ORIGINAL ARTICLE

Lactobacillus Acidophilus Strain L-92 Regulates the Production of Th1 Cytokine as well as Th2 Cytokines

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ABSTRACT

Background: There is growing interest in probiotics such as lactic acid bacteria (LAB), not only for treatment of T helper type (Th) 1-mediated diseases but also for Th2-mediated diseases, including allergic diseases, since lactic acid bacteria may be able to modulate the Th1/Th2 balance, in addition to having an immunomodulative effect through induction of Th1 bias.

Methods: The effect of oral administration of heat-killed *Lactobacillus acidophilus* Strain L-92 (L-92) on ovalbumin (OVA)-specific immunoglobulin (Ig)E production was investigated in BALB/c mice. L-92 was orally administered to mice for 8 weeks from 2 weeks after initiation of OVA-immunization. Patterns of cytokine and Ig production in splenocytes and cells from Peyer's patches (PPs) from these mice were examined after restimulation with OVA *in vitro*.

Results: L-92 significantly suppressed serum OVA-specific IgE levels for a long period. Cytokines such as interferon (IFN)-γ, interleukin (IL)-4 and IL-10 and Igs such as total IgE and OVA-specific IgE were produced at significantly lower levels by splenocytes of L-92-treated mice, compared with those of control mice. In contrast, transforming growth factor (TGF)-β and IgA levels produced by PPs from L-92-treated mice were significantly higher than in those from control mice.

Conclusions: Oral L-92 administration regulated both Th1 and Th2 cytokine responses, suppressed serum OVA-specific IgE, and induced TGF-β production in PPs. TGF-β is known to be associated with activation of regulatory T (Treg) cells. These data suggest that LAB may have immunomodulative effect by Treg cells via TGF-β activity.

KEY WORDS

Lactobacillus acidophilus strain L-92, Peyer's patch, splenocytes, TGF-β, Treg

INTRODUCTION

Probiotics may establish T helper type (Th) 1 bias and predominance of regulatory T (Treg) cells through changes in the composition of intestinal microflora in a host, due to effects or direct action on gut-associated lymphoid tissue (GALT). Recent studies have also documented a relationship between probiotic induction of intestinal microorganisms and resistance to development of allergy.[1-3] Several strains of *Lactobacillus*, including *L. casei* strain Shirota,[4] *L. plantarum* strain L-137[5] and *L. acidophilus* strain L-92 (L-92)[6] have been reported as probiotics that modify antigen-specific serum immunoglobulin (Ig) E levels in animal models. These results suggest the possible usage of such lactic acid bacteria (LAB) in preventing atopic diseases. Little is known, however, about the exact mechanisms underlying the suppressive and immunomodulative effects of LAB.

In contrast to their propensity to counteract allergic development, LAB also exert inhibitory effects on the development of Th1-mediated diseases, including colitis and diabetes in animals and humans,[7-14] suggesting that they may modulate immunity through a mechanism that does not involve polarization of the immune response toward Th1. One possibility is that

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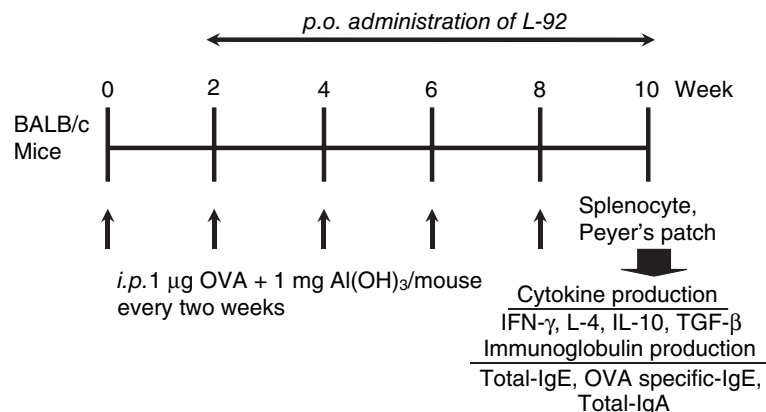Email: cbp10610@pop12.odn.ne.jp

Fig. 1 Experimental schedule 1. Immunization with OVA and oral administration of L-92 in mice, (and measurement of cytokine (IFN-γ, IL-4, IL-10 and TGF-β) and Ig (total IgE, OVA-specific IgE and total IgA) production from splenocytes and cells of PPs in the L92-treated mice.) Sham mice were intraperitoneally injected with 0.1 ml sterile 0.9% saline only in weeks 0, 2, 4, 6 and 8 and also fed daily with 200 μl of sterile 0.9% saline.

