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Preventive effects of a probiotic mixture in an ovalbumin-induced food allergy model

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Running title: Effects of probiotics on allergies

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Abstract

Although there has been a steady increase in the prevalence of food allergies worldwide in recent decades, no effective therapeutic strategies have been developed. Modulation of the gut microbiota composition and/or function through probiotics has been highlighted as a promising target for protection against food allergies. In this study, we aimed to investigate the allergy-reducing effects of a probiotic mixture (P5: *Lactococcus lactis* KF140, *Pediococcus pentosaceus* KF159, *Lactobacillus pentosus* KF340, *Lactobacillus paracasei* 698, and *Bacillus amyloliquefaciens* 26N) in mice with ovalbumin (OVA)-induced food allergy. Administration of P5 significantly suppressed the oral OVA challenge-induced anaphylactic response and rectal temperature decline, reduced diarrhea symptoms. Moreover, P5 also significantly inhibited the secretion of IgE, Th2 cytokines (interleukin [IL]-4, IL-5, IL-10, and IL-13), and Th17 cytokines (IL-17), which were increased in mice with OVA-induced food allergy, and induced generation of CD4⁺Foxp3⁺ regulatory T cells. These results revealed that P5 may have applications as a preventive agent for food allergy.

Keywords: food allergy, probiotics, Th1/Th2 balance, IgE suppression, Treg induction

Introduction

Allergic diseases are caused by hypersensitivity of the immune system to foreign substances (allergens), such as drugs, insect products, pollen, or food ingredients. These diseases include hay fever, food allergies, atopic dermatitis, allergic asthma, and anaphylaxis. Food allergies, which are caused by food allergens, such as ovalbumin (OVA) and ovomucoid from eggs, casein and beta-lactoglobulin from milk, tropomyosins from shrimp, peanut agglutinin from peanuts, and gliadin from wheat [1], occur when the immune system reacts to food allergens after ingestion. Ingested allergens permeate into the gastrointestinal tract via the paracellular diffusion pathway [2] and are then taken up by antigen presenting cells (APCs), which present selected peptides via the major histocompatibility complex (MHC) class II to activate naïve T cells into Th cells, resulting in the activation of Th2 cells. Th2 cells produced cytokines such as interleukin (IL)-4, IL-5, and IL-13 and induce the generation of antigen-specific immunoglobulin E (IgE) from B cells. IgE then binds to FcεRI on mast cell [3]. A second exposure to the allergen leads to crosslinking between IgE and allergen [4]. Allergen-induced mast cells exhibit degranulation, releasing a variety of inflammatory mediators that can elevate allergic symptoms, such as itchiness, tongue swelling, hives, trouble breathing, pruritus, abdominal pain, vomiting, diarrhea, urticaria, hypotension, and lethal anaphylactic shock [5, 6]. IgE-mediated food allergy has been estimated to affect 1–3% of the adult population and up to 3–7% of children [7- 9]. Although there has been a steady increase in the prevalence of food allergy worldwide in recent decades [10], no effective therapeutic strategies have been developed, and the primary management of food allergies consists of strictly avoiding relevant allergens [11]. Recently probiotics has been highlighted as a promising target of protection against allergic disease. Probiotics are defined as live microorganisms that, when administered at adequate amounts, confer health benefits to their human hosts [12]. During last decade, a

growing body of evidence is accumulating to show that probiotics alleviate allergic disease through the induction of immune tolerance [13], the modulation of intestinal immunity [14], the enhancement of the gut mucosal barrier [15], and the production of substances such as short-chain fatty acids [16]. In food allergy, also, a number of previous studies have suggested that specific probiotic strains have the ability to alleviate food allergies. For example, *Lactobacillus casei* shirota prevents food allergic symptoms (rectal temperature, IgE, IL-4, and IL-5) and inhibits dendritic cell (DC) maturation and T cell activation [17]. Additionally, oral administration of *Lactococcus lactis* NCC 2287 for 7 weeks in sensitized mice reduces allergic manifestations and production of IL-12, C-C- motif chemokine ligand (CCL) 11 and CCL17 [18]. *Lactobacillus plantarum* NRIC0380 alleviates allergic symptoms by inducing Tregs and controlling the Th1/Th2 balance [19]. Schiavi et al [20] reported that oral administration of a probiotic mixture containing eight different strains (*Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. bulgaricus, *Lactobacillus casei*, *Lactobacillus plantarum*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium breve*, and *Streptococcus salivarius* subsp. thermophilus) could have beneficial effects in OVA-sensitized mice, with a reduction in symptom severity and downregulation of Th2 cytokine mRNA expression. Moreover, Yang et al [21] reported that *Lactobacillus paracasei* L9 reduces β -lactoglobulin allergic sensitization via inducing regulatory DCs (regDCs) and manipulating the balance of Th1/Th2 responses. These studies suggested that the main inhibitory effects of probiotics in the food allergic response involve restoration of the Th1/Th2 immune balance and acquisition of oral tolerance by inducing regulatory cells, such as Tregs, regulatory B cells, and tolerogenic DCs.

In a preliminary study searching for anti-allergic probiotics, we found five probiotics (*Lactobacillus pentosus* KF 340, *Lactococcus lactis* KF140, *Bacillus amyloliquefaciens* 26N, *Lactobacillus paracasei* 698, and *Pediococcus pentosaceus* KF159 isolated from Korea

traditional foods) that possess the IL-4 suppressive effect and Treg inducing effect via *in vitro* test. Therefore, in this study, we aimed to investigate the effects of a mixture of these five probiotics on alleviation of allergic symptoms, including diarrhea and anaphylactic response, in an OVA-induced food allergy model.

Materials and Methods

Materials

De Man, Rogosa, and Sharpe (MRS) and tryptic soy broth (TSB) medium were purchased from Difco (Sparks, MD, USA). RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, and Dulbecco's phosphate-buffered saline (D-PBS) were purchased from Gibco (Grand Island, NY, USA); OVA (grade VI) and red blood cell lysis buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Isolation and preparation of probiotics

Lactobacillus pentosus KF340 (KCCM11675P), *Lactobacillus paracasei* 698 (KFCC11569P), *Lactococcus lactis* KF140 (KCCM11673P), *Pediococcus pentosaceus* KF159 (KCCM11674P), and *Bacillus subtilis* 26N (KCCM11287P), were isolated from Korean traditional fermented foods and identified by 16S rDNA sequence analysis. *Lactobacillus pentosus* KF340, *Lactobacillus casei* 698, *Lactococcus lactis* KF140, and *P. pentosaceus* KF159 isolated from kimchi (Korean traditional fermented cabbage) were cultured in MRS medium at 37°C for 24 h. *Bacillus amyloliquefaciens* 26N, isolated from Deonjang, a Korean traditional fermented soybean paste, was cultured in TSB medium at 37°C for 24 h. For preparation of live probiotics, cultured cells were collected by centrifugation at $5000 \times g$ for 15 min and then washed twice with phosphate-buffered saline (PBS). Pelleted cells were lyophilized and stored at -80°C for further study. The probiotic mixture (P5) contained 1×10^8 CFU of each of the five stains.

Immunoregulatory effects of P5 *ex vivo*

To evaluate the modulation of the Th1/Th2 balance of P5, BALB/c mice (5 weeks old, female, 18–20 g) were sensitized with 10 µg OVA adsorbed in 1 mg/mL Imject Alum (Pierce, Rockford, IL, USA) by intraperitoneal (i.p.) injection on days 0 and 14. After the second OVA injection (day 28), the mesenteric lymph nodes (mLNs) were collected from OVA-sensitized mice and placed in ice-cold RPMI containing 10% FBS, antibiotics, and 2-mercaptoethanol. The mLNs were ground with the sterile flat bottom of a syringe piston to homogenize the sample, and homogenized single cells were then washed twice with RPMI medium and centrifuged ($415 \times g$, 5 min). For cytokine analysis, the cells were adjusted to 5×10^6 cells/mL in RPMI medium using the trypan blue exclusion method. In 24-well plates, the cells were co-cultured with 1×10^7 CFU of the probiotic mixture (P5) in the presence or absence of OVA (100 µg/mL/well). The plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 72 h. After incubation, the supernatants were collected to analysis IL-4 and IFN-γ. To evaluate the anti-inflammatory and CD4+Foxp3-induction effects of P5, mLNs were obtained from normal BALB/c mice as described above. For analysis of cytokines (IL-10 and IL-12), the cells were adjusted to 5×10^6 cells/mL in RPMI medium using the trypan blue exclusion method. The cells were then co-cultured with 1×10^7 CFU of the probiotic mixture (P5) in the presence of gentamycin (Gibco) at 37°C in a humidified incubator with 5% CO₂ for 72 h. For CD4+Foxp3+ analysis, mLN-derived lymphocytes (1×10^6 cells/well) from BALB/c mice were cultured in 96-well plates in the presence of antigen (100 µg/mL OVA) and T-cell receptor stimuli (10 µg/mL plate-bound anti-CD3 monoclonal antibodies (17A2) and 2 µg/mL soluble anti-CD28 monoclonal antibodies (37.51; Biolegend, San Diego, CA, USA). At that time, the cells were treated with 1×10^7 CFU of the probiotic mixture (P5) and incubated at 37°C in a

humidified incubator with 5% CO₂ for 72 h.

