J. Microbiol. Biotechnol. (2019), 29(9), 1361–1368
https://doi.org/10.4014/jmb.1905.05017

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Immune Enhancement Effects of *Codium fragile* Anionic Macromolecules Combined with Red Ginseng Extract in Immune-Suppressed Mice

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Received: May 9, 2019
Revised: August 2, 2019
Accepted: August 6, 2019

First published online
August 8, 2019

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pISSN 1017-7825, eISSN 1738-8872

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Codium fragile is an edible seaweed in Asian countries that has been used as a thrombolytic, anticoagulant, antioxidant, anti-inflammatory, and immune-stimulatory agent. Ginseng has also been known to maintain immune homeostasis and to regulate the immune system via enhancing resistance to diseases and microorganisms. In this study, anionic macromolecules extracted from *C. fragile* (CFAM) were orally administered with red ginseng extract (100 mg/kg body weight) to cyclophosphamide-induced immunosuppressed male BALB/c mice to investigate the immune-enhancing cooperative effect of *Codium fragile* and red ginseng. Our results showed that supplementing CFAM with red ginseng extract significantly increased spleen index, T- and B-cell proliferation, NK cell activity, and splenic lymphocyte immune-associated gene expression compared to those with red ginseng alone, even though a high concentration of CFAM with red ginseng decreased immune biomarkers. These results suggest that CFAM can be used as a co-stimulant to enhance health and immunity in immunosuppressed conditions.

Keywords: Anionic macromolecules, *Codium fragile*, red ginseng extract, cyclophosphamide, immune-suppressed mice

Introduction

Immunity is the ability to counteract foreign substances such as pathogens by recognizing their presence and removing them to maintain body homeostasis [1]. The largest lymphoid organ, containing one-fourth of the body's lymphocytes, is the spleen [2, 3], which comprises immune cells for mediating immune responses [4]. In the spleen, innate and adaptive immune reactions play an important role in immune homeostasis because they can be mounted efficiently [5]. Activated splenic lymphocytes produce cytokines such as IL-2, IL-6, and IFN-γ as well as other inflammatory mediators for innate and adaptive immune responses [6]. Natural killer (NK) cells, which are also found in the spleen, play a major role in host defense through cytotoxic activity against tumors and virus-infected cells [7]. In addition, NK cells act as primitive killers in the early immune response and are a specialized group of effector cells capable of mediating inflammatory responses through cellular cytotoxicity or cytokine and chemokine discharge [8].

Most anticancer chemotherapeutic drugs kill tumor cells while also damaging normal cells through the involvement of immune-associated cells during the cytokine storm [9]. Cyclophosphamide (CY) is a widely-used alkylating agent for chemotherapeutic treatment of several cancers [10]. The main side effect of CY is the inhibition of hematopoiesis [11], with clinical side effects such as nausea, vomiting, anorexia, bone marrow suppression, and lympho-proliferative disorders [12], often leading to immuno-suppression and cytotoxic effects [13].

Codium fragile is a dark green alga that is widely distributed in the coastal areas of East Asia, Oceania, and Northern Europe [14]. *C. fragile* extracts have been reported

to exhibit pharmacological activities such as antiviral, antiedema, antiallergic, antibacterial, antiprotozoal, anti-carcinogenic [15], anti-inflammatory [16], and immune-enhancing effects on RAW264.7 macrophage cells as an activator of the NF-κB and MAPK pathways [17]. Red ginseng extract has been reported to improve the improvement of blood circulation, suppression of cancer development, and infection-defending actions [18]. Many studies have demonstrated the beneficial effects of red ginseng on various diseases, such as immune system disorders, cancer, cardiovascular diseases, and neuronal disease [19–21].

Previous studies have suggested that combining certain bioactive components may enhance their therapeutic effect compared to the individual compounds [22, 23]. Very recently, our group also reported that *C. fragile* anionic molecules exhibited immune-enhancement effects in CY-induced immunosuppressed mice [24]. The present study investigates the immune-enhancing synergistic effects of CFAM combined with red ginseng extract, and also determines the optimal ratio for enhancing immune activity in immune-suppressed male BALB/c mice.

Materials and Methods

Animals

Male BALB/c mice at 6 weeks old (weighing 21–23 g) were provided by the Central Lab, Animal Inc. (Korea). A standard laboratory diet and water were provided to all mice and experimental protocols were approved by the Gangneung-Wonju National University Committee for Animal Experiments (GWNU-2018-20).

