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Improvement of Immune and Hematopoietic Functions by *Litsea japonica* Fruit Extract in Cyclophosphamide-Treated BALB/c Mice

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Abstract: Loss of immunity is an important cause in the pathology of infectious disease. This study investigates the effect of *Litsea japonica* fruit extract (LJFE) as a potential functional food on immunity and hematopoietic function in immunosuppressed BALB/c mice. Immunity-stimulating activity was observed in mice supplemented with LJFE at low (25 mg/kg), medium (50 mg/kg), and high (100 mg/kg) dosage for seven days after administration of cyclophosphamide. LJFE treatment significantly improved spleen injury score ($p < 0.001$) and body weight ($p < 0.02$) by approximately two-fold with a high dosage of LJFE (100 mg/kg). Spleen-derived lymphocyte analysis demonstrated that the numbers of clusters of differentiation (CD)4^+ and $CD8^+$ T-cells were notably increased by approximately two-fold ($p < 0.001$) with a high dosage of LJFE (100 mg/kg). In mouse splenocytes differentiated into T- and B-lymphocytes, LJFE significantly induced proliferation up to approximately 90% of control for T- ($p < 0.001$) and B-lymphocytes ($p < 0.01$) with a high dosage of LJFE (100 mg/kg). Furthermore, LJFE significantly recovered the numbers of white blood cells, red blood cells, and platelets. Enzyme-linked immunosorbent assay revealed that serum levels of immune-related cytokines, such as tumor necrosis factor-α, interleukin (IL)-1β, IL-6, IL-2, and interferon (IFN)-γ, were notably Increased. In addition, serum levels of immunoglobulin (Ig) A, IgM, and IgG were restored by LJFE treatment. This study provides a reference to use *L. japonica* as a functional food ingredient to improve immunity and hematological function in humans.

Keywords: *Litsea japonica*; immunity; hematopoietic function; cytokines; mice

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1. Introduction

Litsea japonica (Thunb.) Juss is an edible plant that is mainly distributed in East Asia. It contains various bioactive substances such as lactones and terpenoids [1–3]. The biological benefits of *L. japonica* have been mainly explored for osteoarthritis [4,5] and diabetic conditions [6–8]. However, its effectiveness in other pathological conditions remains under investigation. *L. japonica* can enhance immunity. The flavonoids found in this plant exhibit potent inhibitory activity against the complement system [9]. Fruit extracts of *L. japonica* promote the production of proinflammatory mediators and cytokines, such as nitric oxide, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β, compared to that

by ginsenoside treatment in murine macrophage RAW 264.7 [10]. Additionally, the fruit extracts can be beneficially applied to treat neurodegeneration, and their analgesic effects are associated with anti-inflammatory activity [11,12]. Therefore, *L. japonica* is regarded as a potential immunomodulator [10]. The immune system protects the body from foreign pathogens [13,14]. To maintain physiological balance and homeostasis, the immune system recognizes and destroys antigens [14,15]. Therefore, orchestration and maintenance of the immune system are critical for a healthy physiological state, and bioactive substances that modulate the immune response are of immense importance. However, studies on the regulation of immunity and hematopoietic activities by *L. japonica* in animal models are scanty. In this study, for the first time, we aim to evaluate the effects of *L. japonica* fruit extract (LJFE) on immunomodulation and hematopoietic activity in cyclophosphamide (CPA)-induced immunosuppressed BALB/c mice. For this purpose, we measured levels of immunological T-cells, hematological cells, and proinflammatory cytokines.

2. Materials and Methods

2.1. Preparation of LJFE

LJFE used in this study was manufactured and provided by Hurum Co., Ltd. (Seoul, Republic of Korea). The *L. japonica* fruit was collected in Jeju island (Seogwipo, Republic of Korea). To prepare LJFE, the seeds of *L. japonica* were removed and remaining seedless fruits were freeze-dried at −80 °C for three days. The dried fruits were crushed and extracted with 70% ethanol at 60 °C. LJFE produced by concentrating the extracts under reduced pressure and then freeze-drying was stored in a refrigerator (BÜCHI Labortechnik, Flawil, Switzerland) at −20 °C until further use. Red ginseng, used as a positive control, was provided by Kwangdong Pharmaceutical Co., Ltd. (Seoul, Republic of Korea). Red ginseng was extracted at 90 °C by adding 70% ethanol to six-year-old red ginseng raw materials. The red ginseng extract was decompressed by 100 mmHg pressure using a vacuum concentrator (Sunileyela Co., Ltd., Seongnam, Republic of Korea) to prepare a red ginseng extract with a solid content of 38%.

