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Effect of *Chlorella vulgaris* on Immune-enhancement and Cytokine Production *in vivo* and *in vitro*

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Abstract The object of this study was to investigate the immune-enhancing effects of *Chlorella vulgaris* (CV) on a deteriorated immune function by a protein-energy malnutrition (PEM) diet. Unicellular algae, CV were used as a biological response modifier. Male C57BL/6J mice were fed for 15 days with standard diet or a PEM diet, which is associated with decreased host immune defense. After 8 days, mice in the PEM diet group were orally administered by 0.05, 0.1, and 0.15 g/kg body weight of CV or distilled water. Nutritional parameters, and interferon (IFN)-γ levels were significantly increased in the blood serum of the CV (0.15 g/kg)-treated group (29.6±2.8 pg/mL) compared to the non-treated PEM group (4.1±0.4 pg/mL, $p<0.05$). In addition, cell proliferation and production of cytokines were investigated via a CV (0.01, 0.1, and 1 mg/mL) treatment using a human T cell line MOLT-4 cell. The CV treatment (1 mg/mL) significantly increased the production of both IFN-γ and interleukin (IL)-2 (51.3±3.4 and 285.9±18.8 pg/mL, respectively) compared to the control (51.3±3.4 and 442.6±14.3 pg/mL, respectively), but did not affect the production of IL-4. These results suggest that CV may be useful in improving the immune function.

Keywords: *Chlorella vulgaris* (CV), immune enhancement, protein-energy malnutrition (PEM), MOLT-4 T cell, interferon-γ (IFN-γ), interleukin-2 (IL-2)

Introduction

Chlorella vulgaris (CV) is a freshwater unicellular, microscopic alga that is widely used as a food supplement in Japan (1). The supplement is taken as tablets, capsules, extract liquid, or as a food additive; claims for health benefits include the improvement of the immune function as well as improvements in the control of hypertension, fibromyalgia, and ulcerative colitis. Numerous human and animal experiments have documented a variety of pharmacological effects of CV and CV extract, including improvements hypertension levels, lipid metabolism, anti-tumor and antibacterial activities, and the promotion of dioxin excretion (2-7). Kanouchi *et al.* (8) orally treated male Sprague-Dawley rats with CV powder to study its effects on serum antibody levels and antibody production by splenocytes and mesenteric lymphocytes. Their results confirmed that CV enhanced immunoglobulin (Ig) M antibody production by circulating splenocytes and mesenteric lymphocytes as well as IgG antibodies by splenocytes and mesenteric lymphocytes. However, to date, the effects of CV on the immune function when deteriorated by nutrition deficiency has not been reported.

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The interaction of nutrient deficiencies and the immune status has been the focus of increasing research over the last 3 decades. Nutrients derived from dietary proteins and fats as well as micronutrients, vitamins, and minerals impact immune cells systemically in circulating blood, regional lymph nodes, and the specialized immune system of the gastrointestinal tract. As a result, the body's defense mechanisms are affected (9). Protein-energy malnutrition (PEM) affects both cellular and humoral immunity; it influences the phagocytic function, cytokine production, generation of complement factors, and the production of secretory IgA (10). A disproportionately large reduction in spleen weight of young adult female PEM mice compared to other organ weights was determined. The lower spleen weight corresponded to a reduction in the number of splenocyte cells in PEM mice compared to normal mice (11). The capacity of T cells to produce interferon (IFN)-γ is consistently depressed in rodent models of acute protein and energy deficits, while interleukin (IL)-4 production remained unaffected (12,13). T helper (Th) 1 lymphocytes produce IL-2, IFN-γ, and tumor necrosis factor (TNF), and Th2 lymphocytes produce IL-4 (14). The induction of Th1 immune responses plays a critical role in protecting against various intracellular microorganisms and tumors, and is involved in reversing Th2 cell-facilitating diseases, such as allergic inflammation (15). The cytokine productions are directly correlated with immune functions in the T cell; hence, the cytokine levels in T cells and

splenocytes with or without a CV treatment were compared.

