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Dead *Lactobacillus plantarum* Stimulates and Skews Immune Responses toward T helper 1 and 17 Polarizations in RAW 264.7 Cells and Mouse Splenocytes

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This study was undertaken to evaluate the immunomodulatory effect of dead nano-sized *Lactobacillus plantarum* (nLp) in RAW 264.7 cells and murine primary splenocytes. nLp is a dead, shrunken, processed form of *L. plantarum* nF1 isolated from kimchi (a traditional Korean fermented cabbage) and is less than 1 μm in size. It was found that nLp treatment stimulated nitric oxide (NO) production more in RAW 264.7 macrophages than pure live *L. plantarum* (pLp), and that the stimulatory properties were probably largely derived from its cell wall. In addition, nLp induced murine splenocyte proliferation more so than pLp; in particular, a high dose of nLp (1.0×10^{11} CFU/ml) stimulated proliferation as much as lipopolysaccharide at 2 μg/ml. Moreover, according to our cytokine profile results in splenocytes, nLp treatment promoted Th1 (TNF-α, IL-12 p70) responses rather than Th2 (IL-4, IL-5) responses and also increased Th17 (IL-6, IL-17A) responses. Thus, nLp stimulated NO release in RAW 264.7 cells and induced splenocyte proliferation more so than pLp and stimulated Th1 and Th17 cytokine production. These findings suggested that dead nLp has potential use as a functional food ingredient to improve the immune response, and especially as a means of inducing Th1/Th17 immune responses.

Keywords: Dead nano-sized *Lactobacillus plantarum*, nitric oxide, RAW 264.7 macrophages, splenocyte, Th1/Th2/Th17 cytokines

Introduction

Probiotics are defined as living microorganisms that exhibit beneficial effects on the host when adequate doses are administered [13]. Probiotics play important roles in the gastrointestinal tract by modulating immune functions. In particular, *Lactobacillus plantarum*, a major species used for the fermentation of various vegetables, exerts several probiotic effects; for example, it has immunopotentiating activities and ameliorates the symptoms of chronic intestinal inflammation and irritable bowel syndrome [8, 17]. Furthermore, recent studies have reported that heat-killed cells or fractionated cell components (exopolysaccharides or lipoteichoic acid) also have beneficial health effects [1].

Dead nano-sized *L. plantarum* (nLp) is a shrunken processed form of *L. plantarum* nF1 (deposited at the NITE Biological Resource Center, Tokyo; Accession No. NITE-P1462) isolated from kimchi (a traditional Korean fermented cabbage) of size 0.5–1.0 μm. It is manufactured by incubation under harsh conditions and following this with a nano-dispersing process. This process enhances the ability of *L. plantarum* to induce interleukin (IL)-12 and produces cells that are rounder and smaller (<1 μm) than rod-shaped live *L. plantarum* [20]. In our previous studies, we found that nLp exhibited stronger anti-colitis and anti-colorectal cancer effects than pLp in a mouse model, which suggested nLp has potential use as a substitute for live, pure *L. plantarum* (pLp) to alleviate the symptoms of colitis and

colorectal cancer [27, 28].

In older individuals, the immune system is weakened by the aging process, stress, or disease [12]. These deficiencies could be addressed by enhancing the immune responses using natural or chemical adjuvants with immunomodulatory properties, whereas repeated administrations of chemical pharmaceuticals can cause side effects, such as, fever, headache, or hypertension [11]. Evidence shows that probiotics have potential for modulating the immune system. Certain *Lactobacillus* species, such as *L. rhamnosus* (HN001), *L. acidophilus* (HN017), *L. casei*, and *L. rhamnosus* GG, can promote innate and acquired immunity and enhance protection against infections and diseases [7, 12, 19]. However, in severely immunodeficient patients, live probiotics have some potential to cause disease owing to their own pathologies [41], and thus, recently, dead lactic acid bacteria have attracted attention as immunomodulators.

