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Immune-enhancing effects of β-lactoglobulin glycated with lactose following in vitro digestion on cyclophosphamide-induced immunosuppressed mice

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ABSTRACT

β-Lactoglobulin (β-LG) is a major milk protein, making up more than 53% of the total whey proteins, and is seen as a valuable ingredient in food processing because of its high essential amino acid content and diverse functional applications. The Maillard reaction can occur during the storage and processing of food and generate various beneficial effects, including anti-allergenicity, antioxidant, and immunomodulatory effects. The addition of an β-LG-lactose conjugate (LGL) produced by the Maillard reaction was shown to have a strong immune-enhancing effect, increasing both nitric oxide generation and cytokine expression through activation of RAW 264.7 cells, even after in vitro digestion. Furthermore, daily LGL administration resulted in the upregulation of several immune markers in a cyclophosphamide-induced immunosuppressive mouse model, indicating that this treatment stimulates multiple immune cells, including macrophages, natural killer cells, and lymphocytes, enhancing the proliferation and activation of both the innate and adaptive immune responses. Taken together, these findings indicate that consuming LGL on a regular basis can improve immunity by increasing the natural production of various immune cells.

Key words: Maillard reaction, beta-lactoglobulin, in vitro digestion, immunomodulatory, cyclophosphamide

INTRODUCTION

Milk proteins are known for their high nutritional value in the food industry as a key source of protein (Villa et al., 2018), whereas milk-derived allergens, such as casein, can limit their use in food products (Li et al., 2021; Villa et al., 2021). During the heat treatment process (i.e., boiling, sterilization, and drying), chemical changes in milk can occur, which can result in a nonenzymatic browning reaction between sugar and protein known as the Maillard reaction (**MR**; van Lieshout et al., 2020). It has been reported that MR decreases the total allergenicity of β-LG (Perusko et al., 2018; Yang et al., 2020) and generates various benefits including increasing its byproducts' antioxidant and immunomodulatory activities (Yáñez et al., 2018; Kim et al., 2019; Liu et al., 2019; Chun et al., 2020; Xiang et al., 2021). It is also worth mentioning, however, that the MR can lead to the development, by oxidative stress and diabetes (Nooshkam et al., 2020), of advanced glycation end products (**AGE**) such as methylglyoxal-derived AGE. Therefore, the amount of AGE must be minimized.

β-Lactoglobulin, a major whey protein in milk, accounting for more than 53% of whey proteins (Tsermoula et al., 2021), is an important component in food processing due to its high EAA content and functionality, with proven uses in the production of emulsions, foams, and gels (Liu et al., 2019; Nastaj and Sołowiej, 2020; Quevedo et al., 2020; Tsermoula et al., 2021). Previous research has shown that Maillard reaction products (**MRP**) derived from whey proteins have immune-enhancing properties, raising nitric oxide (**NO**) and pro-inflammatory cytokine production through stimulation of RAW 264.7 macrophages (Chun et al., 2016) and that MR-conjugate (**LGL**), derived from β-LG and lactose, is a major immune-enhancing active constituent derived from whey protein (Chun et al., 2020).

Modern humans are vulnerable to an environment that can gradually weaken their immune systems as a result of emerging virus-related infections, drug side effects, and environmental pollution. Furthermore, there is a growing concern in improving immune system preservation for ever-increasing human life span (Lee and Lawrence, 2018; Yang et al., 2019). This indicates that numerous researchers have continued to investigate substances for improving immunity (Yun et al., 2018; Monmai et al., 2019; Yang et al., 2019; Yoo et al., 2019).

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In this study, the immune-enhancing effects of the digested LGL samples were determined using a RAW 264.7 macrophage model after the food-derived MRP from LGL were digested using in vitro human digestion. Furthermore, following oral administration of the LGL, cyclophosphamide (**CY**)-induced immunosuppressive mice showed enhanced growth and activities of peritoneal macrophages, splenic natural killer (**NK**) cells, and splenic lymphocytes.

MATERIALS AND METHODS

Materials and Reagents

The sources of the purchased materials were as follows: concanavalin A (**Con A**), CY, Hanks' balanced salt solution, lactose, β-LG (L-3908), LPS, *o*-phtaldialdehyde (**OPA**), disodium tetraborate decahydrate, DL-dithiothreitol, L-leucine, and neutral red were from Sigma-Aldrich; EZ-Cytox cell viability assay kit was from DoGen; EZ-LDH cytotoxicity assay kit was from Biomax; the murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection; the mouse T cell lymphoma cell line YAC-1 was from the Korean Cell Line Bank; the RNAiso Plus reagent was from TaKaRa Bio; the 2X SYBR Green RT-PCR master mix was from Invitrogen; and the bicinchoninic acid protein assay kit, Roswell Park Memorial Institute-1640 medium (RPMI; phenol red free), Dulbecco's modified Eagle's medium (4.5 g/L glucose), fetal bovine serum (**FBS**), and antibiotics were from Gibco (Thermo Fisher Scientific).

Preparation of MRP Conjugate, LGL Derived from MR Between β-LG and Lactose

β-Lactoglobulin was pretreated to reduce intrinsic endotoxin levels as described by Chun et al. (2020). Next, the β-LG (5 mg) was mixed with 10 mg of lactose in 0.1 *M* sodium phosphate buffer (pH 7.4) and heated in a shaking water bath at 55°C for 24 h with agitation at 60 rpm. After dialysis using 3.5 kDa molecular weight cut-off tubing (Spectrum Medical Industries), these samples were lyophilized and the protein content in the final product, LGL was measured using a bicinchoninic acid protein assay kit. Following that, the samples were used in in vitro digestion and animal feeding.

The degree of glycation was measured by measuring fluorescence intensity (excitation 370 nm/emission 440 nm) and comparing 12% SDS-PAGE gel bands according to method by Chun et al. (2020). On the SDS-PAGE gel, 16 and 0.4 μg of protein were added per lane for staining with Coomassie brilliant blue G-250 and silver, respectively.

Experimental in Vitro Digestion

Simulated Gastrointestinal Digestion of LGL. The LGL was digested using simulated digestive juices, which were generated by incorporating enzymes and proteins into solutions and adjusting the pH as previously defined (Lee et al., 2018). The LGL was artificially digested by incubating it with artificial saliva, gastric juice, duodenal juice, and bile in the following order at 36.5°C and a moderate agitation rate (55 rpm). First, oral digestion was simulated by incubating LGL with 4 mL of saliva for 5 min, followed by gastric digestion by inserting 8 mL of gastric juice and incubating this solution for another 2 h. These samples were then mixed with 8 mL of duodenal juice and 4 mL of bile for 2 h to simulate intestinal digestion. After that, the supernatant of each fraction was obtained by centrifugation at 1,146 × *g* at 4°C for 10 min to yield **O** (incubated with saliva), **OG** (incubated with saliva and gastric juice), and **OGI** (incubated with saliva, gastric juice, and duodenal juice/bile). Using a 20% acrylamide gel, SDS-PAGE was used to determine the level of LGL degradation. Each digested fraction was then diluted in 0.1 *M* sodium phosphate buffer (pH 7.4), and 16 μg of each digested fraction was loaded onto the PAGE gels. After electrophoresis, the gel was stained with Coomassie brilliant blue G-250. The degree of hydrolysis (**DH**) was measured using the OPA assay modified by Adler-Nissen (1979). Each digested fraction was mixed with OPA reagent (160 mg of OPA/4 mL of ethanol, 7.62 g of disodium tetraborate decahydrate, 200 mg of SDS, and 176 mg of DL-dithiothreitol mixed and adjusted to 200 mL) and 0.1 mg/mL of serine reagent as 0.4:3:0.4 (vol/vol/vol) ratio. The DH was determined at 330 nm with L-leucine as the standard and was calculated using the following formula: DH (%) = h/h_{tot} × 100, where h is the number of hydrolyzed bonds and h_{tot} is the total number of peptide bonds per protein equivalent (mEqv/g of protein). The value of h_{tot} for β-LG is 7.20 mEqv/g of protein according to Cheison et al. (2010).