In this study, the effects of CV in a PEM experimental model on nutritional markers, such as plasma total protein (TP), albumin, glucose levels, and on immunological markers, including the plasma IFN-γ level, the numbers of splenocytes and lymphocytes were examined *in vivo*. In addition, the productions of IFN-γ, IL-2, and IL-4 on the CV-treated human T cell line MOLT-4 cells were analyzed in order to investigate the effect of CV on the production of cytokines *in vitro*.

Materials and Methods

Animals Male C57BL/6J mice weighing 16-18 g (Orient Co., Ltd., Seongnam, Korea) were used in these experiments. They were housed under following laboratory conditions: temperature 23±1°C, humidity 40-60%, 12:12-L/D cycle, lights on at 7 am. Food and water were available *ad libitum*. Sixty male C57BL/6J mice were divided into 6 groups of 10 mice each. Two control groups were fed a standard diet, and mice in other 4 groups were fed a PEM diet for 15 days. The components of PEM diet was based on the previous reports (9-12), detailed components are in Table 1. Mice were treated in accordance with the current law and the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals.

Treatment of CV The CV, supplied by Daesang Corp. WellLife (Seoul, Korea), was dissolved in distilled water and used as suspension at 0.05, 0.1, and 0.15 g/kg dosages. Each 10 mice were divided into 6 groups. Two groups were fed by standard diet, and 4 groups were fed by PEM diet. After 8 days from fed by standard or PEM diet, the mice in standard diet group were administered by distilled water (DW) or 0.15 g/kg CV. The mice in PEM diet group were administered by DW, 0.05, 0.1, and 0.15 g/kg CV, respectively. The CV or DW was administered orally for a week. The CV used in this experiment was found to be free from endotoxin as determined within the limits of an endotoxin assay kit (Sigma-Aldrich, St. Louis, MO, USA), performed according to manufacturer's protocol.

Preparation and ingredient analysis of blood serum Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). After anesthetization, blood was withdrawn from the hearts of the mice into syringes. The serum was then prepared by centrifugation at 8,000×g at 4°C for 10 min. The presence of TP, albumin, and glucose were determined using an autoanalyzer (Hitachi 747; Hitachi, Tokyo, Japan).

Table 1. Composition of experimental diets

Component	Standard (g/kg)	PEM[1)] (g/kg)
Casein	230.00	57.50
DL-Methionine	3.00	3.00
Sucrose	431.70	581.80
Corn starch	200.00	200.00
Corn oil	52.30	54.03
Cellulose	37.86	57.01
Vitamin mix	10.00	10.00
Ethoxyquin (antioxidant)	0.01	0.01
Mineral mix	13.37	13.37
Calcium phosphate, dibasic	16.66	22.00
Calcium carbonate	5.10	1.28

[1)]Protein-energy malnutrition.

Counting mononuclear cell suspensions from spleen, superficial and mesenteric lymph nodes After blood was withdrawn from the mice, the spleens, superficial lymph nodes, and mesenteric lymph nodes were removed and placed on ice covered with complete medium RPMI-1640 (Gibco BRL, Grand Island, NY, USA), containing 10% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS, USA), 100 mL penicillin, and 100 μg/mL streptomycin (Gibco BRL). To obtain a single cell suspension, the spleens and lymph nodes were teased, and cells were counted in a hemocytometer.

MOLT-4 cell cultures Human T cell line MOLT-4 cells were grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in the presence of 5% CO_2. Because CV is not dissolved completely in water, for the *in vitro* test, it was prepared by crushing the cell walls in a Dyno Mill (WAB Inc., Maschinenfabrick, Basel, Switzerland) and spray drying. The dried powder was weighed and dissolved in DW. The suspension was used after filtering through a 0.4-μm syringe filter. Cell aliquots (3×10^5 cells/well) were seeded in microplate wells and stimulated with CV (0.01-1 mg/mL) for 24 hr at 37°C in 5% CO_2. Supernatants were harvested after 24 hr for a quantification of the cytokine levels, and the cells were used for viability assay.

MTT assay Cell aliquots (3×10^5 cells/well) were seeded in microplate wells and incubated with 20 μL of a 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium (MTT) solution (5 mg/mL) for 4 hr at 37°C under 5% CO_2 and 95% air. Consecutively, 250 μL of dimethylsulfoxide (DMSO) was added to extract the MTT formazan, and the absorbance of each well was read at 540 nm by an automatic microplate reader.

