

Lactobacillus plantarum HY7712 Ameliorates Cyclophosphamide-Induced Immunosuppression in Mice

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Lactic acid bacteria (LAB) in fermented foods have attracted considerable attention recently as treatment options for immune diseases, the incidence of which has been increasing worldwide. The ability of 500 strains of LAB, isolated from *kimchi*, to induce TNF- α production in peritoneal macrophages was investigated. *Lactobacillus plantarum* HY7712 most strongly induced TNF- α production as well as NF- κ B activation. However, HY7712 inhibited NF- κ B activation in LPS-stimulated peritoneal macrophages. When HY7712 was orally treated in cyclophosphamide (CP)-immunosuppressed mice for 5 or 15 days, it reversed the body and spleen weights, blood RBC and WBC levels, and splenocyte and bone marrow cells that were reduced by CP. Orally administered HY7712 increased concanavalin A-induced T cell proliferation to 84.5% of the normal group on day 15, although treatment with CP alone markedly reduced it to 53.7% of the normal group. Furthermore, orally administered HY7712 significantly induced the expressions of IL-2 and IFN- γ in ConA-induced splenic cytotoxic T cells of CP-treated mice. Orally administered HY7712 restored the CP-impaired phagocytosis of macrophages in mice. Orally administered HY7712 also restored the cytotoxicity of NK and cytotoxic T cells derived from spleen and bone marrow against YAC-1 in CP-immunosuppressed mice. Based on these findings, orally administered HY7712 may accelerate the recovery of cyclophosphamide-caused immunosuppression, without evident side effects, by immunopotentiating NK and Tc cells, and may provide a mechanistic basis for using

HY7712 as an alternative means in lessening chemotherapy-induced immunosuppression in cancer patients.

Key words: Lactic acid bacteria, *Lactobacillus plantarum* HY7712, immunopotentiality, cyclophosphamide

Many anticancer drugs currently used in chemotherapy have cytotoxic side effects and seriously affect the patients' quality of life, regardless of curative effects. Therefore, reducing the side effects of anticancer drugs such as cyclophosphamide and doxorubicin is necessary [6, 24]. Cyclophosphamide is a widely used alkylating anticancer drug with a high therapeutic index and broad spectrum of activity against various cancers [24]. However, cyclophosphamide often results in serious side effects, such as leukopenia, myelosuppression, immunosuppression, and cytotoxic effects [26]. The host immune systems weakened by the chemotherapy of anticancer agents cannot defend against the progressive growth of tumor cells [3, 13]. Hence, immunopotentiating agents, such as levamisole, have been used [22]. However, these also cause severe side effects, such as serious neurological symptoms, gastric hemorrhage, colic, anemia, and vasculitis [1, 17, 23]. Therefore, many attempts have been made to search for safer immunomodulating agents.

Lactic acid bacteria (LAB) are Gram-positive, non-spore forming, non-respiring cocci or rods that ferment carbohydrates and produce lactic acid [7, 8, 25]. The common LAB genera in fermented foods such as cheese, yogurt, and *kimchi*, and in intestinal microflora are *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Pediococcus* sp., *Enterococcus* sp., *Streptococcus* sp., and *Bifidobacterium* sp. The LAB are safe microorganisms that repair disturbances of indigenous microflora [8, 20], aid the development of beneficial microflora [5, 25], have anticolitic effects [5, 19], and

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induce nonspecific activation of the host immune system [8, 11]. For example, *Lactobacillus* sp. and *Bifidobacterium* sp. enhance humoral, cellular, and/or nonspecific immunity in cancer cells-inoculated animals or healthy animals [9, 10, 20]. Therefore, the development of LAB with immunoenhancing properties, and that are free of side effects, is beneficial to humans with impaired immune function. Of the LAB, *Lactobacillus plantarum* isolated from kefir stimulates lymphocyte responses in intact and cyclophosphamide-immunocompromised mice [4]. Nevertheless, studies of the effect of lactic acid bacteria in immunodeficient or immunosuppressed animal or humans are few.

During the screening program to discover immunopotentiating LAB from fermented foods, we selected *Lactobacillus plantarum* HY7712, which activated NF- κ B in murine peritoneal macrophages, but inhibited NF- κ B in LPS-stimulated peritoneal macrophages, from Chinese cabbage *kimchi*. We measured its immunopotentiating effect in cyclophosphamide-immunosuppressed mice.