Cell Culture and Treatment. The RAW 264.7 cells were seeded at 5×10^5 cells/mL in Dulbecco's Modified Eagle Medium containing 10% FBS and grown for 24 h at 37°C under 5% CO_2 and 95% air. These cells were subsequently cultured with medium (control), 10 ng/mL LPS (positive control), 50 μg/mL digested β-LG (**ogi-LG**), or 50 μg/mL LGL (**ogi-LGL**). The ogi-LG and ogi-LGL groups were solely treated with each digest in the absence of LPS.

These cells were then incubated for 4 h for cytokine analysis or 24 h for NO output measurement.

Measurement of NO Production. The amount of NO in RAW 264.7 cells was measured by the method

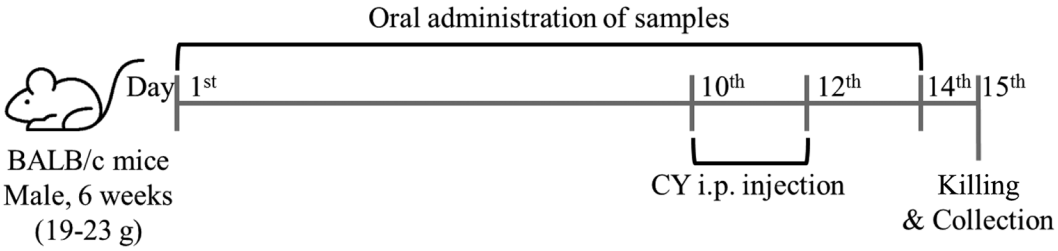
described by Chun et al. (2016). Supernatants from the treated cells were collected and diluted with Griess reagent; the samples were then measured at 540 nm with sodium nitrite as the standard.

Quantitative Reverse-Transcription PCR Analysis. To measure cytokine mRNA expression [i.e., tumor necrosis factor (**TNF**)-α, IL-1β, and IL-6], the total mRNA from each treatment group was isolated using the RNAiso Plus reagent and then subjected to cDNA synthesis. This served as the template for the quantitative reverse-transcription PCR assay, which was carried out at 63.6°C with the appropriate cytokine primers and a SYBR Green RT-PCR master mix. The primers used were as described by Chun et al. (2016) and their expression was reported relative to the β-actin housekeeping gene.

Experimental Animals

Animal Care and Ethics Statements. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Korea University (approval number: KUIACUC-2020–0009). Sample size was decided using G-Power software (version 3.1.9.2; Heinrich-Heine-University Düsseldorf) and was calculated using statistical test (one-way ANOVA), effect size factor (0.56), *P*-value ($P < 0.05$), power (0.8), and number of groups (7 groups). The effect size factor was determined as a comparison to Yun et al. (2018). Male BALB/c mice (6 wk, 19–23 g) were purchased from Orient Bio and housed in an environmentally controlled room with a temperature of 20 to 24°C and a 12-h light:dark period for at least 1 wk before the experiment. Animals had free access to standard diet and water throughout this adjustment period.

Oral Administration and Immunosuppression. The mice were randomly divided into 7 groups (n = 10 each). Each group received a single oral dosage of a predetermined treatment for 14 d: normal control (**NC**) and CY only (as an immunosuppressive control) were treated with water; **CY+LG100** and **CY+LG400** were given 100 or 400 mg of β-LG protein/kg of BW, respectively; **CY+LGL100** and **CY+LGL400** were treated with 100 or 400 mg of LGL protein/kg of BW, respectively; **CY+FC75** received 75 mg of fucoidan/kg of BW (fucoidan; ≥300 kDa) extracted from *Undaria pinnatifida* sporophyll as a positive control (Yoo et al., 2019). Immunosuppression was induced in all groups except the NC group by i.p. injection of CY (80 mg/kg of BW per day) once a day for 3 consecutive days (from d 10 to 12). On d 15, after being anesthetized with CO_2 gas, all of the mice were killed (Figure



Groups	Oral administration of samples (for 14 days; 1st to 14th)	CY i.p. injection (for 3 days; 10th to 12th)
Normal control (NC; *n*=10)	PBS	PBS
CY-only (*n*=10)	PBS	CY 80 mg/kg of BW per day
CY+LG100 (*n*=10)	β-LG 100 mg protein/kg of BW per day	
CY+LG400 (*n*=10)	β-LG 400 mg protein/kg of BW per day	
CY+LGL100 (*n*=10)	LGL 100 mg protein/kg of BW per day	
CY+LGL400 (*n*=10)	LGL 400 mg protein/kg of BW per day	
CY+FC75 (*n*=10)	FC 75 mg DM/kg of BW per day	

Figure 1. Design of the animal experiment protocol. Over a 14-d period, animals were given treatments orally once per day. On d 10 to 12, the animals were given an i.p. injection of cyclophosphamide (CY). LGL = Maillard conjugated β-LG with lactose; FC = fucoidan.

1). The BW of both classes was measured on d 14 after CY injection.

Isolation and Culture of Peritoneal Macrophages. A modified version of the Li et al. (2017) procedure was used to obtain peritoneal macrophages, in which each animal received an i.p. injection of RPMI containing 5% FBS and antibiotics. After that, the peritoneal macrophages were collected, centrifuged, and resuspended in RPMI comprising 10% FBS and antibiotics. These macrophages were then incubated in RPMI with 1 g/mL LPS for 24 h to see if the treatment affected proliferation and NO development. Phagocytosis was evaluated after 3 h of culture in RPMI without LPS.

Isolation and Culture of Splenocytes. After isolating splenocytes from mice as described by Kim et al. (2018), the splenocytes were centrifuged and resuspended in RPMI containing 10% FBS and antibiotics, and their proliferation in response to 10 μg/mL LPS, as a B lymphocyte mitogen, or 5 μg/mL Con A, as a T lymphocyte mitogen, was calculated after 48 h. The activity of splenic NK cells was evaluated using a 4-h coculture of splenocytes and YAC-1 at 37°C.

Proliferation of Peritoneal and Splenic Lymphocytes. The proliferation of each experimental culture was measured using the EZ-Cytox cell viability assay kit according to the manufacturer's instructions. After measuring the absorbance of their supernatant at 450 nm, the values were calculated as a percentage compared with the untreated control.