Treg cells play an important role in regulating the Th1/Th2 imbalance underlying the immunomodulative effects of LAB. In support of this hypothesis, Ohno *et al.* showed that the suppressive effect of specific IgE production of *Bifidobacterium bifidum* G9-1 (BBG9-1) in ovalbumin (OVA)-immunized mice is due to reduction of IgE production via an interferon (IFN)-γ-independent mechanism mediated by Treg cells.[15] Hence, the immunomodulative effects of LAB may not only be due to induction of Th1 bias, as reported to date, but may also occur through modulation of the imbalance of Th1 and Th2 cells.

The presence of two kinds of CD4 + Treg cells, T regulatory type 1 (TR1) cells and T helper type 3 (TH3) cells, has recently been shown in mucosal immunization. Akbari *et al.* report that isolated pulmonary dendritic cells (DCs) produce interleukin (IL)-10 and induce TR1 cells after respiratory exposure to antigen,[16] whereas DCs from the gut produce transforming growth factor (TGF)-β; this may explain why antigen administered via the gut preferentially induces TH3 regulatory cells.[17] Furthermore, *in vitro* studies using the intestinal epithelial cell line Caco-2 suggest that epithelial cells might secrete more TGF-β and α-tolerogenic cytokines in the physiologically inflammatory environment of the intestine in the presence of LAB.[18] Therefore, LAB may be appropriate for treatment of various disorders, including allergic diseases, due to their ability to induce Treg cells and activate these cells through TGF-β production at the mucosal surface. In the current study, we examined the immunomodulative effect of oral administration of L-92 in OVA-immunized mice, and studied the underlying mechanism using splenocytes and cells from Peyer's patches (PPs).

METHODS

MICE
Six-week-old male BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan) and acclimatized for 1 week before the start of experiments. The mice were housed in an air-conditioned animal room at a temperature of 22 ± 1℃ and a humidity of 60 ± 5%, with a 12-hour light/dark cycle under pathogen-free conditions. They were fed a standard diet (Norsan Corporation, Kanagawa, Japan) and allowed free access to water throughout the experimental period. Experiments were undertaken in accordance with the guidelines for the care and use of experimental animals of the Japanese Association for Laboratory Animal Science, published in 1987.

BACTERIA
L-92 was obtained from the R&D Center, Calpis Co. Ltd., Kanagawa, Japan, where the strain was originally isolated. Bacteria were cultured for 48 hours at 37℃, and the cells were then collected by centrifugation, washed with sterile distilled water, heat-killed at 100℃, lyophilized, and suspended in phosphate-buffered saline (PBS).

EXPERIMENTAL SCHEDULE 1 (Fig. 1)
Mice (*n* = 6 per group) were intraperitoneally injected with 1 μg of OVA (Ovalbumin; Sigma-Aldrich. Inc., St. Louis, USA) absorbed onto 1 mg of alum in 0.1 ml sterile 0.9% saline in weeks 0, 2, 4, 6 and 8. These mice were also fed daily with 200 μl of L-92 suspension (0.2, 1.0 or 5.0 mg/day/animal) in sterile 0.9% saline for 8 weeks from 2 weeks after the 1st immunization, using an intragastric stainless steel feeding tube; control mice did not receive this suspension. Sham

mice were intraperitoneally injected with 0.1 ml sterile 0.9% saline only in weeks 0, 2, 4, 6 and 8 and also fed daily with 200 μl of sterile 0.9% saline. Blood was collected from the orbital cavity under anesthesia once a week from day 0. Serum samples were prepared by centrifugation at $10000 \times g$ at 4℃ for 5 minutes, and then frozen at –80℃ until determination of Ig levels. In week 10, six mice from the control group, six mice from the sham group, and six mice from the high-dose L-92 group (5.0 mg/day/mouse) were sacrificed by deep anesthesia, and cells were subsequently obtained from the spleens and PPs of these mice.