MATERIALS AND METHODS

Materials

RPMI 1640, lipopolysaccharide (LPS), and levamisole hydrochloride were purchased from Sigma Co. (St. Louis, MO, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits were from Pierce Biotechnology (Rockford, IL, USA). The protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany).

Selection of LAB

LAB were selected from the Korea Yakult Company Collection (Gyunggi prefecture, Korea), of which 500 strains were LAB isolated from *kimchi* by using MRS agar.

Animals

Male BALB/c mice (5 weeks old, 20–25 g) were supplied from the Orient Experimental Animal Breeding Center (Seoul, Korea). All animals were housed in wire cages at 20–22°C and 50 ± 10% humidity, fed standard laboratory chow (Orient Experimental Animal Breeding Center), and allowed water *ad libitum*. All experiments were performed in accordance with the NIH and Kyung Hee University guidelines for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University.

Isolation and Culture of Peritoneal Macrophages and Immunoblotting

Male ICR mice were intraperitoneally injected with 2 ml of 4% sodium thioglycolate solution [15]. Mice were sacrificed 4 days after injection. The peritoneal cavities were then flushed with 10 ml of RPMI 1640. The peritoneal lavage fluids were centrifuged at 500 ×g for 10 min and the cells were resuspended with RPMI 1640 and plated. After incubation for 2 h at 37°C, the cells were washed three

times and nonadherent cells were removed by aspiration. The cells were cultured in 24-well plates (0.5×10^6 cells/well) at 37°C in RPMI 1640 plus 10% FBS. The attached cells were used as peritoneal macrophages. To examine the immunomodulating effect of inactivated macrophages (heated at 80°C for 1 h), peritoneal macrophages were incubated in the absence or presence of lactic acid bacteria (1×10^5 CFU/0.1 ml) with or without LPS (50 ng/ml). Their cytotoxicities were measured using the crystal violet method.

For the immunoblot analyses of p-p65 and p65, the peritoneal macrophages were resuspended in 1 ml of RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. After centrifugation, the supernatant was used for the immunoblot assay. The proteins from collected cells were subjected to electrophoresis on 8–10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to nitrocellulose membrane. The p65, p-p65, and β -actin were assayed by immunoblotting [15].

Induction of Immunosuppression in Mice and Treatment with Test Agents

Mice were injected intraperitoneally (i.p.) with cyclophosphamide (150 mg/kg/day) twice every second day. Mice were divided into 9 groups (8 mice per group) 24 h after the final injection of cyclophosphamide and received *Lactobacillus plantarum* HY7712 (1×10^8 , 1×10^9 , or 1×10^{10} CFU), orally administered once a day for 5 or 15 days and levamisole (2.5 mg/kg) as a positive control or vehicle as a negative control.

Peripheral Erythrocyte, Leukocyte, Splenocyte, and Bone Marrow Cell Counts

For the assay of numbers of red blood cells (RBC) and white blood cells (WBC), blood was collected into tubes treated with heparin on the day of sacrifice.

For the isolation of splenocytes, the spleen was suspended with the aid of a glass homogenizer and filtered through a sieve mesh. Single-cell spleen suspensions were pooled in serum-free RPMI 1640 medium. Erythrocytes were then lysed with an ammonium chloride solution (0.15 M NH_4Cl , 10 mM KHCO_3 , 0.1 M EDTA, pH 7.2).

For the isolation of bone marrow cells (BMC), BMC suspensions were prepared by flushing a femur with serum-free RPMI 1640 medium. Total numbers of RBC, WBC, splenocytes, and BMC were counted under light microscopy.

Assay of Splenocyte Proliferation Induced by T Cell Mitogen Concanavalin A

Splenocytes were placed into 96-well flat-bottomed microplates in triplicate at 5×10^5 cells/well, and then 2.5 μg /well of concanavalin A was added to the wells. The cells were then incubated in a total volume of 200 μl /well. Serum-free RPMI 1640 medium was used as the control. The proliferated cells were counted by propidium iodide exclusion assay [15]. Thus, propidium iodide (1 $\mu\text{g}/\text{ml}$) was added to the culture medium for 5 min, collected, and then analyzed for propidium iodide exclusion with flow cytometry (BD Accuri C6 Cytometer, San Jose, MI, USA).

Preparation of Natural Killer and Cytotoxic T Cells from Splenocytes and Bone Marrows and Their Cytotoxicity Assays

Splenocytes were prepared as the effector cells for the activity assay of splenic natural killer (NK) and cytotoxic T lymphocytes, as

described previously [21, 30]. NK cells and cytotoxic T cells were isolated from splenocytes (1×10^7 cells/well) and bone marrow cells were prepared from mice by a NK cell and CD8⁺ T cell isolation kit (Miltenyi Biotec, Technology, Teterow, Germany), respectively.