Phagocytotic Activity of Peritoneal Macrophages. The phagocytotic activity of peritoneal macrophages was measured as described by Yun et al. (2018). After washing the cells with PBS to remove unattached cells, 0.1% (wt/vol) neutral red solution was applied, and the cells were incubated at 37°C for 3 h. The cells were then washed in PBS and placed in a cell lysis solution and left overnight at 4°C. After measuring the absorbance of their supernatant at 450 nm, the values were calculated as a percentage compared with the untreated control.

Splenic NK Cell Activity. The cytotoxic activity of splenic NK cells against target cells was evaluated using a commercial colorimetric LDH kit, according to the manufacturer's instructions. Splenocytes and YAC-1 mouse lymphoma cells were cocultured at a ratio of 100:1 for 4 h; then, their supernatants were collected and evaluated the absorbance at 490 nm using the EZ-LDH cytotoxicity assay kit according to the manufacturer's instructions. The percentage of potent NK cell activity was calculated using the following formula: cytotoxic activity (%) $= (A_e - A_s)/(A_m - A_s) \times 100$, where A_e is the absorbance of the experimental medium, A_s is the absorbance of the spontaneous medium, and A_m is the absorbance of the maximum medium.

Statistical Analysis

Data are expressed as the mean ± standard deviation. The differences between the experimental groups were compared by one-way ANOVA followed by Tukey's test in cell experiments or Duncan's multiple range test in in vivo experiments ($P < 0.05$) using SAS software (version 9.4; SAS Institute Inc.).

RESULTS

Characterization of the Digested LGL and Its Immune Stimulatory Effects on RAW 264.7 Macrophage Cells

After glycation, LGL showed a significant ($P < 0.05$) increase in fluorescence intensity (34,332 ± 15,143 arbitrary units) compared with β-LG (4,211 ± 5,510 arbitrary units; Figure 2A). Figure 2B shows an β-LG band (18 kDa) on a Coomassie-stained SDS-PAGE gel in the β-LG fraction; however, a band slightly above the original 18 kDa-band and a band thought to be dimer were seen in the LGL fraction. Figure 2C shows the LGL fraction on a silver-stained gel with multiple bands ranging in size from 18 to 35 kDa. These results of LGL by glycation were observed to be similar to our previous study (Chun et al., 2020).

Figure 3 shows the LGL breakdown pattern following in vitro digestion, as well as the DH of each digested component. The LGL protein band was found to be larger than 15 kDa in the O (nonhydrolyzed), oral fraction after 5 min of incubation with saliva (Figure 3A). However, after 2 h of incubation in gastric juice, this protein band vanished (the OG fraction), indicating that the LGL protein was digested by pepsin-containing gastric juice. The LGL was extensively hydrolyzed to 77.6 ± 5.1% after the gastric stage (the OG fraction), as indicated in the DH result in Figure 3B, and even more hydrolyzed to 84.8 ± 4.0% after the intestinal stage (the OGI fraction). In addition, we investigated the production of NO and the mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in RAW 264.7 macrophage cells treated with digested LGL (the ogi-LGL) in the absence of LPS (Figure 4). Compared with the untreated control (1.5 ± 1.6 μM), ogi-LG treatment increased NO levels by more than 6.1-fold (9.3 ± 1.2 μM). In addition, NO production (12.2 ± 1.1 μM) in cells treated with ogi-LGL was 1.3-fold ($P < 0.05$) higher than in the ogi-LG-treated cells. Moreover, the expression of TNF-α, IL-1β, and IL-6 in the ogi-LGL group was 2.6, 4.6, and 7.9 times higher

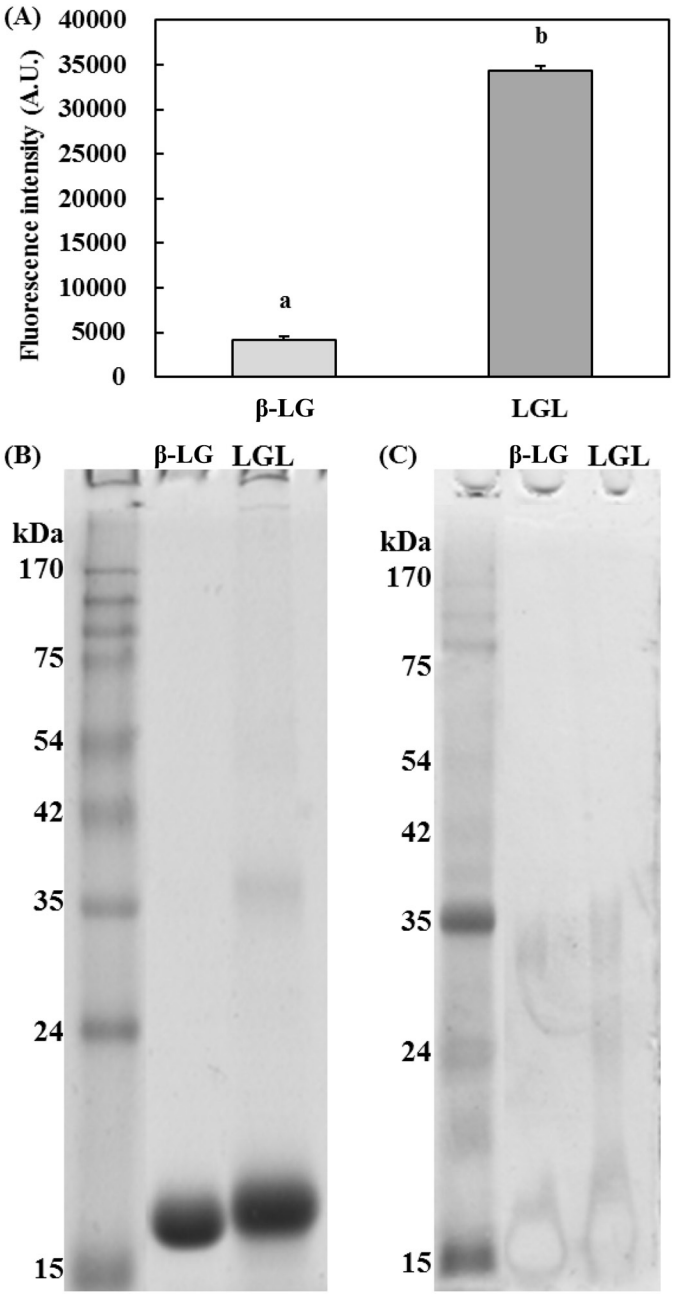


Figure 2. Glycation degree of β-LG-lactose conjugate (LGL). (A) The fluorescence intensity (arbitrary unit, A.U.) was measured at excitation of 370 nm and emission of 440 nm. (B) The 12% SDS-PAGE gels were stained with Coomassie brilliant blue G-250 (16 μg per lane) and (C) with silver (0.4 μg per lane), respectively. The marker's molecular weights (kDa) are shown on the left side of the gel. Values represent the mean ± SD of 3 independent experiments performed in triplicate. Means with different letters are significantly different based on the results of the ANOVA and Tukey's test evaluations ($P < 0.05$).

($P < 0.05$), respectively, than that of the ogi-LG group. These results suggest that the digested LGL may be a more effective macrophage enhancer than the digested β-LG, indicating that digestion can modulate the immune activity of LGL.