Anionic Macromolecules of *Codium fragile* Extract

CFAM was extracted and purified according to a previously reported method [17]. Briefly, milled seaweed was stirred in 80% EtOH for one day. The precipitate was collected by centrifugation, and was washed with EtOH and acetone, followed by centrifugation, filtration, and evaporation. The precipitate was re-dissolved in distilled water, and free proteins in the precipitate were removed using the Savag method [25].

CY-Induced Immunosuppression in Mice

Mice ($n = 5$ per group) were kept in specific pathogen-free animal facilities for one week before the start of experiments. One group as the control group (normal group) was orally administered with saline. The other groups were orally administered with 100 mg/kg body weight (BW) of red ginseng extract (Ginseng; Korea Ginseng Corp., Korea) [24] supplemented with four different concentrations of CFAM (0, 25, 50, 75, and 100 mg/kg BW). Levamisole (LVS) is a synthetic compound used for immunomodulation study and it plays a role as an immune-regulator by modulating macrophage chemotaxis and T-cell lymphocyte function [26]. In this study, LVS (Sigma-Aldrich, USA) was used as a positive control [27] and was orally administered at a concentration of 40 mg/kg BW. All the groups were administered the treatments once a day during the 10 successive days. Mice except the normal group intraperitoneally received 80 mg/kg BW of CY (Sigma–Aldrich) [24] one time per day during the 4^{th} to 6^{th} days following the beginning of administration. Mice (except those in the normal group) were injected intraperitoneally once a day with 80 mg/kg BW of CY [24] on day 4-6 post-administration, and mice were sacrificed at 24 h after the termination of treatments.

Splenocyte Preparation

After dissection of mice, the spleen of each mouse was collected, weighed and placed in ice-cold PBS for splenocyte isolation. Splenocytes were extracted using RBC Lysis Buffer (eBioscience, USA). After centrifugation and washing with PBS, the splenocytes were suspended in RPMI-1640 (Gibco Laboratories, USA) medium containing 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin (Welgene, Korea). The spleen index was calculated by the following equation:

$$\text{Spleen index} = \frac{\text{The weight of spleen (mg)}}{\text{The weight of mouse body (g)}}$$

Splenic Lymphocyte Proliferation

The extracted splenocytes were induced by 5 μg/ml of Concanvalin A (Con A; Sigma-Aldrich, USA) (Con A), T-cell mitogen, or 10 μg/ml of Lipopolysaccharide (Sigma-Aldrich, USA) (LPS), B-cell mitogen. The non-treated cells (cultured in RPMI-1640 alone) served as a normal control. After 48 h of culturing, cellular proliferation was evaluated by EZ-Cytox Cell Viability Assay Kit (Daeillab Service, Korea) and its ratio was measured by the next equation:

$$\text{Splenic lymphocyte proliferation ratio (\%)} = \frac{\text{Absorbance of the test group}}{\text{Absorbance of the control group}} \times 100$$

Splenic Natural Killer (NK) Cell Activity

The activity of NK cell as an effector cell in spleen was evaluated using YAC-1 cells as a target cell (Korean Cell Line Bank). Splenocytes and YAC-1 cells were co-cultured to gain an effector-to-target cell ratio of 50:1. After 4 h of co-culturing, the cultured supernatant was centrifuged at 400×*g* to remove contaminated cells. By using a lactate dehydrogenase (LDH) solution (Promega Co., USA), the cultured supernatant was then mixed [28]. The absorbance at 490 nm of each sample was tested using an EL800 Absorbance Microplate Reader (BioTek, USA), and NK cell activity was calculated using the next formula:

$$\text{Splenic NK cell activity (\%)} = \frac{(\text{Experimental release} - \text{Spontaneous release})}{(\text{Maximum release} - \text{Spontaneous release})} \times 100$$

RNA Extraction and First Strand cDNA Synthesis

Total RNA was isolated from the mitogen-stimulated lymphocytes using Tri reagent (Molecular Research Center, Inc., USA). The extracted concentration was measured by nano-photometer (Implen, Germany). The isolated RNA was used as template to produce first strand cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA).

Immune-Associated Gene Expression Assay

As a template, first strand cDNA (0.1 ng) was used for the real-time quantitative PCR assay to quantify gene expression in lymphocytes of spleen and the gene expression assay was performed in triplicate. Specific primer pairs were used for transcriptional expression analysis of immune-associated genes [24] by using SYBR Premix Ex Taq II (Takara Bio Inc., Japan) detection chemistry and QuantStudio™ 3 FlexReal-Time PCR System (ThermoFisher scientific, USA).