Experimental animals and feeds

Twenty-eight BALB/c mice (5 weeks old, female, 18–20 g) were purchased from Orient Bio Inc. (Gyeonggi, Korea). After acclimating for 1 week, the mice were randomly divided into normal (naïve), untreated (sham), dexamethasone-treated (positive), and P5-treated groups. All mice were maintained in the same room under conventional conditions with a regular 12-h light/dark cycle and temperature and relative humidity maintained at 23 ± 2°C and 50% ± 5%, respectively. Mice were allowed free access to food and water. All animal experimental procedures were reviewed and approved by the Ethics Committee of the Experimental Animal Research Laboratory, Korea Food Research Institute (approval code, KFRI-224).

Induction of experimental food allergies by oral administration of OVA

To evaluate the effects of P5 on the OVA-induced allergic response *in vivo*, mice were sensitized with 20 µg OVA adsorbed on 2 mg/mL Imject Alum (Pierce) by i.p. injection on days 0 and 14. From day 28, mice were orally challenged with 50 mg OVA in saline every 3 days, for a total of five times. To investigate the preventive effects of P5, lyophilized P5 was suspended in saline and orally administered (5×10^8 CFU/head) every day from days 28 to 40 (Fig. 1). Following the final challenge, diarrhea, anaphylaxis, and rectal temperature were measured as indexes of food allergy symptoms. Diarrhea and anaphylaxis were observed by visually monitoring mice for 60 min after challenge. The following criteria were used for scoring diarrhea: 0, normal stools; 1, a few wet and unformed stools; 2, a number of wet and unformed stools with moderate perianal staining of the coat; and 3, severe and watery stools with severe perianal staining of the coat. The following criteria were used for anaphylactic responses: 0, no symptoms; 1, reduced activity, trembling of limbs; 2, loss of consciousness, no activity upon prodding; 3, convulsions; and 4, death. Rectal temperature was measured using

a Thermalert TH5 monitoring thermometer (Physitemp, Clifton, NJ, USA). Body weight and water intake were carefully monitored and measured every week. On day 40, mice were sacrificed, and sera, spleens, and mLNs were collected.

Preparation of primary cells and cell cultures

Spleens and mLNs were obtained from sacrificed mice on day 40 and ground with the sterile flat bottom of a syringe piston to homogenize the sample. Homogenized single cells were then collected and treated with red blood cell lysing buffer (Sigma-Aldrich) for spleens. The cells were then washed twice with RPMI medium and centrifuged ($415 \times g$, 5 min). Subsequently, the cells were adjusted to 5×10^6 cells/mL in RPMI medium using the trypan blue exclusion method. In 24-well plates, the cells were cultured in the presence of OVA (100 $\mu\text{g/mL/well}$). The plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 72 h. After incubation, the supernatants were collected for cytokine analysis.

Measurement of serum immunoglobulins

To measure immunoglobulin levels, serum samples from each group were obtained by collecting blood from the orbital venous plexus. An enzyme-linked immunoassay (ELISA) protocol was used to determine total IgE and OVA-specific IgE antibody levels in the samples. Aliquots (200 $\mu\text{L/well}$) of IgE capture antibody for total IgE (BD Pharmingen, San Diego, CA, USA) or OVA (10 $\mu\text{g/mL}$ dissolved in 0.1 mol/L NaHCO₃, pH 8.2) for OVA-specific IgE were coated on 96-microwell plates (Nunc, Thermo Fisher Scientific, Roskilde, Denmark). The plates were incubated overnight at 4°C and then carefully washed three times with washing buffer (0.5 g/L Tween 20 in PBS). Serum samples were diluted 1:5 for total IgE and OVA-specific IgE determination. One hundred microliters of diluted sample was added to each well. Total IgE and OVA-specific IgE levels were determined using biotin-conjugated rat anti-mouse (BD Pharmingen) according to the manufacturer's protocols. The plates were read using an

ELISA plate reader (Molecular Devices, Inc., Eugene, OR, USA) at 450 nm.

Measurement of cytokines from cultured splenocytes and mLN-derived lymphocytes

A cytokine assay kit (BD Pharmingen) was used to measure cytokine levels (interferon-gamma [IFN- γ], IL-12, IL-4, IL-5, IL-13, IL-10, and IL-17) in the supernatant, according to the manufacturer's protocols. Briefly, recovered supernatants and standard solutions were transferred to 96-well plates precoated with the appropriate monoclonal antibodies raised against each of the target cytokines and then incubated at room temperature for 2 h. After thorough washing with the wash buffer included in the kit, a horseradish peroxidase-conjugated secondary antibody was added to each well, and incubation was continued at room temperature for 2 h. After removal of the secondary antibody, the substrate solution for the enzymatic reaction was added, and samples were incubated for another 30 min in the dark. The reaction was terminated by addition of stop solution, and absorbance was measured at 450 nm using a microplate reader (Molecular Devices, Inc.)

Immunofluorescence staining of CD4 and Foxp3

For CD4+Foxp3⁺ analysis, mLN-derived lymphocytes (1×10^6 cells/well) from BALB/c mice were cultured in 96-well plates in the presence of antigen (100 μ g/mL OVA) and T-cell receptor stimuli (10 μ g/mL plate-bound anti-CD3 monoclonal antibodies (17A2) and 2 μ g/mL soluble anti-CD28 monoclonal antibodies (37.51)(Biolegend, San Diego, CA, USA). The cells were incubated at 37°C in a humidified incubator with 5% CO₂ for 72 h. To stain Foxp3, cells were washed in fluorescence-activated cell sorting(FACS) buffer (1% fetal calf serum and 0.1% NaN₃ in PBS) and incubated with anti-CD16/32 antibody 2.4G2 for 5 min at 4°C to block Fc receptors (clone;2.4G2) (BD pharmingen, San Diego, CA). Surface CD4 molecules were stained with Phycoerythrin-(PE-) labeled anti-CD4 (clone:H129.19)(BioLegend, San Diego, CA) for 30 min at 4°C. The cells were then fixed and permeabilized using Foxp3

fixation/permeabilization concentrate and diluent (BD Biosciences, San Jose, CA) for 1h at 4°C. After Fc receptors were blocked for 15 min, cells were stained using peridinin chlorophyll-a protein-Cy5.5-(PerCP-Cy5.5-) labeled anti Foxp3 (eBioscience, San Diego, CA) in FACS buffer at 4°C for 30 min. Data were acquired by flow cytometry using a BD FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star, San Carlos, CA, USA).

Statistical analysis

Numerical data are expressed as means $\pm$ standard deviations using SPSS software. The significance of differences between experimental conditions was analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Results with *p* values of less than 0.05 were considered significant.

Results

Effects of P5 on cytokine production *ex vivo*

To evaluate the modulatory effects of P5 on the Th1/Th2 balance, we examined the expression patterns of IFN- γ and IL-4 in mLN-derived lymphocytes (isolated from OVA-sensitized mice) cocultured with P5 in the presence of OVA. As a result, co-culture with P5 increased IFN- γ (a Th1 cytokine) production and reduced IL-4 (a Th2 cytokine) production (Table 1). To evaluate the anti-inflammatory effects of P5, we measured the levels of the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokine IL-12 in mLN-derived lymphocytes (isolated from healthy mice) co-cultured with 1×10^7 CFU of P5. Due to the pleiotropic properties of IL-10 produced by many cell types, IL-10 secreted from DCs or Th2 cells in Th2 skewing environment and a low pathogenic environment may act as pro-inflammatory cytokine rather than anti-inflammatory cytokine [22-26]. Thus, we used the mLN-derived lymphocytes isolated from healthy mice to evaluate the anti-inflammatory effect of P5. According to previous studies screening anti-inflammatory probiotics [27-29], there is a close correlation between the IL-

10^{high}/IL-12^{low} phenotype *ex vivo* and *in vivo* immunomodulatory efficacy. Probiotics having the IL-10^{high}/IL-12^{low} phenotype (> 10 for the IL-10/IL-12 ratio) *ex vivo* could suppress diverse immune disorders *in vivo*. In this study, coculture with P5 induced the IL-10^{high}/IL-12^{low} phenotype (ratio of 23.94 for IL-10/IL-12; Table 1). These results suggested that P5 may alleviate allergic disease via modulation of the Th1/Th2 balance and anti-inflammatory properties.