2.2. Animal Treatment and Experimental Design

The effect of LJFE on immune and hematopoietic function was evaluated in BALB/c mice (seven-week-old, male, Samtako Bio Korea, Osan, Republic of Korea). Mice were randomly divided into control (N), CPA-treated (CPA, Sigma-Aldrich Chemical Co., St. Louis, MO, USA), red ginseng-administered positive control (PC), and LJFE at low (25 mg of extract/kg body weight of mice per day)-, middle (50 mg of extract/kg body weight of mice per day))-, and high (100 mg of extract/kg body weight of mice per day)-dosage groups (Figure 1). The CPA, PC, LJFE25, LJFE50, and LJFE100 groups received 100 mg/kg CPA for the first three days. The low-, middle-, and high-dose groups received 25, 50, and 100 mg/kg of LJFE, respectively. The treatment lasted for seven days. During the experiment, mice were free to eat, drink water, and housed under standard laboratory conditions (temperature, 20 °C; humidity, 50–60%, and 12 h light–dark cycle). This study was approved by the Institutional Animal Care and Use Committee of the Dong-eui University (approval No. A2022-016, Busan, Republic of Korea).

2.3. Collection of Spleen and Blood Samples

Seven days after LJFE administration, the body weight of each mouse was measured and recorded. Mice were euthanized by CO_2 inhalation. Immune organs, such as the spleens, were removed for histological and immune cell analysis. Blood was collected via a cardiac puncture, centrifuged at 500× *g* for 15 min at 4 °C, and serum was used for analysis of immunoglobulins (Igs), cytokines, and immune biomarkers [16].

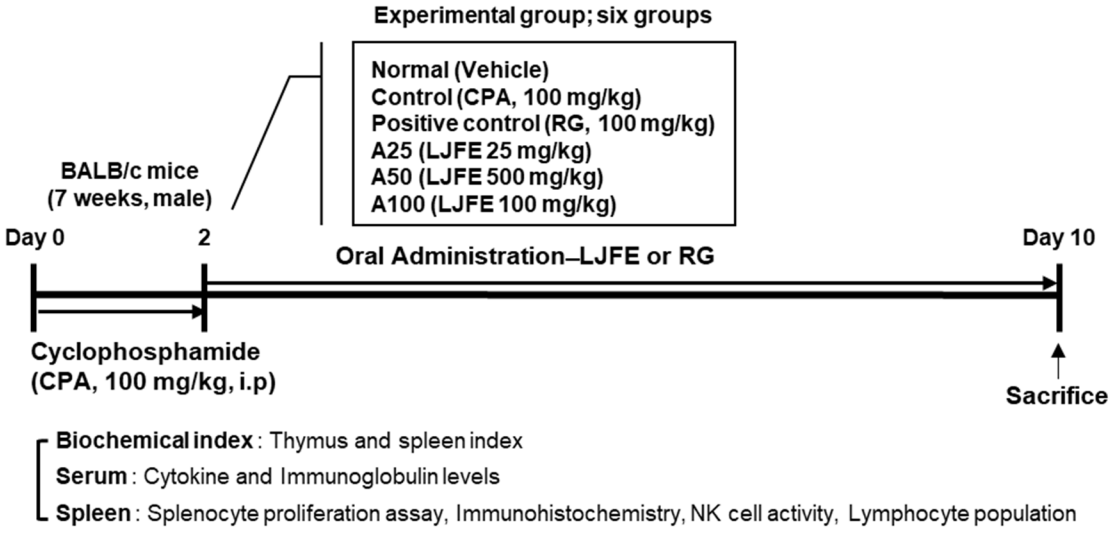

Figure 1. Schematic of the study to assess the effect of *L. japonica* fruit extract (LJFE) in cyclophosphamide (CPA)-induced immunosuppressed mice. BALB/c mice were treated on days 0–3 by an intraperitoneal injection of 100 μL CPA (100 $mg \cdot kg^{-1} \cdot day^{-1}$). LJFE suspended in sterile water was orally administered once daily for seven days. Red ginseng (RG) treatment was used as a positive control.

2.4. Hematological Analysis

Hematological analyses for WBCs, PLTs, lymphocytes, RBCs, hematocrit, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were conducted using a blood cell analyzer (Sysmex Corporation, Kobe City, Hyogo Prefecture, Japan) as previously described [17].

2.5. Immune Marker Analysis

Serum IgM (ab215085), IgA (ab157717), and IgG (ab151276) levels were detected using enzyme-linked immunosorbent assay (ELISA) kits (Abcam Inc., Cambridge, UK). Levels of TNF-α (SMTA00B), IL-6 (M6000B), IL-1β (MLB00C), and interferon (IFN)-γ (MIF00) were examined by ELISA (R&D Systems, Minneapolis, MN, USA). The experiments were performed according to the manufacturers' instructions [18].

2.6. Isolation of Splenocytes

The spleen was washed with RPMI 1640 medium (WelGENE Inc., Gyeongsan, Republic of Korea) containing 1% penicillin-streptomycin and 10% fetal bovine serum as previously described [19]. The spleen was then incubated in 10 mL culture medium and homogenized using a 40 μm nylon cell strainer (Corning Inc., Corning, NY, USA). The obtained splenocytes were centrifuged at 120× *g* for 10 min at 4 °C. Red blood cells in the resulting pellet were lysed using a lysis buffer, and the splenocytes were centrifuged again. The resulting pellet was washed with RPMI 1640 medium and used for further experiments.

