Beneficial immune-stimulatory effect of Chlorella supplementation: the enhancement of Natural Killer cell activity and early inflammatory response

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Beneficial immune-stimulatory effect of Chlorella supplementation: the enhancement of Natural Killer cell activity and early inflammatory response

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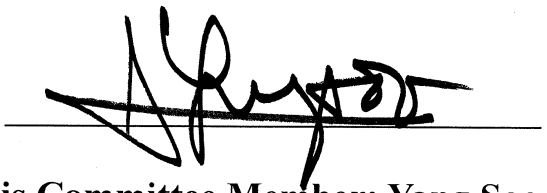

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감사의 글

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먼저, 노화과학협동과정에 입학하여 배움의 길을 열어주시고 항상 열정적인 모습으로 많은 가르침을 주셨던 이종호 교수님께 진심으로 감사 드립니다. 바쁘신 가운데도 많은 가르침 주신 장양수 교수님께 감사 드립니다.

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고 있는 령우, 자기 몸도 돌보지 않고 열심인 희정이, 꼼꼼함과 귀여움으로 꽉 찬 혜원이, 내가 어느 순간 무심하게 변해버려서 미안하고 함께 한 시간을 되돌아보며 감사의 마음을 전합니다. 그리고 언제나 밥도 잘 못 먹고 실험을 같이하던 책임감 강한 미지, 마음이 여린 용희, 외에도 열거 할 수 없을 정도로 많은 선배님, 후배님들에게 감사의 마음을 전합니다. 그리고 항상 친절하게 신경 써주신 정임누나에게 감사의 마음을 전합니다. 그리고 수업시간에만 뵙지만 자애로운 열정을 가지신 조재선 선생님과 항상 많은 관심 가져 주시고 열심히 노력하시는 모습이 존경스러운 서울대병원의 김지영선생님께도 감사의 마음을 전합니다.

오랜 시간을 함께 보내며 항상 응원을 해 준 나의 소중한 친구들에게 감사의 마음을 전합니다. 옆 연구실에서 힘들 때 언제나 변함없이 힘이되 준 태원 형과 최고의 여자친구 인경 누나, 인생을 즐기며 살 줄 아는 멋진 재승 형, 과묵하지만 무한한 정을 갖고 있는 홍규 형, 톡톡 튀는 매력이 있는 사랑스런 승은 누나, 이제는 결혼을 해 아기엄마가 된 하선누나와 매형, 언제나 연구하느라 바빠서 얼굴 보기도 힘든 승한 형, 우리 예무리 선배, 동기, 후배들에게 감사의 마음을 전합니다. 그리고 바쁠 때 서로 고민을 들어주고 의지가 된

학부 동문들인 원영, 재우 모두 성공하길 바랍니다.

마지막으로 세상 무엇과도 바꿀 수 없고 가장 사랑하는 우리 가족에게 감사의 마음을 전합니다. 우리가족을 가장 사랑하시고 언제나 중심에서 든든한 버팀목이 되어주시고 바른길로 이끌어 주시는 가장 존경하는 아버지, 어떤 일이든 다 이해해 주시고 자신보다는 자식을 먼저 생각하시는 사랑하는 어머니, 잘못할 때 쓴 소리로 나를 바로잡아주고 가족만 생각하는 누나에게 감사의 마음을 전합니다.

아쉬움이 많이 남는 대학원 생활이지만 인생의 전환점이었고 많은 것을 배우고 느낄 수 있던 시간이었습니다. 많은 지도와 마음으로 많은 사랑을 주신 이종호 교수님께 다시 한번 감사의 마음을 전합니다.

2010년 12월 31일 지난 2년을 회상하며

우 용 제

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ABSTRACT

Beneficial immune-stimulatory effect of Chlorella supplementation: the enhancement of Natural Killer cell activity and early inflammatory response

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Background: *in vitro* and animal studies have demonstrated that *Chlorella* is potent biological response modifiers which modulate immune responses. However, no direct evidences for the effect of *Chlorella* supplementation on immune response in human subjects were reported.

Objective: We investigated a beneficial effect of *Chlorella* supplementation on immune/inflammatory response in normal Koreans.

Design: This study was designed for an 8-week randomized, double-blinded, placebo-controlled trial: Placebo (n=23) or 5g of *Chlorella* (CL, n=28) as form of tablets. Cytotoxic activities of *Natural killer (NK)* cells and serum concentrations of interleukin-12, interferon-γ and interleukin-1β were measured.

Results: After the 8-week, serum concentrations of interferon-γ ($p<0.05$) and IL-1β ($p<0.001$) significantly increased and that of interleukin-12 ($p<0.1$) tended to increase in CL group. The differences in changed values of these cytokines between the two groups were statistically significant. *NK* cell activities (%) were significantly increased in CL group after the intervention, but not in Placebo group. The differences in changed values between the two groups were also statistically significant. Additionally, changed levels of *NK* cell activity were positively associated with those of serum interleukin-1β ($r=0.280$, $p=0.047$) and interferon-γ ($r=0.271$, $p<0.005$). Signficantly positive correlations were also observed among the changed levels of serum cytokines; betwwen interferon-γ and interleukin-1β ($r=0.448$, $p<0.001$), between

interleukin-12 and interleukin-1β (r=0.416, p=0.003) and between interleukin-12 and interferon-γ (r=0.570, p<001).

Conclusion: These results suggest a beneficial immunostimulatory effect of *Chlorella* supplementation by enhancing the *NK* cell activity and producing interferon-γ and interleukin-12 as well as interleukin-1β, the Th-1 cell-induced cytokines.

Key words: Chlorella, Natural killer cell activity, interleukin-12, interferon-γ, interleukin-1β, beneficial immunostimulatory effect

1. INTRODUCTION

Chlorella is an unicellular green algae which contains essential amino acids, protein, minerals, vitamins, dietary fiber, and a wide range of antioxidants, bioactive substances and chlorophylls [1, 2]. It has been a popular foodstuff worldwide, especially in Japan, Taiwan as well as in Korea.

