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Lactobacillus plantarum HY7712 Protects Against the Impairment of NK-Cell Activity Caused by Whole-Body γ-Irradiation in Mice

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While searching for lactic acid bacteria that can restore aging-impaired immune responses, we isolated the Toll-like receptor (TLR) 2/NF-κB-activating strain *Lactobacillus plantarum* HY7712 from *kimchi* and investigated its immunomodulating effect in whole-body γ-irradiated mice. Exposure to HY7712 strongly activated NF-κB signaling in RAW264.7 cells, but inhibited lipopolysaccharide-stimulated NF-κB activation. Moreover, HY7712 protected against the downregulation of interferon (IFN)-γ and upregulation of interleukin (IL)-13 caused by γ-irradiation in mice. In mice, γ-irradiation impaired NK-cell activity against YAC-1 tumor cells, but following HY7712 exposure, the activity of NK cells was restored to 91.5% of the level measured in control mice ($p < 0.05$). These findings suggest that HY7712 activates the TLR2/NF-κB signaling pathway and protects against the impairment of NK-cell activity caused by γ-irradiation or aging.

Keywords: *Lactobacillus plantarum* HY7712, γ-irradiation, aging, NK cell activity

γ-Irradiation induces the preferential differentiation of helper T cells into Th2 cells rather than Th1 cells [17]: it reduces the levels of interleukin (IL)-12 and interferon (IFN)-γ in the serum. Expression of Th1 cytokines such as IFN-γ has been shown to be markedly reduced in whole-body γ-irradiated mice, resulting in a Th1/Th2 imbalance, as well as the impairment of NK cells, like aged mice [9, 17, 18]. Aging also causes the decline of the immune response, thus a shift toward to a dominance of Th2-related response [16].

Lactic acid bacteria (LAB) are safe microorganisms that repair disturbances of indigenous microflora [1, 12] and induce nonspecific activation of the host immune system [1, 3]. For example, *Lactobacillus plantarum* stimulates monocytes/macrophages *via* Toll-like receptor 2 (TLR2) to produce IL-12 and IFN-γ, which promote the differentiation of naive Th lymphocytes towards Th1 subsets [7, 8]. We also found that *Lactobacillus plantarum* HY7712 restored activity of NK cells and T cells in cyclophosphamide-immunosuppressed mice [5]. However, studies on LAB restoring the immune system impaired by aging have not been performed thoroughly.

Therefore, to search LAB restoring the aging-impaired immune responses, we investigated first the ability of LAB isolated from Chinese cabbage *kimchi* to activate TLR2/NF-κB signaling and then its immune-restoring effect in whole-body γ-irradiated mice.

For the transfection of pNiFty-luc and the assay of luciferase activity, RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Invitrogen Co., Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin, and then the cells (1×10^5 cells) were transiently transfected with the pNiFty-luc (InvivoGen, San Diego, CA, USA)

luciferase reporter plasmid (24 μg/plate) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Two hundred LAB were isolated from Chinese cabbage *kimchi* according to the previous method of Lee *et al*. [10].

Male C57BL/6 mice (23–26 g, 8 weeks old) were supplied by the Central Laboratory Animal Inc. (Seoul, Korea). Mice were divided into 4 groups; 2 sham groups treated with or without HY7712 (1×10^9 CFU/day) and 2 γ-irradiated groups with or without HY7712. Each group consisted of 5 mice. All mice were individually housed and maintained under controlled conditions of humidity (50 ± 10%), light (12/12 h light/dark cycle), and temperature (25 ± 2°C), fed on standard laboratory chow (Samyang, Seoul, Korea) and permitted *ad libitum* access to water. The γ-irradiation (3 Gy) was treated after the final administration of HY7712. A γ-ray generator (IBL 147 C; CIS Bio-International, France) was used for generating irradiation (^{137}Cs, 0.8 Gy/min). Non-irradiated mice were maintained under the same condition except γ-irradiation. The γ-irradiated mice were returned to the cage and sacrificed 3 days after the irradiation. The blood samples were taken from the inferior vena cava. All experiments were performed in accordance with the NIH and Kyung Hee University guides for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in