The tumoricidal activity of the effector cells, NK cells, or cytotoxic T cells was evaluated by measuring the cytotoxicity against YAC-1 cells labeled with a Vybrant CFDA SE Cell Tracer kit according to the manufacturer's protocol (Invitrogen, Grand Island, NY, USA).

Effector cells (5×10^5 per well) in the 96-well microplates were co-cultured with target cells (5×10^5 per well) at an E/T ratio of 1:1 for 24 h. Then, MgCl₂ was added at the final concentration of 1 mM. The cells were washed and stained with propidium iodide and analyzed by flow cytometry. The level of cytotoxicity was determined as the number of CFSE⁺ cells stained with propidium iodide minus the background level by flow cytometry.

Phagocytosis Assay of Peritoneal Macrophages

Peritoneal macrophages were prepared as previously described [15]. The phagocytosis activity of macrophages was measured by the neutral red uptake method as previously described [28]. Peritoneal exudates were harvested at 96 h after mice were intraperitoneally injected with 2 ml of 4% thioglycollate. The cells were cultured in RPMI 1640 with 10% fetal calf serum at 37°C for 20 h (humidified atmosphere of 5% CO₂). The following day, all nonadherent cells were removed by washing with PBS. Adherent cells were detached using 10 mM EDTA in PBS and seeded at a density of 5×10^5 cells/well in the 24-well microplates with complete RPMI 1640 medium and cultured for 24 h. The cells were washed, and neutral red (50 µg/ml) was added and incubated for 3 h, washed to remove the excess dye, and blotted dry. The cells were resuspended in 50% ethanol containing 1% glacial acetic acid and the absorbance at 540 nm was measured.

Determination of Cytokines

To measure IL-2 and IFN-γ by ELISA, the splenocytes were stimulated with concanavalin A (5 µg/ml) or LPS (10 µg/ml) in 96-well plates according to Won *et al.* [29]. The supernatants were harvested at 24 h and then transferred to 96-well ELISA plates. IL-2 and IFN-γ concentrations were determined using commercial ELISA kits (Pierce Biotechnology, Rockford, IL, USA) [16].

Statistics

All data were expressed as the mean ± standard deviation, with statistical significance analyzed using one-way ANOVA followed by a Student–Newman–Keuls test.

RESULTS

Isolation of Immunopotentiating LAB from *Kimchi*

To isolate immunopotentiating LAB, we selected LAB from *kimchi*, a Korea fermented food, and measured their TNF-α productivity and NF-κB activation in peritoneal macrophages stimulated with or without LPS. Of the LAB, we selected *L. plantarum* HY7712, which potently induced TNF-α expression in peritoneal macrophages, but inhibited NF-κB activation in LPS-stimulated peritoneal macrophages (Fig. 1).

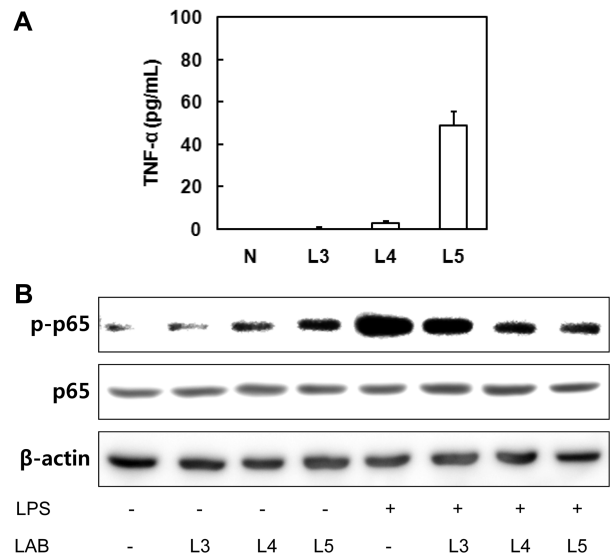


Fig. 1. Effect of *Lactobacillus plantarum* HY7712 on the expression of TNF-α (A) and the activation of NF-κB on peritoneal macrophages stimulated with or without lipopolysaccharide (LPS) (B).

