Immune-Enhancing Effect of Nanometric *Lactobacillus plantarum* nF1 (nLp-nF1) in a Mouse Model of Cyclophosphamide-Induced Immunosuppression

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Nanometric *Lactobacillus plantarum* nF1 (nLp-nF1) is a biogenics consisting of dead *L. plantarum* cells pretreated with heat and a nanodispersion process. In this study, we investigated the immune-enhancing effects of nLp-nF1 in vivo and in vitro. To evaluate the immunostimulatory effects of nLp-nF1, mice immunosuppressed by cyclophosphamide (CPP) treatment were administered with nLp-nF1. As expected, CPP restricted the immune response of mice, whereas oral administration of nLp-nF1 significantly increased the total IgG in the serum, and cytokine production (interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF- α)) in bone marrow cells. Furthermore, nLp-nF1 enhanced the production of splenic cytokines such as IL-12, TNF- α , and interferon gamma (IFN- γ). In vitro, nLp-nF1 stimulated the immune response by enhancing the production of cytokines such as IL-12, TNF- α , and IFN- γ . Moreover, nLp-nF1 given a food additive enhanced the immune responses when combined with various food materials in vitro. These results suggest that nLp-nF1 could be used to strengthen the immune system and recover normal immunity in people with a weak immune system, such as children, the elderly, and patients.

Keywords: Nanometric *Lactobacillus plantarum* nF1, immune enhancement, interleukin-12, macrophage, immunoglobulin G, cyclophosphamide

Introduction

The recent years have seen an increase in various infectious disorders, including respiratory diseases, due to rapid industrial development, environmental pollution, and climate changes [1, 2]. To overcome these health problems, effective vaccines have been developed. However, there is a risk of infection with viruses and pathogens that have not yet been identified. An answer to this problem could be to strengthen the immunity, which could respond to infections by unknown viruses or pathogens [3, 4]. To this end, many researchers are exploring materials with immune-enhancing

properties and are studying the mechanisms behind these properties. For example, *Panax ginseng*, also called Korean red ginseng, has been extensively used in traditional medicine and as a functional food. It can boost the immune system by enhancing the phagocytic ability of macrophages, reinforcing the activity of natural killer cells, and inducing the production of mucosal secretory immunoglobulin (Ig) A [5–7]. Moreover, various immune-enhancing materials such as *Aloe vera* and propolis are being developed as health functional foods [8–11].

Probiotics (defined as live microorganisms) are known to mostly function by improving the intestinal environments

through increasing the viability of beneficial bacteria and reducing the growth of harmful bacteria [12]. In addition, probiotics used as functional foods have been found to play an important role in controlling immune reactions, which has resulted in various health benefits being claimed, including enhancement of the immunity, control of hypersensitivity (allergy), and inhibition of inflammation [12–15]. The probiotic mixture VSL#3 (*Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus bulgaricus*, and *Streptococcus thermophilus*), for example, has been found to have multiple functions, and it has been indicated for the enhancement of the immune system, attenuation of allergic responses, and treatment of inflammatory bowel disease and irritable bowel syndrome [16–19].

Recently, many studies have reported that heat-killed microorganisms or fractionated cellular components, such as cytoplasmic extracts, membrane fragments, exopolysaccharides, and lipoteichoic acids, can modulate various immune responses, alleviating symptoms of colitis, autoimmune disorders, and allergies [20, 21]. For this kind of substances, Mitsuoka [22] suggested the term “biogenics,” which is defined as “food ingredients which beneficially affect the host by directly immunostimulating or suppressing mutagenesis, tumorigenesis, peroxidation, hypercholesterolemia, or intestinal putrefaction,” and includes the administration of nonviable probiotic bacteria to obtain some “probiotic” effects [23]. Biogenic materials, including nonviable probiotic bacteria, have been actively developed as health supplements or functional foods because they possess stability to processes involving heat or acids [24, 25].

Nanometric *Lactobacillus plantarum* (strain name: nF1) (nLp-nF1) is a biogenics consisting of dead (nonviable) *L. plantarum* cells pretreated with heat and a nanodispersion process. The nLp-nF1 has a shrunken, granular shape and a size of 0.5–1.0 μm , and it has been registered in the DNA Data Bank of Japan (Accession No. NITE-P1462). In vitro studies using splenocytes of BALB/c mice have shown that nLp-nF1 has immunostimulating activities, involving the production of interleukin (IL)-12 and interferon (IFN)- α [26–28]. Furthermore, administration of nLp-nF1 effectively attenuates dextran sulfate sodium (DSS)-induced colitis symptoms and DSS-azoxymethane-induced colorectal cancer [29, 30]. These anti-inflammatory and tumor suppressive effects might be derived from the induction of T helper (Th) 1 cells by IL-12, and from the induction of tumor necrosis factor- α (TNF- α) production.