Many of *in vitro* and animal studies have demonstrated that *Chlorella* or *Chlorella* extract are potent biological response modifiers which modulate immune responses against tumors [3-5], bacterial and viral infection [6-10] as well as native Ags i.e. casein [11]. Oral administration of *Chlrorella* extract enhanced the resistance to infection with *Listeria monocytogenes*, an intracellular bacterium, through the augmentation of T-helper-1 (Th1) cell response both in normal and immunocompromised hosts [8, 9]. In addition to the boost of immune function, it also showed the considerable antioxidant effect and the reduction of blood glucose levels in diabetic animal models and stress-induced ulcer mice [12-18].

Various pharmacological effects of *Chlorella* have been expressed not only in animal models [19, 20] but also in human experiments [21-23]. According to Okudo *et al*. [21], the blood cholesterol levels were reduced by

Chlorella intakes in hypercholesterolemic patients. Fujiwara *et al*. [22] also long-term administration of *Chlorella* tablets showed a beneficial effect on hyperlipemia. Nakamura *et al*. demonstrated that *Chlorella* intakes reduced blood pressure in mildly hypertensive subjects [23]. *Chlorella* supplementation for 6-week, brought a favorable impact on antioxidant status in male smokers [24].

However, there were no direct evidences on the effect of *Chlorella* supplementation on immune response in human subjects, particularly in uninfected normal people. Therefore, this study aimed to investigate if *Chlorella* supplementation gives a beneficial effect on immune and inflammatory response in uninfected normal people.

2. BACKGROUND

2.1. Chlorella

2.1.1. The characteristic of chlorella

Chlorella spp. are green algae that are members of the phylum Chlorophyta. They are eukaryotic unicellular microorganisms that have been documented to exist at least 1 billion years ago. The cells are spherical and nonmotile. Cell size varies between 2 and 8μm. Under optimum conditions *Chlorella* spp. cells can reproduce in less than 2 h. Protein and lipid content are variable. Protein content can be as little as 15% to as much as 60 + % dw. Similarly, the lipid content can be controlled, depending upon the media, by environmental conditions such as photoperiod, and species. Genetic engineering has not been pursued extensively at this time but represents a potential for tapping these microorganisms to produce fine chemicals and, possibly, valuable Pharmaceuticals [25].

The *Chlorella* spp. are viewed as having a protein quality value greater than other spinach, milk, chicken egg (Table 1.). And comparison of amino

acid composition of chlorella and beef, amino acid composition of chlorella greater than beef (Table 2.) [26].

Table 1. Comparison of biochemical properties and composition of chlorella, spinach, milk, and chicken egg (per 100 g)

Component	Chlorella*	Spinach	Milk	Chicken egg
Protein	60.6 g	3.3 g	2.9 g	12.3 g
Carbohydrate	3.7 g	3.6 g	4.5 g	0.9 g
Fat	12.8 g	0.2 g	3.2 g	11.2 g
Ash	4.5 g	1.7 g	0.7 g	0.9 g
Fiber	13.0 g	3.5 g	-	-
Vitamin A	58,900 IU	2,900 IU	1,100 IU	640 IU
Vitamin B1	1.29 mg	0.13 mg	0.03 mg	0.08 mg
Vitamin B2	4.55 mg	0.23 mg	0.15 mg	0.48 mg
Nicine	32.1 mg	0.6 mg	0.1 mg	0.1 mg
Vitamin C	74 mg	65 mg	0 mg	0 mg
Vitamin E	22.8 mg	2.1 mg	0.1 mg	1.1 mg
Energy	372 kcal	25 kcal	59 kcal	162 kcal

*Chlorella made by pure culture using a fermenter

Adapted from Min-Sook Kang *et al.*

Table 2. Comparison of amino acid composition of chlorella and beef (per 100 g)

Amino acids	Chlorella** [A]	Beef (Fat free)*** [B]	Relative Content (%) [A/B×100]
Arginine	3.51 g	1.20 g	292.5
Lysine*	4.88 g	1.70 g	287.1
Histidine	1.16 g	0.75 g	155.0
Phenylalanine*	2.48 g	0.77 g	322.1
Tyrosine	1.64 g	0.63 g	260.3
Leucine*	4.52 g	1.60 g	282.5
Isoleucine*	1.04 g	0.88 g	118.2
Methionine	1.20 g	0.22 g	545.5
Valine*	3.14 g	0.92 g	341.3
Alanine	4.38 g	1.10 g	398.2
Treonine*	1.38 g	0.89 g	155.1
Tryptophan	1.01 g	0.21 g	481.0
Cysteine*	0.71 g	0.76 g	93.4
Glutamic acid	6.60 g	2.90 g	227.6
Aspartic acid	4.86 g	1.80 g	270.0

*Essential amino acid

** Chlorella made by pure culture using a fermenter

Adapted from Min-Sook Kang *et al.*

2.1.2. The function of chlorella

Administration of Chlorella in animals has demonstrated effects on numerous biochemical functions, such as remarkable antioxidant and anticataract effects in streptozotocin-induced diabetic rats [15] and lowered blood glucose in diabetic animals [16]. Also, anti-inflammatory and immunomodulatory activities have been reported [27]. In addition to producing cytokines and boosting immune function [12, 14, 28, 29], Chlorella can decrease oxidative stress and stressinduced ulcers in mice [13, 17, 18]. In rabbits fed a high cholesterol diet for 10 wk, Chlorella vulgaris showed antilipidemic and antiatherosclerotic actions [30]. These results were also proven in a human study where Okudo *et al*. [21] demonstrated that Chlorella intake decreases cholesterol levels in patients with hypercholesterolemia. These potential health benefits of Chlorella have been attributed to the effects of specific ingredients in Chlorella, such as minerals, dietary fiber, and a wide range of antioxidants and chlorophylls.