Effects of P5 administration on OVA-induced food allergy symptoms

Food allergy symptoms induced by OVA were evaluated and scored by the criteria for diarrhea, anaphylactic response, and rectal temperature (Fig. 2). Severe symptoms of OVA-induced food allergy, such as reduced mobility (score after 1 h, 2.29), increased explosive diarrhea (score after 1 h, 2.64), and decreased rectal temperature (Δ temp after 1 h, -9.74) were observed in the sham group. In contrast, the P5-treated group showed significant inhibition of OVA-induced anaphylactic response (score after 1 h, 0.53) and diarrhea (score after 1 h, 1.36). We also measured rectal temperature for 1 h after the fifth challenge with OVA. After 1 h, rectal temperature in the sham group was decrease to -9.74°C. However, P5 administration ameliorated the decrease in the rectal temperature by OVA to -3.5°C. These results demonstrated that P5 administration could alleviate food allergy symptoms through inhibition of systemic and intestinal allergic reactions.

Effects of P5 administration on total and specific IgE levels in serum

To investigation the effects of oral administration of P5 on total IgE and specific IgE levels in an OVA-induced food allergy model, sera were obtained from each group after the fifth OVA challenge. Total IgE and OVA-specific IgE were dramatically increased in the sham group. However, total IgE and OVA-specific IgE in the P5-treated group were significantly reduced by about 28% and 49%, respectively, compared with that in the sham group (Fig. 3).

Effects of P5 on cytokine patterns in splenocytes and mLN-derived lymphocytes from mice with OVA-induced food allergy

In order to determine the immunomodulatory effects of P5, we investigated the cytokine patterns in spleens and mLNs isolated from mice with OVA-induced food allergy. In splenocytes **re-stimulated with OVA**, all Th2-related cytokines (IL-4, IL-5, IL-10, and IL-13) showed increased levels following induction of food allergy and were significantly inhibited by administration of P5 (Fig. 5A–D). In contrast, Th1-related cytokines (IFN- γ , IL-12) were significantly increased in the P5-treated group compared with those in the sham group (Fig. 5E, F). We also investigated the role of IL-17, a Th17-related cytokine, in food allergy. IL-17 secretion was found to increase following induction of food allergy and was significantly suppressed by administration of P5 (Fig. 5G). These cytokine patterns were also observed in mLNs **re-stimulated with OVA** (Fig 4. A-G).

Effects of P5 on the CD4⁺Foxp3⁺ T-cell population in mice with OVA-induced food allergy and normal BALB/c mice

In order to determine whether suppression of the Th2 and Th17 response was caused by Tregs, we measured the CD4⁺Foxp3⁺ T-cell population in mLNs from mice with OVA-induced food allergy. The CD4⁺Foxp3⁺ T-cell population increased by about 1.6 fold in the P5-treated group compared with that in the sham group (Fig. 6A,B). Additionally, secretion of transforming growth factor (TGF)- β , a Treg-related cytokine, was increased in the P5-treated group compared with that in the sham group (Fig. 5H). In addition, in an *ex vivo* analysis using lymphocytes from mLNs of healthy BALB/c mice, co-culture with P5 induced a 3.1-fold increase in the CD4⁺Foxp3⁺ T-cell population (Fig. 6C,D) and a 3.7-fold increase in the expression of IL-10, an anti-inflammatory cytokine (Table 1), compared with those in PBS-treated cells. These data indicated that the suppressive effects of P5 on the Th2 and Th17

responses were related to induction of Tregs.

Discussion

In this study, we confirmed that the probiotic mixture P5, which was composed of five strains (i.e., *Lactobacillus paracasei* KF711, *Lactobacillus pentosus* KF340, *Bacillus amyloliquefaciens* KF26, *Pediococcus pentosaceus* KF159, and *Lactococcus lactis* KF140) inhibited Th2 and Th17 immune responses through regulation of the Th1/Th2 balance and Treg induction, thereby alleviating OVA-induced food allergy symptoms. This mechanism is similar to the findings of studies showing that oral administration of the probiotic mixture VSL#3 [20] and *Lactobacillus plantarum* NRIC0380 [19] alleviated symptoms of allergy through regulation of the Th1/Th2 balance and Treg induction.

In IgE-dependent food allergies, food allergy symptoms are known to be caused by the predominant Th2 immune response. Th2 cytokines, such as IL-4, IL-5, IL-10 and IL-13, play an important role in the manifestation of allergic symptoms by inducing the production of antibodies, such as IgE or IgG1, and by promoting eosinophil proliferation, mast cell degranulation, and B-cell isotype switching [3]. Inhibition of the Th2 immune response can be controlled by enhancing the Th1 immune response. Many probiotics, such as *Lactobacillus brevis* HY7401 [30], *Pediococcus pentosaceus* Sn26 [31], and *Bifidobacterium lactis* [32], have been shown to have anti-allergic activities in various food allergy models through inhibition of the Th2 immune response by promoting the Th1 immune response. In the present study, analysis of the cytokine secretion pattern in splenocytes isolated from OVA-induced mice showed that in the P5-treated group, all Th2-related cytokines, including IL-4, IL-5, IL-10, and IL-13, decreased compared with those in the sham group, and Th1-related cytokines, such as IL-12 and IFN- γ , increased. This pattern was also observed in mLNs (Fig. 4.). Furthermore, both the

anaphylactic response and the diarrhea score decreased. Thus, the Th1/Th2 immunoregulatory activity of P5 appeared to affect both systemic and local immunity. The P5-mediated Th1/Th2 balance-regulating activity was also confirmed in an *ex vivo* experiment; co-cultivation of lymphocytes from mLNs of OVA-sensitized mice and P5 at a concentration ratio of 1:10 resulted in increased IFN- γ and decreased IL-4.

Foxp3⁺ Tregs, which suppress the Th17, Th1, and Th2 immune responses, have been reported as immune cells capable of inhibiting the Th2 immune response. These cells are known to secrete the anti-inflammatory cytokines IL-10 and TGF- β and to play important roles in alleviating food allergies [33]. Tregs have been reported to act directly or indirectly on the sensitization and effector phases of food allergy development through various routes, e.g., inhibition of allergen-induced degranulation of effector T cells [34], inhibition of the entry of eosinophils and other effector T cells into inflamed tissues [35], induction of tolerogenic DCs [36], inhibition of direct Th2 cell activation [33], and inhibition of IgE secretion by acting on B cells [37], thereby alleviating the symptoms of food allergy. Recent studies have shown that Foxp3⁺ Tregs induced by commensal intestinal bacteria play important roles in regulating allergic responses and balancing Th1/Th2 polarization [38-40]. It has been reported that these regulatory effects of Tregs in alleviating food allergy is by the stabilization of mast cells via balance of IL-4(or IL-17) and TGF- β [41, 42] as well as the suppressive effect of IL-10 secreted from Treg. Thus, Treg induction is an important mechanism for the prevention and treatment of allergic diseases caused by probiotic ingestion. Treg induction in immune organs, such as mLNs, Peyer's patches, and the spleen, through ingestion of probiotics, such as *Lactobacillus gasseri* OLL2809 [43], probiotic mixture VSL#3 [20], and *Lactobacillus plantarum* NRIC0380 [19], has been reported to alleviate allergy in many animal models.

To confirm that the inhibitory effects of P5 on the Th2 and Th17 immune responses

were caused by Treg induction, P5 and mLN s were co-cultivated. The results showed that the CD4⁺Foxp3⁺ cell population and IL-10 secretion increased by P5 treatment, suggesting that P5 inhibited Th2 and Th17 immune responses through Treg induction. In addition, in our OVA-induced food allergy model, the population of CD4⁺Foxp3⁺ cells and TGF- β secretion in mLN s from P5 administrated group increased compared with those in the sham group, and the levels of Th2-related cytokines and the Th17-related cytokine IL-17 decreased compared with those in the sham group. Although the specific role of Th17 in the maintenance and development of food allergy symptoms needs to be studied further, IL-17 may be associated with food allergy development based on previous reports showing upregulation of IL-17 mRNA expression in food allergy-induced mice [44], increased IL-17 in antigen-restimulated lymphocytes [45], and decreased allergic symptoms and IL-17 expression in *Fc ϵ RI* gene-knockout mice [46]. In normal immune responses, Th17-dominant immune responses can be regulated by Treg induction because Th17 cells maintain a balance with Tregs [42]. Therefore, IL-17 inhibition by oral administration of P5 is also thought to be caused by Treg induction.