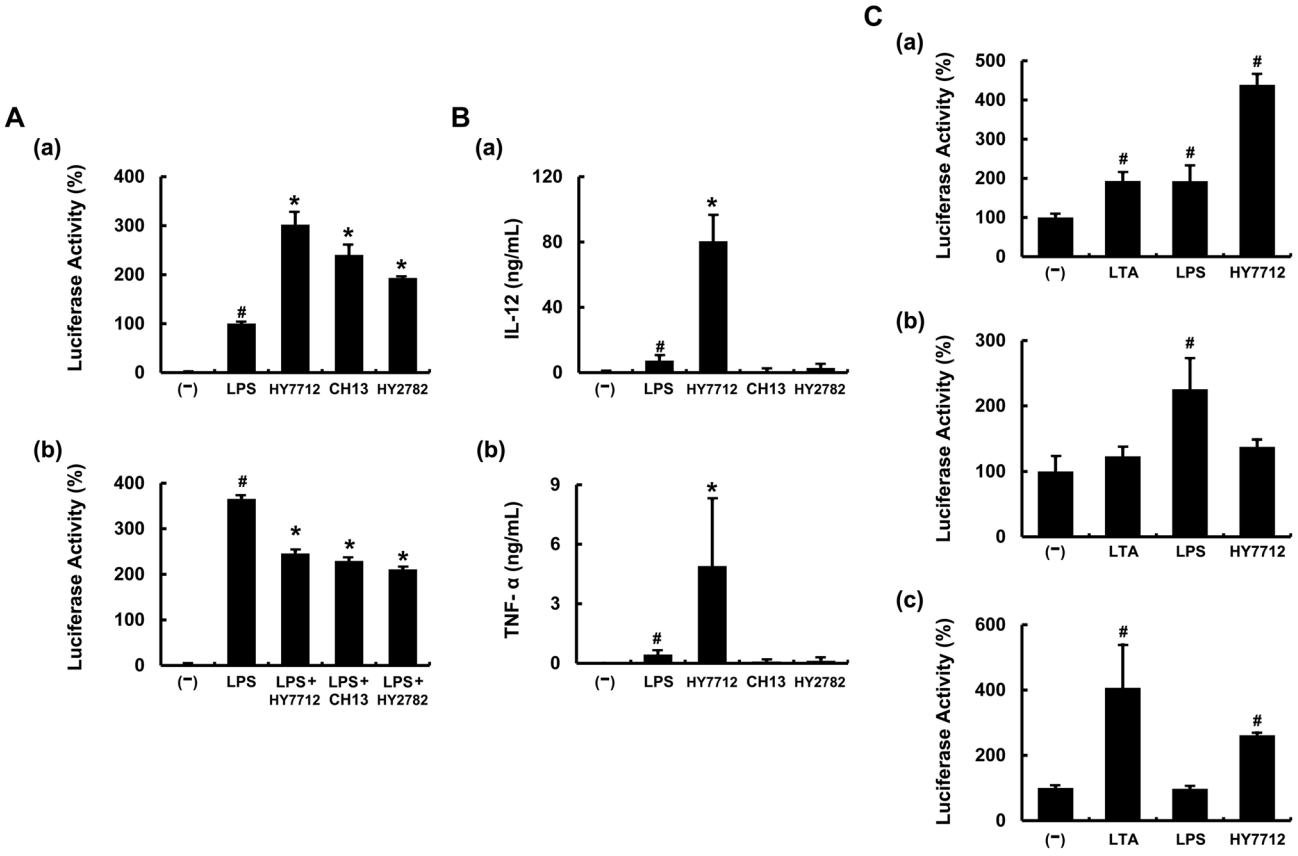

Fig. 1. Immunomodulating effects of *Lactobacillus* strains isolated from *kimchi* on RAW264.7 cells.