2.2. Immune response

2.2.1. *Natural killer (NK)* cells in innate immunity

NK cells have emerged as pivotal players in immune responses against pathogens and tumors. Research during the past decade has focused on the identification of the cell surface receptors and effecter molecules that *NK* cells use in target-cell recognition and destruction. Attention now turns to determining both the role of *NK* cells in vivo in innate immunity and their contribution to adaptive immunity. Although many of the *NK*-cell activating and inhibitory receptors, their ligands and signaling pathways have been discovered, the biological relevance of these molecules in host defense, how they are regulated during development, and elucidation of the interactions between *NK* cells and other hematopoietic cells are critical issues to address.

2.2.1.1. *NK* cell–dendritic cell crosstalk

It has long been appreciated that *NK* cells can kill immature dendritic cells (DCs), in the same way as they kill other target cells in vitro, but more recently it has been shown that *NK* cells and DCs reciprocally activate one another during an immune response (Figure 1). The original description of NK–DC crosstalk was published in a report by Fernandez *et al*. in 1999, in which the anti-tumor response of mouse *NK* cells was shown to be enhanced by DCs in vivo. These authors also described co-culture conditions in which DCs enhance the cytotoxicity of *NK* cells against ‘hird party’targets and also induce *NK* cell secretion of IFN-γ. This type of coculture system has since been exploited to define the requirements for NK–DC crosstalk.

2.2.1.2. Activation of *NK* cells by dendritic cells

The induction of IFN-γ production, cytotoxicity, CD69 expression and proliferation in resting *NK* cells in vitro has been documented by using mouse DC cell lines, mouse bone-marrow-derived DCs, human monocyte-derived DCs and human cord-blood-derived DCs. The mechanism by which DCs activate resting *NK* cells in vitro requires direct cell contact, but probably also involves soluble factors. These in vitro studies suggested that a range of cytokines produced by DCs, including IL-12, IL-18 and type I IFN, are required for the induction of the various *NK* effecter functions, but the data are conflicting and no general consensus has emerged. Interestingly, IL-2 is produced by DCs and is necessary for DC-induced IFN-γ production by *NK* cells in vitro and in vivo. In addition, the maturation state of the DCs might influence their ability to activate *NK* cells. Several studies have shown that immature DCs require a maturation stimulus to activate *NK* cells, whereas others have shown that immature and mature DCs are equivalent in their ability to activate *NK* cells. The in vivo relevance of *NK* activation by DCs has been demonstrated in murine tumors and viral models, both implicating the $CD8a^+$ DC subset. During infection of C57Bl/6 mice with mouse cytomegalovirus (MCMV), the expansion of *NK* cells induced by DCs was

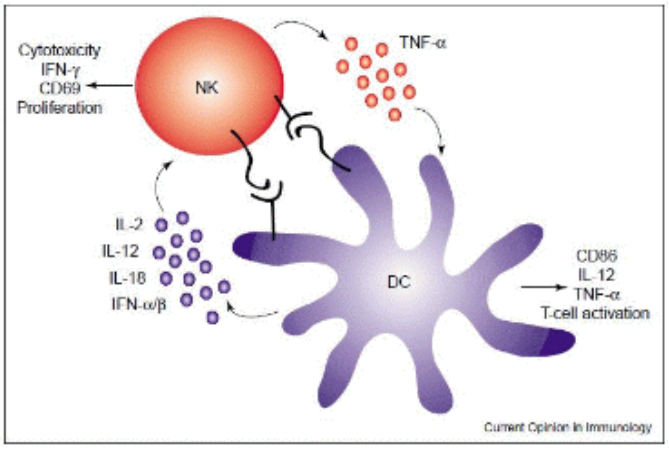
shown to specifically involve the Ly49H receptor on *NK* cells and the cytokines IL-12 and IL-18.

2.2.1.3. Activation of dendritic cells by *NK* cells

The other side of the reciprocal NK–DC interaction is how the DCs are activated or matured by interactions with *NK* cells, as measured by DC cytokine production, induction of co-stimulatory molecules or ability to stimulate T-cell responses. Although fewer studies have investigated the mechanism of this process, it is known that optimal DC activation by *NK* cells in vitro requires both cell contact and TNF-a production. It is unclear whether resting *NK* cells can activate DCs, but *NK* cells pre-activated with IL-2 are potent DC activators, both alone or in synergy with inflammatory stimuli, such as lipopolysaccharide. Interestingly, Piccioli *et al*. showed that the outcome of the NK–DC interaction is tightly regulated by the ratio of the two cell types; at low NK:DC ratios, DC maturation prevails, whereas at high NK:DC ratios, *NK* killing of DC prevails. Two in vivo studies demonstrate DC activation by *NK* cells. The MCMV study discussed above shows that *NK* cells are necessary for the maintenance of $CD8a^{+}$DCs during viral infection, again through the Ly49H *NK* receptor. Mocikat *et al*. reported that, in an in vivo study of tumor rejection, *NK* cells were required for IL-12 production by DCs, and this interaction was then responsible for the generation of a protective $CD8^{+}$ T-cell response. In these in vivo models, *NK* cell activation presumably

occurs through virally induced cytokines or by the direct interaction of the tumor cells with *NK* cells [31].

Figure 1. Crosstalk between NK cells and dendritic cells.



Crosstalk between NK cells and dendritic cells. NK cells and dendritic cells (DCs) have the ability to reciprocally activate one-another, both *in vitro* and *in vivo*. This crosstalk includes cell contact involving unknown receptor-ligand pairs and soluble mediators produced by the two cells. The cytokines, TNF-α, IL-2, IL-12, IL-18 and IFNs, have all been implicated in this process. The end result of these interactions is NK cells activated for cytotoxicity, IFNγ production, and proliferation and DC that have matured and are capable of cytokine production and T cell activation.