In the present study, we investigated whether nLp-nF1 enhances the immune response in primary splenocytes isolated from mice. Furthermore, we examined whether administration of nLp-nF1 has immunostimulating effects in the cyclophosphamide (CPP)-induced immunosuppressed mouse model. Finally, we assessed whether nLp-nF1 induces IL-12 production when used as a functional food ingredient in combination with various general foods.

Materials and Methods

Materials

To measure IL-12, TNF- α , IL-1 β , and IFN- γ , enzyme-linked immunosorbent assay (ELISA) kits were purchased from BD Biosciences (USA), and an IgG ELISA kit was obtained from Santa Cruz Biotechnology (USA). Anti-CD3 (FITC), anti-CD11b (APC), anti-CD11c (PE), and anti-CD335 (APC/Fire 750) antibodies were purchased from Biolegend (USA). CPP was purchased from Tokyo Chemical Industry Co. (Japan), and concanavalin A (Con A) was obtained from Sigma-Aldrich (USA).

Sample Preparation

The nLp-nF1 (deposited at the NITE Biological Resource Center, Tokyo; Accession No. NITE-P1462) used in this study was obtained from Biogenics Korea Co., Ltd. (Korea). Briefly, nLp-nF1 was manufactured by incubating *Lactobacillus plantarum* for 20 h under pH control, followed by incubation at high temperature (40°C), high salinity (1.0% (w/w)), and low pH (pH 5.0) for 4 h. Then, the sample was sterilized (80°C, 10 min) and nanodispersed by high-pressure homogenization [27, 28].

Animals

Five-week-old female BALB/c mice for in vitro experiments and 6-week-old male ICR mice for in vivo experiments were purchased from OrientBio Inc. (Korea). The animals were housed in an air-conditioned room (23 $\pm$ 2°C) with a 12 h light/dark cycle in accordance with the guidelines for animal use and care of the Korea Food Research Institute (Permit No. KFRI-M-16044).

In Vivo Experiments Using an Immunosuppressed Mouse Model

ICR mice were divided into four groups consisting of naive, sham, low-dose nLp-nF1 (4×10^9 CFU/kg body weight), and high-dose nLp-nF1 (4×10^{10} CFU/kg body weight) ($n = 5$). Mice of all groups, except the naive group, were intraperitoneally injected with CCP (150 mg/kg/day) twice, once on day 0 and again on day 2, for induction of immunosuppression (Fig. 1). Mice in the nLp-nF1 group were orally administered with nLp-nF1 once a day on days 3 to 17 (15 days). All mice were killed on day 18. For analysis of IgG levels, serum samples were obtained by collecting blood from the orbital venous plexus. Spleens and bone marrows were removed and were used for analysis of cytokine production.

Fig. 1. Experimental schedule of the cyclophosphamide (CPP)-induced immunosuppressed mouse model.

Culture of Splenocytes and Bone Marrow Cells

Splenocytes were isolated from the spleens of ICR mice (immunosuppressive mouse model), and cells were seeded at 1×10^6 cell/ml with Con A ($2 \mu\text{g/ml}$) in 12-well plates. After 48 h, the cell supernatants were harvested to measure cytokines levels.

Bone marrow cells were obtained from the long bones of ICR mice, and cells were seeded at 5×10^6 cell/ml in 12-well plates in RPMI medium without Con A and containing 10% fetal bovine serum (FBS). The cell supernatants were harvested after 72 h for evaluation of cytokine production.

Measurement of Cytokines and Serum IgG Levels Using ELISA

The levels of cytokines (IL-12, TNF- α , and IFN- γ) and serum IgG were measured using ELISA kits according to the manufacturer's protocols. Briefly, recovered supernatants and standard solutions were transferred to 96-well plates precoated with the appropriate monoclonal antibodies against each of the target cytokines, and then incubated at room temperature for 2 h. After thorough washing with buffer, horseradish peroxidase-conjugated secondary antibodies were added to each well, and incubation was continued at room temperature for 2 h. After removal of the secondary antibody, the substrate 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 the absorbance was measured at 450 nm using a microplate reader (Molecular Devices, USA).