Despite the our results (Increase of Foxp3⁺ cell and TGF-beta, decrease of IL-17) supporting Treg induction activity of P5, the decrease in IL-10 secretion in the *in vivo* model in contrast to that *in vitro* can be attributed to the pleiotropic properties of IL-10. IL-10 is a pleiotropic cytokine that is produced by many cell types, including macrophages, epithelial cells, mast cells, and Tregs, and has both pro- and anti-inflammatory effects during immune responses [22-24]. The function and activity of IL-10 are affected depending on the type of stimulus, the cell type producing IL-10, the immunological environment, and the genetic background of the cells or animal used. IL-10 secreted from immune regulatory cells such as Tregs and tolerogenic DCs, inhibits the Th1, Th2, and Th17 immune responses by acting as anti-inflammatory cytokine [47], while IL-10 secreted from DCs or Th2 cells promotes the

development of Th2 immune response and the mast cell response [25,26,48]. Although the role of IL-10 remains controversial in allergic environment [47], many studies have been demonstrated that the IL-10 is associated with the development of allergic inflammation; Laouini and colleagues [49] reported that DC-derived IL-10 plays an important role in the Th2 responses to antigen and in the development of skin eosinophilia in a murine model of allergic dermatitis; Polukort and colleagues [50] demonstrated that IL-10 plays an essential role in the progression of allergy in the OVA-induced murine food allergy model using BALB/C mice, suggesting that allergic symptoms, e.g. intestinal anaphylaxis, diarrhea, mast cell activation, and Th2 cytokine production, were alleviated in IL-10^{-/-} mice compared with that in wild-type controls. In contrast to these reports, Ryan and colleagues reported that IL-10 prevents IgE-mediated activation of mast cells, inhibits *FcεRI* expression in model using C57BL/6 mice [51-53]. These discrepancies in the role of IL-10 during inflammatory conditions *in vivo* are thought to be due to the immune microenvironment and the genetic background of used animal. Kolawole and Ryan recently reported the mast cell responsiveness to IL-10 may be altered by genetic background [54]. Polukort and colleagues [50] reported that IL-10 can enhance mast cell expansion and promote allergic responses in BALB/C mice, which are known to have a strong Th2 bias.

In our experiment, we used the BALB/C mice to establish the OVA-induced food allergy model, and administration of P5 started under Th2 skewing environment (after 2nd immunization). In light of these, IL-10 detected *in vivo* may act a Th2-related cytokine secreted from DCs or Th2 cells, rather than as an anti-inflammatory cytokine secreted from Treg. Indeed, the increased secretion of IL-10 in the sham group, which showed severe symptoms of food allergy was observed. Therefore, the decrease of IL-10 *in vivo* is assumed to result from the mitigation of Th2 dominant immune response by P5. In contrast to *in vivo*, the Treg population

and IL-10 secretion by co-culture with P5 were increased *ex vivo* analysis using lymphocytes from mLN of healthy BALB/c mice, while IL-12 was decreased. Considering the decrease of IL-12, IL-10 detected *ex vivo* might act as an anti-inflammatory cytokine. Thus, it is thought these discrepancy between *in vivo* and *ex vivo* in IL-10 secretion pattern may be due to differences in immunological environment.

In summary, although further studies should be conducted to fully understand the regulatory mechanisms of P5 *via* Tregs induction in food allergy, our results indicated that oral supplementation with the probiotic mixture P5 attenuated OVA-induced allergic symptom by regulation of the Th1/Th2 balance and suppression of Th2 and Th17 responses *via* induction of Tregs. Thus, our study provided evidence that P5 may be a potential candidate probiotic mixture for the prevention of food allergy disorder.

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References

1. Tanabe S. 2007. Epitope peptides and Immunotherapy. *Curr. Protein Pept. Sci.* **8**:109-118.
2. Kaminogawa S. Hachimura S. Nakajima-adachi H, Totsuka M. 1999. Food allergens and mucosal immune systems with special reference to recognition of food allergens by gut-associated lymphoid tissue. *Allergol. Int.* **48**:15-23.

3. Georas SN, Guo J, De Fanis U, Casolaro V. 2005. T-helper cell type 2- regulation in allergic disease. *Eur. Respir. J.* **26**:1119-1137.
4. Galli SJ, Tsai M, Piliponsky AM. 2008. The development of allergic inflammation. *Nature* **454**:445-454.
5. Ben-Shoshan AH, Clarke AE. 2011. Anaphylaxis: past, present and future. *Allergy* **66**:1-14.
6. Benhamou AH, Caubet JC, Eifemann PA, Nowak-Wegrzyn A, Marcos CP, Reche M. *et al.* 2010. State of the art and new horizons in the diagnosis and management of egg allergy. *Allergy* **65**: 283-289.
7. Sicherer SH, Sampson HA. 2014. Food allergy: epidemiology, pathogenesis, diagnosis, and treatment. *J. Allergy Clin. Immunol.* **133**:291–307.
8. Longo G, Berti I, Burks AW, Krauss B, Barbi E. 2013. IgE-mediated food allergy in children. *Lancet.* **382**:1656–1664.
9. De Silva D, Geromi M, Panesar SS, Muraro A, Werfel T, Hoffmann-Sommergruber K, *et al.* 2014. Acute and longterm management of food allergy: systematic review. *Allergy* **69**:159–167.
10. Branum AM, Lukacs SL. 2009. Food allergy among children in the United States. *Pediatrics* **124**:1549–1555.
11. Sicherer SH, Sampson HA. 2010. Food allergy. *J. Allergy Clin. Immunol.* **125**:s116-125.
12. FAO/WHO. 2001. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Available from <http://www.fao.org/3/a-a0512e.pdf>. Accessed July. 5. 2016
13. Berni Canani R, Gilbert JA, Nagler CR. 2015. The role of the commensal microbiota in the regulation of tolerance to dietary antigens. *Curr. Opin. Allergy Clin. Immunol.* **15**:243–

249.

14. Ohnmacht C, Park JH, Cording S, Wing JB, Atarashi K, Obata Y, *et al.* 2015. The microbiota regulates type 2 immunity through ORgt β T cells. *Science* **349**:989–993.
15. Isolauri E, Kalliomäki M, Laitinen K, Salminen S. 2008. Modulation of the maturing gut barrier and microbiota: a novel target in allergic disease. *Curr Pharm Des.* **14**:1368-1375.
16. Berni Canani R, Sangwan N, Stefka AT, Nocerino R, Paparo L, Aitoro R, *et al.* 2016. *Lactobacillus rhamnosus* GG supplemented formula expands butyrate producing bacterial strains in food allergic infants. *The ISME Journal* **10**:742–750.
17. Shida K, Takahashi R, Iwadata E, Takamiz-awa K, Yasyui H, Habu S, *et al.* 2002. *Lactobacillus casei* Shirota sup-presses serum immunoglobulin E and immunoglobulin G1 responses and systemic anaphylaxis in a food allergy model. *Clin. Exp. Allergy* **32**:563–570.
18. Zuercher AW, Weiss M, Holvoet S, Moser M, Moussu H, van Overtvelt L, *et al.* 2012. *Lactococcus lactis* NCC 2287 alleviates food allergic manifestations in sensitized mice by reducing IL-13 expression specifically in the ileum. *Clin. Dev. Immunol.* **2012**:485750.
19. Yoshida T, Fujiwara W, Enomoto M, Nakayama S, Matsuda S, Sugiyama H, *et al.* 2013. An increased number of CD4+CD25+cell induced by an oral administration of *Lactobacillus plantarum* NRIC0380 Are Involved in antiallergic activity. *Int. Arch. Allergy Immunol.* **162**:283-289.
20. Schiavi E, Barletta B, Butteroni C, Corinti S, Boirivant M, Di Felice G. 2011. Oral therapeutic administration of a probiotic mixture suppresses established Th2 responses and systemic anaphylaxis in a murine model of food allergy. *Allergy* **66**:499–508.
21. Yang Jing, Ren F, Zhang H, Jiang L, Hao Y, Luo X. 2015. Induction of regulatory dendritic