Cytokine assay A sandwich enzyme-linked immunosorbent assay (ELISA) was carried out for IFN-γ, IL-2, and IL-4 in duplicate using a 96-well format. ELISA plates (Nunc, Roskilde, Denmark) were each coated with 100 μL aliquots of anti-human (or mouse) IFN-γ, IL-2, and IL-4 monoclonal antibodies at 1.0 μg/mL in phosphate-buffered saline (PBS) at pH 7.4 and were then incubated overnight at 4°C. The plates were washed in PBS containing 0.05% Tween-20 and blocked with PBS containing 1% BSA, 5% sucrose, and 0.05% NaN_3 for 1 hr. After additional washes, 100 μL sample or each standard was added and the plates were then incubated at 37°C for 2 hr. After 2 hr incubation at 37°C, the wells were washed and 0.2 μg/mL of biotinylated anti-human (or mouse) IFN-γ, IL-2, and IL-4 were then added to each and they were incubated again at 37°C for 2 hr. After washing the wells, avidin-peroxidase was added and plates were incubated for 20 min at 37°C. Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated

microplate ELISA reader. A standard curve was run on each assay plate using recombinant IFN-γ, IL-2, and IL-4 in serial dilutions.

Statistical analysis Results were expressed as the mean ±standard error (SE) of independent experiments, and statistical analyses were performed by a one-way analysis of variance (ANOVA) with Tukey's and Duncan's post hoc tests to express the differences among the groups. All statistical analyses were performed using SPSS v 12.0 statistical analysis software. A value of $p<0.05$ was considered to indicate statistical significance.

Results and Discussion

Effect of CV on blood nutritional parameters After 8 days fed by PEM diet, the mice in 3 groups among PEM diet group were administered by 0.05, 0.1, and 0.15 g/kg CV, and the mice in the other group were administered by DW for a week. The mice administered by DW in PEM diet group (0.46±0.21 g) gained significantly less body weight than standard diet mice (3.8±0.32 g), and the body weights of the CV-treated PEM diet groups were similar to those of the PEM diet group. The CV-treated groups (0.05, 0.1, and 0.15 g/kg) gained 0.11±0.19, 0.04± 0.13, and 0.47 ±0.22 g, respectively. To clarify the effective mechanism of CV, the levels of blood nutritional parameters were assessed in mice after the PEM diet. Plasma TP, albumin, and glucose levels were significantly decreased in PEM mice (4.8±0.1 g/dL, 3.06±0.0 g/dL, and 179±4.9 mg/dL, respectively) compared to the mice fed the standard diet (5.3±0.8 g/dL, 3.4±0.1 g/dL, and 275.7±4.1 mg/dL, respectively; $p<0.05$). The levels of TP, albumin, and glucose in the 0.05, 0.1, and 0.15 g/kg CV-treated groups (5.5±0.1 g/dL, 3.4±0.0 g/dL, and 282.7±5.9 mg/dL in 0.15 g/kg CV-treated group) were significantly increased compared to these levels in the PEM diet group ($p<0.05$; Table 2). Each levels of CV-treated standard diet group were not different from DW-treated standard diet group.

Effect of CV on mononuclear cell numbers in the spleen and the superficial and mesenteric lymph nodes To examine the effect of CV on the deteriorated immunity caused by the PEM diet, the number of mononuclear cells in the crucial immune organs were counted. The cell numbers for the spleen, superficial lymph node, and mesenteric lymph node were $(37.6\pm3.4)\times10^6$, $(82.0\pm6.1)\times10^5$, and $(86\pm6.1)\times10^5$ in standard diet group. Those numbers in PEM diet group were $(10.4\pm1.6)\times10^6$, $(46.0\pm4.9)\times10^5$, and $(51.7\pm9.7)\times10^5$. As expected, in each case, this number in the PEM mice was significantly decreased compared to the mice fed the standard diet ($p<0.05$). Each numbers of CV-treated standard diet groups was not different from DW-treated standard diet group. The cell numbers for the spleen and superficial lymph node were increased in a dose-dependent manner in the CV-treated groups [$(153.0\pm1.7)\times10^6$ cells, $(66\pm2.9)\times10^5$ cells, respectively, in 0.15 g/kg CV-treated group]. However, although the cell number for the mesenteric lymph node increased significantly, this did not show dose-dependency (Table 3).