$CD4^+$ T helper (Th) cells play a major role in modulating immune responses. Classically, these cells are subdivided into Th1 and Th2 based on their cytokine production patterns. Th1 cells are responsible for cellular immunity and defense against intracellular pathogens, and produce tumor necrosis factor (TNF)-α, IL-12, or interferon (IFN)-γ, whereas Th2 cells regulate humoral immunity and defense against extracellular pathogens by producing IL-4, IL-5, or IL-10 [26]. Dysregulated Th1/Th2 polarization can cause several immunological diseases, such as rheumatoid arthritis (Th1), allergy (Th2), or cancer (Th2), and thus, it is important to maintain a balance between Th1 and Th2 responses [23]. Recently, a third subset of Th cells, termed Th17 cells, were discovered that defend against pathogens that were not cleared by Th1 or Th2 cells and secrete IL-6, IL-17, or IL-22 [5, 38].

In the present study, we compared the immunostimulatory properties of pLp and nLp in RAW 264.7 macrophages and mouse primary splenocytes. The effects of pLp and nLp on nitric oxide (NO) release were examined and the abilities of the cytoplasm and cell wall extracts of pLp to induce immunostimulatory effects in RAW 264.7 macrophage cells were compared. In addition, the effects of pLp and nLp on the proliferation of mouse primary splenocytes were compared and the levels of cytokines (Th1, Th2, and Th17) released by nLp treatment were investigated in splenocyte culture supernatants.

Materials and Methods

Microorganisms and Sample Preparation

L. plantarum nF1 (deposited at the NITE Biological Resource Center, Tokyo; Accession No. NITE-P1462) was provided by Biogenics Korea (Seoul, Korea) and cultured in MRS broth at 37°C for 18 h. To measure NO release and splenocyte proliferation, *L. plantarum* nF1 cells were harvested by centrifugation at 4,000 ×*g* for 20 min and then washed twice with sterile saline solution. Cell pellets were then suspended in RPMI medium at concentrations of 1.0×10^{10} CFU/ml (low dose) or 1.0×10^{11} CFU/ml (high dose). To prepare cytoplasmic and cell wall crude fractions, after the above-mentioned wash process, pellets were sonicated and centrifuged at 30,000 ×*g* for 20 min to obtain cytoplasmic extract (supernatant) and cell wall crude extract (pellets) fractions [2, 37]. Protein concentrations of the extracts were determined using the Bradford assay.

nLp was manufactured by Biogenics Korea by incubating *L. plantarum* for 20 h under pH control, then incubating it at high temperature (40°C), high salinity (1.0% (w/w)), and low pH (pH 5.0) for 4 h, and following this with sterilization and nano-dispersion (high pressure homogenization) stages [20].

In Vitro Assay of the Immunostimulatory Properties of nLp

Cell lines. RAW 264.7 murine macrophages were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Gyeongsan, Korea) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 100 units/ml of penicillin/streptomycin (Gibco BRL, Rockville, MD, USA) in a humidified 5% CO_2/95% air incubator at 37°C.

Determination of NO production. RAW 264.7 cells (1×10^5 cells/well) were incubated in 96-well plates containing PBS, 2 μg/ml lipopolysaccharide (LPS; Sigma, St Louis, MO, USA) or different concentrations of samples (pLp, nLp, or cytoplasmic or cell wall crude fractions) for 24 h. NO contents were measured by adding 100 μl of Griess reagent (Sigma) to 100 μl of culture supernatant and measuring the absorbance at 570 nm using $NaNO_2$ as the standard [9].

Ex Vivo Assay of the Immunostimulatory Properties of nLp and Induced Cytokine Profiles

Animals. Male C57BL/6J mice were obtained from Samtaco Bio Korea (Osan, Korea) at 6 weeks of age. Animals were maintained under controlled conditions (RH 55 ± 5%, 22 ± 2°C under a 12-h light/dark light cycle) with feed and water supplied ad libitum. The study protocol was approved by the Institutional Animal Care and Use Committee of Pusan National University (Busan, Korea) (PNU-IACUC; approval number PNU-2015-0798).

