB for 5 days and then sensitized by applying 150 µl of 5% (w/v) PiCl dissolved in ethanol:acetone (4:1) on the shaved, bare abdominal skin and hind paws. Five days later, weekly challenges with 150 µl of 0.8% (w/v) PiCl dissolved in olive oil and applied on the shaved dorsolateral skin and the sides of both ears were started and continued for another 46 days.

Clinical skin severity score The clinical severity of dermatitis of the auricle and dorsolateral skin was scored using previously described methods (10). Five factors (pruritus, rubor/hemorrhage/erosion, edema, abrasion/tissue deficit, and crust formation/xerosis) were considered. The total clinical score was calculated as the sum of the individual scores graded as 0 (subclinical), 1 (slight), 2 (moderate), and 3 (severe).

Measurement of total IgE levels The total IgE levels were assays using a sandwich ELISA kit (Shibayagi, Gunma, Japan) according to the manufacturer's instructions.

Cell culture Human intestinal epithelial cell line Caco-2 cells were cultured in DMEM (with glutamine, high glucose) supplemented with 1% MEM-NEAA, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% decomplemented FBS (56°C, 30 min). Murine macrophage cell line RAW 264.7 cells were cultured in DMEM (with glutamine, low glucose) supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Murine fibrosarcoma cell line L929 cells were cultured in MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cell cultures were incubated at 37°C in a humidified 5% CO₂ incubator.

Transepithelial electrical resistance measurement The transepithelial electrical resistance (TER) value was measured using a Millicell-ERS instrument (Millipore, Eschborn, Germany), in keeping with a previous study (16).

Co-culture system A co-culture system was constructed as described by Tanoue et al. (16). Caco-2 cells were seeded at a density of 1.3×10^5 cells/well on Transwell insert plates (4.67 cm², 0.4-µm pore size; Corning CoStar Corp., Cambridge, MA, USA). The cell culture medium was changed every 3 days until the cells were completely differentiated (TER value >400 Ωcm²). RAW264.7 cells

were seeded at a density of 0.5×10^5 cells/well in 24-well tissue culture plates and incubated overnight to allow them to completely adhere to the wells. After replacing all media with RPMI1640, the Transwell insert plates on which the Caco-2 cells had been cultured were placed in the wells preloaded with RAW264.7 cells (Fig. 2). To evaluate the effect of the LABs isolated from kimoto, the lyophilized LAB cells were applied to the apical side of the wells at a final concentration of 1 or 100 µg/ml. After incubation for another 3 h, culture supernatants from the basolateral side of the well were collected for TNF-α measurement.

TNF-α content measurement The TNF-α content was determined using a cytotoxicity assay with L929 cells, wherein murine rTNF-α was used as the standard, as described by Takada et al. (17).

Statistical analyses Dennett's method was used for multiple comparisons with a control group. Statistical significance was defined as $p < 0.05$ or 0.01. Data are presented as means ± standard error of mean (M ± S.E.). The clinical severity score was compared between the control group and LK-117 group by using the Wilcoxon test. For this test, statistical significance was defined as $p < 0.05$.

RESULTS

Suppression of the anti-TNP IgE-mediated hypersensitivity reaction by oral administration of LABs isolated from kimoto

Oral administration of LAB lead to significant differences in body weight or amount of water consumed between the control group and any group throughout the experiment (data not shown). The hypersensitivity reaction was evoked by applying PiCl on the earlobe of the mice. As shown in Fig. 3, the change in ear thickness after the PiCl challenge averaged 26.0 ± 7.3 µm in the LK-117 group compared to 74.0 ± 6.2 µm in the control group, and the strong suppression of ear swelling was observed with statistically significant improvement in any of four groups consisting of *L. sakei* and *L. mesenteroides*. In contrast, *L. curvatus* LK-141 showed no such effects, and the average ear thickness of mice administered this lactobacillus was 92.7 ± 7.2 µm. *L. mesenteroides* and *L. sakei*, which are typical LABs present in the traditional seed mash used in Japanese sake brewing, seemed to suppress edema considerably.