Adapted from Jessica A Hamerman *et al.*

2.2.2. Cytokines

Interleukin-12 (IL-12) is a heterodimeric cytokine produced primarily by antigen-presenting cells (monocytes, macrophages, dendritic cells, and B cells). Its production is stimulated by bacteria, bacterial products, and intracellular parasites and enhanced by priming with granulocytemacrophage colony-stimulating factor (CM-CSF) and interferon-y (IFN-γ) or inhibited by IL-b. The major biological activity of IL-12 is on T and *NK* cells in which it increases cytokine production, proliferation, and cytotoxicity. Its production occurs several hours after exposure to infectious agents, which induces a rapid production of IFN-y by *NK* and later by T cells. This IFN-γ potentiates antigen-presenting cell functions important in clearing infectious agents (phagocytosis, oxidative burst, and production of nitrous oxide) and also increases further production of IL-12. IL-12 has been clearly demonstrated to be important in the generation of CD4 and CD8 type 1 T cells both in vivo and in vitro. Our data reveals that IL-12 primes naive T cells for high IFN-γ and IL-b production, whereas IL-4 is required for IL-4 priming, thus suggesting that these genes and possibly others are independently regulated. IL-12 is therefore involved in the skewing of cytokine production toward a type 1 and has been implicated in being involved in selective mechanisms of established T cells. It is now becoming clear that the IL-12 acts as both a proinflammatory

cytokine and an immunomodulator and therefore bridges the innate and adaptive immune responses [32].

3. SUBJECT AND METHOD

3.1. Study Subject

3.1.1. Study population

Study subjects were recruited from the Health Service Center of Yonsei Hospital. Subjects were excluded if they have any history of the following: 1) clinical or electrocardiographic evidence of CAD, stroke, myocardial infarction, or peripheral arterial occlusive disease; 2) diabetes mellitus (fasting glucoses≥126mg/dL or 2hr serum glucose ≥200mg/dL after a 75g oral glucose tolerance test); 3) abnormal liver or renal function; 4) thyroid or pituitary disease; 5) acute or chronic inflammatory/immune disease including malignant tumor, lung disease, leukemia, autoimmune disease 6) lactose intolerance, 7) orthopedic limitations; 8) body weight loss/gain in the past 1 year; 9) regular use of any medications that could affect cardiovascular function and/or metabolism; and 10) the number of white blood cells>$8\times10^3/\mu L$. The aim of the study was carefully explained to the volunteers, and their written informed consent was obtained. Finally, 60 people were enrolled in this study.

3.1.2. Study design and *Chlorella* source

This study was designed in an 8-week randomized, double-blinded, placebo-controlled trial. Sixty subjects were randomly assigned to receive placebo (n=30) or 5g of *Chlorella* (n=30) as form of tablets. Test-product pills contained only dried *Chlorella* (97% purity, Daesang Corp., Seoul, Korea) as an active ingredient and placebo-product pills contained lactose in identical-looking tablets. Subjects were instructed to consume 5 g/d of *Chlorella* (12 pills/d) or placebo (12 pills/d) by taking 4 pills after each main meal. All participants were encouraged to maintain their usual lifestyle and dietary habits. Compliance was assessed at the end of the study using pill counting and food records.

3.2. Method

3.2.1. Anthropometric parameters and blood collection

Body weight and height were measured unclothed and without shoes in the morning. Body mass index (BMI) was calculated as body weight in kilograms divided by height in square meters (kg/m^2). Blood pressure (BP) was obtained from the left arm of seated patients with an automatic blood pressure monitor (TM-2654, A&D, Tokyo, Japan) after 20 min of rest. After overnight fast (12-hours), venous blood specimens were collected in EDTA-treated and plain tubes. Tubes were immediately placed on ice until they arrived at the analytical laboratory (within 1-3h). Blood specimens in EDTA tubes were used for the isolation of peripheral blood mononuclear cells (PBMC) and those in plain tubes were separated into serum and stored at -70℃ until analysis.

3.2.2. Isolation of peripheral blood mononuclear cells (PBMC)

Whole blood was mixed with the same volume of RPMI 1640 (Gibco, Invitrogen Co, USA) and gently laid on a histipaque-1077(Sigma, CA, USA), then centrifuged at condition in 2000rpm, 20 min, 10℃. After the separation, a thin layer of buffy coat indicating PBMC was isolated, washed twice with RPMI 1640 and resuspended in RPMI 1640 with streptomycin. They were used for *Natural killer (NK)* cell cytotoxicity assay.

3.2.3. Cytotoxic activities of *Natural killer (NK)* cells

The cytolytic activities of NK cells were determined by CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega Co., WI, USA). For NK cell cytotoxic activity, PBMCs isolated from each subject were incubated with K562 cells. Briefly, PBMC cells (effecter cell, E) were seeded in the well in a ratio of 5:1 and 1.25:1 with the K562 cells (2×10^4cells/well) (targeted cell, T). The plates treated at different ration of E:T were incubated at 37℃ with 5% CO_2 for overnight according to the manufacturer's instructions. Finally, NK cell activity of effecter cells was measured with 2030 multilable reader (Victor™ x5, PerkinElmer, USA) at 490nm and was calculated with this formula.

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental - Effecter Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum - Target Spontaneous}} \times 100$$

3.2.4. Cytokine assay for serum concentrations of interleukin-12, interferon-γ and interleukin-1β

Serum concentrations of interleukin-12 (IL-12), interferon-γ (IFN-γ) and interleukin-1β (IL-1β) were measured using Bio-Plex Pro™ Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions.

3.2.5. Serum lipid profile, blood glucose and white blood cell count

Fasting serum levels of total cholesterol and TG were measured using commercially available kits on a Hitachi 7150 Autoanalyzer (Hitachi Ltd. Tokyo, Japan). After precipitation of serum chylomicron, low density lipoprotein (LDL) and very low density lipoprotein (VLDL) with dextran sulfate-magnesium, HDL cholesterol (HDL-C) left in the supernatant was measured by an enzymatic method. LDL cholesterol (LDL-C) was estimated indirectly using the Friedewald formula for subjects with serum TG concentrations <400 mg/dL (4.52 mol/L). In subjects with serum TG concentrations≥400 mg/dL (4.52 mol/L), LDL-C was measured by an enzymetic method on a Hitachi 7150 Autoanalyzer directly. Fasting glucose was measured by a glucose oxidase method using the Glucose Analyzer (Beckman Instruments, Irvine, CA, USA). White blood cell (WBC) count was determined using the HORIBA ABX diagnostic (HORIBA ABX SAS, Parc Euromedicine, France).