Statistical Analysis

All the results was statistically analyzed using Statistix 8.1 Statistics Software (Statistix, USA). All the data were also compared with the control group using one-way analysis of variance, and Tukey's post-hoc test was used to identify significant differences with $p < 0.05$.

Results

Effect of CFAM Combined with Red Ginseng on the Spleen Index

Fig. 1 shows the change of body weight, spleen index, and spleen size of mice tested in this study. The spleen size and spleen index show the significant reduction in the CY-treated mice compared to normal mice (Figs. 1B and 1C). Treatment with red ginseng alone significantly increased the spleen index of CY-treated mice, and the supplementation of red ginseng solution with four different concentrations of CFAM was observed to promote the spleen index compared to treatment with red ginseng alone. As shown in Fig. 1A, no significant differences in body weight were observed among experimental groups.

Effect of CFAM Combined with Red Ginseng on Splenic Lymphocyte Proliferation

To evaluate the proliferation of splenic lymphocytes, splenocytes from each treatment group were stimulated by T-cell (Con A) and B-cell (LPS) mitogens for 48 h.

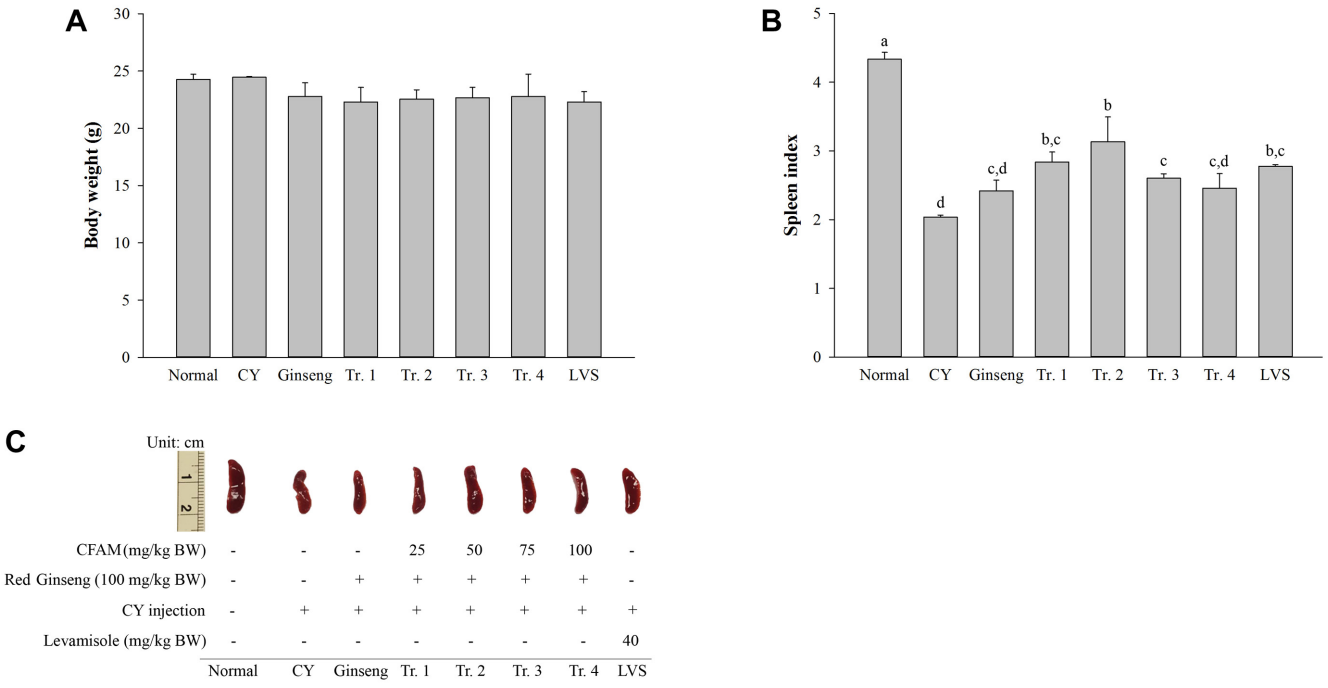

Fig. 1. Effects of various concentrations of CFAM coupled with red ginseng extract.
(**A**) Effect on body weight; (**B**) Effect on spleen index; (**C**) Effect on spleen size. Data are presented as means ± standard deviation. The letters a, b, c, and d indicate a significant difference ($p < 0.05$) between treatment groups. CY = Cyclophosphamide (negative control); Tr. = Treatment (different concentrations of CFAM: 25, 50, 75, and 100 mg/kg BW); LVS = Levamisole (positive control).