Proliferation of mouse splenocytes. Splenocytes were isolated from normal mice sacrificed after an acclimation period of 1 week. Spleens were aseptically removed and single spleen cell suspensions were prepared in RPMI-1640 medium (Welgene) containing 10% FBS and 100 units/ml penicillin/streptomycin, as previously described with slight modification [2, 16]. To quantify splenocyte proliferation, splenocytes were incubated at a density of 1×10^6 cells/ml in 96-well plates with PBS, LPS (2 μg/ml), pLp, or nLp for 72 h.

Cell viability was determined using a 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) assay.

Enzyme-linked immunosorbent assay (ELISA) of cytokines. Mouse primary splenocytes were cultured on 24-well plates at 2×10^6 cells/ml, and then incubated with PBS, LPS (2 μg/ml), concanavalin A (ConA, Sigma; 2 μg/ml), or two concentrations of nLp for 72 h. Levels of Th1 cytokines (TNF-α, IL-12 p70), Th2 cytokines (IL-4, IL-5), and Th17 cytokines (IL-6, IL-17A) in culture supernatants were measured using appropriate ELISA kits (BioLegend, San Diego, CA, USA).

Statistical Analysis

Results are expressed as means ± SDs. One way analysis of variance and Duncan's multiple range test were used to determine the significances of differences between amounts of NO released and splenocyte proliferation. The Student's *t*-test was used to determine the significance of difference between measured cytokine levels. The analysis was conducted using PASW Statistics ver. 18 (IBM Co., Armonk, NY, USA), and values of $p < 0.05$ were considered to be statistically significant.

Results

In Vitro Assay of the Immunostimulatory Properties of nLp

Effect of nLp and cell wall extracts of pLp on NO production in RAW 264.7 cells. The levels of NO release in LPS-treated controls increased dramatically to 20.3 ± 1.4 μM (Fig. 1A). Both doses of pLp and of nLp enhanced NO production, particularly at the high dose. At the high dose (1.0×10^{11} CFU/ml), nLp stimulated significantly more NO release (15.0 ± 0.9 μM) than pLp (12.3 ± 0.4 μM), but at low dose (1.0×10^{10} CFU/ml), NO releases were nonsignificantly different ($p < 0.05$).

To determine which cell fraction most affected the immunostimulatory activities of pLp and nLp, we compared the abilities the cytoplasmic and cell wall extracts of pLp to stimulate NO production (Fig. 1B). At all concentrations examined, cytoplasmic and cell wall extracts induced NO release and these releases occurred in a dose-dependent manner. However, the cell wall extract enhanced NO production significantly more than the cytoplasmic extract ($p < 0.05$). Furthermore, the NO concentrations for cell wall extract treatment at 50 or 100 μg protein/ml were 17.3 ± 1.0 μM and 18.6 ± 1.0 μM, respectively, which were comparable to those induced by 2 μg/ml LPS (19.6 ± 1.4 μM; $p < 0.05$). These results show that NO release was stimulated more by nLp than pLp, and suggest that these activities were largely derived from cell walls.

Ex Vivo Assay of the Immunostimulatory Properties of nLp and Induced Cytokine Profiles

Effect of nLp on splenocyte proliferation. To evaluate the immunopotentiating effects of pLp and nLp, splenocyte cell proliferation was observed after 72 h of exposure to low or high concentrations of pLp or nLp and compared with that induced by LPS (Fig. 2). LPS significantly increased proliferation by 28.9% ($p < 0.05$), and pLp or nLp dose-dependently stimulated splenocyte proliferation.