EXPERIMENTAL SCHEDULE 2

OVA-primed splenocytes were used for *in vitro* studies. BALB/c mice were injected intraperitoneally with 1 μg of OVA adsorbed onto 1 mg of alum in 0.1 ml sterile 0.9% saline. Injections were given twice on day 0 and day 14, and mice were sacrificed on day 28 to obtain splenocytes.

PRODUCTION OF CYTOKINES AND IgS FROM SPLENOCYTES *IN VITRO*

Spleens were removed from mice after sacrifice by deep anesthesia. After lysis of erythrocytes, splenocytes were suspended at a concentration of 2.5×10^6 cells/ml in RPMI medium (Invitrogen Corporation, N.Y., USA) supplemented with 10% fetal calf serum (FCS; Thermo Trace, Melbourne, Australia) and penicillin/streptomycin (Invitrogen Corporation). The splenocytes (2.5×10^6 cells/ml) were seeded on 48-well microplates (Nunc, Roskilde, Denmark) and then stimulated with OVA at a final concentration of 100 μg/ml at 37℃, in the presence of heat-killed bacteria (0.01 to 1 μg/ml) or in the absence of bacteria. The effect of oral administration of L-92 on cytokine and IgE production was tested by culturing the splenocytes in the same concentration of OVA. Cytokine levels, IgE and OVA-specific IgE levels were determined after 7 days of the splenocyte cultures, as previously described.[19]

PRODUCTION OF TOTAL IgA AND CYTOKINES FROM PPS *IN VITRO*

PPs were removed from three mice of each group after sacrifice by deep anesthesia. Seven to 10 PPs were obtained from each mouse, and were gently crushed and filtered through a 40-μm nylon mesh (BD Bioscience, Bedford, USA). Cells from PPs were subsequently prepared after organ digestion in RPMI medium supplemented with collagenase (Roche, Indianapolis, IN, USA) and DNase 1 (Sigma-Aldrich Co, Zwijndrecht, The Netherlands) for 60 minutes at 37℃ and pooled. The cells were resuspended at a concentration of 2.5×10^6 cells/ml in serum-free medium consisting of RPMI medium supplemented with Nutridoma SP (1% vol/vol; Roche) and penicillin/strep-

tomycin. The cells (2.5×10^6 cells/ml) were seeded on 48-well microplates (Nunc, Roskilde, Denmark) and then stimulated with OVA at a final concentration of 100 μg/ml at 37℃ for 72 hours. Cytokine and total IgA levels produced from the cell cultures were determined after three days, as previously described.[20]

MEASUREMENT OF CYTOKINES AND IgS

Measurement of IFN-γ, IL-4, TGF-β, IL-10, total IgA, total IgE and OVA-specific IgE was performed using a sandwich enzyme-linked immunosorbent assay (ELISA). Commercial ELISA kits were used for IFN-γ, IL-4 and TGF-β (Bio Source International, Camarillo, CA, USA), IL-10 (R & D, Minneapolis, MN, USA), and total IgA (Bethyl Laboratories, Inc., Montgomery, TX, USA). OVA-specific IgE and total IgE in mouse serum and the culture supernatant were measured using ELISAs, as previously described.[21] In brief, measurement of OVA-specific IgE was performed in flat-bottom 96-well microliter plates (Nunc, Roskilde, Denmark) coated with anti-mouse IgE (Serotec, Oxford, UK). After blocking with 1% bovine serum albumin (BSA; Sekagaku Kogyo, Tokyo, Japan), 100 μL of diluted sample was added to each well, followed by incubation at room temperature for 1 hour. After washing with PBS containing 0.1% Tween 20 (T-PBS), 100 μl of diluted biotinylated-OVA was added to each well and incubated at room temperature for 1 hour. Then, after extensive washing with T-PBS, 100 μl of diluted peroxidase-conjugated streptavidin (Dakocytomation, Glostrup, Denmark) was added to each well. The enzymatic reaction was stopped by addition of 100 μl of 2 M H_2SO_4 after incubation at room temperature for exactly 30 minutes. The optical densities of the enzymatic reaction solutions were read using an automatic ELISA plate reader (Multiscan MS, ver 8; Labsystems Oy, Helsinki, Finland) at 450 nm (reference 690 nm) and analyzed using Deltasoft 3 (Biometallics Inc., Princeton, NJ, USA). Sequentially diluted anti-OVA IgE monoclonal antibody (mAb; donated by Dr M. Kiniwa, Taiho Pharmaceutical Co. Ltd., Saitama, Japan) was used to prepare a standard curve. To measure the total IgE, similar procedures were carried out using peroxidase-labeled polyclonal anti-mouse IgE (Nordic Immunological Laboratories, Tilburg, Netherlands). The detection limit for IgE and OVA-specific IgE was 1 ng/ml.