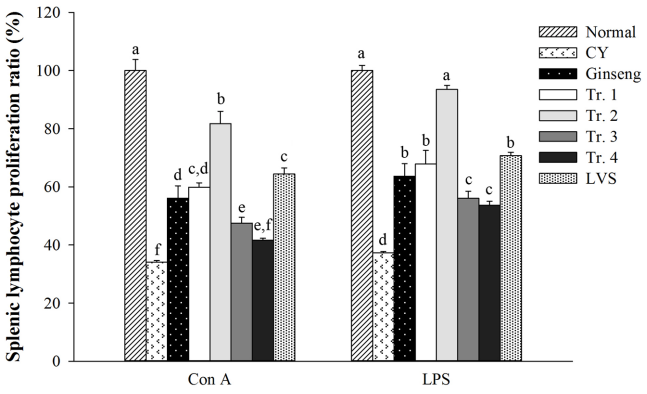

Fig. 2. Effects of various concentrations of CFAM coupled with red ginseng extract on splenic lymphocyte proliferation.
Data are presented as means ± standard deviation. The letters a, b, c, d, e, and f indicate a significant difference ($p < 0.05$) between treatment groups. CY = Cyclophosphamide (negative control); Tr. = Treatment (different concentrations of CFAM: 25, 50, 75, and 100 mg/kg BW); LVS = Levamisole (positive control).

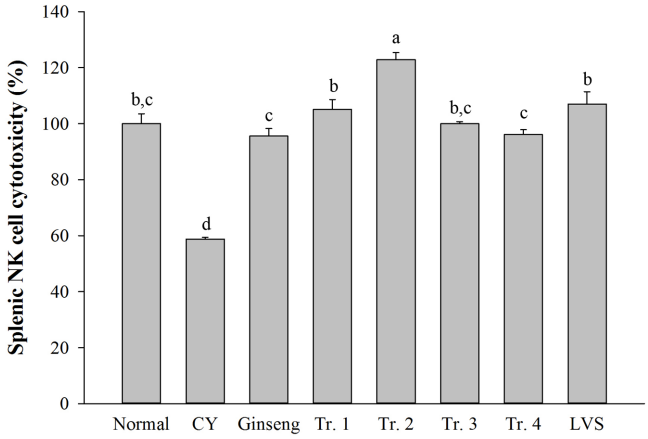

Fig. 3. Effects of various concentrations of CFAM coupled with red ginseng extract on splenic NK cell cytotoxicity.
Data are presented as means ± standard deviation. The letters a, b, c, and d indicate a significant difference ($p < 0.05$) between treatment groups. CY = Cyclophosphamide (negative control); Tr. = Treatment (different concentrations of CFAM: 25, 50, 75, and 100 mg/kg BW); LVS = Levamisole (positive control).

Compared to the normal group, splenic lymphocyte proliferation in response to both T-cell and B-cell mitogens remarkably decreased in the CY-treated group (Fig. 2). However, the red ginseng-treated group exhibited enhanced proliferation of splenic lymphocytes in response to both T- and B-cell mitogens. Furthermore, treatment with up to 50 mg/kg BW of CFAM coupled with red ginseng significantly boosted T- and B-cell proliferation compared to the red ginseng group, unlike groups with higher concentrations of CFAM (75 and 100 mg/kg BW).

Effect of CFAM Combined with Red Ginseng on Splenic NK Cell Activity

Splenic NK cell activity was evaluated via co-culturing of YAC-1 cells. The cultured cell supernatant was used to determine cytotoxicity via the LDH assay. As shown in Fig. 3, splenic NK cell activity was suppressed by CY treatment compared to normal mice. However, red ginseng treatment markedly recovered the NK cell activity of splenocytes in CY-treated mice. Moreover, the highest splenic NK cell activity was observed in the group treated with 50 mg/kg BW of CFAM supplemented in red ginseng solution, rather than in higher concentration CFAM groups (75 and 100 mg/kg BW).