(**A**) The effect of 3 *Lactobacillus* strains isolated from fermented foods on the activation of NF-κB signaling in pNiFty-luc-transfected RAW264.7 cells. (**B**) Expression of IL-12 and TNF-α in PBMCs. (**C**) NF-κB activation in psiTLR2- and psiTLR4-transfected RAW264.7 cells. (**A**) The pNiFty-luc-transfected RAW264.7 cells (1×10^5 cells/well) were incubated with heat-inactivated lactic acid bacteria (1×10^6 CFU/ml) in the absence (a) or presence (b) of lipopolysaccharides (LPS, 1 μg/ml) for 24 h and then luciferase activity was assayed using the Bright-Glo Luciferase Assay System purchased from Promega (Madison, WI, USA). (**B**) The levels of IL-12 (a) and TNF-α (b) were analyzed using the Bio-plex assay system. (**C**) RAW264.7 cells (1×10^5 cells/well) were transfected with pNiFty-luc alone (a), pNiFty-luc and psiTLR2 (b), or pNiFty-luc and psiTLR4 (c) and then incubated with HY7712 (1×10^6 CFU/ml) or with HY7712 lipoteichoic acid (LTA, 1 μg/ml; Invivogen) in the absence or presence of LPS (1 μg/ml) for 24 h, after which luciferase activity was measured. The cells were transfected with siRNAs (100 nM) targeting TLR2 or TLR4 by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Each value indicates the mean ± SD (n = 5); $^{\#}$Significantly different vs. control group ($p < 0.05$), and *significantly different vs. group treated with LPS alone ($p < 0.05$).