- cells by *Lactobacillus parcasei* L9 prevents allergic sensitization to bovine β -lactoglobulin in mice. *J. Microbiol. Biotechnol.* **25**:1687-1696.
22. Thompson-Snipes L, Dhar V, Bond MW, Mosmann TR, Moore KW, Rennick DM. 1991. Interleukin 10: a novel simulatory factor for mast cells and their progenitors. *J. Exp. Factor Med.* **173**:507-510.
23. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. 1993. Interleukin-10. *Annu. Rev. Immunol.* **11**: 165–190.
24. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* **19**: 683–765.
25. Iwasaki A, Kelsall BL. 1999. Freshly isolated peyer patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *J. Exp. Med.* **190**:229-239
26. Palma JP, Yauch RL, Kang HK, Lee HG, Kim. 2002. Preferential induction of IL-10 in APC correlates with a switch from Th1 to Th2 response following infection with a low pathogenic variant of Theiler's virus. *J. immunol* **168**:4221-4230.
27. Kang HJ, Im SH. 2015. Probiotics as an immune modulator. *J. Nutr. Sci. Vitaminol.* **61**:S103-S105.
28. Kim JE, Chae CS, Kim GC, Hwang W, Hwang JS, Hwang SM, *et al.* 2015. *Lactobacillus helveticus* suppresses experimental rheumatoid arthritis by reducing inflammatory T cell responses. *J. Funct foods* **13**:350-362.
29. Kwon HK, Lee CG, So JD, Chae CS, Hwang JS, Sahooa, *et al.* 2010. Generation of regulatory dendritic cells and CD4⁺Foxp3⁺ T cells by probiotics administration suppresses immune disorders. *Proc. Natl. Acad. Sci. USA* **107**:2159-2164.

30. Lee J, Bang J, Woo HJ. 2013. Effect of orally administered *Lactobacillus brevis* HY7401 in a Food allergy mouse model. *J. Microbiol. Biotechnol.* **23**:1636-1640
31. Masuda T, Kimura M, Okada S, Yashi H. 2010. *Pediococcus pentosaceus* Sn26 inhibits IgE production and the occurrence of ovalbumin induced allergic diarrhea in mice. *Biosci. Biotechnol. Biochem.* **74**:329-335.
32. Kim JY, Choi YO, Ji GE. 2008. Effect of oral probiotics (*Bifidobacterium lactis* AD011 and *Lactobacillus acidophilus* AD031) administration on ovalbumin-induced food allergy mouse model. *J. Microbiol. Biotechnol.* **18**:1393–1400.
33. Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis M, Akdis CA. 2010. Role of Treg in immune regulation of allergic diseases. *Eur. J. Immunol.* **40**:1232-1240.
34. Kashyap M, Thornton AM, Norton SK, Barnstein B, Macey M, Brenzovich J, *et al.* 2008. Cutting edge: CD4 T cell-mast cell interactions alter IgE receptor expression and signaling. *J. Immunol.* **180**:2039-2043.
35. Ring S, Schafer SC, Mahnke K, Lehr HA, Enk AH. 2006. CD4+ CD25+ regulatory T cells suppress contact hypersensitivity reactions by blocking influx of effector T cells into inflamed tissue. *Eur. J. Immunol.* **36**:2981-2992.
36. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, *et al.* 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* **322**:271-275.
37. Meiler F, Klunker S, Zimmermann M, Akdis CA, Akdis M. 2008. Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors. *Allergy* **63**:1455-63.
38. Round JL, Mazmanian SK. 2010. Inducible foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *PANS* **107**:12204-9.
39. Kanjarawi R, Dy M, Bardel E, Sparwasser T, Dubois B, Mecheri S, Kaiserlian D. 2013.

- Regulatory CD4⁺Foxp3⁺ T cells control the severity of anaphylaxis. *PLOS one* **8**: e69183.
40. Lyons A, O'Mahoby D, O'Brein F, MacSharry J, Sheil B, Ceddia M, *et al.* 2010. Bacterial strain-specific induction of Foxp3⁺ T regulatory cells in protective in murine allergy models. *Clin. Exp. Allergy* **40**:811-819.
41. Macey MR, Sturgill JL, Morales JK, Falanga YT, Morales J, Norton SK, *et al.* 2010. IL4 and TGF-beta1 counterbalance one another while regulating mast cell homeostasis. *J. Immunol.* **189**: 4688-4695.
42. Hanidziar D, Koulmanda M. 2010. Inflammatory and the balance of Treg and Th17 cells in transplant rejection and tolerance. *Curr. Opin. Organ Transplant.* **15**:411-415.
43. Yoshida AA, Yamada K, Hachimura S, Sahihara T, Ikegami S, Shimizu M, *et al.* 2016. Enhancement of oral tolerance induction in DO11.10 Mice by *Lactobacillus gasseri* OLL2809 via Increase of effector regulatory T cells. *PLoS ONE* **11**:e0158643.
44. Yamaki K, Yoshino S. 2012. Preventive and therapeutic effects of rapamycin, mammalian target of rapamycin inhibitor, on food allergy in mice. *Allergy* **67**:1259-1270.
45. Perrier C, Thierry AC, Mercenier A, Corthesy B. 2010. Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice. *Clin Exp Allergy* **40**:153–162.
46. Wang M, Takeda K, Shiraishi Y, Okamoto M, Dakhama A, Joetham A, *et al.* 2010. Peanut induced intestinal allergy is mediated through a mast cell-IgE-FcepsilonRI-IL-13 pathway. *J Allergy Clin. Immunol.* **126**:306–316.
47. Bijjiga E, Martino AT. 2013. Interleukin 10 Regulatory cytokine and its Clinical consequences. *J. Clin. Cell. Immunol.* doi:10.4172/2155-9899.S1-007
48. Tangye SG, Ferguson A, Avery DT, Ma CS, Hodgkin PD (2002) Isotype switching by

- human B cells is division-associated and regulated by cytokines. *J. Immunol.* **169**: 4298-4306.
49. Laouini D, Alenius H, Bryce P, Oettgen H, Tsitsikov E, Geha RS. 2003. IL-10 is critical for Th2 responses in a murine model of allergic dermatitis. *J. Clin. Invest.* **112**:1058-1066
50. Polukort SH, Rovatti J, Carlson L, Thompson C, Ser-Dolansky J, Kinney SRM, *et al.* 2016. IL-10 Enhances IgE-mediated mast cell responses and is essential for the development of experimental food allergy in IL-10 deficient mice. *J. immunol.* **196**:4865-4876.
51. Mirmonsef P, Shelburne CP, Fitzhugh Yeatman C, II, Chong HJ, Ryan JJ. 1999. Inhibition of Kit expression by IL-4 and IL-10 in murine mast cells: role of STAT6 and phosphatidylinositol 39-kinase. *J. Immunol.* **163**: 2530–2539.
52. Kashyap M, Thornton A M, Norton SK, Barnstein B, Macey M, Brenzovich J, *et al.* 2008. Cutting edge: CD4 T cell-mast cell interactions alter IgE receptor expression and signaling. *J. Immunol.* **180**: 2039–2043.
53. Yeatman CF, II, Jacobs-Helber SM, Mirmonsef P, Gillespie SR, Bouton LA, *et al.* 2000. Combined stimulation with the T helper cell type 2 cytokines interleukin (IL)-4 and IL-10 induces mouse mast cell apoptosis. *J. Exp. Med.* **192**: 1093–1103.
54. Kolawole M, Ryan J. 2011. Mast cell responsiveness to IL-10 is altered by genetic background. *J. Immunol.* **186**: 151.14

Figure legends

Fig. 1. Timeline of experimental procedures. Female BALB/c mice (5 weeks old) were divided into naïve (n = 7), Dexa (n = 5), sham (n = 8), and P5 groups (n = 8). To induce food allergy, mice were immunized with 20 µg OVA and 2 mg alum by intraperitoneal (i.p.) injection on day 0; from day 28, mice were orally challenged with 50 mg OVA in saline every 3 days, for a total of five times. Simultaneously (from day 28), the naïve and sham groups were provided saline (0.9%), and the P5 group was provided 5×10^8 CFU of the probiotic mixture. As a positive control, dexamethasone (Dexa; 1 mg/kg BW) was administered orally every day from day 28 to 40. Diarrhea score, anaphylaxis symptoms, and rectal temperature were measured for 1 h after challenge as an index of food allergy symptoms.