Immune-Enhancing Effects of the Oral Administration of LGL in CY-Induced Immunosuppressed Mice

On d 14, mouse BW varied between 22.1 and 23.8 g, and only a significant ($P < 0.05$) difference in the CY-treated groups was observed in BW as compared with the NC (Figure 5A). In BW, including the high-dose LGL group (CY+LGL400), there was no significant difference in the groups treated with CY. The weight changes in the spleen, an important immune-regulating organ, were seen in Figure 4B and 4C for each group during the experiment. A significantly ($P < 0.05$) smaller spleen was shown in the CY-treated groups than in the NC group, which is easily visible. Figure 4C also indicates a significant ($P < 0.05$) decline of the spleen index in the CY-only group (18.3 ± 0.9 mg/10 g of BW) compared with the NC (31.2 ± 1.5 mg/10 g of BW). The groups co-treated with CY and LGL (CY+LGL100 and CY+LGL400) showed a significant ($P < 0.05$) improvement in their splenic index in comparison to the other CY-treated groups. In this study, high-molecular-weight fucoidan was used as a positive control because it has been shown to improve immune function by activating splenic NK cells in immunosuppressed mice (Yoo et al., 2019).

The proliferation of peritoneal macrophages reduced in the CY group only (31.3 ± 3.3%), relative to the NC groups, whereas in the CY+LGL400 group, the proliferation was significantly ($P < 0.05$) increased by 50.7 ± 11.5% compared with the NC group (Figure 6A). In addition, the NO production (3.2 ± 1.0 μM), which was significantly ($P < 0.05$) decreased in the CY-only group, was increased in the CY+LGL400 group (7.6 ± 1.5 μM). In addition, the NO production (7.6 ± 1.5 μM) in the CY+LGL400 group was slightly higher ($P < 0.05$) than in the CY only group (3.2 ± 1.0 μM). Next, the phagocytic activity of the peritoneal macrophages has also been examined to determine the effect of these treatments on the innate immune response. As seen in Figure 6B, the phagocytic activity was reduced to 17.8 ± 7.9% in the CY only group; however, it was significantly ($P < 0.05$) increased in the CY+LGL400 group (38.7 ± 8.1%). Taken together, these findings suggest that the administration of high dose of LGL exerts an immunomodulatory effect on CY-induced immunosuppression in peritoneal macrophages.

In addition, we confirmed this immune enhancement mechanism in immunosuppressed mice by evaluating splenic NK cells, lymphocytes, and cytokines. The cy-

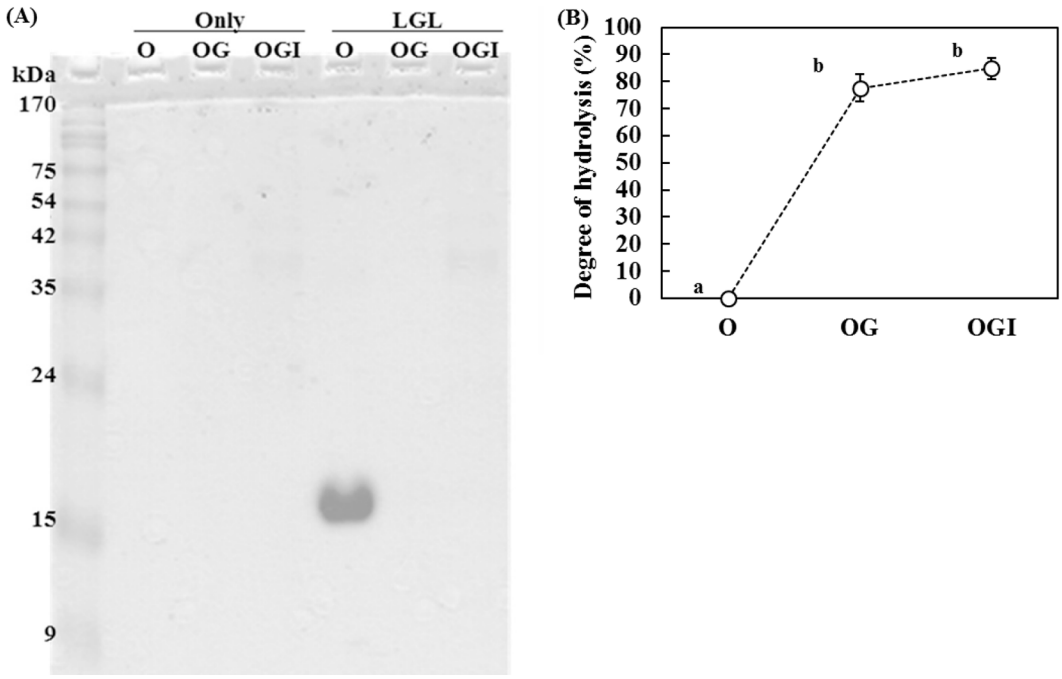


Figure 3. Degradation of β-LG-lactose conjugate (LGL) during in vitro digestion. (A) Coomassie brilliant blue G-250 staining of gel shows degradation patterns of LGL at different levels of digestion on a gel layer. The marker's molecular weights (kDa) are shown on the left side of the gel. A total of 16 μg of each sample was loaded into the 20% gel. (B) Degree of hydrolysis was measured using a modified *o*-phtaldialdehyde (OPA) assay. Values represent the mean ± SD of 3 independent experiments performed in triplicate. Means with different letters are significantly different based on the results of the ANOVA and Tukey's test evaluations ($P < 0.05$). O = oral fraction incubated with saliva; OG = oral-gastric fraction incubated with saliva and gastric juice; OGI = oral-gastric-intestinal fraction incubated with saliva, gastric juice, and duodenal juice/bile.

totoxicity result of splenic NK cells (Figure 7A) reveals that the CY-only group (18.4 ± 4.5%) was 2.3 times less than the NK group (42.2 ± 4.5%), whereas the CY+LGL400 group (44.3 ± 6.7%) totally restored the cytotoxic activities. Also, the CY+LGL100 group, which received a lower dose of LGL, showed enhanced NK cytotoxic activity that was not significantly ($P < 0.05$) different from the CY+LGL400 group. Also, after stimulation with LPS and Con A, there was a significant ($P < 0.05$) increase in the proliferation of splenic B lymphocytes and splenic T lymphocytes, respectively, in the LGL-treated groups compared with the CY-only group (Figure 7B and 7C). Furthermore, as compared with the NC group, the high-dose, CY+LGL400 group displayed even higher proliferation in both sets of lymphocytes.

Finally, when compared with the NC group, the CY-only group showed a significant ($P < 0.05$) decrease in the mRNA levels of several pro-inflammatory cytokines in the spleen (Figure 8), including an 86.7 ± 8.4% decrease for TNF-α, an 88.7 ± 19.2% decrease for IL-1β, and an 89.9 ± 21.0% decrease for IL-6. The LGL-treated groups, on the other hand, appeared to mitigate this effect, demonstrating lower reductions in each cytokine, 33.9–46.7% for TNF-α, 42.2–52.4% for IL-1β, and 31.8–40.1% for IL-6. Although there was no significant difference between these 2 groups (CY+LG vs. CY+LGL), splenic cytokine expression was more likely to respond to LGL than to LG.