STATISTICAL ANALYSIS

All values are expressed as means ± S.E.M. An unpaired Student's *t*-test (two-tailed) was used to evaluate the significance of the difference between two independent groups with equal variance, as assessed using an *F* test. In all other cases, Welch's *t*-test (two-tailed) was employed. One-way analysis of variance (ANOVA) and Fisher's test were used for multiple comparisons with controls. Values of $P < 0.05$ were considered to indicate statistically significant differ-
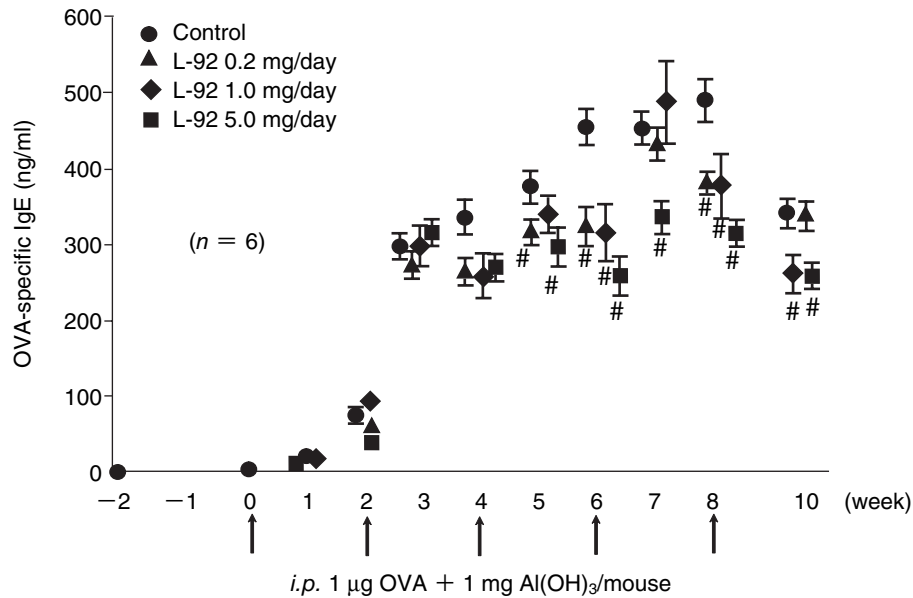
Fig. 2 Effect of oral administration of L-92 on OVA-specific IgE production in mice. Blood was collected from the orbital cavity once a week from day 0, and the serum level of OVA-specific IgE was determined by ELISA. #$P < 0.05$ compared with the control group, assessed by ANOVA and Fisher test.

ences.

RESULTS

ORAL ADMINISTRATION OF L-92 SUPPRESSED ANTI-OVA IgE ANTIBODY PRODUCTION *IN VIVO*

The time course of serum OVA-specific IgE levels in OVA-immunized mice is shown in Figure 2. To evaluate the immunomodulative effect of oral administration of L-92, BALB/c mice were injected with OVA at 2-week intervals for 8 weeks. This immunization schedule resulted in a marked increase in serum OVA-specific IgE levels (Fig. 2, control). Test groups were fed heat-killed L-92 (0.2, 1.0 or 5.0 mg/day/mouse) during the 8-week period. Serum samples from all groups were collected weekly. The OVA-specific IgE levels were significantly lower in mice receiving L-92, and particularly in those given the highest dose (5.0 mg/day), compared with the levels in control mice.