2.7. Measurement of Natural-Killer (NK)-Cell Activity

The activity of NK cells was measured by monitoring lactate dehydrogenase (LDH) activity in YAC-1 cells, a mouse tumor lymphocyte cell line (American Type Culture Collection, Manassas, VA, USA) that releases LDH when attacked by NK cells. NK cells from splenocytes were isolated using a Mouse NK-Cell Enrichment Kit (StemCell Technology, Inc., Vancouver, BC, Canada) according to the manufacturer's instructions. Isolated NK cells were seeded into 96-well plates at a density of 1×10^5 cells/well. YAC-1 cells were reacted with NK cells at 1:100 ratio and incubated at 37 °C under 5% CO_2 for 4 h. Then,

the activity of NK cells was measured using a Colorimetric LDH Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), following manufacturer's instructions.

2.8. Measurement of Splenocyte Proliferation

Splenocytes were cultured in RPMI 1640 medium. Splenocytes were diluted to 1×10^7 cells/mL and aliquoted into 100 µL aliquots in a 96-well plate. Cells were treated with lipopolysaccharide (LPS, 1 µg/mL) and concanavalin A (1 µg/mL) (Sigma-Aldrich Chemical Co.) and incubated at 37 °C with 5% CO_2 for 48 h. Cell proliferation was then measured at 460 nm with a microplate reader (Beckman Coulter Inc., Brea, CA, USA) using Cell Counting Kit (CCK)-8 (Sigma-Aldrich Chemical Co.), as previously described [20].

2.9. Lymphocyte Subpopulation Analysis

The activity of $CD4^+$ and $CD8^+$ T-cells was analyzed in splenocytes isolated from mice. Splenocytes were stained with fluorescein isothiocyanate-conjugated rat anti-mouse CD8a and PE-conjugated rat anti-mouse CD4 antibodies (Thermo Fisher Scientific Inc.) for 30 min at 4 °C. Stained splenocytes were washed with phosphate-buffered saline and lymphocytes were analyzed by flow cytometry (Accuri C6, BD Biosciences, Ann Arbor, MI, USA) at the Core-Facility Center for Tissue Regeneration, Dong-eui University.

2.10. Hematoxylin and Eosin (H&E) Staining

H&E staining was performed to observe histological changes in the spleen. Excised spleen sections were fixed with 4% formaldehyde (Thermo Fisher Scientific Inc.), and the tissues were embedded in paraffin. The embedded tissues were cut to a thickness of 4 µm to prepare slide specimens. The prepared slides were stained with H&E (Sigma-Aldrich Chemical Co.) as previously described [21], and histological changes were observed using a high-resolution microscope (EVOS FL Auto 2 imaging system; Thermo Fisher Scientific Inc.).

2.11. Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) using GraphPad Prism V.5.0 (GraphPad Inc., San Diego, CA, USA). $p < 0.5$ was considered significant using Tukey's post hoc test. Values are represented as means ± standard deviation.

3. Results

3.1. Effect of LJFE on Spleen and Body Weight

To evaluate the effect of LJFE on immune function, we investigated histology, spleen injury score, and body weight. As shown in Figure 2A, the spleens of mice treated with LJFE at low, medium, and high doses had increased white pulp (WP), red pulp (RP), marginal zone (MZ), and central arteries (CA) compared to those of the CPA-treated group. In addition, LJFE at 100 mg/kg significantly alleviated the spleen injury score by 1.3-fold in comparison to that of the CPA group (Figure 2B). Moreover, the LJFE-treated groups showed reduced body weight changes compared with those in the CPA group (Figure 2C). Changes in these indicators were superior or similar to those observed in the red ginseng-treated group that was used as a positive control. These results indicated that LJFE exerted beneficial effects on both immune organ and body weight changes.