DISCUSSION

Maillard reaction has been confirmed to have various benefits for its MRP, including increased antioxidants and immunomodulatory effects (Chevalier et al., 2001; Chun et al., 2016; Pyo et al., 2016; Liu et al., 2017). Our previous in vitro study reported that LGL, which is an β-LG-lactose conjugate produced via MR, exerts several immune-enhancing effects, including an increase in NO and pro-inflammatory cytokine expression in RAW 264.7 macrophages (Chun et al., 2020). Food-derived MRP, on the other hand, are exposed to some degree of gastrointestinal digestion after oral intake (Rufián-Henares and Delgado-Andrade, 2009). Maillard reaction products have been shown to resist digestion by decreasing their susceptibility to digestive enzymes (Corzo-Martínez et al., 2010), whereas other studies have shown that MRP increase digestion (Li et al., 2013). To the best of our knowledge, this is the first study to investigate whether the immune-enhancing ef-

fects of LGL generated by MR could be sustained even after in vitro-simulated digestion and in vivo experiment.

After sequential digestion of LGL with saliva, gastric juice, and duodenal juice/bile, potential immune-enhancing effects of in vitro-digested LGL were found using RAW 264.7 macrophages (Figure 4). This suggests that the enhancing effects of MRP may be maintained or even improved following in vitro digestion. Furthermore, in our animal experiment, orally administered LGL was shown to increase immunity by stimulating immune cells (Figures 6–8). The mRNA levels of TNF-α, IL-1β, and IL-6 in RAW 264.7 cells were detected by quantitative reverse-transcription PCR in the present study; however, the production of these cytokines in the macrophage supernatant was not determined by ELISA. According to several studies, when RAW 264.7 cells were stimulated with test substances, there was a positive relationship between mRNA expression levels and the production of the cytokines, TNF-α, IL-1β, and IL-6 (Cai et al., 2020; Guo et al., 2020; Park et al., 2020; Fan et al., 2021; Feng et al., 2021). Therefore, in this study, the immune cells were assumed to have higher cytokine protein levels if the corresponding mRNA levels were higher.

Kim et al. (2019) reported that fermented products from MR-conjugated whey protein using *Lactobacillus gasseri* 4M13 have improved anti-inflammatory effects on RAW 264.7 cells, reducing NO production and inflammatory cytokine expression compared with the nonfermented MRP. Other studies also found that fermented MRP-derived whey protein hydrolysate improved antioxidant activity, cardiovascular prevention activity, and NK cell activity (Oh et al., 2014; Kang et al., 2017). As a consequence, it is possible that the effects of these MRP will be maintained or increased after digestion.

Considering the amount of β-LG or LGL (400 mg/kg of BW per day) dosages used in this study, the dosage can be converted to 32.4 mg/kg of BW per day for humans using the body surface area factor reported by Reagan-Shaw et al. (2008). This amount is about 3.6% of the recommended daily protein intake for Korean adults (0.91 g/kg of BW per day), and is 6.8 times smaller than the β-LG content (0.22 g/kg of BW) in various commercial items, including a single serving of whey protein (25 g/60 kg of BW). In this study, the CY treatment via i.p injection was used to induce immune suppression. In animal experiments, CY is also used to reduce peritoneal macrophages, splenic NK cells, and

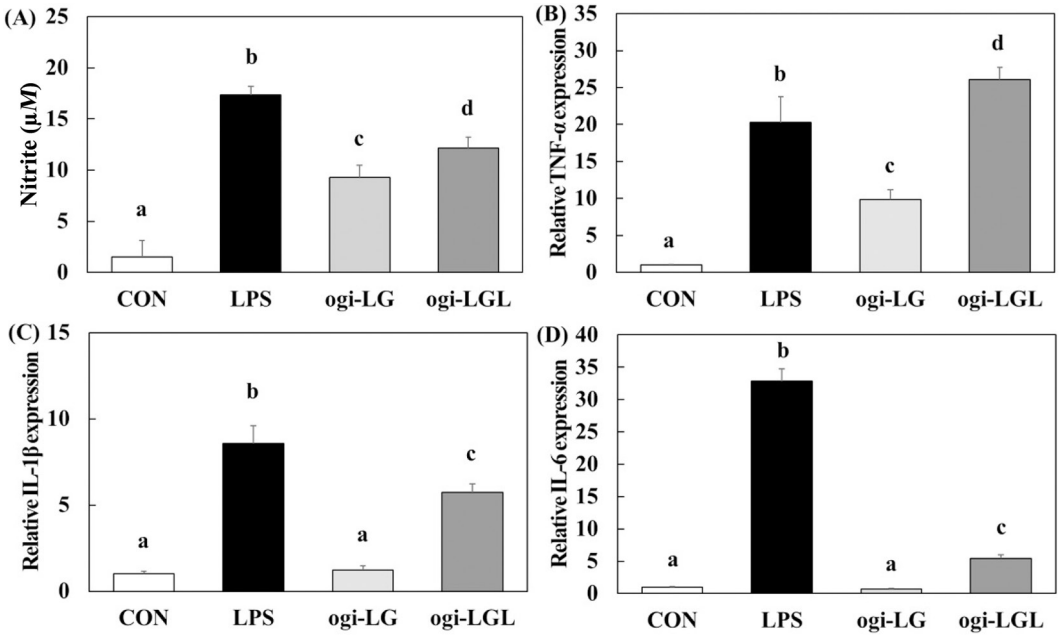


Figure 4. β-Lactoglobulin-lactose conjugate (LGL) stimulation on the RAW 264.7 macrophages following in vitro digestion. (A) Nitric oxide (NO) production in RAW 264.7 cells in response to digested LGL. Using a Griess assay, NO production was measured after 24 h of treatment. (B–D) Tumor necrosis factor (TNF)-α, IL-1β, and IL-6 mRNA expression levels were determined using quantitative reverse-transcription PCR after 4 h of treatment. Each group was treated as follows: nontreated control (CON); LPS (positive control), 10 ng/mL; in vitro-digested β-LG (ogi-LG), 50 μg/mL; in vitro-digested LGL (ogi-LGL), 50 μg/mL. Values represent the mean ± SD of 3 independent experiments performed in triplicate. Means with different letters are significantly different based on the results of the ANOVA and Tukey's test evaluations ($P < 0.05$).