3.2.6. The assessment of dietary intake and physical activity level

The subjects' usual diet information was obtained using both a 24-hour recall method and a semi-quantitative food frequency questionnaire (SQFFQ) of which the validity had been previously tested [33]. We used the former to carry out analyses and the latter to check if the data collected by 24-hour recall methods was representative of the usual dietary pattern. All the subjects were given written and verbal instructions by a registered dietitian on completion of a 3-day (2 week days and 1 weekend) dietary record every 4 weeks. Dietary energy values and nutrient content from a 3-day food records were calculated using the Computer Aided Nutritional analysis program (CAN-pro 2.0, Korean Nutrition Society, Seoul, Korea). Total energy expenditure (TEE) (kcal/day) was calculated from activity patterns including basal metabolic rate, physical activity for 24 hours [34], and specific dynamic action of food. Basal metabolic rate for each subject was calculated with the Harris-Benedict equation [35].

3.2.7. Statistical analysis

Statistical analyses were performed using SPSS version 15.0 for Windows (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL, USA). For intra-group tests, we conducted paired *t*-tests. For inter-group comparison, student *t* -test was used to compare initial value or absolute (net) differences. Frequencies were tested by chi-square test among groups. Pearson correlation coefficients were used to examine relationships between variables. We determined whether each variable was normally distributed before statistical testing, and logarithmic transformation was performed on skewed variables. For descriptive purposes, mean values are presented using untransformed values. Results are expressed as mean ± S.E or %. A two-tailed value of $P<0.05$ was considered statistically significant.

4. RESULTS

Among enrolled subjects (n=60), 9 dropped out for personal reasons and 51 subjects completed the study. Among the drop-outs, 7 were in the placebo group, 2 was in *Chlorella* supplement group. Thus, compliance was high (85%) and no serious adverse reactions due to *Chlorella* supplementation were noted. Participants were supplied with 360 tablets of placebo pills or *Chlorella* pills at 0-week and at 4-week respectively. They were also asked to return unconsumed tablets at the next visit (4-week and 8-week respectively). All the participants are reinforced to regularly consume the pills by a dietitian through every 2 week's phone call check during the intervention period. Compliance was verified by counting the remained tablets. If the tablets are consumed more than 80%, compliance was considered good. Among the 9 dropped-out, 2 in the placebo group, 2 in *Chlorella* supplementation group had personal problems, and 5 in the placebo group showed no good-compliance on capsule consumption.

4.1. General and biochemical characteristics of study subjects

Table 2 presents general and biochemical parameters measured at baseline and after the 8-week *Chlorella* supplementation. There were no significant differences in the baseline levels and changed levels such as gender distribution, age, body mass index, WHR, blood pressure, WBC count, lipid profiles and blood glucose between CL group and Placebo group. In addition, no significant differences were found in daily dietary intake and energy expenditure between the two groups.

Table 2. General characteristics of study subjects before and after the 8-wk intervention

	Test (n=28)		Placebo (n=23)	
	0 week	Δ change	0 week	Δ change
Male /female (n)	10 / 18		10 / 13	
Age (year)	36.3 ± 1.82		32.6 ± 1.77	
Weight (kg)	62.7 ± 1.89	-0.26 ± 0.24	63.6 ± 2.63	-0.54 ± 0.25
BMI (kg/m^2)	22.8 ± 0.53	-0.11 ± 0.09	22.7 ± 0.72	-0.20 ± 0.09
WHR	0.84 ± 0.01	0.00 ± 0.00	0.84 ± 0.01	0.00 ± 0.00
SBP (mmHg)	114.5 ± 3.29	-0.38 ± 1.88	118.2 ± 2.52	-0.33 ± 1.73
DBP (mmHg)	77.2 ± 2.32	0.04 ± 1.40	82.0 ± 2.07	-3.09 ± 1.71
WBC (x10^3/uL)	4.72 ± 0.16	0.24 ± 0.17	5.03 ± 0.17	0.18 ± 0.15
Triglyceride (mg/dL)	104.0 ± 11.8	10.6 ± 7.02	129.2 ± 24.2	-8.78 ± 17.0
Total cholesterol (mg/dL)	183.0 ± 6.42	-5.50 ± 6.13	181.3 ± 6.65	-2.61 ± 3.07
HDL-cholesterol (mg/dL)	48.1 ± 2.75	0.46 ± 1.90	49.1 ± 2.64	2.17 ± 1.51
LDL-cholesterol (mg/dL)	114.1 ± 6.76	-8.08 ± 5.65	106.6 ± 5.46	-4.89 ± 4.32
Glucose (mg/dL)	86.2 ± 1.38	-2.69 ± 1.68	82.5 ± 1.54	1.95 ± 1.94
Daily dietary intake and energy expenditure				
TEE (kcal)	2323 ± 64.5	25.1 ± 28.5	2424 ± 85.0	46.7 ± 29.7
TCI (kcal/d)	2284 ± 47.2	4.21 ± 16.1	2221 ± 93.6	-34.1 ± 26.9
TEE/TCI	1.02 ± 0.02	0.01 ± 0.02	1.13 ± 0.07	0.04 ± 0.02
Carbohydrate (%)	61.6 ± 0.15	0.02 ± 0.16	61.6 ± 0.23	0.07 ± 0.22
Protein (%)	16.7 ± 0.17	0.05 ± 0.21	16.3 ± 0.17	0.33 ± 0.23
Fat (%)	22.0 ± 0.18	-0.17 ± 0.26	22.3 ± 0.23	-0.26 ± 0.23
Fiber (g)	22.6 ± 1.38	-0.21 ± 1.40	21.8 ± 1.60	0.60 ± 1.66

Mean±SE

tested by independent t-test (intergroup comparison for initial value and Δ change)

*P<0.05, compared with Δ change value of Test group

BMI: body mass index, WHR: waist to hip ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, TEE: total energy expenditure, TCI: total calorie intake

4.2. Serum concentrations of cytokines before and after the intervention

Figure 2 shows serum concentrations of IL-12, INF-γ and IL-1β between CL group and Placebo group before and after the intervention. None of these cytokines at initial levels were significantly different between the two groups. After the intervention, serum concentrations of INF-γ ($p<0.05$) and IL-1β ($p<0.001$) significantly increased and that of IL-12 ($p<0.1$) tended to increase in CL group. The differences in changed values of IL-12 ($p<0.01$), INF-γ ($p<0.001$) and IL-1β ($p<0.01$) between the two groups were also statistically significant.