Fig. 2. Effects of the probiotic mixture (P5) on OVA-induced food allergy symptoms. Food allergy symptoms induced by OVA were evaluated and scored by criteria for diarrhea symptoms, anaphylactic response, and rectal temperature for 1 h after challenge with OVA. Diarrhea score (A), anaphylactic response score (B), and rectal temperature (C) were evaluated on the fifth challenge. Each values is presented as the mean $\pm$ SD (naïve, n = 7; dexa, n = 5; sham and P5, n = 8). Bars represent significant differences from the sham group at * p = 0.05 and ** p = 0.01. Data were analyzed using ANOVA followed by Duncan's multiple range test.

Fig. 3. Effects of the probiotic mixture (P5) on production of total IgE (A) and OVA-specific IgE (B) in serum from mice with OVA-induced food allergy. Serum was collected after the fifth challenge with OVA. Each values is presented as the mean $\pm$ SD (naïve, n = 7; sham and P5,

n = 8). Bars represent significant differences from the sham group at $*p = 0.05$ and $** = 0.01$.

Data were analyzed using ANOVA followed by Duncan's multiple range test.

Fig. 4. Immunomodulatory effects of the probiotic mixture (P5) on cytokine patterns in splenocytes. Mice were sacrificed by cervical dislocation on day 40, and spleens were removed. Splenocytes were isolated from the spleens and cultured in RPMI medium containing 10% FBS for 72 h in the presence of OVA (100 $\mu\text{g/mL}$). Cytokines secreted from splenocytes were measured by ELISA. IL-4 (A), IL-5 (B), IL-10 (C), and IL-13 (D) were measured as Th2 cytokines, and IFN- γ (E) and IL-12 (F) were measured as Th1 cytokines. IL-17 (G) was measured as a Th17 cytokine, and TGF- β (H) was measured as a Treg-related cytokine. Each values is presented as the mean $\pm$ SD. Bars represent significant differences from the sham group at $*p = 0.05$ and $** = 0.01$. Data were analyzed using ANOVA followed by Duncan's multiple range test.

Fig. 5. Immunomodulatory effects of the probiotic mixture (P5) on cytokine patterns in lymphocytes derived from mesenteric lymph nodes (mLNs). Mice were sacrificed by cervical dislocation on day 40, and mLNs were removed. Lymphocytes were isolated from the mLNs and cultured in RPMI medium containing 10% FBS for 72 h in the presence of OVA (100 $\mu\text{g/mL}$). Cytokines secreted from mesenteric lymphocytes were measured by ELISA. IL-4 (A), IL-5 (B), IL-10 (C), and IL-13 (D) were measured as Th2 cytokines, and IFN- γ (E) and IL-12 (F) were measured as Th1 cytokines. IL-17 (G) was measured as a Th17 cytokine, and TGF- β (H) was measured as a Treg-related cytokine. Each values is presented as the mean $\pm$ SD. Bars represent significant differences from the sham group at $*p = 0.05$ and $** = 0.01$. Data were

analyzed using ANOVA followed by Duncan's multiple range test.

Fig. 6. Effects of the probiotic mixture (P5) on induction of CD4+Foxp3+ T cells. (A and B)

The CD4+Foxp3+ T-cell population in mesenteric lymph nodes (mLNs) from mice with OVA-

induced food allergy. (C and D) The CD4+Foxp3+ T-cell population in mLNs co-cultured with

P5 (1×10^7 CFU) from BALB/c mice. Each values is presented as the mean $\pm$ SD. Bars

represent significant differences from the sham group at $*p = 0.05$ and $** = 0.01$. Data were

analyzed using ANOVA followed by Duncan's multiple range test.

589 **Table 1. Cytokine secretion in mLN-derived lymphocytes co-cultured with the probiotic mixture (P5) from OVA-sensitized mice and**
590 **BALB/c mice**

	OVA-sensitized mice			BALB/c mice	
	PBS	P5 (1×10^7 CFU)		PBS	P5 (1×10^7 CFU)
IFN- γ (pg/mL)	2648.76 $\pm$ 113.95	3910.33 $\pm$ 13.81**	IL-10(pg/mL)	139.16 $\pm$ 2.06	517.43 $\pm$ 18.11 **
IL-4(pg/mL)	482.54 $\pm$ 14.54	235.26 $\pm$ 22.95 **	IL-12 (pg/mL)	ND	21.61 $\pm$ 4.26
Ratio(IFN- γ /IL-4)	5.4	16.62	Ratio(IL-10/IL-12)	ND	23.94

591 Each value is presented as the mean $\pm$ SD (n = 3). Bars represent significant differences from the PBS group at * $p = 0.05$ and ** $p = 0.01$. Data
592 were analyzed using ANOVA followed by Duncan's multiple range test.

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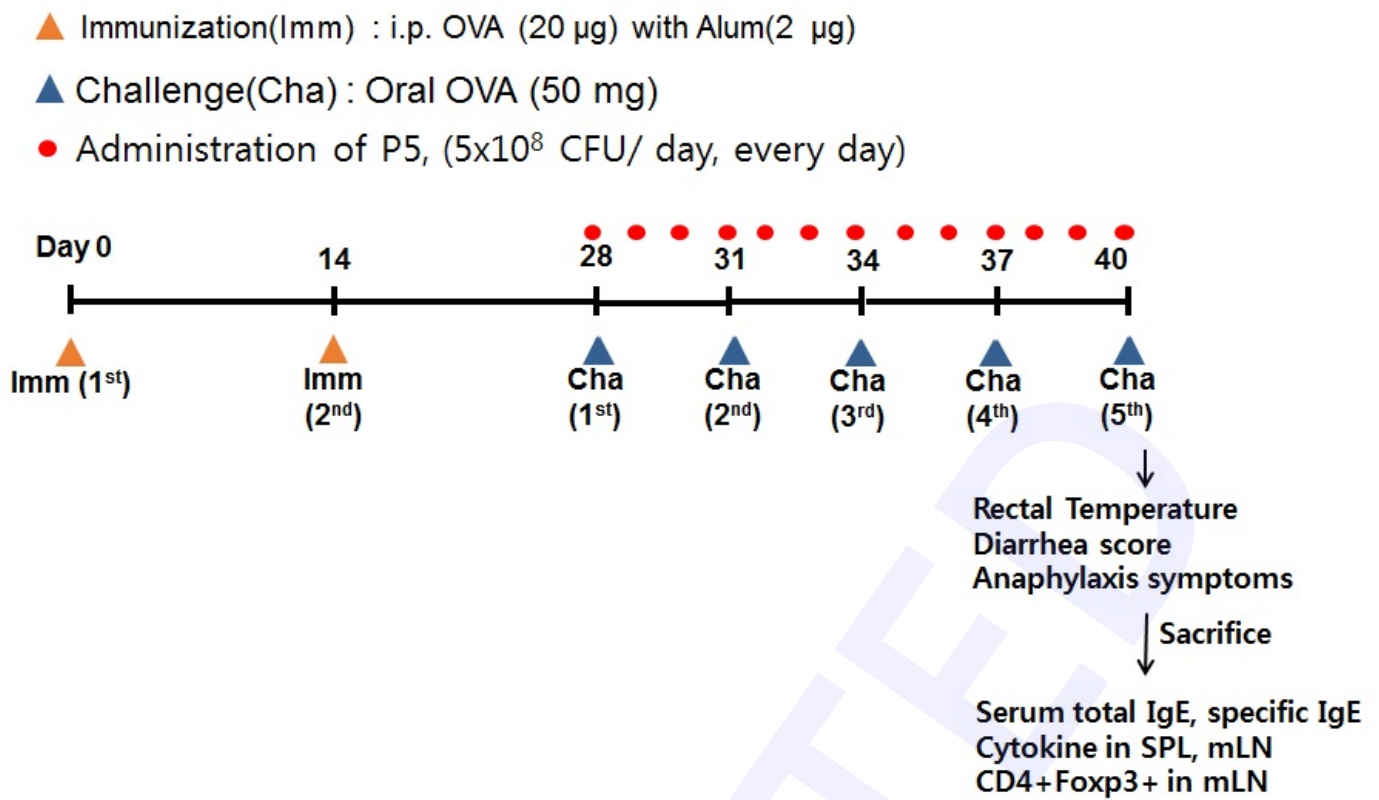