Analysis of the Population of Bone Marrow Cells Using Flow Cytometry

Bone marrow cells were gathered and suspended in Hank's balanced salt solution containing 10% FBS, 10% sodium azide, and 8% sodium bicarbonate. The cells were incubated on ice with Fc Block (anti-CD16/32) (BD Pharmagen, USA), and then they were stained with anti-CD3 (FITC), anti-CD11b (APC), anti-CD11c (PE), and anti-CD335 (APC/Fire 750) antibodies for 30 min. The presence of single cells was confirmed by flow cytometry, which was performed using CytoFLEX (Beckman Coulter, USA).

In Vitro Experiments Using Splenocytes

Splenocytes were prepared by aseptically removing the spleens from BALB/c mice. The tissue was homogenized, and cells were collected and treated with red blood cell lysis buffer. Cell counting was performed by the trypan blue exclusion method, and the

number of splenocytes was adjusted to a cell density of 5×10^6 cells/ml in RPMI 1640 medium (10% FBS). Splenocytes were then cultured in the absence or presence of nLp-nF1 at 37°C for up to 72 h in a humidified incubator with 5% CO_2 . The culture supernatants were harvested and used to evaluate the effects of nLp-nF1 on cytokine production by ELISA.

Treatment of Products Combined with nLp-nF1

For in vitro experiment of products combined with nLp-nF1, each product was diluted in PBS as follows: porridge, 1/20; instant coffee, 1/20; beverage, 1/20; dry milk, 1/20; sauce, 1/2,000. Next, nLp-nF1 was added to the products at a dose of 5×10^7 CFU/ml, and then splenocytes were treated with the products combined with nLp-nF1 in culture media. After 72 h, the culture supernatants were harvested and used to evaluate the effects of nLp-nF1 on cytokine production by ELISA.

Statistical Analysis

Results are expressed as the mean $\pm$ standard deviation (SD). Statistical analysis of the differences between experimental groups were assessed by one-way analysis of variance followed by F-protected Fisher's least significant difference tests.

Results and Discussion

nLp-nF1 Increases Serum IgG Levels in CPP-Induced Immunosuppressed Mice

There are five classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM [31]. They have different roles in the immune response. IgG is the most common immunoglobulin in blood, lymph fluid, cerebrospinal fluid, and peritoneal fluid, and it is a key player in humoral immunity [32]. IgG can interact with macrophages, neutrophils, and natural killer cells, leading to activation of these cells [33]. In this study, we assessed serum IgG levels as an indicator of immune stimulation or immune enhancement. To investigate the immune-enhancing effect of nLp-nF1, the immunosuppressed mouse model (CPP-induced) was used. CPP is an immunosuppressor used in neoplastic and severe autoimmune disorders because it reduces a wide variety of immune responses such as those from macrophages,

Fig. 2. Immune-enhancing effect of nLp-nF1 on serum IgG levels in cyclophosphamide (CPP)-induced immunosuppressed ICR mice.

Serum from blood of the orbital venous plexus of CPP-treated mice was used to measure total IgG levels by ELISA. Data are presented as the mean $\pm$ SD. Asterisks (*) indicate significant differences ($p < 0.05$) between the sham and nLp-nF1 groups.

neutrophils, and effector T cells [34–36]. As expected, the CPP-injected group had reduced levels of IgG in sera compared with the control group, whereas the IgG levels in the group orally administered with nLp-nF1 were significantly higher than that in the CPP-treated group (Fig. 2). In general, an increase of IgG levels is able to connect with Th1-dominant immune responses, and the immune system maintains immune balance with Th1- and Th2-related immune responses [37]. Thus, we additionally investigated the levels of serum IgE as a Th2-mediated main response. As a result, the serum IgE was generally detected at a very low level, and the administration of nLp-nF1 could not change the IgE levels (data not shown). The result demonstrates that administration of nLp-nF1 can contribute to stimulation of the systemic immune system, leading to immune enhancement by increasing IgG levels (Th1-mediated immune response) without the regulation of IgE levels (Th2-mediated immune response).

nLp-nF1 Induces Production of IL-12 and TNF- α in Bone Marrow Cells from CPP-Induced Immunosuppressed Mice

The bone marrow is a primary immune organ that is important in the production of B lymphocytes and the precursor of T lymphocytes. Furthermore, in the bone marrow, immature myeloid cells are generated and differentiated to granulocytes, monocytes, macrophages, natural killer cells, and dendritic cells [38, 39]. Macrophages, natural killer cells, and dendritic cells originated in the

Fig. 3. Immune-enhancing effect of nLp-nF1 on cytokine production by bone marrow cells isolated from cyclophosphamide-induced immunosuppressed ICR mice.