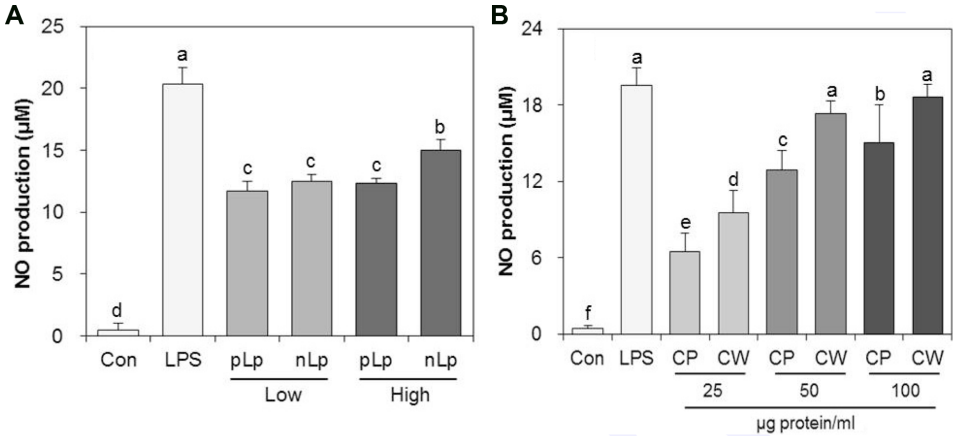

Fig. 1. Effect of nanometric *Lactobacillus plantarum* (nLp) (**A**) and cell extracts of pLp (**B**) on nitric oxide (NO) production by RAW 264.7 cells.

Results are presented as means ± SDs ($n = 8$). Results followed by different lowercase letters (a–d) were significantly different ($p < 0.05$). Con, cells treated with PBS alone; LPS, cells treated with lipopolysaccharide (2 μg/ml); pLp-low, cells treated with pure live *L. plantarum* (pLp) at 1×10^{10} CFU/ml; pLp-high, cells treated with pLp at 1×10^{11} CFU/ml; nLp-low, cells treated with nLp at 1×10^{10} CFU/ml; nLp-high, cells treated with nLp at 1×10^{11} CFU/ml; CP, cytoplasmic extract of pLp at 25, 50, or 100 μg protein/ml; CW, cell wall extract of pLp at 25, 50, or 100 μg protein/ml.

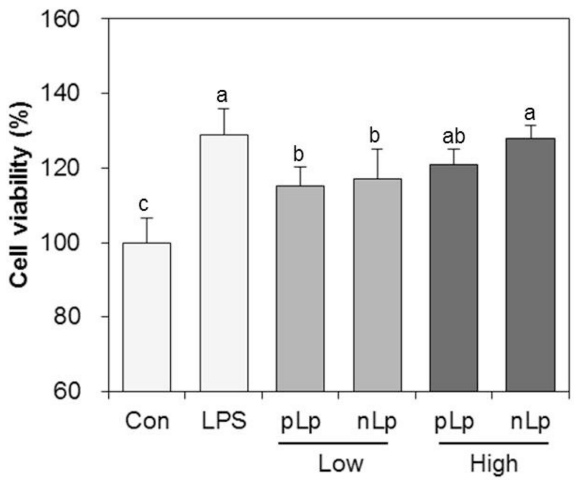

Fig. 2. Effect of nLp on splenocyte proliferation.
Results are presented as means ± SDs ($n = 8$). Results followed by different lowercase letters (a–c) were significantly different ($p < 0.05$). Group abbreviations are as described in the legend of Fig. 1.

Although no significant difference was found between low-dose pLp and nLp, high-dose nLp promoted splenocyte proliferation by 27.8% versus untreated controls, which was 5.9% higher than that achieved by pLp and similar to that achieved by LPS at 2 μg/ml ($p < 0.05$). We found that nLp enhanced splenocyte proliferation more than pLp at the high dose, and such enhancement was as strong as LPS treatment (2 μg/ml). We then focused on the immunopotentiating properties of nLp rather than pLp and investigated its ability to induce cytokines.