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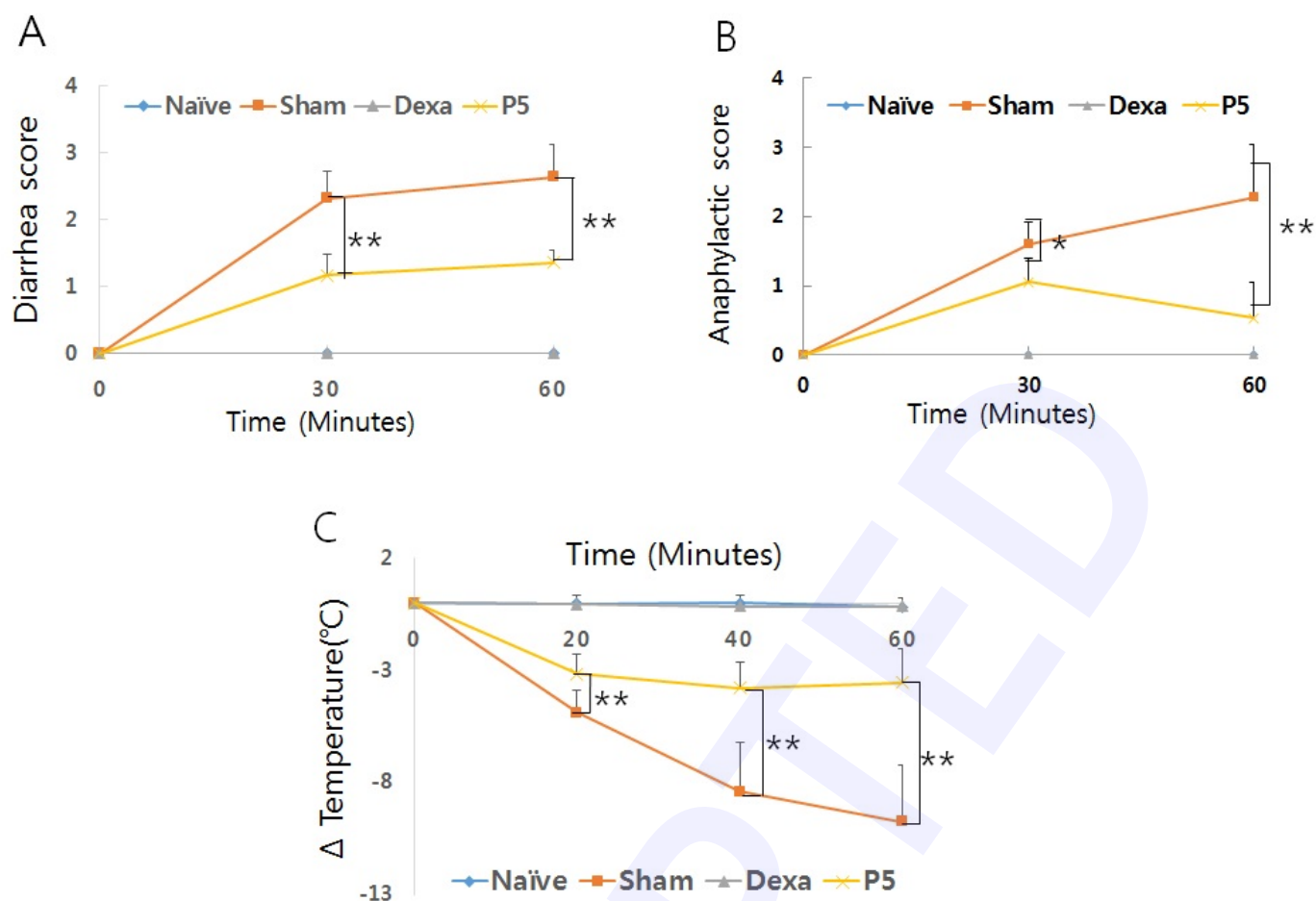


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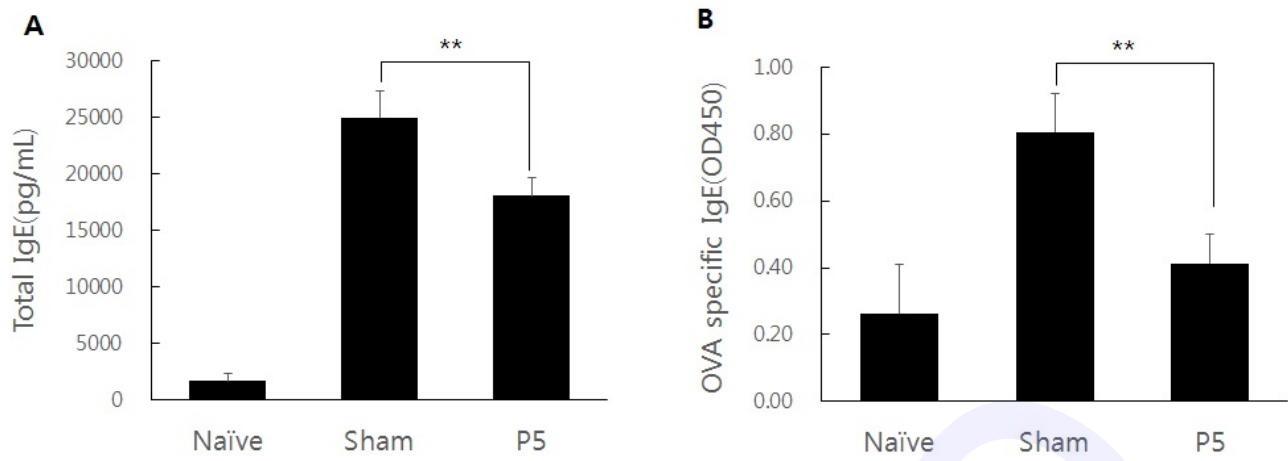


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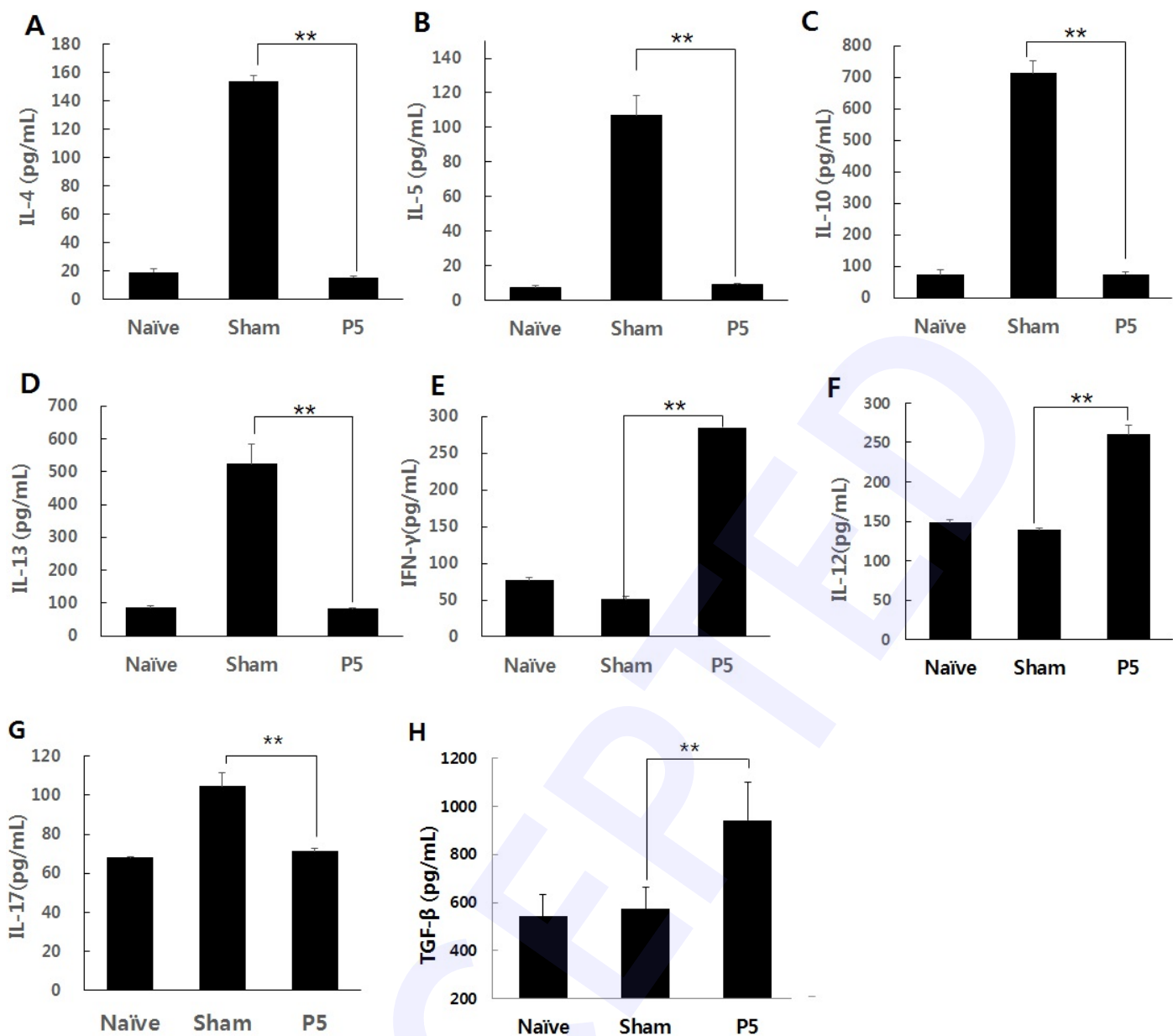