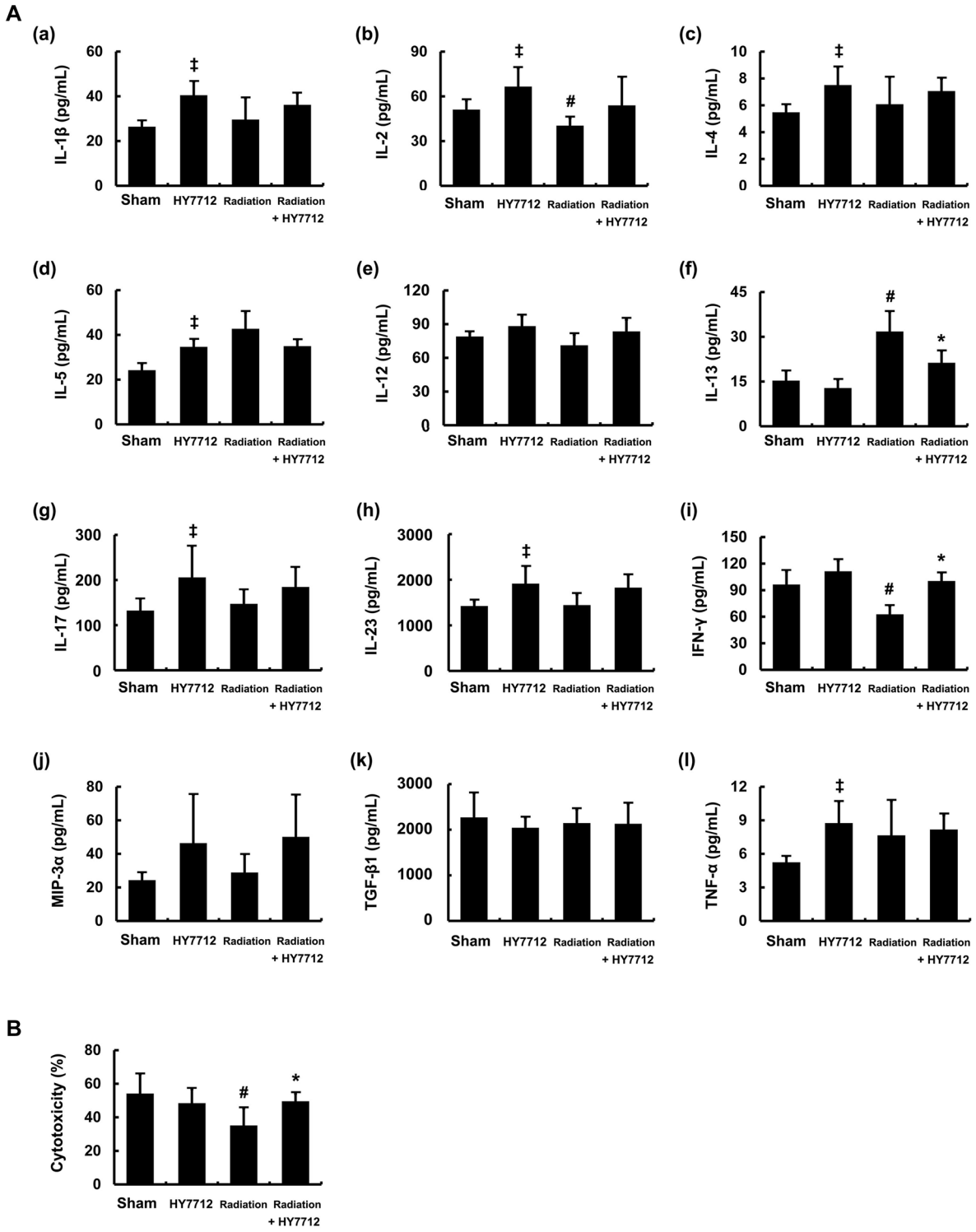

Fig. 2. Effect of *L. plantarum* HY7712 on blood cytokine expression (**A**) and NK-cell activity against YAC-1 cells (**B**) in control and γ-irradiated mice.

Blood cytokine levels were measured using the Quantibody array kit. *L. plantarum* HY7712 suspended in sterilized PBS was orally administered once a day (1×10^9 CFU/day) for 3 weeks. Mice in the control group were administered the vehicle alone. The cytotoxic activity of NK cells against YAC-1 cells was measured at an effector-to-target ratio of 50:1. Each value indicates the mean ± SD ($n = 5$). ‡Significantly different vs. sham-treated control group ($p < 0.05$). #Significantly different vs. sham-treatment group ($p < 0.05$). *Significantly different vs. γ-irradiation-treated group ($p < 0.05$).

the Korea Yakult Institutional Animal Use Committee (#KYIACUC-11-01-0311-Y).

The amount of blood cytokines were measured by using the Quantibody Mouse TH17 Array from Ray Biotech (Norcross, GA, USA). The amount of IL-12 and tumor necrosis factor (TNF)-α in the supernatant of LAB or LPS-stimulated human peripheral blood mononuclear cells (PBMC) were determined by the Bio-plex assay system (Bio-Rad, Hercules, CA, USA). NK-cell activity was measured by the previously reported flow cytometry method [17].

All data are expressed as the means ± standard deviation (SD), with the statistical significance between groups analyzed using the unpaired Student's *t*-test. A p value <0.05 was considered statistically significant.

When NF-κB-activating activities of 200 LAB isolated from *kimchi* were measured in RAW264.7 cells stimulated with or without LPS, *L. plantarum* HY7712 most potently induced NF-κB activation in RAW264.7 cells stimulated without LPS, but inhibited NF-κB activation in LPS-stimulated cells (Fig. 1A). HY7712 also induced the expression of IL-12 and TNF-α, the transcription factor of which is NF-κB, in human PBMCs (Fig. 1B).