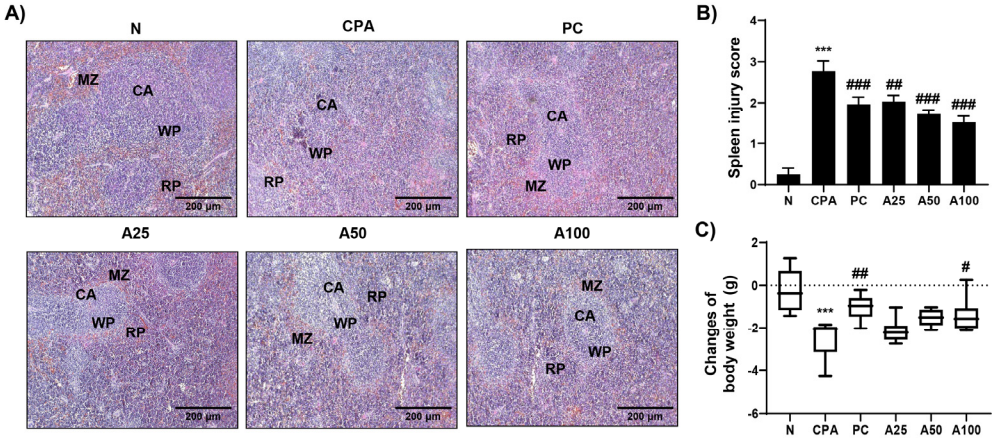

Figure 2. Effect of LJFE on spleen index and body weight in CPA-induced immunosuppressed mice. (**A**) Hematoxylin and eosin (H&E) staining was performed using spleen sections of mice, and representative images are shown, scale bar, 200 μm. (**B**) Spleen injury score. (**C**) Changes in body weight of mice. *** $p < 0.05$ vs. control group. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$ vs. CPA group. A25, 25 mg/kg. A50, 50 mg/kg. A100, 100 mg/kg. CPA, cyclophosphamide-treated group. N, control group. WP, white pulp. RP, red pulp. MZ, marginal zone. CA, central arteries. N, control.

3.2. LJFE Enhanced the Numbers of CD4$^+$ and CD8$^+$ Lymphocytes

Lymphocyte subtypes are distinguished according to their molecular structure on the cell surface, known as CD. In general, lymphocytes are classified as CD4$^+$ and CD8$^+$ T-lymphocytes, which promote B-cell activation and mediate cytotoxicity, respectively [22]. LJFE at 100 mg/kg significantly increased the numbers of CD4$^+$ and CD8$^+$ T-cells in splenocytes ($p < 0.0001$), which were decreased in CPA-treated mice (Figure 3A–D). These effects of LJFE at doses of 50 and 100 mg/kg were comparable or superior to those of red ginseng. Typically, the CD4$^+$/CD8$^+$ T-cell ratio was close to 2 in this study [23]. Although the effect of LJFE was not statistically significant here ($p > 0.05$), it tended to restore the CD4$^+$/CD8$^+$ ratio compared to that of the CPA group, but not of the red ginseng-treated group (Figure 3E). These results suggested that LJFE promoted the number of CD4$^+$ and CD8$^+$ lymphocytes, to potentially increase immunity.

3.3. LJFE Increased Lymphocyte Proliferation and NK-Cell Activity

LJFE at 25, 50, and 100 mg/kg concentrations significantly stimulated T-lymphocyte proliferation in comparison to that in the CPA group after treatment of splenocytes with concanavalin A (1 μg/mL) (Figure 4A). In addition, 100 mg/kg LJFE notably promoted B-lymphocyte proliferation in comparison to that in the CPA group (Figure 4B) after treatment of splenocytes with LPS (1 μg/mL). Furthermore, LJFE at 50 and 100 mg/kg doses induced NK-cell activity in comparison to that of the CPA group (Figure 4C). These effects of LJFE on lymphocyte proliferation and NK-cell activity at a dose of 50 or 100 mg/kg were comparable to those of the red ginseng-administered group. The results indicated that LJFE effectively promoted immunity by stimulating lymphocyte proliferation and NK-cell activity, which was similar to that observed in the red ginseng group.