ORAL ADMINISTRATION OF L-92 SUPPRESSED CYTOKINE PRODUCTION IN SPLENOCYTES *IN VITRO*

Upon stimulation with OVA *in vitro*, splenocytes from OVA-immunized mice produced IFN-γ, IL-4 and IL-10 (Fig. 3). Each cytokine production was significantly suppressed in the splenocytes of mice given L-92 at 5.0 mg/day, compared with those in control mice (Fig. 3).

ORAL ADMINISTRATION OF L-92 SUPPRESSED TOTAL AND OVA-SPECIFIC IgE PRODUCTION IN SPLENOCYTES *IN VITRO*

Upon stimulation with OVA *in vitro*, splenocytes from OVA-immunized mice produced Igs (total IgE and OVA-specific IgE) (Fig. 4). Each Igs production was significantly or completely suppressed in the splenocytes of mice given L-92 at 5.0 mg/day, compared with those in control mice (Fig. 4).

ORAL ADMINISTRATION OF L-92 INDUCED TGF-β AND TOTAL IgA PRODUCTION IN CELLS OF PPS *IN VITRO*

As shown in Figure 5, TGF-β and total IgA production by cells of PPs from OVA-immunized mice given L-92 at 5.0 mg/day was significantly higher than in control mice.

L-92 INDUCED IFN-γ AND SUPPRESSED IL-4 AND IL-10 PRODUCTION IN CULTURED OVA-STIMULATED SPLENOCYTES

Isolated splenocytes from OVA-primed BALB/c mice were cultured with the same antigen in the absence or presence of L-92. As shown in Figure 6, L-92 from 0.01 to 1.0 μg/ml induced IFN-γ production in a dose-dependent manner, and the highest dose of L-92 (1.0 μg/ml) induced significantly higher IFN-γ production, compared with control. In contrast, IL-4 and IL-10 production was significantly suppressed by the highest dose of L-92 (1.0 μg/ml), but not in a dose-dependent manner (lower doses of L-92 did not have a significant effect). Induction of IFN-γ by L-92
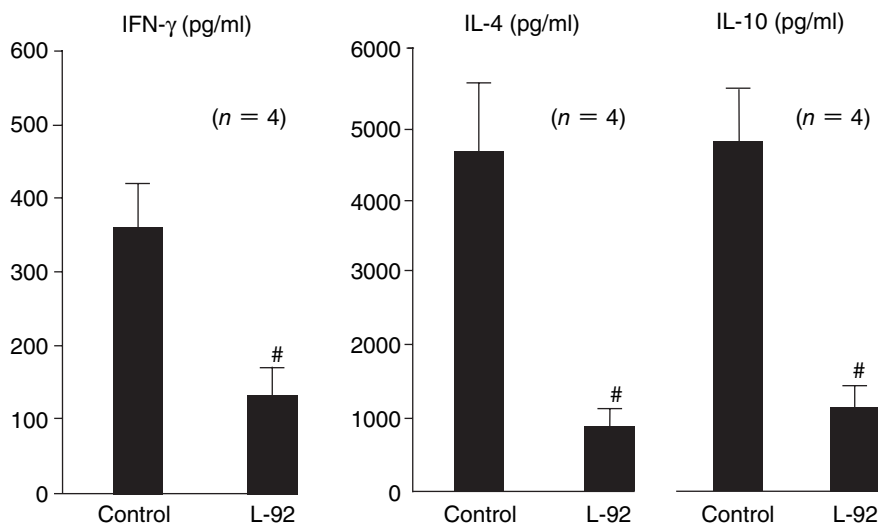
Fig. 3 Effect of oral administration of L-92 on cytokine (IFN-γ, IL-4 and IL-10) production in splenocytes from mice *in vitro*. As in Figure 1, splenocytes were removed from four mice each of the control group, sham group and high-dose L-92 group (5.0 mg/day). Splenocytes (2.5×10^6 cells/ml) were stimulated with OVA at a final concentration of 100 μg/ml for 7 days at 37°C, and the cytokine levels in the culture medium were then determined by ELISA. #$P < 0.05$ compared with the control group, assessed by unpaired Student's *t*-test or Welch's *t*-test.
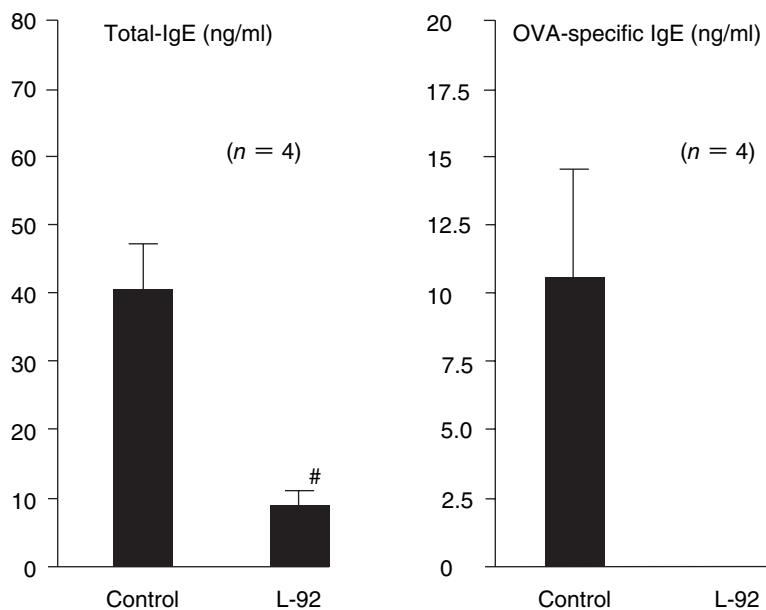