The peritoneal macrophages were incubated with heat-inactivated *L. plantarum* HY7712 (N, vehicle alone; L3, 1×10^3 CFU/ml; L4, 1×10^4 CFU/ml; L5, 1×10^5 CFU/0.1 ml) in the absence or presence of LPS (50 ng/ml) for 90 min (for the assay of NF-κB) or 24 h (for the assay of TNF-α). Protein expression of levels p-p65, p65, and β-actin were assayed by immunoblotting and those of TNF-α by ELISA. * $p < 0.05$ vs. normal group.

Effect of *L. plantarum* HY7712 on Peripheral RBC, WBC, NK, and T Cells in Cyclophosphamide-Treated Mice

To evaluate the immunopotentiating effect of HY7712 *in vivo*, we administered it to CP-immunosuppressed mice and measured the weights of the body and spleen and the numbers of peripheral RBC and WBC, splenocytes, and BMC (Fig. 2). Treatment with CP significantly reduced body weight to 78.1% and 80.9% of the normal group on days 5 and 15 post treatment, respectively. However, treatment with HY7712 (1×10^{10} CFU) reversed the body weight to 86.6% and 86.0% of the normal group on days 5 and 15, respectively. Treatment with CP also reduced the numbers of RBC and WBC to 77.1% and 55.8% of the normal group on day 5 after CP treatment, respectively, and reduced them to 97.9% and 76.6% of the normal group on day 15. Treatment with HY7712 reversed the numbers of CP-reduced RBC and WBC to 77.3% and 81.6% of normal mice on day 5 after CP treatment in CP-treated mice, respectively, and reversed them to 100.3% and 93.1% of normal mice on day 15, respectively. Treatment with HY7712 reversed the numbers of CP-reduced splenic T and NK cells and bone marrow T and NK cells to normal mice on day 15. The reversing effect of HY7712 in CP-immunosuppressed mice was comparable to that of levamisole, which is used commercially to increase the numbers of RBC and WBC in cancer drugs-treated patients [22]. No severe symptoms,