Effect of LK-117 on the development of AD-like skin lesions

LK-117 had the highest ability to suppress the hypersensitivity reaction, NC/Nga mice fed LK-117 showed no significant difference in body weight and amount of water consumed compared to NC/Nga mice that were not. The total clinical scores calculated for at the 46 days after sensitization are shown in Fig. 4A. The average total clinical score was 8.0 ± 0.6 in LK-117 group and 10.0 ± 0.6 in the control group. This result indicates that the development of atopic lesions was suppressed in NC/Nga mice fed with LK-117 to a statistically significant extent. We assessed the total plasma IgE levels in the NC/Nga mice 46 days after

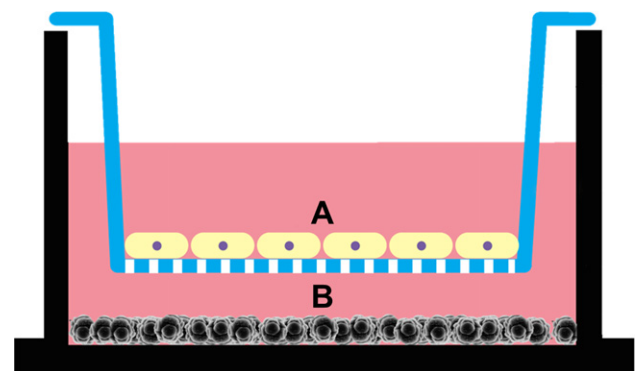


FIG. 2. *In vitro* intestinal gut model constructed with Caco-2 cells in the apical compartment (A) and RAW264.7 cells in the basolateral compartment (B). The cell populations co-cultivated in different compartments stay physically separated but may communicate via paracrine signaling through the pores of the membrane separating the compartments. Lyophilized LAB cells suspended in RPMI1640 were placed on the apical side.

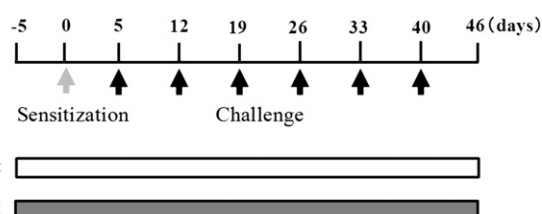


FIG. 1. Schematic representation of the experiment using NC/Nga mice to investigate the effects of LK-117 administration for the prevention of AD-like skin lesions. The time points of sensitization and challenges are indicated by arrowheads.

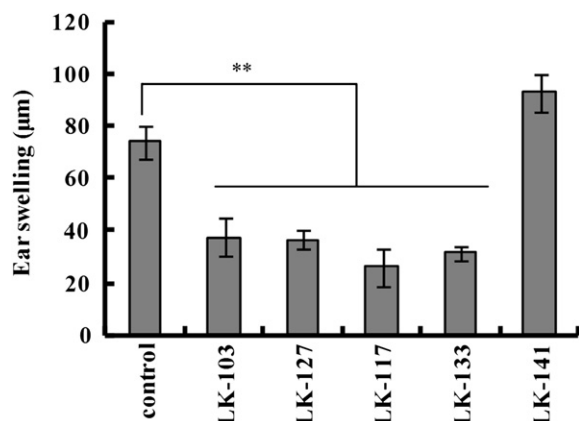


FIG. 3. Effects of LAB on IgE-mediated cutaneous anaphylaxis reaction on the ears of BALB/c mice. LAB suspension (2 mg in 200 μ l) was administered to BALB/c mice through a gastric tube once a day for 4 days, and IgE-mediated cutaneous anaphylaxis was then induced using PiCl. Edema was measured before and after the PiCl challenge. The values represent the means $\pm$ S.E. of 5 mice in each group. **Statistically different at the one percent level from the values of the control group.

sensitization but found no significant difference between mice fed with LK-117 and those that were not (Fig. 4B). This suggested that LK-117 intake suppressed dermatitis in skin lesions via a mechanism independent of IgE production.

Effect of LABs on RAW264.7 cells in the co-culture system As shown in Fig. 3, oral administration of some strains of LABs suppressed the hypersensitivity reaction. Therefore, we assumed that this effect was mediated by intestinal immunity. To ascertain whether LABs affect the response of dendritic cells (DCs) and macrophages, which is mediated by intestinal epithelial cells, Caco-2 cells and cells of the mouse macrophage cell line RAW264.7 were co-cultured using the Transwell system (Fig. 2). *L. sakei* LK-117 and *L. curvatus* LK-141, which had the most effect and no effect in the hypersensitivity reaction, respectively, were applied in the co-culture system. Treatment of Caco-2 cells with LK-117, but not LK-141, significantly increased TNF- α production in a dose-dependent manner (Fig. 5). However, LK-117 did not directly induce TNF- α production from RAW264.7

cells (data not shown). The integrity of the Caco-2 monolayer was maintained in the co-culture experiments, for the TER values throughout all experiments fall in the range of 492–515 Ω cm² at the start-up of co-culture, and in the range of 484–512 Ω cm² at the end. These results indicate that LABs can affect cytokine production from RAW264.7 interacting with Caco-2 cells.