Effect of CV on the production of IFN-γ in blood serum Plasma IFN-γ, IL-2, and IL-4 levels were assayed in the PEM mice. Although the production levels of IL-2 and IL-4 remained unaffected by the PEM diet (101±2.4, 94±7.5% compared to standard diet group, respectively), the plasma IFN-γ level was decreased in the PEM mice (4.1±0.4 pg/mL) compared to the standard diet mice (22.73±3.1 pg/mL). However, the IFN-γ level was significantly increased when the mice were administered 0.15 g/kg CV

Table 2. Effect of CV on blood nutritional parameters in mice[1)]

Diet	Treatment CV (g/kg)	TP (g/dL)	Albumin (g/dL)	Glucose (mg/dL)
CON	0	5.33±0.88	3.43±0.09	275.67±4.06
CON	0.15	5.56±0.12	3.40±0.57	284.00±5.50
PEM	0	4.80±0.05*	3.06±0.03*	179.00±4.93*
PEM	0.05	5.30±0.06^{o}	3.20±0.06^{o}	202.67±3.71^{o}
PEM	0.1	5.70±0.06^{o}	3.30±0.06^{o}	232.00±5.78^{o}
PEM	0.15	5.50±0.06^{o}	3.40±0.04^{o}	282.67±5.92^{o}

[1)]CV (0.05, 0.1, and 0.15 g/kg/day, p.o. for a week) was administered orally to mice, we have measured the levels of TP, albumin, and glucose in the serum. Each level was determined by the autoanalyzer. Each data value indicates the mean±SE (n=10). *$p<0.05$ compared to CON 0; $^{o}p<0.05$ compared to PEM 0.

Table 3. Effect of CV on mononuclear cell numbers in mice[1)]

Diet	Treatment CV (g/kg)	Spleen ($\times10^6$)	Superficial lymph node ($\times10^5$)	Mesentric lymph node ($\times10^5$)
CON	0	37.60±3.45	82.00±6.06	86.00±6.10
CON	0.15	44.48±2.73	79.57±5.15	94.28±7.16
PEM	0	10.40±1.57*	46.0±4.90*	51.67±9.71*
PEM	0.05	16.57±0.87^{o}	97.00±8.66^{o}	84.00±14.58^{o}
PEM	0.1	28.80±1.84^{o}	126.50±23.23^{o}	55.20±6.44^{o}
PEM	0.15	42.80±3.20^{o}	153.00±1.73^{o}	66.00±2.97^{o}

[1)]CV (0.05, 0.1, and 0.15 g/kg/day, p.o. for a week) was administered orally to mice, we have counted the each cell numbers. Each data value indicates the mean±SE (n=10). *$p<0.05$ compared to CON 0; $^{o}p<0.05$ compared to PEM 0.

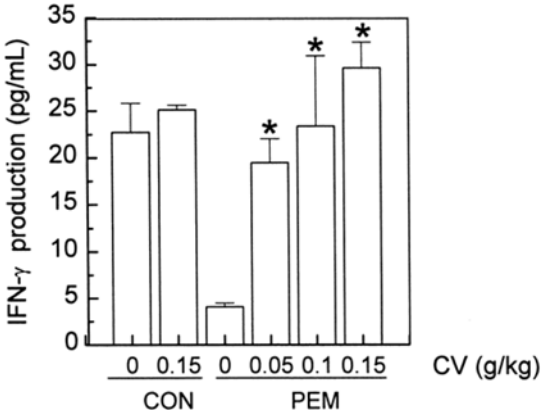

Fig. 1. Effect of CV on plasma IFN-γ production in mice. Values represent the mean±SE. *$p<0.05$, significantly different from the PEM 0 diet group.

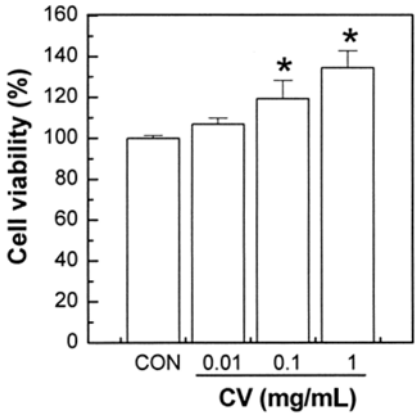

Fig. 2. Effect of CV on the cell viability. Human T cell line MOLT-4 cells (3×10^5 cells/well) were treated with various concentrations of CV for 24 hr. Cells were then collected and assessed for viability using MTT. Values represent the mean±SE of duplicate determinations from 3 separate experiments. *$p<0.05$, significantly different from the media control.