Effect of nLp on Th1, Th2, and Th17 cytokine production by splenocytes. *Th1 Cytokines:* Amounts of Th1, Th2, and Th17 released were measured to determine the type of immune response affected by nLp in splenocytes. As controls, cells were treated or not with LPS or ConA without nLp. Levels of TNF-α and IL-12 p70 were measured to gauge the Th1 response, IL-4 and IL-5 for the Th2 response, and IL-6 and IL-17A for the Th17 response. At low and high concentrations, nLp significantly and dose-dependently induced the secretion of TNF-α and IL-12 p70 in untreated and in LPS or ConA treated splenocytes (Fig. 3A). In particular, high-dose nLp significantly increased TNF-α secretion by 71.0-fold versus untreated splenocytes ($p < 0.001$), by 33.8% versus LPS-induced splenocytes ($p < 0.05$), and by 42.3% versus ConA-induced splenocytes ($p < 0.001$). In addition, high-dose nLp dramatically increased IL-12 p70 secretion by 4.8-, 2.8-, and 2.8-fold versus untreated ($p < 0.001$), LPS-treated ($p < 0.01$), and ConA-treated ($p < 0.001$) controls, respectively. Thus, nLp was found to be a potent stimulator of TNF-α and IL-12 p70 secretion, which are both related to Th1 immune response, in splenocytes.

Th2 Cytokines: As shown in Fig. 3B, in untreated splenocytes, only low-dose nLp induced IL-4 production, but low-dose nLp significantly reduced IL-4 levels in LPS-induced controls ($p < 0.05$), and high- and low-dose nLp significantly reduced IL-4 levels in ConA-treated controls ($p < 0.01$). Furthermore, high- and low-dose nLp did not affect IL-5 production in untreated or LPS-treated splenocytes, but did so in ConA-induced splenocytes. IL-5 secretion was profoundly reduced by low-dose nLp versus ConA-treated controls (250.1 ± 6.0 pg/ml) to 75.2 ± 1.0 pg/ml and by high-dose nLp to 51.4 ± 0.7 pg/ml. Accordingly, nLp appeared not to specifically affect IL-4 and IL-5 secretion, although when co-treated with ConA it did dose-dependently suppress these secretions.

Th17 Cytokines: IL-6 and IL-17A levels were measured to evaluate the effect of nLp on Th17 cytokine release (Fig. 4). nLp significantly promoted the secretion of IL-6 at low and high doses in untreated and ConA-treated splenocytes, but induced no notable change in LPS-treated splenocytes. In addition, IL-17A secretion was also increased by low and high doses of nLp in untreated and LPS-treated splenocytes, but not in ConA-treated controls. In particular, IL-17A production was dramatically increased by 2.7-fold by low-dose nLp and by 10.3-fold by high-dose nLp versus treatment of naive splenocytes ($p < 0.01$). These results show that nLp generally induces Th17 cytokine release, but that its effects are dependent on mitogen type. It would appear that nLp affects cytokine profiles by enhancing Th1 and Th17 immune responses and suppressing (or maintaining) the Th2 immune response in splenocytes in a mitogen-type-dependent manner.

Discussion

Several reports have been issued on the effects of dead probiotics on immunomodulation, infection prevention, the improvement of intestinal environment, and cancer suppression [14]. In particular, heat-killed probiotics, such as *Enterococcus faecalis* or bifidobacteria, and fractionated cell components, such as cell wall preparations from *E. faecalis* or *Lactobacillus* spp. or lipotechoic acids from *L. casei* or *L. fermentum*, have been reported to augment host resistance and stimulate innate immune responses [1, 21, 30, 36]. Accordingly, it has been recently suggested that the definition of probiotics could be revised to "microbial cell preparations and microbial cell components that confer a health benefit to the host" rather than placing focus on