DISCUSSION

The degranulation of mast cells caused by antigen-antibody reactions triggers type I allergic conditions such as rhinitis, sinusitis, atopic dermatitis, asthma, pollinosis, and food allergies. Key process components required for IgE responses, such as Th2 lymphocytes, IgE, IL-4, and IL-13, play a central role in the initiation of mast cell degranulation. Many studies have attempted to improve the Th1/Th2 balance using LABs and suppress mast cell degranulation using polyphenols in foods, as a practical approach for anti-allergy treatment using foods (7,18). The Th1 response is enhanced in order to suppress IgE production in humans, and autoimmune diseases may be initiated by this increased Th1 response. Examining the PCA reaction is a standard method used to measure allergen-specific IgE levels in mouse allergy models (19) and for evaluating the suppression of mast cell degranulation by anti-allergic drugs and orally administered compounds (12,13). We used mouse ear swelling assays for IgE-dependent allergic inflammation to examine whether LABs, like some polyphenols from citrus foods and tea leaves, can reduce inflammation. As shown in Fig. 3, LABs isolated from the traditional seed mash used to brew sake effectively prevented the immediate hypersensitivity reaction. To our knowledge, studies dealing with the anti-allergic effects of LABs without dependence on IgE level are extremely few and inconclusive. Moreover, as shown in Fig. 4, this suppressive effect was also observed in NC/Nga mice fed with LK-117, independent of the total IgE levels, resulting in a significant reduction in the development of AD-like skin lesions. The mechanism underlying the probiotic effects on allergies has not been identified thus far. Some reports have shown that the symptoms of allergies improve despite no improvement in the Th1/Th2 balance, while other reports have shown improvements in allergy symptoms with concomitant improvements in the Th1/Th2 balance. Sawada et al. reported that heat-treated *Lactobacillus rhamnosus* GG had the

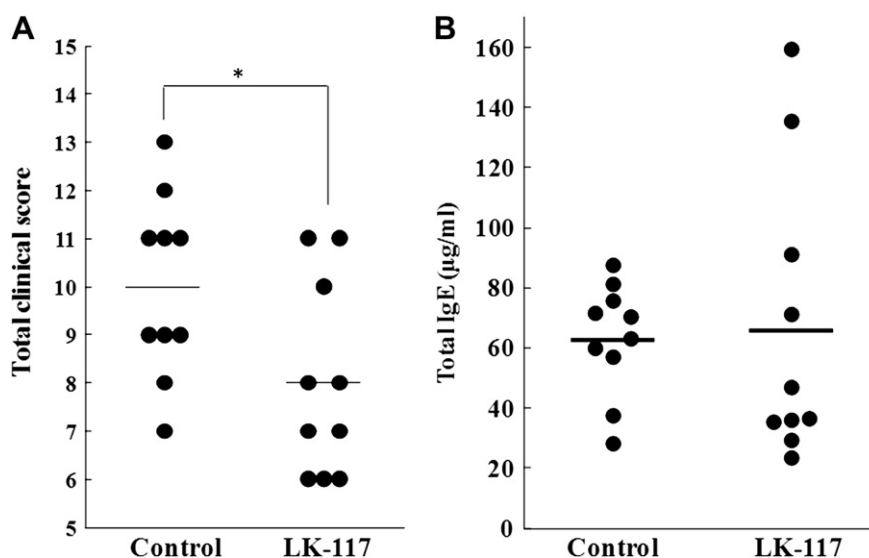


FIG. 4. Effect of LK-117 on PiCl-induced AD-like symptoms in NC/Nga mice. For 51 days, mice were fed chow containing *Lactobacillus sakei* LK-117 or that lacking the probiotic. The total clinical scores (A) and total plasma IgE levels (B) are shown. Each point represents the individual clinical score, and each line represents the mean value of each group (n = 10). *Statistically different at the five percent level from the values of the control group.