(29.6±2.8 pg/mL; $p<0.05$; Fig. 1). As shown in Fig. 1, plasma IFN-γ production was not significantly increased by CV (0.05 and 0.1 g/kg) treatment.

Effect of CV on human T cell line MOLT-4 cell proliferation An MTT assay was performed to assess the effect of CV on the proliferation of cells. It was found that the proliferation of MOLT-4 cells was increased compared to that of the control in a dose-dependent manner. In particular, proliferation in the 0.1 and 1 mg/mL CV-treated cells increased 19.4±8.9, 34.5±8.2%, respectively, compared to control ($p<0.05$; Fig. 2).

Effect of CV on the production of IFN-γ, IL-2, and IL-4 in human T cell line MOLT-4 cells To assess the effect of CV on the production of cytokines, MOLT-4 cells were treated with various concentrations of CV for 24 hr. The levels of IFN-γ, IL-2, and IL-4 were analyzed by the ELISA method. As shown in Fig. 3A, CV (0.01-1 mg/mL; $p<0.05$) significantly increased the IFN-γ level compared to media. Figure 3B shows that the IL-2 level was

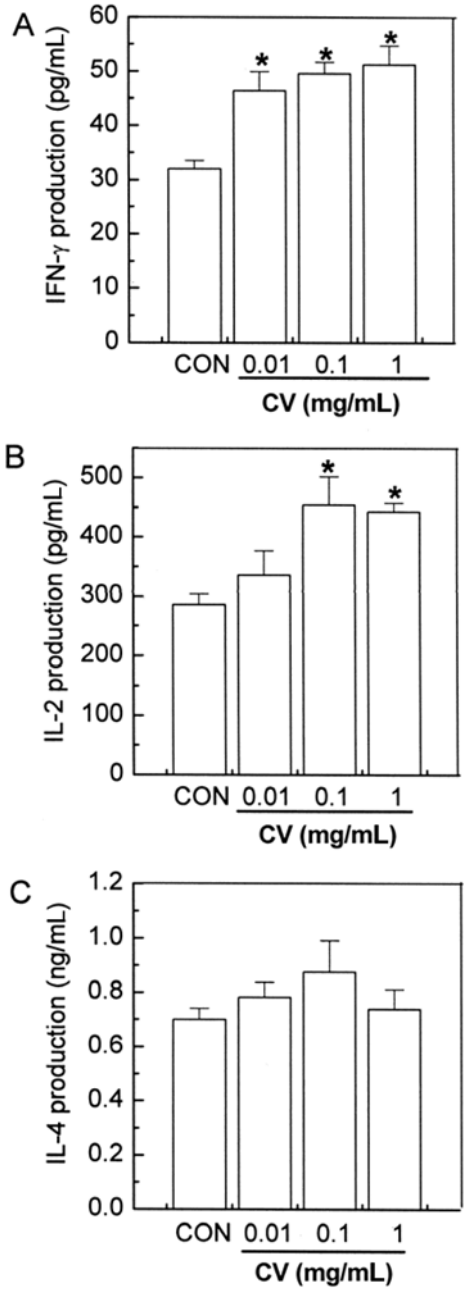

Fig. 3. Effect of CV on IFN-γ (A), IL-2 (B), and IL-4 (C) production in human T cell line MOLT-4 cells. Culture supernatant was collected from none or from CV-treated human T cell line MOLT-4 cells, which were cultured for 24 hr. Cytokine levels in the culture supernatant were measured using ELISA. Values represent the mean±SE of duplicate determinations from 3 separate experiments. *$p<0.05$, significantly different from the media control.

significantly increased by the CV treatment compared to the media (0.1 and 1 mg/mL; $p<0.05$); however, CV did not affect the production of IL-4 (Fig. 3C).