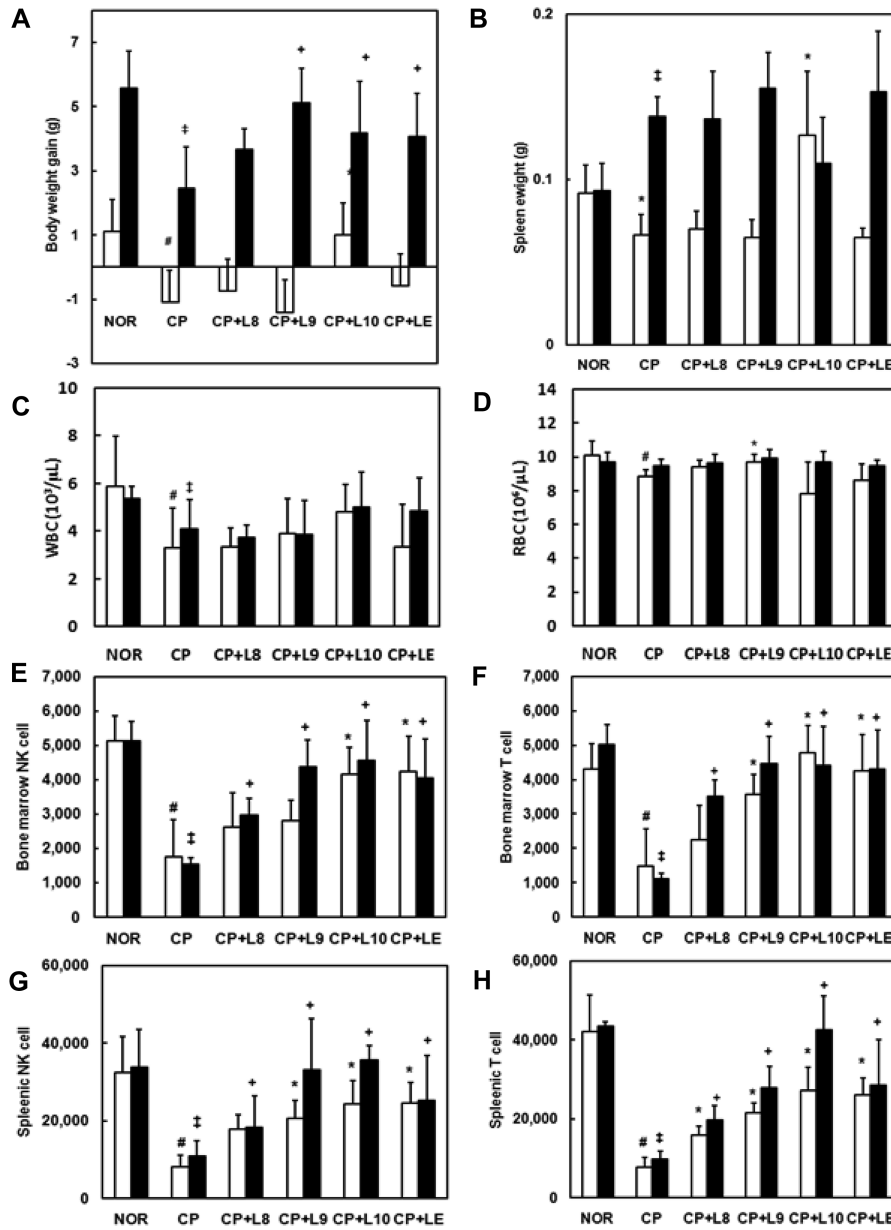


Fig. 2. Effect of orally administered *Lactobacillus plantarum* HY7712 on the weights of body (A) and spleen (B) and numbers of white blood cells (WBC, $\times 10^3/\mu\text{l}$) (C) and red blood cells (RBC, $\times 10^6/\mu\text{l}$) (D), bone marrow NK (E) and T cells (F), and splenic NK (G) and T cells (H) and in CP-immunosuppressed mice.

The mice were sacrificed on days 5 (white bar) and 15 (black bar) after treatment with CP. Data were expressed as mean \pm SD of six mice. NOR, normal mice; CP, treated with CP alone; CP+L8, treated with 1×10^8 CFU of HY7712 in the presence of CP; CP+L9, treated with 1×10^9 CFU of HY7712 in the presence of CP; CP+L10, treated with 1×10^{10} CFU of HY7712 in the presence of CP; CP+LE, treated with levamisole hydrochloride in the presence of CP. # and †, $p < 0.05$ vs. normal group on days 5 and 15, respectively. * and †, $p < 0.05$ vs. CP-alone treated group on days 5 and 15, respectively.

such as death, bleeding, and diarrhea, were observed in HY7712-treated mice during these experiments.

Effect of HY7712 on ConA-Induced Lymphocyte Proliferation

We measured the effect of HY7712 on concanavalin A-induced lymphocyte proliferation in the splenocytes of CP-

immunosuppressed mice (Fig. 3). Treatment with CP reduced markedly the proliferative responses of lymphocytes to ConA, a T cell mitogen, on days 5 and 15 in CP-treated mice, to 41.3% and 53.7% of the normal group, respectively. Treatments with HY7712 (1×10^{10} CFU) increased concanavalin A-induced T cell proliferation to 80.3% and 84.5% of the normal group on days 5 and 15, respectively, whereas the

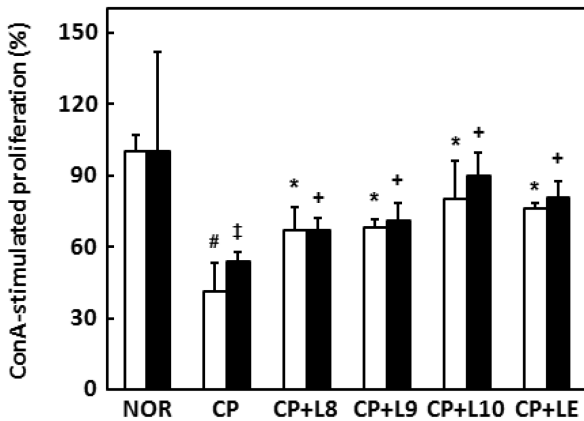


Fig. 3. Effect of orally administered *Lactobacillus plantarum* HY7712 on concanavalin A-induced cytotoxic T cell proliferation in the splenocytes of CP-immunosuppressed mice.

The mice were sacrificed on days 5 (white bar) and 15 (black bar) after treatment with CP and the splenocytes were isolated. Data were expressed as mean \pm SD of six mice. NOR, normal mice; CP, treated with CP alone; CP+L8, treated with 1×10^8 CFU of HY7712 in the presence of CP; CP+L9, treated with 1×10^9 CFU of HY7712 in the presence of CP; CP+L10, treated with 1×10^{10} CFU of HY7712 in the presence of CP; CP+LE, treated with levamisole hydrochloride in the presence of CP. [#] and [‡], $p < 0.05$ vs. normal group on days 5 and 15, respectively. * and ⁺, $p < 0.05$ vs. CP-alone treated group on days 5 and 15, respectively.