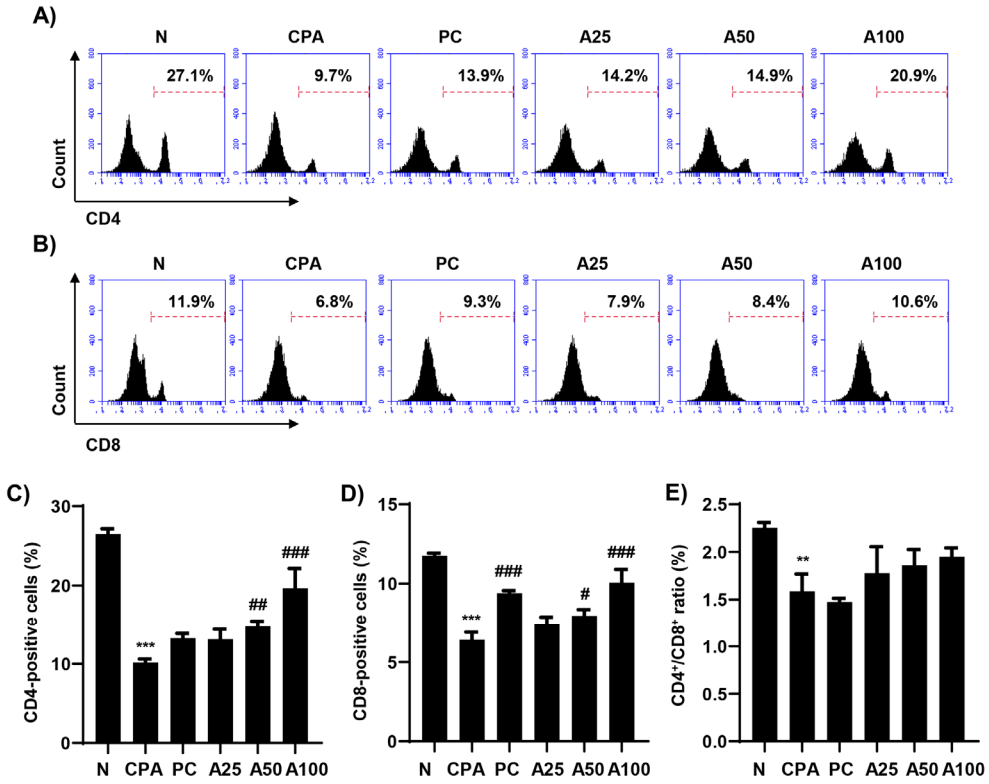

Figure 3. Effect of LJFE on proliferation of cluster of differentiation (CD) 4^+ and $CD8^+$ T-lymphocytes in splenocytes of CPA-induced immunosuppressed mice. (**A**) $CD4^+$ T-cells and (**B**) $CD8^+$ T-cells in splenocytes. (**C**,**D**) Quantitative results of flow cytometry analyses. (**E**) Percentage of splenic $CD4^+$ and $CD8^+$ T-ratio. ** $p < 0.01$ and *** $p < 0.001$ vs. control group. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$ vs. CPA group. A25, 25 mg/kg. A50, 50 mg/kg. A100, 100 mg/kg. CPA, cyclophosphamide-treated group. N, control group.

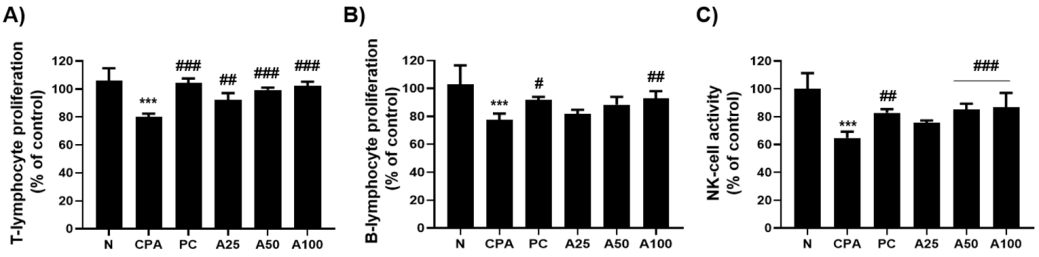

Figure 4. Effect of LJFE on numbers of T- and B-lymphocytes and natural-killer (NK)-cell activation in splenocytes of CPA-induced immunosuppressed mice. (**A**) T-lymphocytes. (**B**) B-lymphocytes. (**C**) NK-cell activity. *** $p < 0.001$ vs. N group. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$ vs. CPA group. A25, 25 mg/kg. A50, 50 mg/kg. A100, 100 mg/kg. CPA, cyclophosphamide-treated group. N, control group.

3.4. LJFE Increased the Number of Immunological Peripheral Blood Cells

To investigate hematopoietic function, WBCs, PLTs, RBCs, lymphocytes, hematocrit, hemoglobin, MCV, MCH, and MCHC were measured in whole blood from mice. As indicated in Figure 5, LJFE significantly enhanced the numbers of WBCs, PLTs, and RBCs at 50 and 100 mg/kg doses, which were comparable or superior to those of the red ginseng-administered group. These results suggested that LJFE enhanced immunity by increasing the number of immunological peripheral blood cells.