Effect of CFAM along with Red Ginseng on Immune-Associated Gene Expression

The immune-associated gene expression levels in mitogen-induced splenic lymphocytes were evaluated using qRT-PCR analysis. Fig. 4 shows that the expression of immune-associated genes was significantly reduced in the CY-treated group. Nonetheless, treatment with different concentrations of CFAM (up to 50 mg/kg BW) combined with red ginseng enhanced T- and B-cell responses by increasing the immune-associated genes expression levels in splenic lymphocytes. Most of the gene (*IL-4*, *IL-10*, *TNF-α*, *IFN-γ*, and *TLR-4*) were more highly upregulated by B-cell mitogens than by the T cell mitogens in splenic lymphocytes. However, the expression levels of *IL-1β* and *IL-6* were higher in T cell mitogen-stimulated cells than in B-cell mitogen-stimulated cells.

Discussion

The immune system is important for the protection of host cells against harmful substances such as bacteria, fungi, and viruses, as well as for the prevention of cancer cell growth [29]. The immune system is divided into innate or adaptive immunity due to their functional activities, and the two usually act together. The first line of host defense is the innate response, while the adaptive response is acquired immunity that reacts to foreign antigens in a highly specific manner [29, 30]. CY is an anticancer drug that is widely used for the treatment of various cancers [31, 32]. However, it induces side effects in the immune system and causes immunosuppression [33]. In this study, we

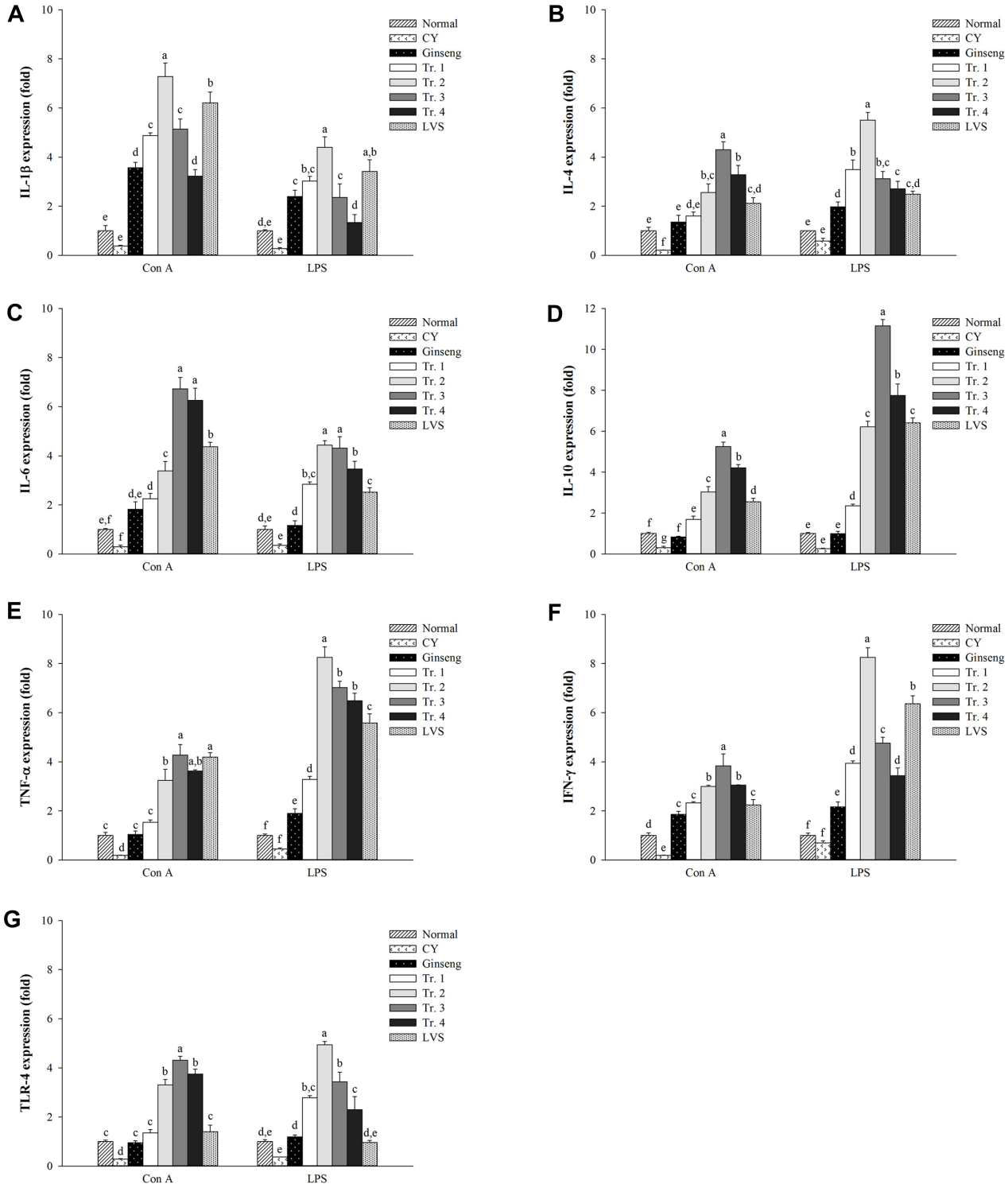

Fig. 4. Effects of various concentrations of CFAM coupled with red ginseng extract on the relative expression levels (fold-change) of immune genes in mitogen-stimulated splenic lymphocytes.