T cell proliferation in the vehicle groups was significantly lower than the normal group.

Next, we measured the effect of HY7712 on the expression of immunomodulating cytokines, IL-2 and IFN- γ , in ConA-stimulated splenocytes isolated from CP-treated mice (Fig. 4). Treatment with CP significantly reduced the expression of IL-2 and IFN- γ by 72.0% and 47.8%, respectively. However, treatment with HY7712 in CP-treated mice reversed their expressions to 46.1% and 87.3% of the normal mice.

Effect of HY7712 on Macrophage Phagocytosis in CP-Treated Mice

The effect of HY7712 on the phagocytosis of macrophages isolated from mice was measured by the neutral red uptake method (Fig. 5). Treatment with CP in mice significantly reduced the phagocytosis of macrophages. However, treatment with HY7712 or levamisole significantly restored the CP-impaired phagocytosis of macrophage. The effect of HY7712 was comparable to that of levamisole.

Effect of HY7712 on the Cytotoxicity of NK and Cytotoxic T Cells in CP-Treated Mice Against YAC-1 Cells

We orally administered HY7712 to CP-treated mice, isolated the NK and cytotoxic T cells from the spleen and bone marrow and measured their cytotoxicities against YAC-1 cells (Fig. 6). Treatment with CP alone significantly reduced the NK cell and cytotoxic T cell cytotoxicities on days 5 and 15, compared with normal mice. Treatment with

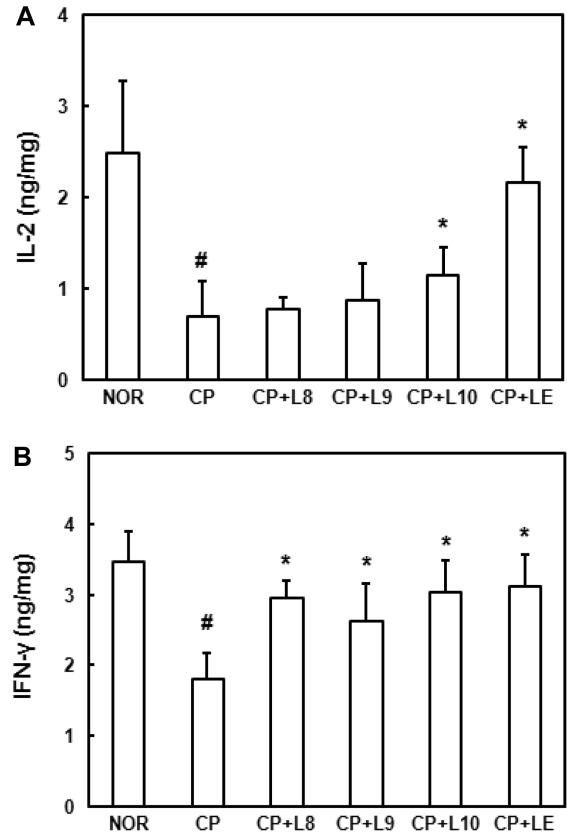


Fig. 4. Effect of orally administered *Lactobacillus plantarum* HY7712 on IL-2 (A) and IFN- γ cytokines (B) secreted in concanavalin A-treated splenic cytotoxic T cells of CP-immunosuppressed mice.

Mice were orally administered *L. plantarum* HY7712 for 15 days. Splenocytes were cultured with concanavalin A (5 μ g/ml) for 24 h. Cytokine levels in the supernatant were measured by ELISA. NOR, normal mice; CP, treated with CP alone; CP+L8, treated with 1×10^8 CFU of HY7712 in the presence of CP; CP+L9, treated with 1×10^9 CFU of HY7712 in the presence of CP; CP+L10, treated with 1×10^{10} CFU of HY7712 in the presence of CP; CP+LE, treated with levamisole hydrochloride in the presence of CP. Data are shown as means \pm SD (n = 6). [#] $p < 0.05$ vs. normal group. * $p < 0.05$ vs. CP-alone treated group.

HY7712 restored the cytotoxicity of NK and cytotoxic T cells derived from spleen against YAC-1 cells to 98.2% and 99.1% of the normal mice on day 5, and 98.1% and 99.6% on day 15, respectively. The cytotoxicity of NK and cytotoxic T cells from bone marrow that was reduced by CP was reversed to 98.9% and 100.2% of the normal mice on day 5, respectively, and 99.0 and 98.1% of normal mice on day 15, respectively. The effect of HY7712 was comparable to that of levamisole.