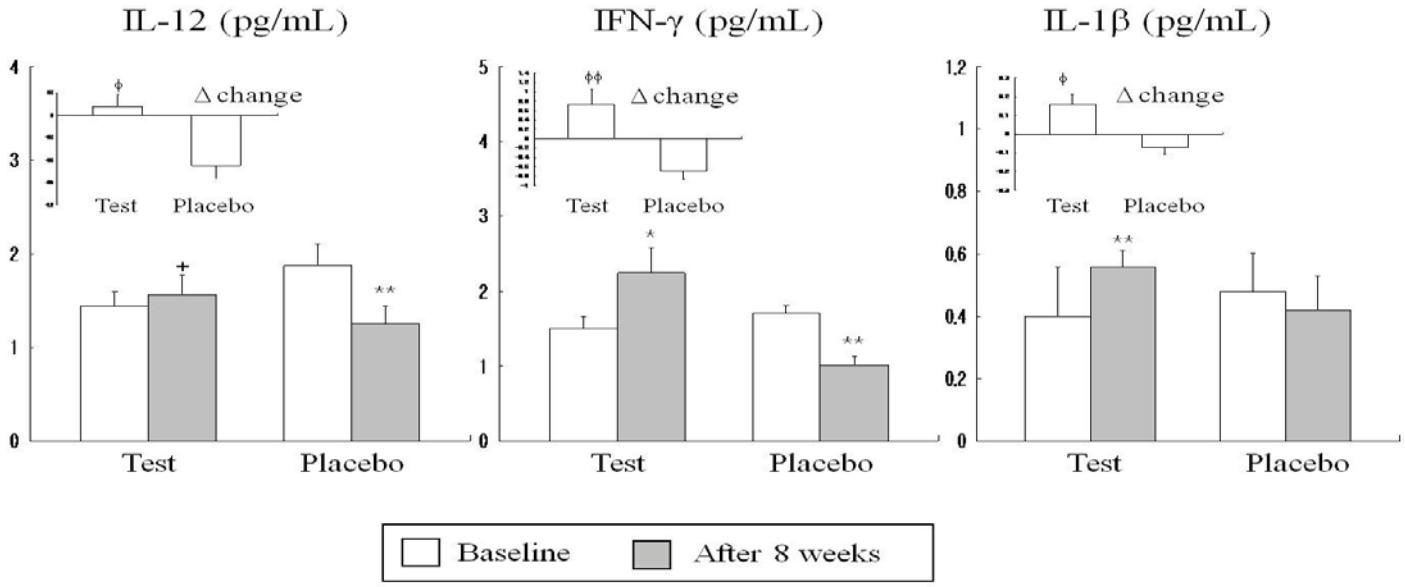


Figure 2. Serum concentration of IL-12, IFN-γ and IL-1β before and after the 8-week Chlorella supplementation

Mean±SE. tested by paired t-test (intra group comparison) or independent t-test (intergroup comparison)

$^{+}P<0.1$, $^{*}P<0.05$, $^{**}P<0.01$ compared with the value at baseline in each group

$^{\phi}P<0.01$, $^{\phi\phi}P<0.001$ compared with Δ change value of Test group

4.3. *NK* cell activity

NK cell activities (%) were measured based on the ratios of effecter cells (PBMC) from subjects to Target cell (K562 cells) as 5:1 or 1.25:1. As shown in Figure 3, *NK* cell activities from both conditions were significantly increased in CL group after the 8-week *Chlorella* supplementation ($p<0.05$, $p<0.05$, respectively), but which was not found in Placebo group. The differences in changed values between the two groups were statistically significant ($p<0.05$, $p<0.05$, respectively).

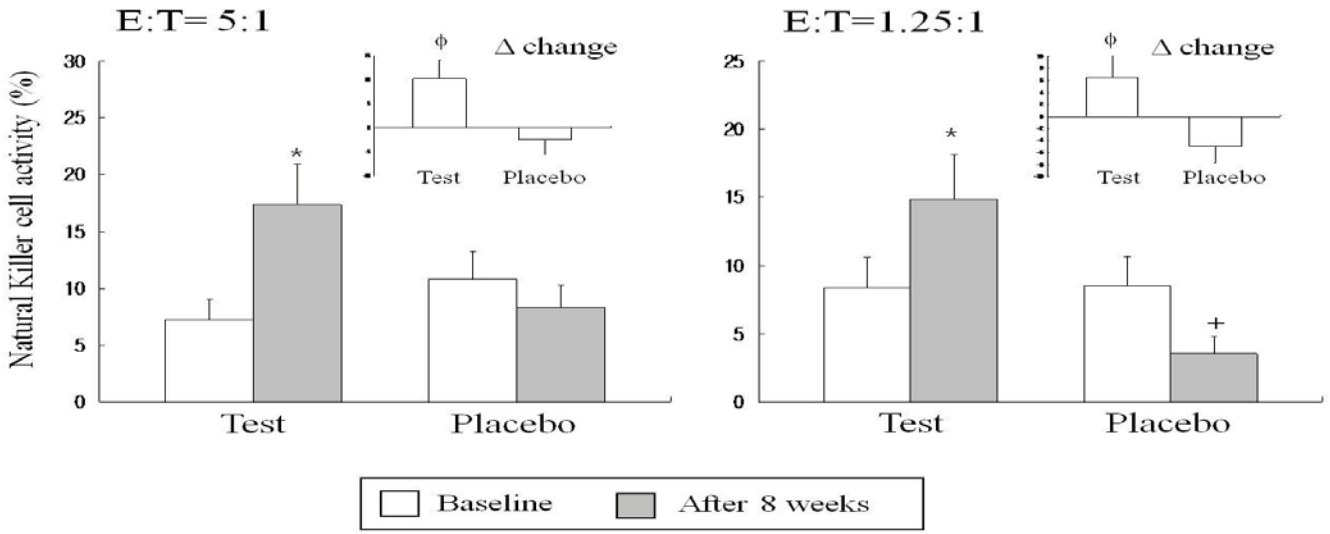


Figure 3. NK cell activity of effecter cells before and after the 8-week Chlorella supplementation