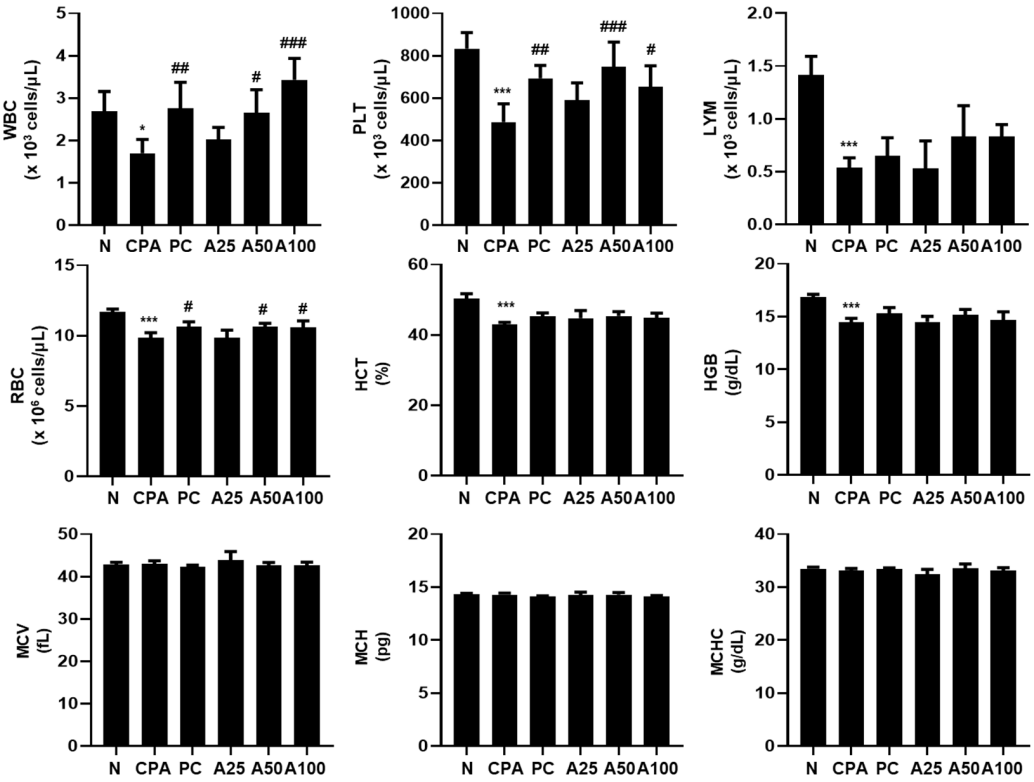

Figure 5. Effect of LJFE on hematopoietic function in whole blood of CPA-induced immunosuppressed mice. Numbers/levels of white blood cells (WBCs), platelets (PLTs), lymphocytes (LYMs), red blood cells (RBCs), hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) have been presented. * $p < 0.05$ and *** $p < 0.001$ vs. control group. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$ vs. CPA group. A25, 25 mg/kg. A50, 50 mg/kg. A100, 100 mg/kg. CPA, cyclophosphamide-treated group. N, control group.

3.5. LJFE Promoted the Production of Immune-Related Cytokines

Cytokines play an important role in mediating the transition from innate to adaptive immunity [24]. Therefore, we investigated the effects of LJFE on the production of immune-related cytokines, including IL-1β, TNF-α, IL-6, IFN-γ, IL-2, and IL-4, in the blood serum of mice. As shown in Figure 6A–C, LJFE notably increased the serum levels of IL-1β and TNF-α at 50 and 100 mg/kg doses, which were statistically similar or superior to those of the red ginseng-administered group. IL-6 increased at a concentration of 100 mg/kg LJFE, which was superior to that of the red ginseng group. In addition, LJFE significantly increased the serum levels of T-helper cell type 1 (Th1) immune factor cytokines, IFN-γ, and IL-2, at 50 and 100 mg/kg doses, which were similar or superior to those of the red ginseng-administered group; however, IL-4 level was slightly increased by LJFE treatment (Figure 6D–F). These results suggest that LJFE potentially stimulated immunity by influencing immune-related cytokines, similar to the effect of red ginseng treatment.

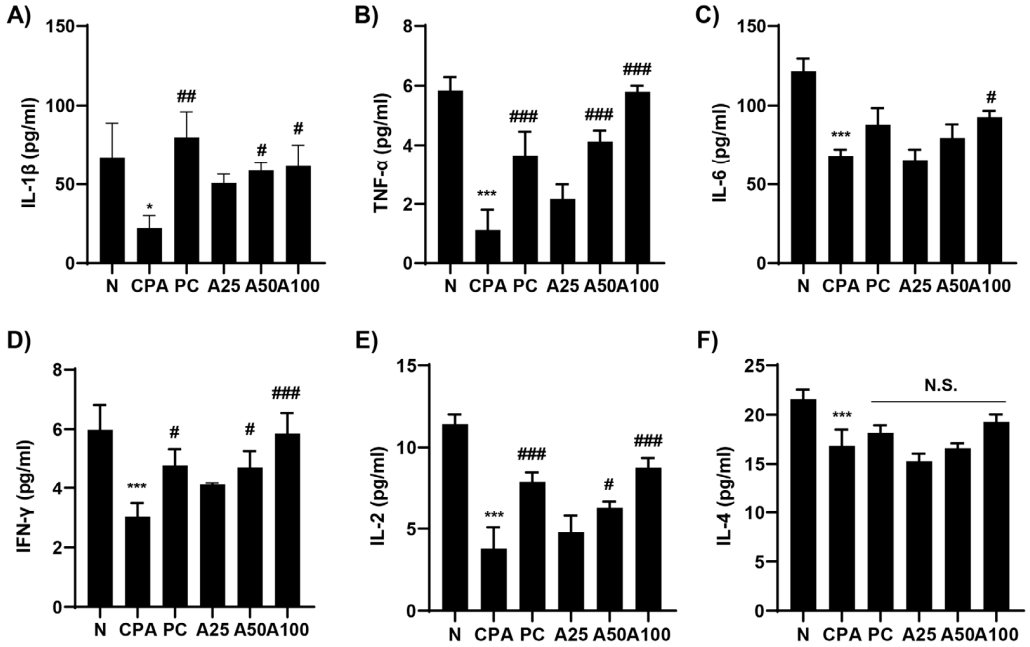

Figure 6. Effect of LJFE on serum levels of immune-related cytokines in CPA-induced immunosuppressed mice. (**A**) Interleukin (IL)-1β. (**B**) Tumor necrosis factor (TNF)-α. (**C**) IL-6. (**D**) Interferon (IFN)-γ. (**E**) IL-2. (**F**) IL-4. * $p < 0.05$ and *** $p < 0.001$ vs. control group. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$ vs. CPA group. A25, 25 mg/kg. A50, 50 mg/kg. A100, 100 mg/kg. CPA, cyclophosphamide-treated group. N, control group. N.S., not significant.