To confirm whether HY7712 activates NF-κB *via* TLR2, we prepared psiTLR2- or psiTLR4-transfected RAW264.7 cells and investigated the effects of LPS or HY7712 on NF-κB activation in these cells (Fig. 1C). LPS activated NF-κB in wild-type and psiTLR2-knockout cells, but did not activate it in psiTLR4-knockout cells. HY7712 activated NF-κB in wild-type and psiTLR4-knockout cells, but did not activate it in psiTLR2-knockout cells.

Next, we orally administered HY7712 to mice for 3 weeks, exposed the whole body to γ-irradiation, and measured the body weight and blood cytokine levels. Body weight was not influenced by treatment with HY7712 (data not shown). However, treatment with HY7712 in sham normal control mice increased the blood levels of IL-1β, IL-2, IL-5, IL-17, IL-23, and TNF-α, but did not affect the levels of IL-12, MIP-3α, and transforming growth factor (TGF)-β1 (Fig. 2A). γ-Irradiation reduced IFN-γ expression to 65.1% of the sham group, but upregulated IL-13 expression by 2.1-fold. Treatment with *L. plantarum* HY7712 protected the downregulation of IFN-γ expression by γ-irradiation to 104.1% of sham normal control, but inhibited the expression of IL-13 by 33.1%. γ-Irradiation reduced splenic NK-cell activity to 64.8% of the sham normal control mice (Fig. 2B). Treatment with *L. plantarum* HY7712 significantly protected the reduction of NK-cell activity by irradiation to 91.5% of the sham normal control group, whereas it did not induce NK-cell activity in the sham normal mice.

Signaling by TLRs is closely linked to the immune-system gene expression that is regulated through the activation of the NF-κB pathway [11]. Certain *Lactobacillus* strains induce the production of IL-12 by activating the TLR2/NF-κB signaling pathway. The lipoteichoic acids present in these bacteria induce the expression of Type I cytokines such as IL-12, IL-18, TNF-α, and IFN-γ [4, 6, 14, 15]. In this study, we determined that *Lactobacillus plantarum* HY7712 activated the TLR2/NF-κB signaling pathway in RAW264.7 cells as described before and induced the expression of IL-12 and TNF-α in PBMCs, similarly as other *Lactobacillus* strains reported previously [8]. Furthermore, administering HY7712 orally to mice upregulated the expression levels in their blood of IL-1β, IL-2, IL-5, IL-17, IL-23, and TNF-α, but did not influence the expression of IL-12, IL-13, MIP-3α, TGF-β, and IFN-γ. These results suggest that HY7712 stimulates the immune system, at least in part, by affecting the expression of Type I and Type II cytokines such as IL-2 and IL-5. Whole-body γ-irradiation in mice upregulated the expression in blood of IL-5 and IL-13, but downregulated the expression of blood IFN-γ. However, exposure of mice to *L. plantarum* HY7712 inhibited the increase in blood IL-13 expression and the reduction in IFN-γ expression caused by γ-irradiation, but did not affect IL-5 expression. Although whole-body γ-irradiation induced a shift toward Th2 dominance in the Th1/Th2 immune balance, as observed in aged mice [9], exposure to *L. plantarum* HY7712 strongly protected against this shift in the immune balance. Furthermore, HY7712 attenuated the impairment of NK-cell activity caused by γ-irradiation, which could be because of the activation of the TLR2/NF-κB signaling pathway and the induction of IFN-γ expression resulting from HY7712 exposure. Administering HY7712 also induced the expression of IL-17 and IL-23 in sham-treated control mice, as reported previously for another *Lactobacillus* strain [2]. However, HY7712 did not protect against γ-irradiation-induced reduction in IL-17 and IL-23 expression, which suggests that HY7712 did not regulate Th17 cells in immunosuppressed hosts.

Based on these findings, we conclude that HY7712 activates the TLR2/NF-κB signaling pathway and protects against the impairment of NK-cell activity caused by γ-irradiation or aging.

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