Fig. 4 Effect of oral administration of L-92 on total-IgE and OVA-specific IgE production in splenocytes from mice *in vitro*. As in Figure 1, splenocytes were removed from four mice each from the control, sham and high-dose L-92 (5.0 mg/day) groups. Splenocytes (2.5×10^6 cells/ml) were stimulated with OVA at a final concentration of 100 μg/ml for 7 days at 37°C, and then total IgE and OVA-specific IgE levels in the culture medium were determined by ELISA. #$P < 0.05$ compared with control group, assessed by unpaired Student's *t*-test or Welch's *t*-test.
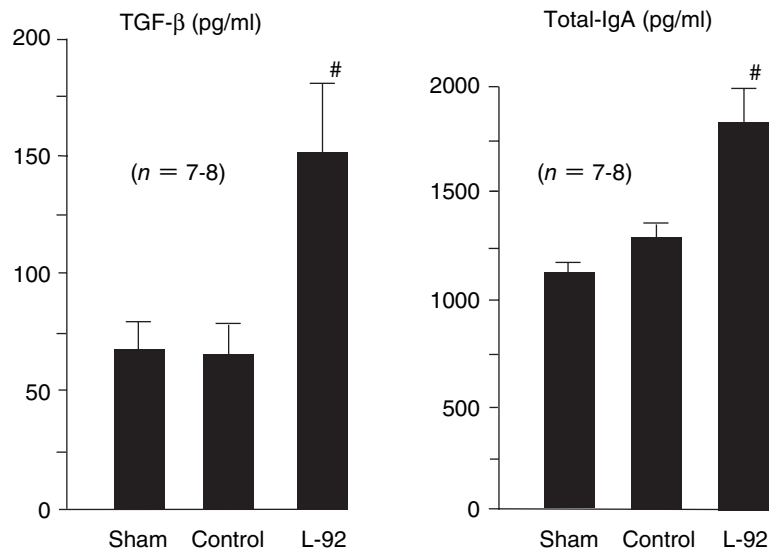
Fig. 5 Effect of oral administration of L-92 on TGF-β and total IgA productions in cells of PPs from OVA-immunized mice *in vitro*. As in Figure 1, cells from PPs were removed from three mice each from the control, sham and high-dose L-92 (5.0 mg/day) groups. The cells (2.5×10^6 cells/ml) from PPs were stimulated with OVA at a final concentration of 100 μg/ml for 3 days at 37°C before measurement of TGF-β and Total IgA levels using ELISA in each case. Sham mice were intraperitoneally injected with 0.1 ml sterile 0.9% saline only in weeks 0, 2, 4, 6 and 8 and also fed daily with 200 μl of sterile 0.9% saline. #$P < 0.05$ compared with control group, assessed by unpaired Student's *t*-test or Welch's *t*-test.