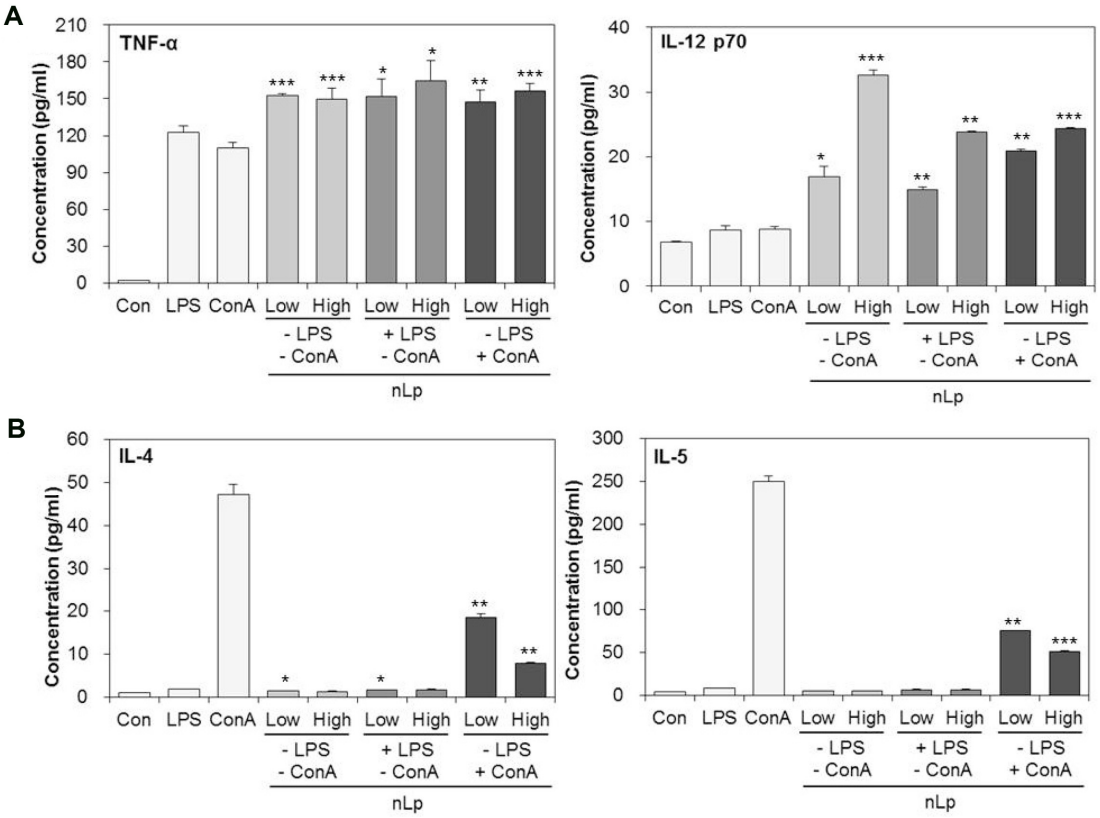

Fig. 3. Effect of nLp on Th1 cytokine (TNF-α, IL-12 p70) (**A**) and Th2 cytokine (IL-4, IL-5) (**B**) production by splenocytes.
Results are presented as means ± SDs (n = 8). Con, cells treated with PBS; LPS, cells treated with 2 μg/ml lipopolysaccharide; ConA, cells treated with 2 μg/ml concanavalin A; nLp-low, cells treated with nLp at 1×10^{10} CFU/ml; nLp-high, treated with nLp at 1×10^{11} CFU/ml. For splenocytes treated with nLp in the absence of LPS and ConA, statistical significance was determined versus PBS-treated splenocytes (Con). For splenocytes treated with nLp in the presence of LPS, statistical significance was determined versus LPS-treated splenocytes (LPS). For splenocytes treated with nLp in the presence of ConA, statistical significance was determined versus ConA-treated splenocytes (ConA). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus Con, LPS, or ConA.