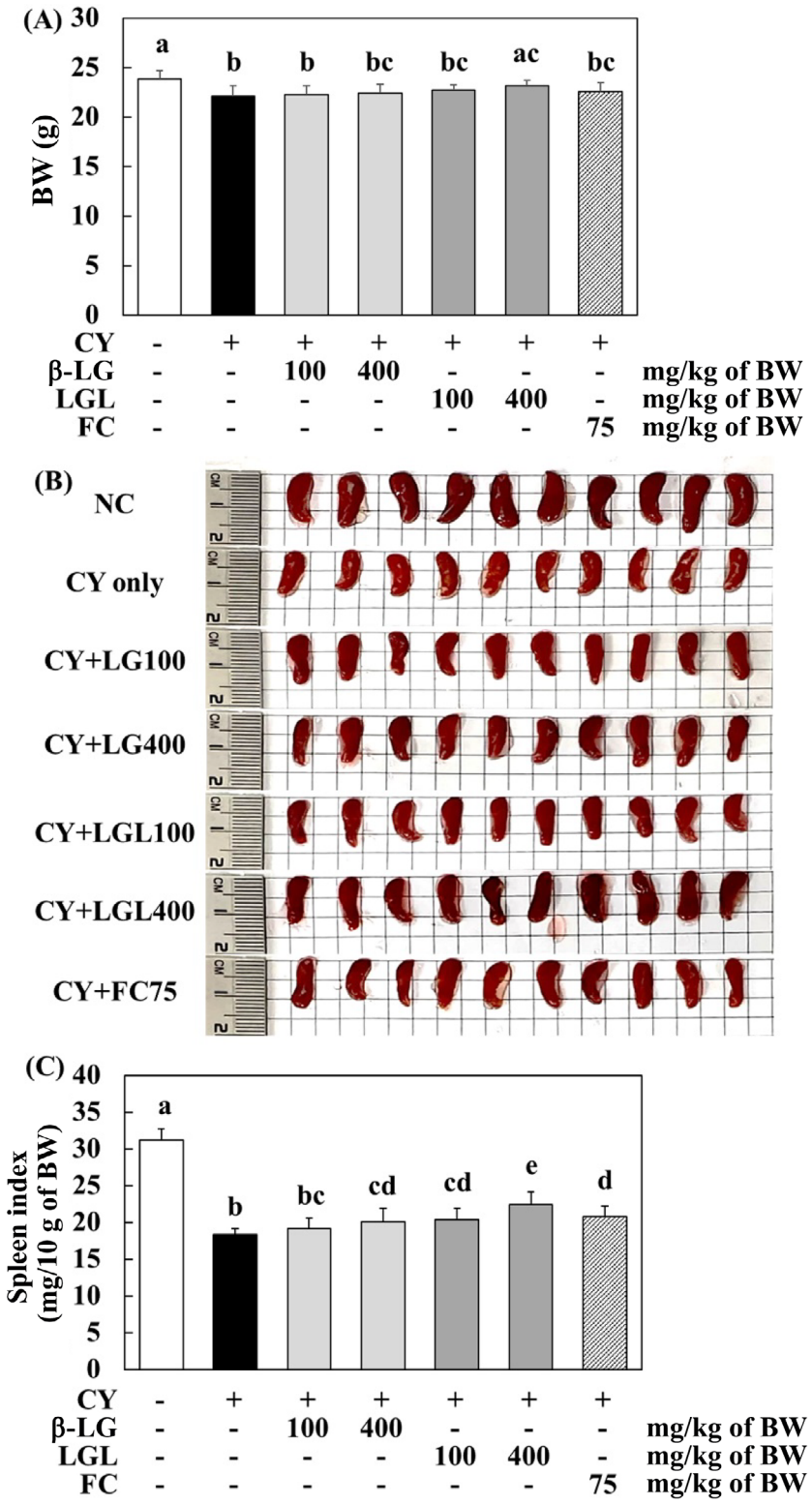


Figure 5. Effects of β-LG-lactose conjugate (LGL) on BW and immune organs in immunosuppressed mice. (A) The BW of mice in each group were measured on d 0 (initial BW) and 14 (final BW) of the oral treatment regimen. (B) Spleens were removed from each group of mice. (C) The spleen weight was divided by the final BW to reveal the organ index for each group. Each group of mice was administrated water, β-LG, LGL, or fucoidan (FC; positive control) via the oral gavage for 14 d and received i.p. injections of saline or cyclophosphamide (CY; 80 mg/kg of BW) on d 10, 11, and 12. Values represent the mean ± SD (n = 10). Means with different letters are significantly different as determined by ANOVA and Duncan's multiple range test ($P < 0.05$).