DISCUSSION

In the present study, treatment with cyclophosphamide (300 mg/kg, i.p.) in mice caused immunosuppression, markedly

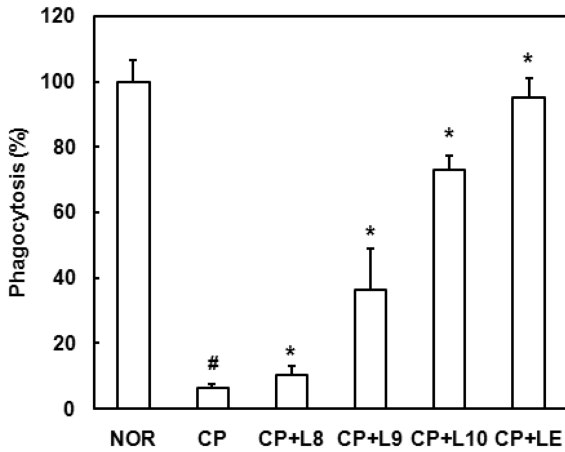


Fig. 5. Effect of orally administered *Lactobacillus plantarum* HY7712 on macrophage phagocytosis indicated by neutral red assay in CP-treated mice.

The mice were sacrificed on day 15 and then peritoneal macrophages were isolated. NOR, normal mice; CP, treated with CP alone; CP+L8, treated with 1×10^8 CFU of HY7712 in the presence of CP; CP+L9, treated with 1×10^9 CFU of HY7712 in the presence of CP; CP+L10, treated with 1×10^{10} CFU of HY7712 in the presence of CP; CP+LE, treated with levamisole hydrochloride in the presence of CP. Data are shown as means \pm SD (n = 6). # $p < 0.05$ vs. normal group. * $p < 0.05$ vs. CP-alone treated group.

reducing the number of peripheral RBC, WBC, splenocytes, and bone marrow cells, and inhibiting lymphocyte proliferative responses to T mitogen, and NK cell activity. These results are consistent with previously reported studies [2, 12, 30]. Therefore, the clinical use of cyclophosphamide is limited by its immunosuppressing activity. To overcome this, levamisole with cyclophosphamide has been used to reduce severe side effects [1, 17, 23].

LAB, such as *L. acidophilus* and *Bifidobacterium bifidum*, have been shown to influence one or several components of humoral, cellular, or nonspecific immunity [9, 10, 18, 20]. For example, *L. casei*, *L. acidophilus*, and yogurt enhanced the number of IgA-producing plasma cells in a dose-dependent manner in animals and humans. LAB also increased splenocyte proliferation in response to mitogens for T and B cells in mice and the cytokine production of TNF- α , IL-1 β , IL-6, and IFN- γ in immune cells [29]. Therefore, we tested the ability of lactic acid bacteria to improve cyclophosphamide-induced immunosuppression in mice.

First, we selected HY7712, which induced TNF- α production via NF- κ B activation in the macrophage, but inhibited NF- κ B