Mean±SE. tested by paired t-test (intra group comparison) or independent t-test (intergroup comparison)

$^{+}P<0.1$, $^{*}P<0.05$, compared with the value at baseline in each group

$^{\phi}P<0.05$ compared with Δ change value of Test group

E: effecter cells (peripheral blood mononuclear cells from subjects), T: target cells (K562 cells)

4.4. Correlations among changed levels of IL-12, IFN-γ and IL-1β in serum, and *NK* cell activity

As shown in Figure 4, changed levels of NK cell activity (%) (E:T=5:1) were positively associated with those of IL-1β (r=0.280, p=0.047) and IFN- γ (r=0.271, p<0.005) in serum. Signficantly strong positive correlations were also observed among the changed levels of serum cytokines; betwwen IFN-γ and IL-1β (r=0.448, p<0.001), between IL-12 and IL-1β (r=0.416, p=0.003) and between IL-12 and IFN-γ (r=0.570, p<001).

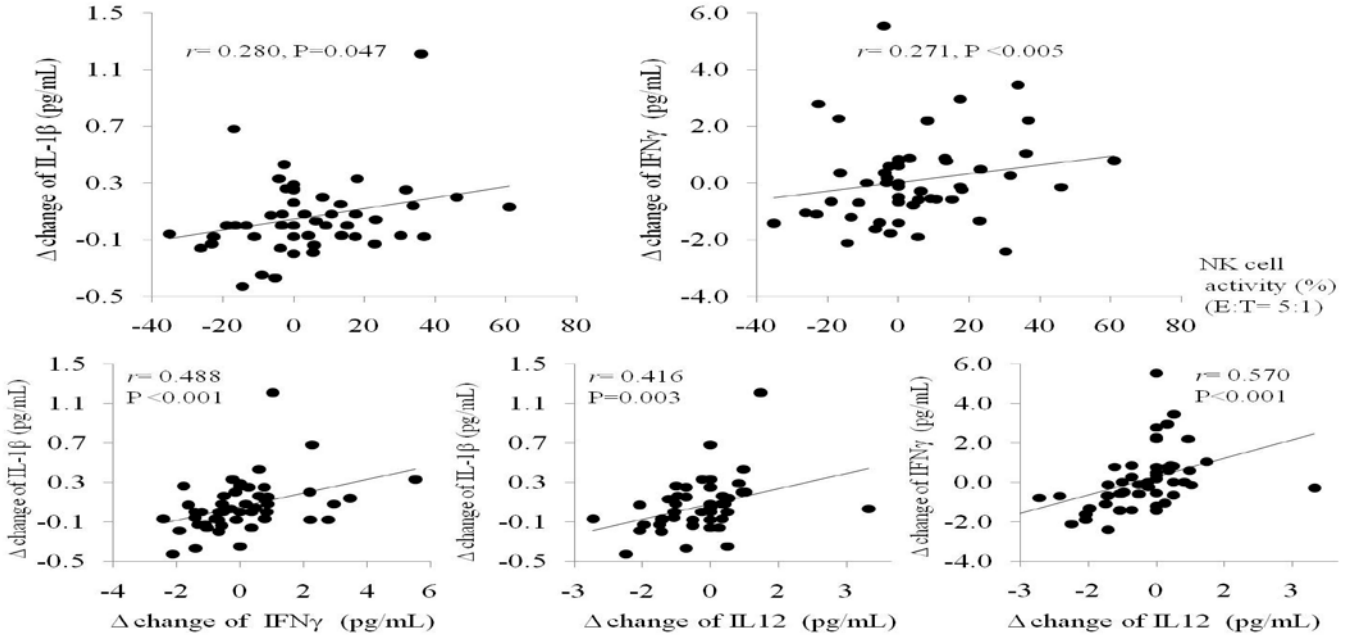


Figure 4. Relationship among changed levels of NK cell activity of effecter cells, IL-12, IFN-γ and IL-1β

r :Pearson correlation coefficient,

E: effecter cells (peripheral blood mononuclear cells from subjects), T: target cells (K562 cells)

5. DISCUSSION

This present study shows that 8-week supplementation of *Chlorella* tablets may give a beneficial immuno-stimulatory effect to normal (uninfected) people by enhancing the *NK* cell activity and producing INF-γ and IL-12 as well as IL-1β, the Th-1 cell-induced cytokines.

Helper T lymphocytes (Th) are divided into two functional subclasses, Th-1 and Th-2 cells based upon the cytokines that they produce, and their effects on cell mediated and humoral immunity [28]. Th-1 cells produce IL-2, INF-γ, TNF-α and IL-12, enhance cell-mediated immunity and also can inhibit cell-mediated immunologic activities. On the other hand, Th-2 cells produce IL-4, IL-5, IL-6 and IL-10, and upregulate humoral immunity. Th-1 and Th-2 derived cytokines also cross-regulate each other in various clinical conditions. Hasegawa *et al*. reported in his mice model infected with *Listeria monocytogenes* that *Chlorella* extract (from *Chlorella vulgaris, CVE*) augmented IL-12 and IFN-γ [29], both of which promote the differentiation of naive CD4+ T cells into Th-1 cells producing IFN-γ, but inhibited the generation of Th-2 cells producing IL-4. In his another report, oral administration of *CVE* inhibited immunoglobulin E (IgE) production against

casein with an impaired Th-2 response [11]. Ewart *et al*. shows that *Chlorella* extract (from *Chlorella pyrenoidosa*, CPE) induces a Th-1 patterned cytokine response (IFN- γ and TNF-α) and a strong anti-inflammatory regulatory cytokines response (IL10) in human PBMC stimulated *ex vivo* [30].