seemed to be associated with decreased IL-4 and IL-10 production.

DISCUSSION

L-92 used in this study has been shown to improve the symptoms of allergic rhinitis patients in a double-blind placebo-controlled clinical study.[22] Therefore, L-92 is thought to be effective for allergic diseases. To examine the underlying mechanism, in this study we observed the effect of direct L-92 administration in splenocytes of OVA-immunized mice, confirmed the effect of oral L-92 administration that suppresses the blood OVA-specific IgE, and determined the mechanism of cytokine and Ig production by splenocytes and cells of PPs after oral administration of L-92 in mice.

Addition of heat-killed L-92 and OVA directly to OVA and alum-immunized splenocytes of mice caused a significant increase in IFN-γ, a Th1 cytokine, and a significant decrease in IL-4 and IL-10, which are both Th2 cytokines (Fig. 6). These *in vitro* results are similar to those of previous reports regarding Th1 cell induction by LAB, and L-92 is thought to suppress Th2 cells through Th1 cell activation. Cell wall components of LAB and other probiotics, such as peptidoglycans and lipoteichoic acid, induce maturation and activation of DCs via toll-like receptor (TLR)

2, as well as producing various proinflammatory cytokines and chemokines.[23-25] In addition, immune-related cytokines, including IL-12, that induce Th1 type responses are produced. Cytosine-guanosine dinucleotide (CpG) sequence motifs composed of unmethylated CpG dinucleotides are the immunostimulatory components of bacterial DNA.[26] Oligodeoxynucleotides (ODNs) containing CpG motifs can directly or indirectly activate DCs, murine macrophages, B lymphocytes, natural killer cells, and T lymphocytes. This action leads to the secretion of various proinflammatory cytokines, including IL-6, IL-12, tumor necrosis factor (TNF), and IFN-γ via TLR9, and finally induces a Th1-biased immune response.[27-29] LAB are known to contain CpG-like immunostimulatory oligonucleotides,[30-32] and ligation of TLR2 and TLR9 directly by some lactobacilli species has also been reported.[33,34] Based on these reports, L-92 may be able to stimulate TLR2 and TLR9 directly, induce a Th1 type response, and suppress the predominance of OVA-immunized Th2 cells.

Next, we confirmed that oral administration of L-92 suppressed OVA-specific IgE in the blood of OVA-immunized mice. To examine the underlying mechanism, splenocytes and cells of PP were examined after oral administration of L-92 to mice. Splenocytes repeatedly immunized with OVA and alum showed
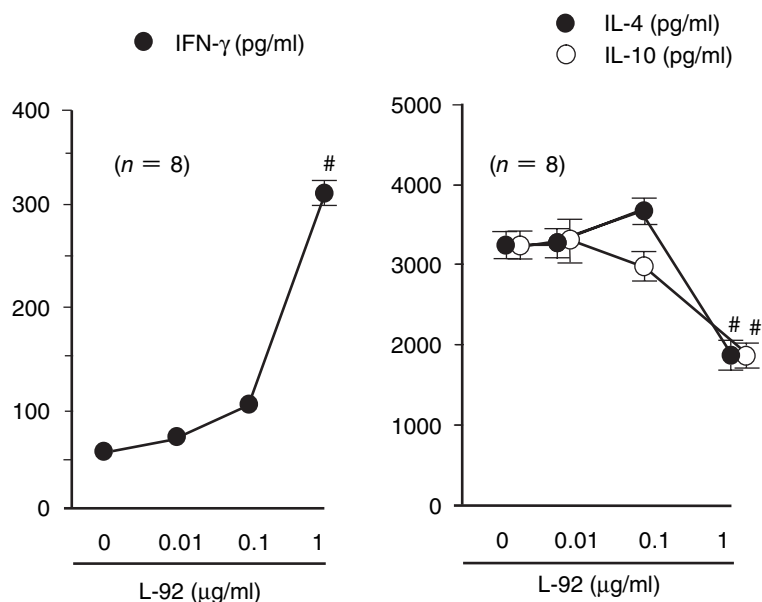
Fig. 6 Direct effect of L-92 on OVA-induced cytokine (IFN-γ, IL-4 and Il-10) production by OVA-immunized splenocytes *in vitro*. Splenocytes (2.5×10^6 cells/ml) from OVA-immunized BALB/c mice were cultured with OVA at a final concentration of 100 μg/ml in the absence (control) or presence of L-92 (0.01 to 1 μg/ml) for 7 days at 37°C, and the cytokine levels in the culture medium were then determined by ELISA. #*P* < 0.01 compared with the control group, assessed by ANOVA and Fisher test.