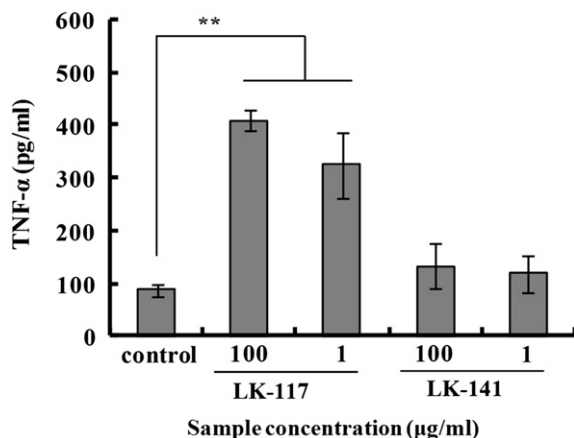


FIG. 5. Effect of LAB on the production of TNF- α from the mouse macrophage-like cell line RAW264.7 via Caco-2 cells in the co-culture system. RAW264.7 cells were cultured overnight in 24-wells plate in 5% CO₂ to allow them to adhere to the bottom of the plate. Then, the Transwell inserts on which the Caco-2 cells had been cultured were placed into the 24-well plates preloaded with RAW264.7 cells. Next, 1 or 100 μ g/ml of lyophilized LK-117 or LK-141 cells was applied to the apical side of the wells and incubated for 3 h. The culture media were then collected for the measurement of TNF- α content. Values represent the means $\pm$ S.E. (n = 3). **Statistically different at the one percent level from the values of the control group.

ability to suppress the development of AD probably because of strong induction of IL-10 independent of the IgE levels (20). Ishida et al. also reported that in clinical tests, oral administration of *Lactobacillus acidophilus* L-92 could alleviate the symptoms of perennial allergic rhinitis, although no significant differences were found in the serum IgE levels or the Th1/Th2 ratio (21). These results indicate that factors apart from the regulation of the Th1/Th2 balance may be involved in the reduction of allergy symptoms.

The co-culture model constructed with the human intestinal epithelial cell line Caco-2 and the murine macrophage cell line RAW 264.7 could imitate gut inflammation *in vivo* (22). Therefore, this co-culture system could be regarded as an intestine. The following question arose because of the high degree of genetic relatedness between *L. sakei* and *L. curvatus*: what contributes to the suppressive effect of LABs on the immediate hypersensitivity reaction (as shown in Fig. 3)? Considering the result shown in Fig. 5, it appears that LK-117 indirectly activates antigen-presenting cells such as macrophages by interacting with the intestinal epithelial cells. Recently, Bouike et al. reported that hydrogen peroxide produced by Caco-2 plays the important role of a secondary messenger in the production of TNF- α from macrophages (23). We found that LK-117 cells upregulated the production of TNF- α from macrophages via intestinal epithelial cells, but they did not directly stimulate macrophages. Bergey's manual described some strains of *L. curvatus* as motile initially; motility is lost on subculture (24). The atypical surface structure of *L. curvatus* might underlie the difference in the immune response it produces as compared to *L. sakei*.

From this observation the question arose: what is the possible link between the suppression of allergy and the enhancement of proinflammatory cytokine? Apart from its well-characterized proinflammatory role, there is increasing evidence for various anti-inflammatory properties of TNF- α . TNF- α was found to be a key factor in the regulation of intestinal glucocorticoids (GCs) synthesis, as absence of TNF- α resulted in an almost complete lack of steroidogenic enzyme expression and intestinal GC synthesis (25). GCs are steroid hormones with potent antiinflammatory properties (26). Thus, a variety of inflammatory disorders are successfully treated with GCs. Noti et al. have reported that repetitive administration of TNF- α restored colonic GC synthesis during oxazolone-

induced colitis and resulted in an amelioration of all clinical parameters and reduced inflammation, and that the induction of GC synthesis does not seem to depend on the inflammation per se but on the type of inflammation. Over the last decade, there has been accumulating evidence that synthesis of bioactive GCs is not restricted to the adrenal glands but extends to other extraadrenal sources, such as the thymus, brain, skin, and vascular endothelium (27). Similarly, TNF- α production enhanced by orally-administrated LK-117 might affect the induction of local GC synthesis in skin diseases. It has been reported that dextran sodium sulfate and 2,4,6-trinitrobenzenesulphonic acid, both promoting a Th1-type inflammation including a strong production of TNF- α , induced a pronounced and transient GC synthesis in the colon. In marked contrast, the Th2 cytokine-dominated inflammation induced by oxazolone failed to induce TNF- α and intestinal GC synthesis (25). The production of the Th1-associated cytokines IFN- γ and IL-12 were also higher in the mice with dermatitis, although NC/Nga mice kept under conventional conditions spontaneously develop AD-like lesions with an elevation of serum IgE levels (28). It is likely that the therapeutic effects for eczematous skin lesions of LK-117 might be linked with cytokine production in NC/Nga mice.

In conclusion, this paper demonstrates that LABs isolated from sake seed mash, especially LK-117, have anti-allergic effects and the ability to suppress AD-like skin lesions when administrated orally, and this effect is not dependent on the total IgE levels. Further investigations are necessary to clarify how LAB cells ameliorate AD-like skin lesions.

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