In our study, subjects who consumed *Chlorella* tablets for 8 weeks had significantly increases in serum levels of INF-γ and IL-12 compared with those who consumed Placebo, which indicates that *Chlorella* may induce the production of INF- γ and IL-12 favoring a Th-1 mediated immune reaction. We also found increased levels of serum IL-1β. It may be partially in accordance with the report of Ishikawa *et al*. that a polysaccharide-rich fraction from *Chlorella pyrenoidosa* induced IL-1β and TNF-α in macrophage cells [31]. Thus, taken together, *Chlorella* cells appear to contain immunostimulatory principles that stimulate a Th-1 based response. However, we did not measure the Th-2 derived cytokines such as IL-4 or IL-6, which may need to be confirmed in a further study.

In our study, subjects supplemented with *Chlorella* had a significant increase in the Natural Killer (*NK*) cell activities. This result might be partly in accordance with the report of Dantas *et al*. showing the oral administration of *Chlorella* extract (*CVE)* significantly increased the *NK* cell activity both in normal (non-infected) mice and in *Listeria monqtogenes* infected mice [32].

The *CV*-treated animals also presented a dose-related increased survival rate. *NK* cells were first identified by their cytotoxic activity against tumor cells, suggesting a role in immunological surveillance against neoplasia. Emerging evidences demonstrated that *NK* cells are important mediators of innate resistance against a variety of pathogenic intracellular microorganisms [33]. Their main function is the production of early IFN-γ, which is crucial to activate antimicrobial macrophage functions [34-39]. The kinetics of IFN-γ production by *NK* cells following infection is extremely fast, providing a source of functional cytokine at the critical time of the T-cell expansion [36,40,41]. In our study, changed level of NK cell activity after the intervention shows a significant positive relationship with that of serum IFN-γ. Signficantly strong positive correlations were also observed among the changed levels of IFN-γ, IL-12 and IL-1β, Th-1 patterned cytokines.

This study specifically focused on a normal healthy (non-infected) Koreans, so the results cannot be generalized to patients, other ethnic or geographical groups whose biochemical characteristics may differ from those in our subjects. Despite these limitations, 8-weeks of *Chlorella* intake in healthy Koreans increased the *NK* cell activity and produced INF-γ and IL-12 as well as IL-1β, the Th-1 cell-induced cytokines. In addition, changes in the *NK* cell activity positively correlated with changes in the cytokines after the

intervention. These results add to the growing literature on beneficial immunostimulatory effect of *Chlorella* supplementation through a clinical human study.

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국문 초록

면역자극에 대한 클로렐라 보충의 긍정적 효과:
자연살해세포와 초기면역반응의 증가

본 연구에서는 한국 건강한 성인에서 클로렐라를 섭취했을 때 면역력 증진과의 관계에 대해 알아보고자 하였다. 20-70세 건강한 성인에서 혈중 백혈구수치가 $8\times10^3/\mu L$ 미만인 자와 과 피험자 선정기준에 적합한 자 60명을 대상으로 총 8주의 연구를 진행하였다. 탈락자(순응도 및 개인사정 등)를 제외하고 Test군(28예)과 placebo군(23예) 간에 식사력, 인체계측, 지방산 조성 등의 차이는 나타나지 않아 안전성을 확인할 수 있었다.

피험자에게 섭취 전 (0주) 섭취후 (8주)에 채혈을 했고 혈액으로부터 혈청을 분리해 싸이토카인 (cytokine) 을 측정했고, 말초단핵구세포(PBMC)를 분리한 후 자연살해세포 활성도 (Natural Killer cell activity)를 측정하였다. 혈청중의 interleukin-12 (IL-12)는 test군에서 증가하는 경향을 보였고, placebo군에서 유의적으로 감소했다. Interferon-γ (IFN-γ)는 test군에서 유의적으로 증가했고, placebo군에서 유의적으로

감소했다. IL-1β는 test군에서 유의적으로 증가했고, placebo군에서는 변화가 없었다. 따라서 IL-12, IFN-γ, IL-1β의 Δ change값이 test군에서 유의적으로 증가함을 알 수 있다.

NK cell activity는 effecter cell (PBMC) : target cell (K562)의 비율이 5:1에서 test군에서 유의적으로 증가했고, placebo군에서는 변화가 없었다. 또한 E:T의 비율이 1.25:1에서 test군에서 유의적으로 증가했고, placebo군에서 감소하는 경향을 보였다. 따라서 NK cell activity Δ change값이 test군에서 유의적으로 증가함을 알 수 있다.

각 면역지표간에 상관관계를 확인 했을 때, NK cell activity의 Δ change와 IL-1β의 Δ change ($p=0.047$) 및 NK cell activity의 Δ change와 IFN-γ의 Δ change ($p < 0.005$) 간에 양의 상관관계를 보였다. 또한, IFN-γ Δ change와 IL-1β Δ change ($p < 0.001$) IL-12 Δ change와 IL-1β Δ change ($p = 0.003$), IL-12 Δ change와 IFN-γ Δ change ($p < 0.001$) 간에 높은 양의 상관관계를 보였다.

결론적으로 본 연구는 건강한 성인에서 클로렐라를 섭취했을 때 cytokine 생성과 NK cell activity를 높여주어 면역력 증진에 효과 (immunostimulatory effect)가 있음을 밝혔다.

핵심어: 클로렐라, 싸이토카인, 자연살해세포 활성도, 면역력, interleukin-12, Interferon-γ, interleukin-1β