high production of IFN-γ, the Th1 cytokine, and the Th2 cytokines IL-4 and IL-10, by the stimulation of OVA and significant suppression of the levels of both Th1 and Th2 cytokines was apparent in splenocytes from mice given L-92 orally. Coincidently, L-92 administration inhibited production of total IgE and OVA-specific IgE in both *in vivo* (Fig. 2) and *in vitro* (Fig. 4) studies. Based on these data, it appears that LAB suppress IgE production through a mechanism that does not involve a shift to Th1-dominant immunity.

Furthermore we examined how oral administration of LAB affects gut immunity in mice using cells from PP, a tissue that is often used to examine the gut immune response. Oral administration of L-92 significantly increased TGF-β and total IgA in cells from PP. We suggest that L-92 may induce TH3 regulatory cells which subsequently spread through the immune system, including to the spleen and modulate the Th1/Th2 balance all over the body.

TGF-β is known to induce an isotype switch of IgA, and TGF-β secreted from DCs of PP is thought to be related to increased IgA production in the intestine. Furthermore, TGF-β secreted from DCs of PP induces oral tolerance through induction of Treg cells,[17,35] and Akbari *et al*. have suggested that DCs of the intestine can induce TH3 regulatory cells, which also produce TGF-β itself and induced by autocrine.[16] TH3 regulatory cells suppress effector T

cell function by production of TGF-β and cell to cell contact.[36]

Smits *et al*. assessed the mechanism of Treg cell activation in human DCs using Lactobacilli of different species.[37] *L. reuteri* and *L. casei* stimulated monocyte-derived DCs and facilitated Treg cell activation, although *L. plantarum* had no such functions. *L. reuteri* and *L. casei* both interact with the C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), and blocking antibodies for DC-SIGN suppressed Treg cell activation by these probiotic bacteria. Based on these data, some species of LAB are thought to exert their function through DC-SIGN, which in turn is thought to be involved in Treg cell activation.

We were unable to determine which components of L-92 induced the significant increase in TGF-β production in PPs and which was the cell population concerned with the increase of TGF-β. We are now studying the change of cell population of PPs and the specificity of IgA. Orally administered LAB are initially incorporated into M cells in the intestine and transferred to PP, which plays a leading role in gut immunity by stimulating DCs. The initial role of the M cells is uncertain, although Tohno *et al*. have suggested that TLR2 develops in intestinal M cells of swine and that TLR2 of M cells may be related to ligand-specific transcytosis and transport of LAB.[38] The initial role of the M cells that may be related to

ligand-specific transcytosis and transport of LAB clearly remain to be clarified. Our results suggest that the immunomodulative effect of oral administration of L-92 may involve Treg cells, and treatment based on functions specific to various types of LAB may therefore be effective for both Th1- and Th2-mediated diseases such as allergic diseases, autoimmune diseases and chronic inflammatory diseases.

ACKNOWLEDGEMENTS

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