3.6. LJFE Increased the Amount of Immunological Igs

To investigate the immunity status, IgA, IgG, and IgM levels were measured in mouse serum. As shown in Figure 7A, LJFE stimulated serum levels of IgA at 50 and 100 mg/kg doses, which was superior to that in the red ginseng-treated and CPA groups. In addition, compared to that of the CPA group, the serum level of IgG increased by 50 mg/kg LJFE treatment, which was similar to that in the red ginseng-treated group (Figure 7B). Furthermore, LJFE stimulated serum levels of IgG at 50 and 100 mg/kg doses in comparison to that of the CPA-treated group, which was similar to the red ginseng-treated group. (Figure 7C). These results indicated that LJFE promoted immunity by increasing the amount of immunological Igs.

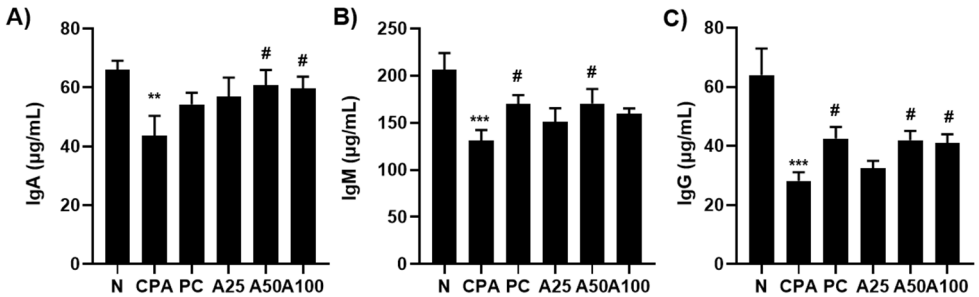

Figure 7. Effect of LJFE on serum levels of immunoglobulins (Igs) in CPA-induced immunosuppressed mice. (**A**) IgA. (**B**) IgM. (**C**) IgG. ** $p < 0.01$ and *** $p < 0.001$ vs. control group. $^{\#}$ $p < 0.05$ vs. CPA group. A25, 25 mg/kg. A50, 50 mg/kg. A100, 100 mg/kg. CPA, cyclophosphamide-treated group. N, control group.

4. Discussion

The immune system protects the body from harmful agents and external invasion; therefore, immune function is important to maintaining a healthy physiological condition. The immune system responds to, recognizes, and removes antigenic foreign substances and removes them to maintain physiological homeostasis [13,14]. Therefore, compounds that modify immune responses have attracted considerable interest. In this study, for the first time, we evaluated the immunomodulatory effects of LJFE in CPA-induced immunosuppressed mice.

In this study, 70% ethanol extracts of *L. japonica* were selected which are approved effective raw materials for health-functional food in certain indications and it was considered that these extracts could be extended to immunostimulating effects. Regarding active substances of *L. japonica* fruit extract, previous studies have reported lactones, terpenoids, and flavonoids [1–3]. Although full analysis of bioactive substances was not conducted for our extract, we selected Hamabiwalactone B as the indicator bioactive substance and its presence was confirmed using the high-performance liquid chromatography (HPLC) method. Regarding mechanism of action, 70% ethanol extract *L. japonica* may promote immunostimulatory activity potentially through proinflammatory transcription factors such as NF-κB as was reported to increase its target gene cytokines such as TNF-α, IL-6, and IL-1β [10]. However, an additional underlying mechanism study using Hamabiwalactone B will be subject to our further study.

The spleen is one of the largest lymphoid organs in the body, and it mediates immunological functions. Therefore, the spleen injury score was used to measure the function of the immune organ [25]. In this study, LJFE significantly reduced the spleen injury score at 25, 50, and 100 mg/kg, suggesting that LJFE recovered damages to the spleen structure and function (Figure 2). In the spleen, RP extracts aged cells from the blood circulation and detects pathogens, and WP is made up of immune cells. The marginal zones are regions between RP and WP capture antigens from the blood [25,26]. LJFE treatment recovered the architecture and these immune regions of the spleen, which were damaged by CPA treatment.