The biological effects of CV on tumors, bacteria, and mitogenes are described in available literature (5,6). Most of these studies provide strong evidence for the hypothesis that CV may enhance immunity in immunocompromised hosts. Using PEM mice, it was demonstrated that CV is

beneficial in terms of improving nutritional and immunological parameters.

In the present study, the levels of plasma TP, albumin, and glucose in immune-deteriorated PEM mice were measured. TP is a rough measure of serum protein, whereas albumin, the main protein in blood, is the key to the regulation of the osmotic pressure of blood. Protein measurements can reflect the nutritional state, kidney disease, liver disease, and many other conditions. Energy is derived initially from the breakdown of glycogen and later from circulating glucose released by the liver and from non-esterified fatty acids (16). Therefore, TP, albumin, and glucose levels are important markers representing the nutritional state in the host defense mechanism. The levels of TP, albumin, and the glucose levels in the serum of the PEM mice were significantly decreased compared to those levels in mice fed with a standard diet ($p<0.05$). Following CV administration, TP, albumin, and glucose levels were significantly increased. These results suggest that CV acts as a source of protein and as an energy supply.

Severe PEM affects cellular immunity, antibody production, the concentrations of secretory Ig A, the phagocytic function, the complement system, and the production of cytokine (17). Several authors have shown that malnutrition is the primary cause of immunodeficiency in the world. Malnutrition has been shown to be related to changes in cellular immunity and peripheral-lymphocyte subsets (mainly $CD3^+$, $CD4^+$, and $CD8^+$). Nevertheless, results from studies investigating this topic are controversial. In some studies, an increase of the lymphocyte proportion was observed, while other studies show a reduction of this (18). In a study by Najera *et al.* (19) no changes in lymphocyte T subsets was found when malnourished and well-nourished children with bacterial infections were compared. These controversial results may be influenced by several factors, such as the methods used, the type and degree of malnutrition, and the infection type. For this reason, obvious markers, such as cell number and cytokine levels, were compared in mice serum. As expected, the mononuclear cell numbers in the spleen and lymph nodes were reduced by the PEM diet while they were increased by an administration of CV. Also assayed were the plasma IFN-γ, IL-2, and IL-4 levels with or without the PEM diet. The IL-2 and IL-4 levels were not affected by the PEM diet, while the IFN-γ level in PEM mice decreased. Following CV (0.15 g/kg) administration, the IFN-γ level was significantly increased ($p<0.05$).

Immunoregulatory cytokines play an important role in determining the nature and strength of an immune response (20,21). Recent studies indicate that the ratio of these 2 Th cell types, Th1 and Th2, is closely correlated with the outcome of many diseases; controlling the Th1/Th2 ratio has been demonstrated as a therapeutic strategy for various diseases (22-24). Many cancer vaccines, particularly in combination with immune adjuvants, elicit strong cellular immune responses, leading to the production of Th1-type cytokines such as IFN-γ, IL-2, and TNF-α (25). IFN-γ is also an important cytokine in the host defense against infection by viral and microbial pathogens (26). IL-2, together with other factors and in conjunction with antigens, mitogens, or anti-Ig antibodies, controls B cell proliferation and differentiation into antibody-producing plasma cells (27). The natural killer and lymphokine-activated killer cells, monocytes and macrophages all have the ability to respond to IL-2 with increased activity or proliferation (28). In this study, it was shown that CV strongly increased the production of IFN-γ and IL-2 while not affecting the production of IL-4 in MOLT-4 cells (Fig. 3). The CV was free of endotoxin (<0.5 ng/mL), indicating that the effects of CV were not caused by lipopolysaccharide (LPS) contamination. This shows that CV may activate Th1 cells as well as help the immune system and host defense. There is no dose dependency for the actions of CV *in vitro*, except with IFN-γ production in MOLT-4 cells. Despite the absence of dose dependency, those changes are, however, noteworthy as evidence of CV affecting the immune system. The results of this study may aid in the understanding of the pharmacological function of the CV for future *in vivo* studies. In this study, it was shown that CV increased immunological markers in a PEM diet model. CV also strongly induced the proliferation and production of IFN-γ and IL-2 of MOLT-4 cells. These results suggest that the increase of the immunological markers caused by CV administration in the PEM diet test may be mediated through immune enhancement.

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