Bone marrow cells were cultured for 72 h, and production of the cytokines (A) IL-12 and (B) TNF- α in culture supernatants was measured by ELISA. Data are presented as the mean $\pm$ SD of three independent experiments. Asterisks (*) indicate significant differences ($p < 0.05$) between the sham and nLp-nF1 groups.

bone marrow play a key role in enhancing or regulating the innate immunity. Therefore, we investigated whether administration of nLp-nF1 induces the production of immune-enhancing cytokines such as IL-12 and TNF- α by bone marrow cells isolated from CPP-induced immunosuppressed mice. As expected, the CPP-injected group showed a reduced production of IL-12 and TNF- α by bone marrow cells compared with the naïve group. However, the reduced production of cytokines was significantly recovered by oral administration of high-dose nLp-nF1 (Fig. 3). In bone marrow, IL-12 can be produced from monocytes, macrophages, and dendritic cells, whereas TNF- α can be produced by monocytes, macrophages, dendritic cells, lymphoid cells, and natural killer cells [40, 41]. To examine which of these cells was responsible for IL-12 and TNF- α production in our model, the population of immune cells in bone marrow, including CD3⁺ cells,

Fig. 4. Effect of orally administered nLp-nF1 on the population of CD11b⁺ cells in bone marrow cells isolated from cyclophosphamide-induced immunosuppressed ICR mice.

Bone marrow CD11b⁺ cells were stained with anti-CD11b and were analyzed by flow cytometry. Data are presented as the mean $\pm$ SD of three independent experiments. Asterisks (**) indicate significant differences ($p < 0.01$) between the sham and nLp-nF1 groups.

CD11b⁺ cells, CD11c⁺ cells, and CD335⁺ cells, was investigated by flow cytometry. We found that high-dose administration of nLp-nF1 resulted in an increase in the CD11b⁺ cell population compared with the CPP-injected group (Fig. 4), but no changes in the CD3⁺ cell, CD11c⁺ cell, and CD335⁺ cell populations were detected (data not shown). In bone marrow, there are many kinds of CD11b-expressing cells such as monocytes, macrophages, dendritic cells, and granulocytes, and the proportion of CD11b⁺ cells accounts for 44% of bone marrow cells [42, 43]. The cells have been known to be able to produce IL-12 and TNF- α as stimulatory factors [44, 45]. These stimulatory factors are able to activate immune cells through autocrine and paracrine signaling, resulting in an increase of proliferation or induction of differentiation. In the present study, Figs. 3 and 4 show that IL-12 and TNF- α were increased by nLp-nF1, and only the population of CD11b⁺ cells was increased. Therefore, we suggest that nLp-nF1 might activate the IL-12- and TNF- α -producing cells and then the produced stimulatory factors activate the CD11b⁺ cells, resulting in an increase in both the cell population and cytokine levels. These results indicate that nLp-nF1 administration enhances the immune system by inducing IL-12 and TNF- α production by CD11b⁺ cells in bone marrow.

nLp-nF1 Increases the Production of IL-12, TNF- α , and IFN- γ in Splenocytes Isolated from CPP-Induced Immunosuppressed Mice

The spleen is the largest secondary immune organ in the body, and it has a filtering function for circulating blood. It is also very important for the maintenance of immune homeostasis, affecting both the innate and the adaptive arms of the systemic immune system [46, 47]. Therefore, we investigated the effect of nLp-nF1 on splenocytes isolated from CPP-induced immunosuppressed mice. Administration of high-dose nLp-nF1 strongly increased the production of IL-12 and TNF- α by splenocytes (Figs. 5A and 5B). IL-12 and TNF- α produced by innate immune cells lead to activation of adaptive immune cells, such as CD4⁺ and CD8⁺ T cells, resulting in the production of IFN- γ . Therefore, we measured the levels of IFN- γ to assess the activation of the adaptive immunity. We found that IFN- γ levels were significantly enhanced by the administration of high-dose nLp-nF1 (Fig. 5C). These results demonstrate that administration of high-dose nLp-nF1 can enhance not only the innate immunity (IL-12 and TNF- α) but also the adaptive immunity (IFN- γ). In this study, therefore, we suggest that the enhancing effects of nLp-nF1 have been verified in both arms of the systemic immune system.