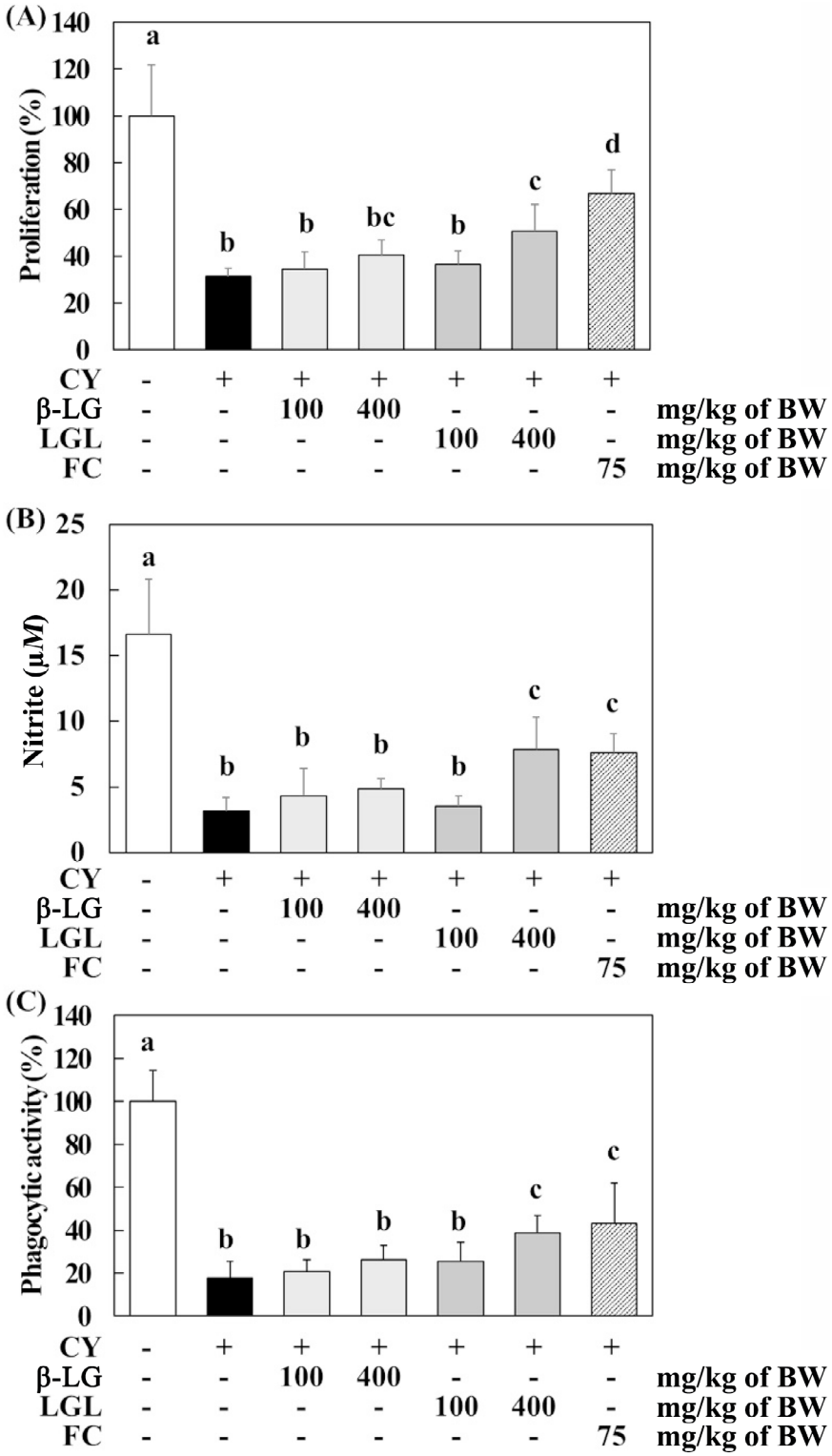


Figure 6. Effect of β-LG-lactose conjugate (LGL) treatment on peritoneal macrophage activities in immunosuppressed mice. (A) The cell proliferation was measured using the Ez-Cytox cell viability assay (DoGen) after 24 h of stimulation with LPS (1 μg/mL). (B) The NO production in peritoneal macrophages was measured using Griess reagent after 24 h of stimulation with LPS (1 μg/mL). (C) The phagocytic activity was measured using a neutral red assay in the absence of LPS stimulation. Each group of mice was administrated water, β-LG, LGL, or fucoidan (FC; positive control) via oral gavage for 14 d and received i.p. injections of saline or cyclophosphamide (CY; 80 mg/kg of BW) on d 10, 11, and 12. Values represent the mean ± SD (n = 10). Means with different letters are significantly different as determined by ANOVA and Duncan's multiple range test ($P < 0.05$).

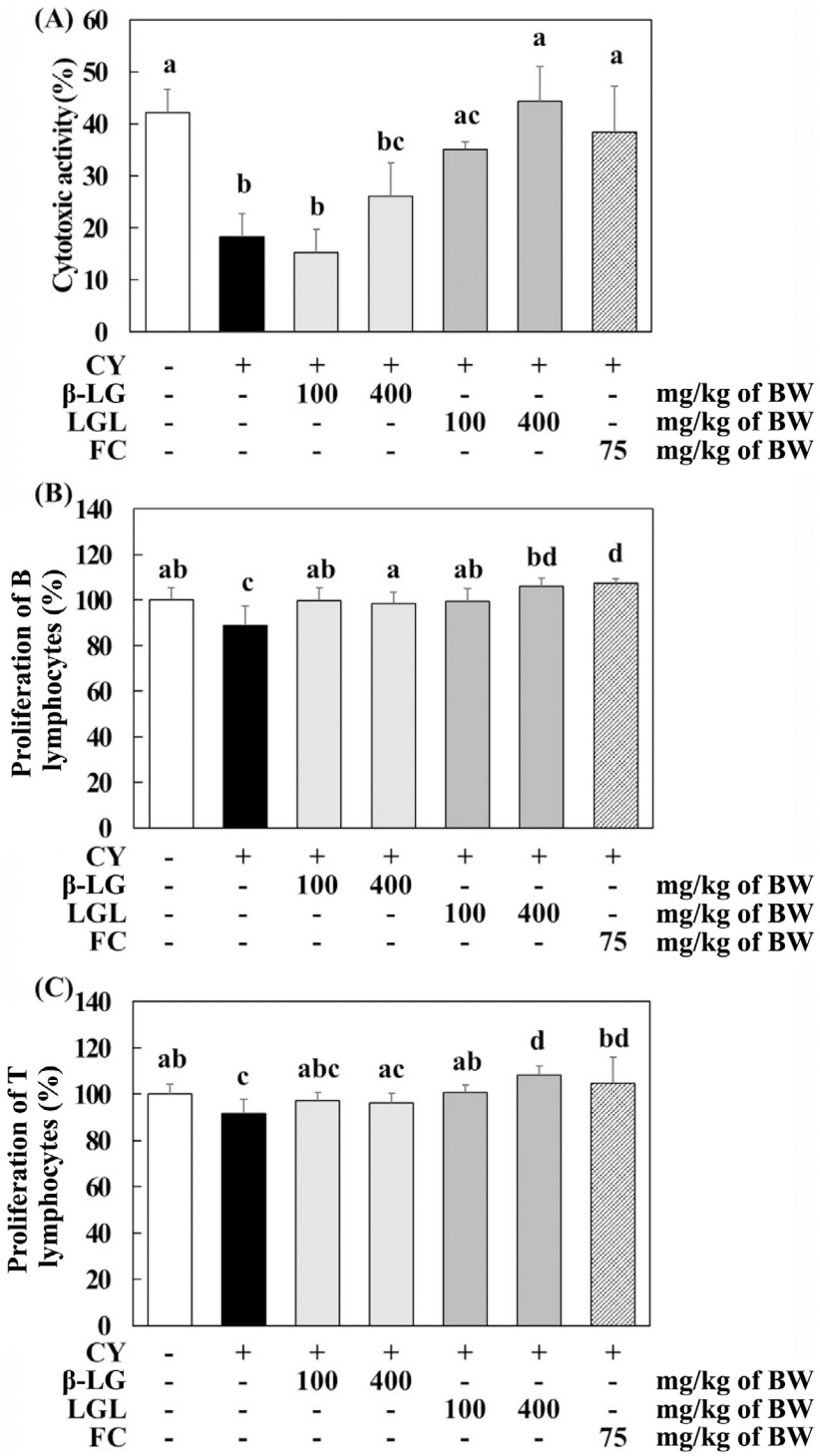


Figure 7. Effect of β-LG-lactose conjugate (LGL) on splenocytes in immunosuppressed mice. (A) The cytotoxicity of the isolated splenocytes was determined following their coculture with YAC-1 (target) cells. This assay measured the cytotoxic activity of splenic natural killer cells (effector cells). The ratio of effector and target cells was 100:1, and the cytotoxic activity was measured using the EZ-LDH cytotoxicity assay kit (Biomax). (B) The isolated splenocytes were also incubated with 10 μg/mL of LPS, which acts as a mitogen for B lymphocytes, for 48 h and lymphocyte production was then measured. (C) The isolated splenocytes were incubated with 5 μg/mL of concanavalin A (Con A), which acts as a mitogen of T lymphocytes, for 48 h and lymphocyte production was then measured. The proliferation of splenic lymphocytes was measured using the Ez-Cytox cell viability assay kit (DoGen). Each group of mice was administrated water, β-LG, LGL, or fucoidan (FC; positive control) via the oral gavage for 14 d and received i.p. injections of saline or cyclophosphamide (CY; 80 mg/kg of BW) on d 10, 11, and 12. Values represent the mean ± SD (n = 10). Means with different letters are significantly different as determined by ANOVA and Duncan's multiple range test ($P < 0.05$).