T-lymphocytes play an important role in immune response. T-cells are distinguished from other lymphocytes by the presence of T-cell receptors on their surfaces. $CD4^+$ cells are T-helper cells, which are functional in adaptive immune response [27,28]. For example, $CD4^+$ cells release cytokines and help in activating other immune cells such as $CD8^+$ cells or cytotoxic T-cells [29]. In our study, LJFE significantly increased the numbers of $CD4^+$- and $CD8^+$-positive cells at 100 mg/kg, suggesting that LJFE notably recovered the cellular immune function of lymphocytes, which was suppressed by CPA treatment (Figure 3). However, the $CD4^+/CD8^+$ ratio improved slightly and did not significantly recover. This may be because of the short treatment duration and low dosage of LJFE.

Lymphocyte activation and proliferation are adaptive immune responses [30]. Based on our results, the mechanisms by which LJFE stimulates the immune system involve the enhancement of T- and B-cell proliferation and NK-cell activation. T-helper cells help B-cells to multiply and produce antibodies and release cytokines. T-helper cells promote the activation of NK cells, another key cytotoxic lymphocyte that protects the body from pathogens and foreign substances [31,32]. LJFE promoted the proliferation of T- and B-lymphocyte and NK-cell activity at 100 mg/kg (Figure 4). NK-cell-derived cytokines and their cytotoxic functions can regulate immune responses and pathogenesis of many immune-related diseases. The activation of cytotoxic T- and NK cells promotes the production of cytokines such as IFN-γ and TNF-α. In our study, the serum levels of IFN-γ and TNF-α were significantly increased by LJFE treatment.

The function of hematopoietic cells reflects immune function [33,34]. Hematopoietic stem cells differentiate and generate blood cells throughout life. Under stress, progenitor cells modify the number of hematopoietic cell types and enhance cytokine production [35,36]. Hematopoietic cells generate blood cells, and changes in the abundance of blood cells determine the status of the immune system [37]. Blood transcriptional analysis results could

be used to investigate a range of autoimmune diseases including multiple sclerosis, diabetes, inflammatory bowel diseases, and more [37]. In this study, LJFE increased the numbers of WBCs, PLTs, and RBCs (Figure 5). These blood cells are important modulators of immune response. WBCs are produced in the bone marrow and circulate through the body, looking for foreign bodies such as bacteria, viruses, and fungi in the circulatory system and tissues [38]. PLTs participate in immune function by secreting inflammatory and bioactive molecules that regulate effector cells of the innate immune system [39]. RBCs interact with and respond to chemokines, nucleic acids, and foreign substances in the blood circulation [39]. Therefore, LJFE-induced immune function may result from the increase in the numbers of WBCs, PLTs, and RBCs; however, the underlying mechanisms require further studies.

Igs are produced by WBCs and regulate immune response by binding to specific antigens, such as bacteria and viruses, leading to their destruction. As the antibody-mediated immune response is highly specific, the Ig class and subclasses differ in their biological interactions and effects [40,41]. IgA deficiency is associated with autoimmune diseases and other pathological conditions such as asthma and allergies [42]. IgM is the largest antibody and the first response to an initial exposure to antigens [43]. IgM deficiency is rare but is associated with suppressed immune function. IgG generally controls the tissue infection, and its deficiency is associated with an enhanced occurrence of infection [44]. In this study, LJFE recovered IgA, IgM, and IgG at 50 mg/kg and 100 mg//kg (Figure 7). Therefore, an increase in the number of Igs may contribute to the improvement of immune function by LJFE.

In summary, most of major immunological and hematological parameters in our results showed greatest effectiveness at high dosage (100 mg/kg), although numbers of measurement also showed efficacy at lower dosage. Regarding cell cytotoxicity, previous studies report that *L. japonica* fruit extracts (30% and 70% ethanol) or Hamabiwalactone B, a main bioactive substance, showed no or low cytotoxicity in murine macrophage RAW 264.7 cells [10,12]. Therefore, *L. japonica* could be potentially supplemented as effective and safe immunostimulatory functional food or supplementation. However, in order to develop *L. japonica* as functional supplementation in market, clinical study should be conducted to confirm efficacy and safety in humans.

5. Conclusions

This study demonstrated that LJFE significantly stimulated the immune response by influencing cytokine secretion in mice. Based on results of LJFE administration, amount of $CD4^+$ and $CD8^+$ T-lymphocytes were notably recovered in splenocytes at high dosage in immunosuppressed mice. In addition, T-lymphocyte proliferation rate and natural-killer-cell activity was ameliorated. Furthermore, LJFE enhanced hematological function parameters such as WBCs, PLTs, and RBCs. In most immunological and hematological functional parameters, high dosage (100 mg/kg) of LJFE was observed to be the most effective.

In conclusion, LJFE could be potentially used as an immunostimulatory compound in functional food development, as demonstrated by the improved immune cell proliferation, activation, cytokine secretion, and hematopoietic functions. However, the current study does not show the underlying mechanism by which LJFE stimulates immune function and this subject needs further investigation.

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