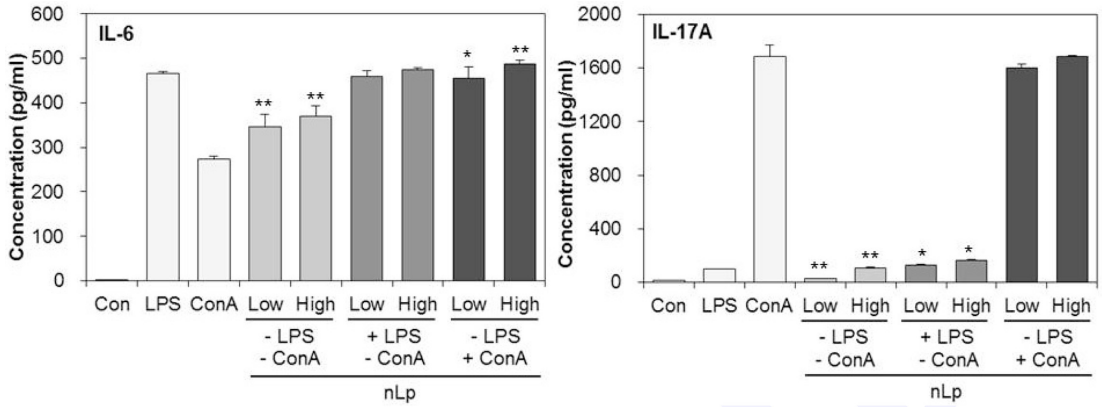

Fig. 4. Effect of nLp on Th17 cytokine (IL-6, IL-17A) production by splenocytes.
Group abbreviations are as described in the legend of Fig. 3. For splenocytes treated with nLp in the absence of LPS or ConA, statistical significance was determined versus PBS-treated splenocytes (Con). For splenocytes treated with nLp in the presence of LPS, statistical significance was determined versus LPS-treated splenocytes (LPS). For splenocytes treated with nLp in the presence of ConA, statistical significance was determined versus ConA-treated splenocytes (ConA). *$p < 0.05$, **$p < 0.01$ versus Con, LPS, or ConA.

live cells [14].

The mechanisms whereby probiotics stimulate immune reactions are not clear. It is possible that probiotics are taken up by M cells and directly influence Peyer's patch immunocompetent cells to stimulate intestinal immune responses. Alternatively, probiotics could be transported to systemic lymphoid tissues, such as mesenteric lymph nodes or spleen, and exert immunomodulatory effects indirectly [11, 12]. Our previous findings regarding direct immunostimulation showed that nLp can inhibit colon carcinogenesis by enhancing fecal secretory IgA levels more so than pLp in a Balb/c mouse colon cancer model, which suggested that the anticancer activity of nLp is associated with its easier uptake by M cells than pLp, and that this results in the subsequent elicitation of stronger intestinal immune responses [28]. In addition, the administration of LAB with small particle sizes was found to increase the production of cytokines, such as IL-12 and IFN-α, and to enhance Th1 cell proliferation [14]. Furthermore, the particle size of nLp becomes less than 1 μm (nano-sized) and tends not to form cell aggregations as much as pLp through the nanometric process [20]. In the present study, we demonstrated the alternative mechanisms of dead probiotic nLp to modulate the immune response by evaluating its capability in macrophages and splenocytes.

Macrophage activation is an important aspect of the host defense response and immune enhancement. When macrophages are activated, some cell factors, such as NO, TNF-α, reactive oxygen intermediates, and other substances required for host defense, are released [39]. NO plays a major role in the host immune system and has antimicrobial and antitumor activities, modulates cytokine production, and stimulates Th cell differentiation [3]. Our findings show that nLp possesses immunostimulatory activity and induces more NO release than pLp in Raw 264.7 macrophage cells. Furthermore, we found the cell wall extracts of pLp contributed more to immunostimulatory activities of pLp and nLp than cytoplasmic extracts, which is in accord with our previous finding that the cell wall fraction of *L. plantarum* PS-21 (also isolated from kimchi) has stronger mitogenic activity than its cytoplasmic fraction [4]. Furthermore, our results suggest that the greater immunostimulatory properties of nLp, as compared with pLp, are due to its smaller size and tendency not to aggregate, and thus to its substantially greater specific cell wall surface area.