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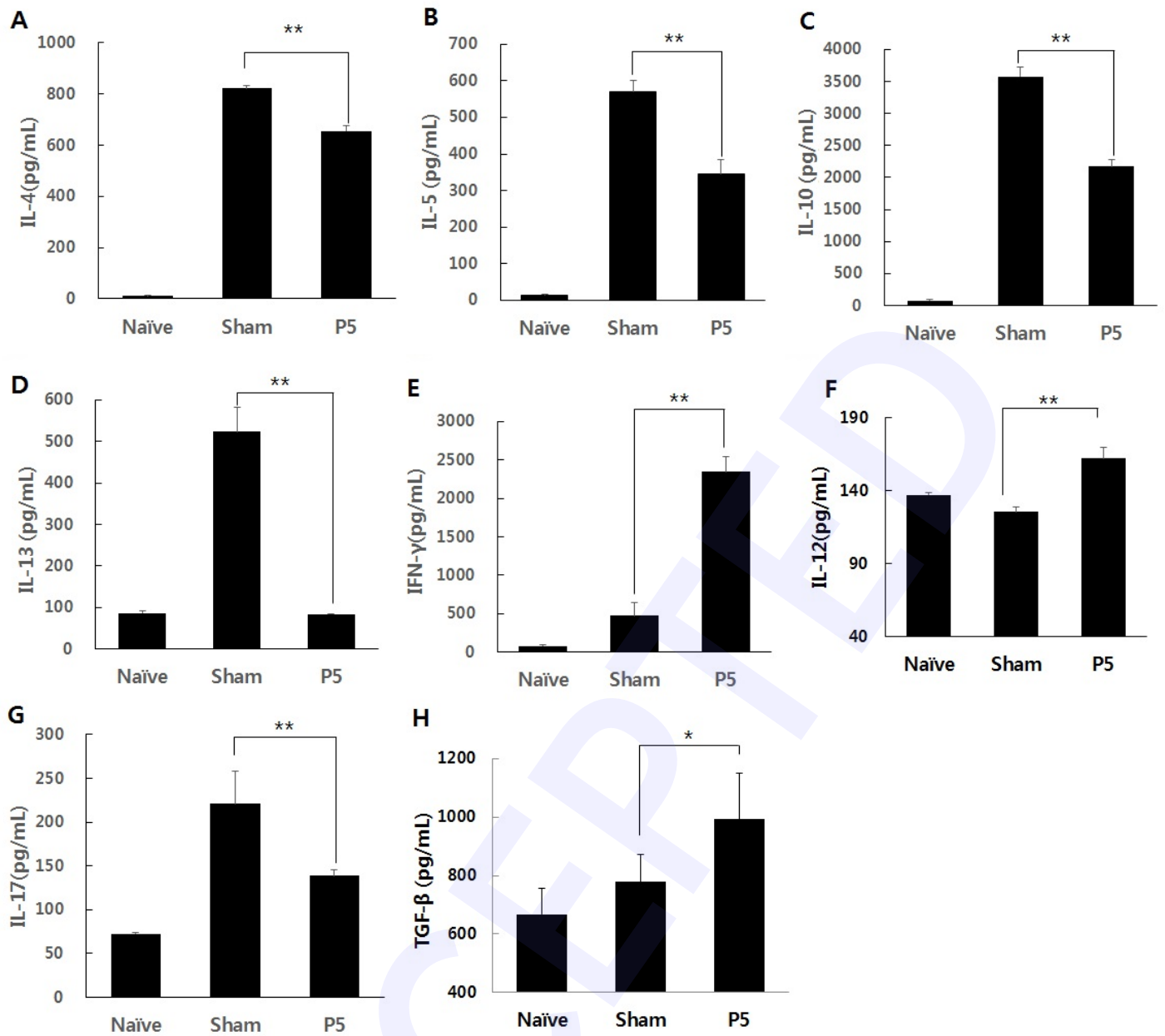


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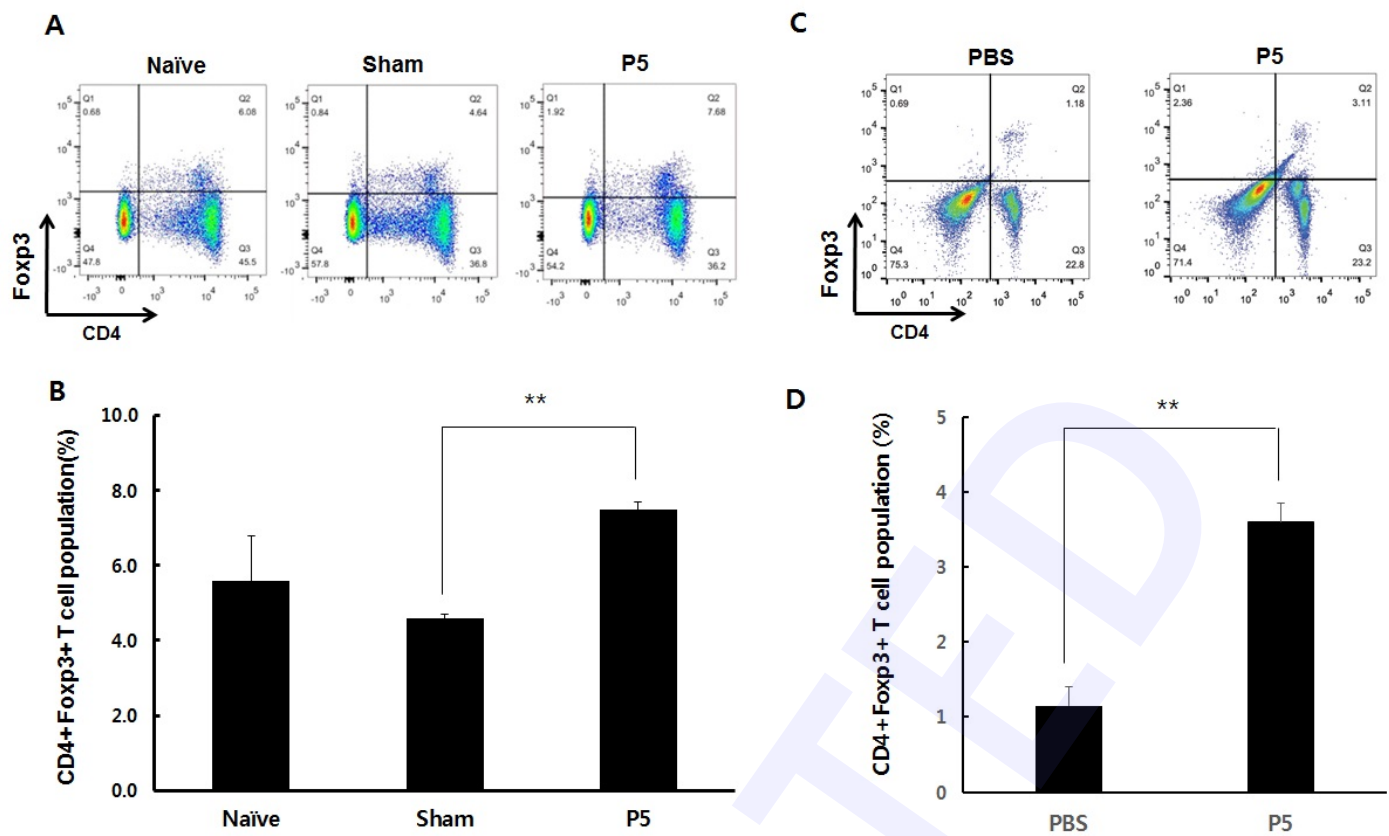


Fig. 6. Effects of the probiotic mixture (P5) on induction of CD4+ Foxp3+ T cells. (A and B) The CD4+ Foxp3+ T-cell population in mesenteric lymph nodes (mLNs) from mice with OVA-induced food allergy. (C and D) The CD4+ Foxp3+ T-cell population in mLNs co-cultured with P5 (1