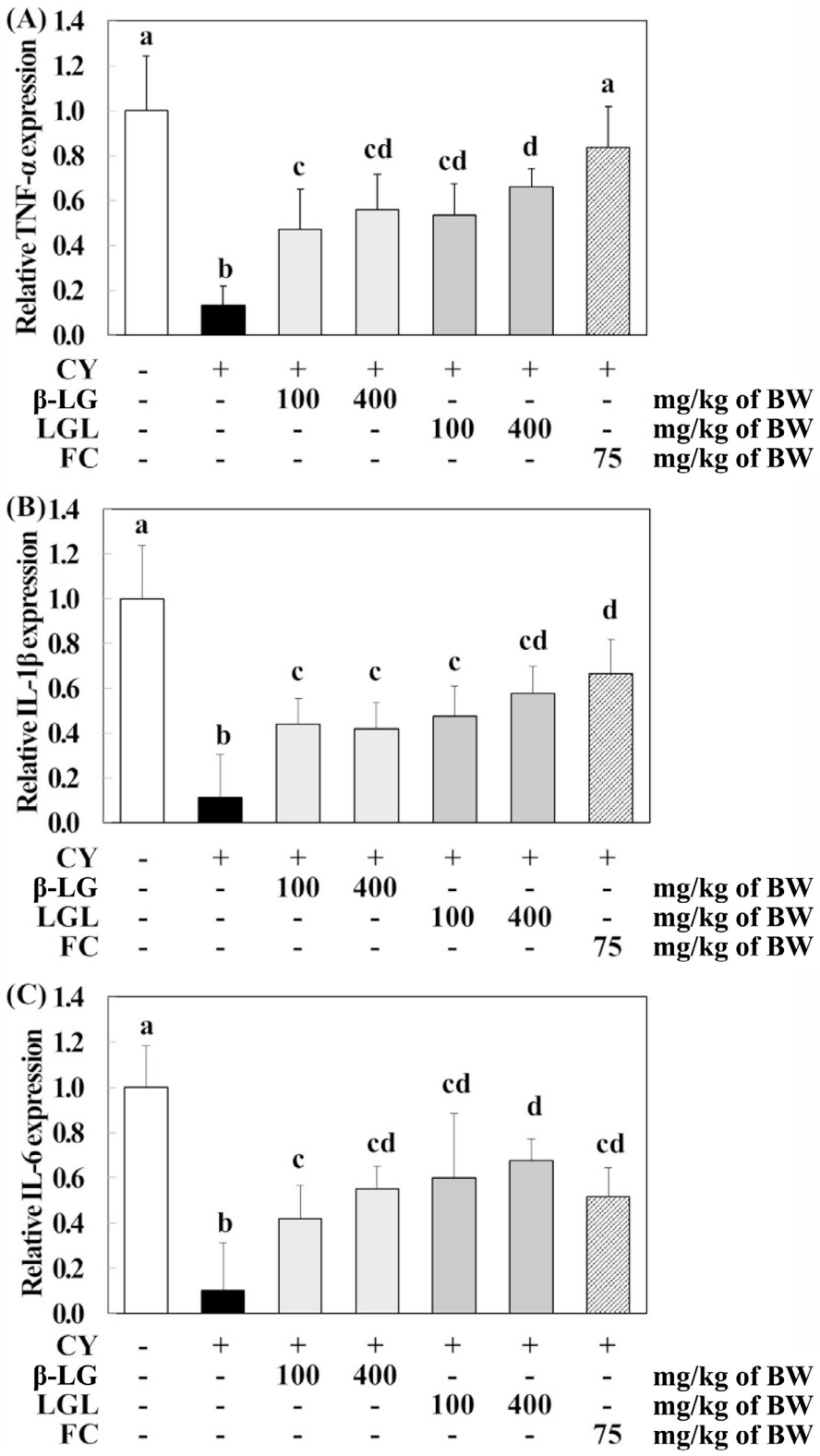


Figure 8. Expression of cytokines in splenic tissues from immunosuppressed mice. The mRNA level of TNF-α (A), IL-1β (B), and IL-6 (C) was evaluated using quantitative reverse-transcription PCR. Each group of mice was administrated water, β-LG, β-LG-lactose conjugate (LGL), or fucoidan (FC; positive control) via oral gavage for 14 d and received i.p. injections of saline or cyclophosphamide (CY; 80 mg/kg of BW) on d 10, 11, and 12. Values represent the mean ± SD (n = 10). Means with different letters are significantly different as determined by ANOVA and Duncan's multiple range test ($P < 0.05$).

splenic lymphocytes (Yun et al., 2018; Monmai et al., 2019).

Macrophages are essential components of the innate immune system, functioning as a primary defense and secreting NO and pro-inflammatory cytokines (Hirayama et al., 2017). This indicates that the increased NO development and phagocytic activity of macrophage cells protects host cells from infection (Uthaisangsook et al., 2002; Chen et al., 2010). In the present study, LGL administration increased NO development and phagocytic activity in a CY-induced immunosuppressed mouse model by inducing peritoneal macrophage proliferation and activation.

The spleen produces several different cells, which control the immune response according to the stimuli through the secretion of different cytokines (Lori et al., 2017; Yoo et al., 2019). The NK cells, which account for approximately 3 to 4% of all splenocytes, contribute a significant function in the innate immune system by facilitating the direct killing of invading pathogens and cancer cells as well as the expression and secretion of various cytokines (Kantakamalakul et al., 2003; Vivier et al., 2004). The most direct measure of host immunity is splenic lymphocyte proliferation, and T lymphocytes and B lymphocytes stimulated by mitogens are involved in nonspecific immune regulation (Tu et al., 2008). According to Mahmud et al. (2004), β-LG digests produced with digestive enzymes including pepsin, trypsin, chymotrypsin, and pancreatin can increase splenocyte proliferation in mice. In the current study, the administration of LGL inhibited the depletion of splenic lymphocytes after the CY treatment. This indicates that digested LGL may enhance immunity by increasing splenic immune cell activity and proliferation. Moreover, cytokines, which affect the proliferation, differentiation, and activity of immune cells, regulate cellular and humoral immune responses (Xu et al., 2009). Tumor necrosis factor-α and IL-1β interact with lymphocytes to induce Th-1 cells and enhance their activity against pathogens and tumor cells (Striz et al., 2014). Interleukin-6 promotes the activation of NK cells and differentiation of bone marrow and T-cells (Striz et al., 2014).

Moreover, despite being an essential component in food processing, β-LG′s usage in the food industry has been limited (de Wit, 1998; Monaci et al., 2006). As a result, the potential of β-LG derived from whey proteins as a functional material would broaden the range of use in the dairy industry. Our earlier research has shown that MR improves the immune-enhancing activity of whey protein concentrate (Chun et al., 2016; Chun et al., 2020). Chun et al. (2020) reported that β-LG is a major protein responsible for the effect of glycated whey protein concentrate. Furthermore, in the current study, the potential immune-enhancing effect of LGL, an β-LG-lactose conjugate generated by the MR, is increased following in vitro digestion, which might contribute to an increase in milk intake demand and public health promotion.

In summary, a digested MR-type LGL conjugate stimulated in vitro RAW 264.7 macrophages, causing them to produce NO and increase mRNA expression of immune-related cytokines, showing that LGL therapy can excite macrophages. Furthermore, LGL oral supplementation improved CY-induced immunosuppression by boosting NO generation, peritoneal macrophage phagocytic activity, splenic NK cell cytotoxicity, immune cell proliferation, and splenic cytokine gene expression, indicating that LGL administration can activate immune cells. To the best of our knowledge, the immune-enhancing effects of LGL generated by MR can be maintained or improved during in vitro digestion and in vivo administration for the first time. We conclude that ingesting LGL on a regular basis can enhance immunity, and its hydrolysates can contribute to several aspects of innate and adaptive immune cell proliferation and function.

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