Increases in splenocyte numbers indicate enhancement of the immune system by relevant cytokine releases and the stimulation of other immune accessory cells [6]. Several probiotic strains, such as *Bifidobacterium longum* or *L. johnsonii*, have been reported to possess immunostimulatory properties, although the majority are isolated from dairy or animal products [18, 33]. nLp is a processed form of *L. plantarum* nF1 isolated from kimchi that could be used in foods rather than live probiotics to increase product shelf life and ease storage and transportation considerations. Our findings showed that nLp stimulated splenocyte proliferation more than pLp, which is in accord with our NO production results. Furthermore, treatment with nLp at high concentration induced splenocyte proliferation as much as LPS at 2 μg/ml. These results suggest that nLp has potential use as an immunostimulant in immunodeficient subjects, and thus, in the latter part of this study, we focused on nLp rather than pLp and measured cytokine profiles induced by nLp.

We found that nLp evokes the Th1 cytokine profile rather than the Th2 cytokine profile in mouse splenocytes. We used two types of mitogens, LPS for B-cell mitogens and ConA for T-cell mitogens. nLp induced the secretions of Th1 cytokines (TNF-α, IL-12 p70) regardless of mitogen type, but it either decreased or did not affect Th2 cytokine production in a mitogen-type-dependent manner. In addition, we found that the IL-12 to IL-10 ratio was increased in nLp-treated splenocytes (data not shown). IL-12 and IL-10 are primary cytokines that play a role in bridging the gap between innate and acquired immunity [15]. IL-12 activates T cells and NK cells to proliferate and secrete interferon (IFN)-γ to lyse target cells [40], whereas IL-10 downregulates these functions and stimulates B-cell maturation and antibody production [35]. A low IL-12/IL-10 ratio is a feature of some types of cancer in humans [23].

A balance of Th1/Th2 cells is crucial to maintain homeostasis of the host immune system [24]. In accord with our results, several probiotics have been reported to enhance the immune response and maintain homeostasis in the host immune system, and some strains of probiotics have been found to alter the balance of Th cells from Th2 to Th1 [2, 16]. *Lactobacillus* species have been reported to enhance the ability of mouse splenic leukocytes to induce IL-12 and IFN-γ, which contributes to Th1 response, but were not found to affect IL-4 or IL-5 production [11, 12, 22]. In addition, heat-killed probiotics, such as *L. plantarum* L-137 or *L. casei* Shirota, have also been reported to possess immunomodulatory activities and to stimulate Th1 cytokine production, but not Th2 cytokine production [22, 31, 34]. Cell components, particularly peptidoglycans, β-glucans, extracellular polysaccharides, and lipoteichoic acids, of probiotics has been reported to stimulate immune response [10, 29, 30, 32]. These findings indicate that whole live cells may not be needed to modulate the immune system via

Th1 cytokine stimulation. Moderate Th1 cytokine stimulation could usefully promote immunological balance and enhance resistance to infections, but it should be noted that excessive increases are undesirable [1], and thus careful screening is probably required before nLp is administered.

According to our Th17 cytokine results, nLp enhanced IL-6 and IL-17A release in a mitogen-type-dependent manner. Th17 cells mediate inflammation and tissue destruction, and thus control human inflammatory conditions and the symptoms of autoimmune diseases by secreting IL-17, IL-6, and GM-CSF [26, 38]. Th17 cytokines play roles in immune regulation and oncogenesis and in defense against bacteria by recruiting and activating neutrophils and macrophages [25]. Our findings suggest that the preferred modulation of the Th1 and Th17 immune response rather than the Th2 response by nLp could be beneficial for treating Th2-associated diseases, such as allergies, asthma, systemic lupus erythematosus, and cancer [23].

In summary, we found that dead nLp has immunostimulatory properties greater than live pLp by inducing higher levels of NO production in RAW 264.7 macrophages, and that its activity is probably derived primarily from the cell wall rather than the cytoplasm. nLp also enhanced mouse splenocyte proliferation more than pLp and favored the Th1 and Th17 immune response rather than the Th2 immune response via relevant cytokine release. Thus, we consider nLp a potential functional food ingredient that could improve the immune response in immunodeficient individuals and enhance Th1 and Th17 immune responses. Owing to the complexity of bacterial cell walls at the molecular level, additional studies are needed to identify the entity responsible for the immunomodulatory properties of nLp.

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