Fig. 5. Immune-enhancing effect of nLp-nF1 on cytokine production by splenocytes isolated from cyclophosphamide-induced immunosuppressed ICR mice.

Splenocytes were cultured with Con A (2 µg/ml) for 48 h, and production of (A) IL-12, (B) TNF-α, and (C) IFN-γ was measured in the culture supernatants by ELISA. Data are presented as the mean ± SD of three independent experiments. Asterisks (**) indicate significant differences ($p < 0.01$) between the sham and nLp-nF1 groups.

Interestingly, we found a difference between low-dose and high-dose administration of nLp-nF1 on immune enhancement in our CCP-induced immune suppressive mouse model. The administration of low-dose nLp-nF1 could not enhance the IgG, IL-12, TNF-α, and IFN-γ levels. We thought that since the immunosuppression induced by CCP was too strong, the immune enhancing effect of nLp-nF1 at low dose did not seem to be effective in this model. Nevertheless, the administration of high-dose nLp-nF1 strongly increased the levels of immunoglobulin and cytokines. We suggest that the high-dose nLp-nF1 can be effective as an immune enhancer, where the remarkable effect of nLp-nF1 may be derived from its nanometric property.

Enhancing Effect of nLp-nF1 on the Immune Response in an In Vitro System

In the present study, we have shown that oral administration

of nLp-nF1 can contribute to the enhancement of the immune response, including both humoral and cellular immunities (innate and adaptive immunities). Unlike probiotics (live microorganisms), since nLp-nF1 is a heat-killed microorganism, its properties can resist the heat and acid treatments of various processes in the manufacturing of end products. Therefore, we examined whether treatment with nLp-nF1 or with other materials, on their own or in combination with nLp-nF1, enhances the immune response in an in vitro system.

First, we investigated the effects of nLp-nF1 on whole splenocytes isolated from mice. We found that production of the cytokines IL-12, TNF-α, and IFN-γ increased significantly with nLp-nF1 treatment in a dose-dependent manner (Fig. 6). This result indicates that nLp-nF1 treatment could enhance the immune response in both in vitro and in vivo systems.

Next, we examined the enhancing effects of various

Fig. 6. Immune-enhancing effect of nLp-nF1 on cytokine production by splenocytes in vitro.

Splenocytes were cultured with or without nLp-nF1 for 72 h and production of (A) IL-12, (B) TNF-α, and (C) IFN-γ was measured in the culture supernatants by ELISA. Data are presented as the mean ± SD of three independent experiments. Asterisks (**) indicate significant differences ($p < 0.01$).

Fig. 7. Effects of food materials in combination with nLp-nF1 on the production of the cytokine IL-12.

Splenocytes were cultured with various food materials on their own or in combination with nLp-nF1 for 72 h. IL-12 cytokine levels were measured by ELISA in the culture supernatants of cells treated with (A) nLp-nF1 plus porridge, (B) nLp-nF1 plus sauce, (C) nLp-nF1 plus coffee, (D) nLp-nF1 plus beverage, and (E) nLp-nF1 plus dry milk. Data are presented as the mean $\pm$ SD of three independent experiments. Asterisks (**) indicate significant differences ($p < 0.01$) between treatments with and without nLp-nF1.

products (porridge, sauce, instant coffee, beverage, and dry milk) on their own or in combination with 5×10^7 CFU/ml of nLp-nF1. All products when combined with nLp-nF1 induced the production of IL-12 in vitro system (Fig. 7). This result demonstrates that nLp-nF1 can enhance the immune response by inducing IL-12, TNF- α , and IFN- γ production even in combination with a variety of products. This suggests that nLp-nF1 used as a food additive would be able to give the functionalities of nLp-nF1 to general foods.

In conclusion, we have shown that nanometric *L. plantarum* (nLp-nF1) has immune-enhancing effects both in vitro and in vivo. This suggests that uptake of nLp-nF1 could contribute to enhancement of the immune system and to the recovery of normal levels of immunity in people with reduced immunity, such as children, the elderly, and patients. Furthermore, nLp-nF1 could be used as a functional food, a probiotic drug, and a functional food additive to stimulate the immune response.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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