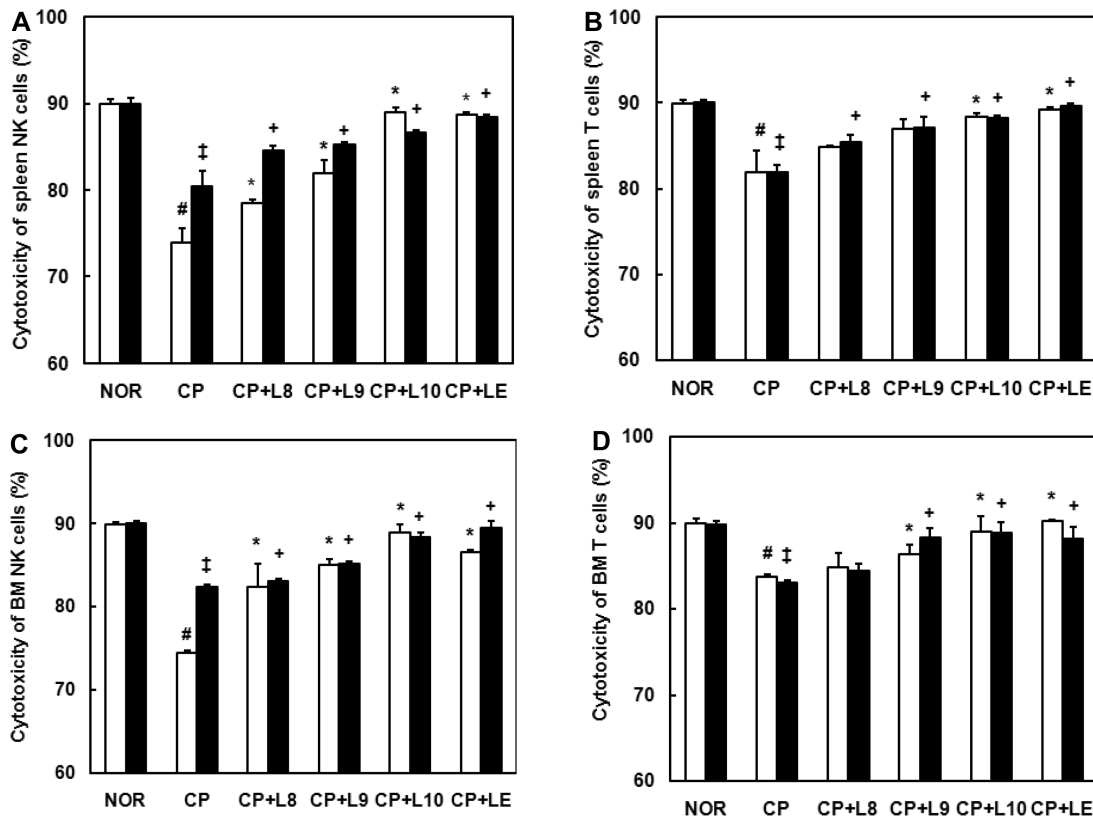


Fig. 6. Effect of orally administered *Lactobacillus plantarum* HY7712 on the cytotoxicities of splenic NK (A) and cytotoxic T cells (B), and bone marrow NK (C) and cytotoxic T cells (D) in CP-treated mice on day 5 (white bar) and 15 (black bar) against YAC-1 cells.

Effectors:targets = 1:1. NOR, normal mice; CP, treated with CP alone; CP+L8, treated with 1×10^8 CFU of HY7712 in the presence of CP; CP+L9, treated with 1×10^9 CFU of HY7712 in the presence of CP; CP+L10, treated with 1×10^{10} CFU of HY7712 in the presence of CP; CP+LE, treated with levamisole hydrochloride in the presence of CP. Data are shown as means \pm SD (n = 6). # $p < 0.05$ vs. normal group. * $p < 0.05$ vs. CP-alone treated group.

activation in LPS-stimulated macrophages, and investigated its immunopotentiating effect in cyclophosphamide-immunosuppressed mice. HY7712 enhanced the activity of immunological effector cells and hematopoiesis in cyclophosphamide-immunosuppressed mice. Thus, HY7712 accelerated the recovery of RBC and WBC in the blood, and NK cells and CTL in the spleen and bone marrow, and enhanced CTL cytotoxicity and NK cell activity against tumor cells, and augmented macrophage phagocytosis. None of the mice treated with HY7712 died, nor did their body weights change significantly during the experiment period. Thus, *in vivo* treatment with HY7712 accelerates the recovery of immunosuppressed mice from leukopenia, myelosuppression, and immunosuppression, the common conditions associated with cancer chemotherapy. These findings suggest that HY7712 affords the greatest protection for immune effector cells and hematopoietic progenitors against cyclophosphamide-induced immunosuppression.

HY7712 promoted ConA-induced Tc lymphocyte proliferation, as previously report by Won *et al.* [29]. HY7712 also significantly increased IL-2 production from ConA-stimulated splenocytes (Fig. 3A). IL-2 is a necessary cytokine for the survival and proliferation of T lymphocytes. Therefore, IL-2 secretion increased by HY7712 may induce T lymphocyte proliferation and IFN- γ production, which could enhance the immune response against cancer and/or pathogen-infected cells. IL-2 is an inducer for natural killer cell activation, which inhibits the growth and metastases of tumors [13]. Tc and NK cells isolated from spleens and bone marrows of mice fed HY7712 exhibited more potent cytolytic activity against target YAC-1 cells than those from control mice. Thus, HY7712 accelerated the recovery of cytotoxic T and NK cell activities, which have been shown to play a role in immunoregulation and tumor surveillance as antitumor effector cells [27, 30], in cyclophosphamide-treated mice.

Based on these findings, HY7712 may accelerate the recovery of cyclophosphamide-caused immunosuppression, without evident side effects, by immunopotentiating cytotoxic T and NK cells, and may provide a mechanistic basis for using HY7712 as an alternative means in lessening chemotherapy-induced immunosuppression in cancer patients.

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