(**A**) IL-1β; (**B**) IL-4; (**C**) IL-6; (**D**) IL-10; (**E**) TNF-α; (**F**) IFN-γ; and (**G**) TLR-4. Data are presented as means ± standard deviation. The letters a, b, c, d, e, f, and g indicate a significant difference ($p < 0.05$) between treatment groups. CY = Cyclophosphamide (negative control); Tr. = Treatment (different concentrations of CFAM: 25, 50, 75, and 100 mg/kg BW); LVS = Levamisole (positive control).

evaluated the efficacy of CFAM combined with red ginseng extract for enhancing immunological functions in immunosuppressed BALB/c mice.

Since the spleen function is highly associated with the immune response, the spleen index is used as a representative immunity indicator [34, 35]. Fig. 1 shows that a combination of CFAM and red ginseng promoted the recovery of spleen size and spleen index in immune-suppressed mice, indicating that host immunity was activated by antigens or mitogens via lymphocyte proliferation variation [36, 37]. In this study, the proliferation of T- and B-lymphocytes was up- or downregulated by Con A and LPS, respectively. Treatment with CFAM combined with red ginseng promoted T- and B-lymphocyte proliferation at CFAM concentrations up to 50 mg/kg BW, in contrast to higher concentration of CFAM (Fig. 2). In addition, Fig. 3 shows that red ginseng significantly enhanced NK cell activity in CY-treated mice, and supplementation of 25 and 50 mg/kg BW of CFAM with red ginseng caused an even more significant increase in NK cell activity than red ginseng treatment alone. These results suggest that a treatment of CFAM combined with ginseng may contribute to regulation of NK cells, which are critical for the natural suppression of cancer cell proliferation, resistance to microbial or parasitic infection, acute graft rejection, neoplasia, and metastasis [38,39].

T cells are defined by the surface expression of T-cell receptors and comprise many different subtypes, such as T helper (Th) cells, cytotoxic T cells, suppressor T cells, and effector T cells. Type 1 T helper (Th1) cells are stimulated during cell-mediated immune responses, while Type 2 T helper (Th2) cells are activated during humoral or allergic responses [40]. Th1 and Th2 cells generate specific and diverse cytokines to directly and indirectly regulate specific immune responses. Th1 cells control the regulation of IL-2, IFN-γ, and TNF-α, while Th2 cells control the regulation of IL-4, IL-6, and IL-10 [41]. Fig. 4 shows that the expression levels of cytokines in CY-treated mice were upregulated following treatment with a mixture of up to 50 mg/kg BW CFAM and red ginseng. This result indicates that CFAM coupled with red ginseng stimulates the secretion of Th1 and Th2 cytokines to regulate and restore immunosuppression. The comitogenic effect did not reach maximum values in a dose-dependent manner in a mixture of 50 mg/kg BW of CFAM with 100 mg/kg BW of red ginseng. Therefore, the ratio of CFAM to red ginseng might be important and should be considered when the two are mixed for use at an industrial level [42].

The current study was performed to evaluate the synergistic immune-enhancing activity of CFAM and red ginseng. Treatment with CFAM combined with red ginseng resulted in increases in spleen size, spleen index, splenic NK activity, splenic lymphocyte proliferation, and splenic lymphocyte immune-associated gene expression, with a maximum concentration of 50 mg/kg BW of CFAM with red ginseng. Moreover, it was confirmed that a mixture of CFAM and red ginseng exhibited greater immune-enhancing effects than those with red ginseng alone. Therefore, these results suggest that CFAM can be used, at the appropriate concentration, as a co-stimulant with red ginseng to enhance health and immunity.

Acknowledgments

This study was supported by the Marine Bio-Regional Specialization Leading Technology Development Program (D11413914H480000100), funded by the Ministry of Oceans and Fisheries in Korea. This research project was also supported by the University Emphasis Research Institute Support Program (No.2018R1A61A03023584) from the National Research Foundation of Korea.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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