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Proliferation and Differentiation of Hematopoietic Cells and Preservation of Immune Functions

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1. Introduction

A limited number of pluripotent stem cells are mainly located in the bone marrow, and give rise to all blood cell 1ineages. Because of their relatively short lifespan, circulating cells must be continually replaced in living body throughout the life. The task performed by hematopoietic stem cells is shared in two main features, that is, the capacity of regeneration which prevents depletion of the cells and the ability of preservation of blood homeostasis. The mechanisms behind the critical choice between lineage-commitment and maintenance of the stem-cell pool involve a number of complex interactions between hematopoietic progenitor cells at different stages of maturation, stromal cells and their extracellular matrix, as well as a variety of stimulatory or inhibitory cytokines provided by the microenvironment.

Hematopoietic growth factors were first identified in the 1960s as soluble agents produced in spleen, uterus or lung, and found to maintain the formation of differentiated colonies from hematopoietic progenitor cells in semisolid culture systems. Hence they were named colony-stimulating factors, CSFs (Schneider and Dy, 1999). Most of these molecules have been purified and their genes have been sequenced. They are currently available in recombinant form and have been used with success in clinical trials.

Hematopoietic growth factors or CSFs can be divided into two categories, according to their target cell specificity (Figure 1). One group comprises the factors whose activity is relatively restricted to particular cell types, such as macrophage colony-stimulating factor (M-CSF) for macrophages, granulocyte colony-stimulating factor (G-CSF) for neutrophils, interleukin-5 (IL-5) for eosinophils and B cells, and thrombopoietin (Tpo) for megakaryocytes and erythropoietin (Epo) for the erythroid lineage. The second category of growth factors has a

relatively wide spectrum of activities, such as IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). The factors target a heterogeneous population of cells, including both primitive and lineage-committed progenitors. Action of these two molecules can be modulated by a number of cytokines which are not essentially growth factors. Among these, IL-1, IL-6, IL-9, IL-1l and leukemia inhibitory factor (LIF) are involved. An interleukin 6 class cytokine or stem cell factor (SCF) plays a particularly important role in the amplification of early stem cell commitment. IL-7 is also noteworthy in this context, with respect to its crucial role in lymphopoiesis, as evidenced by the strong lymphopenia in IL7-deficient mice. Hematopoiesis can be also regulated negatively by a heterogeneous set of molecules, such as interferon, tumor necrosis factor-alpha (TNF-α), transforming growth factor beta (TGFβ) and compounds like prostaglandins, ferritin and lactoferrin.

The precise function of cytokines during constitutive hematopoiesis in a healthy organism is still unclear, although much evidence has been accumulated from the study using genetically modified mice. The purpose of hematopoiesis, however, is not only the maintenance of homeostasis, but also a rapid and controlled response to stress situations. The immune response induced by infection, the number of circulating white blood cells can be remarkably increased (Schneider and Dy, 1999). In the process, the cytokines generated by sensitized lymphocytes and activated cells of the immune system play a crucial role in the recruitment and the differentiation of hematopoietic cells.
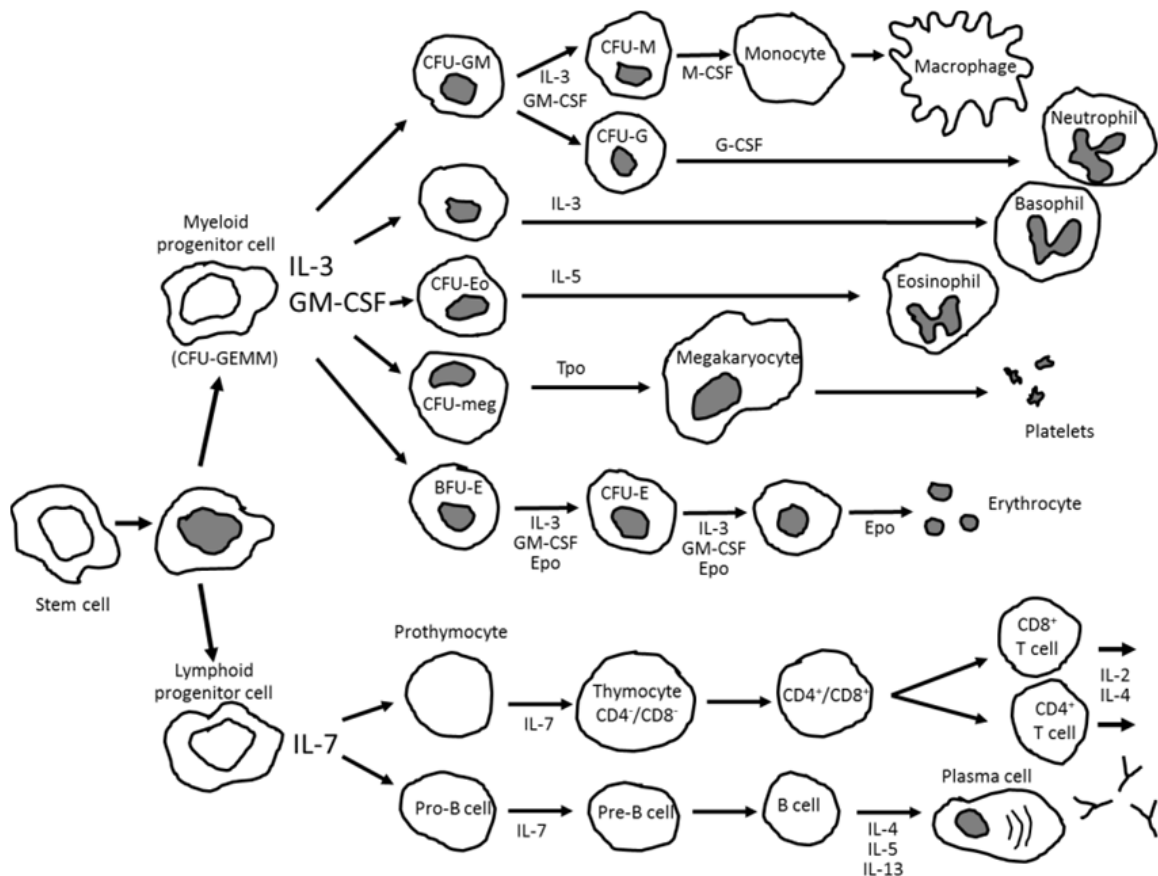
Figure 1. Simplified haematopoietic differentiation scheme and cytokines (modified from Elk and Dy, 1999)

In the chapter, we focus on relations and networks of cytokines, induced by ingesting *in-vivo* study of *Spirulina* and by *in-vitro* cultured cells, to differentiation of hematopoietic cells and preservation of immune functions, and discuss the possibility of their medicinal application for sustaining a healthy state.

2. *Spirulina*

Spirulina platensis is a helicoidal filamentous blue-green alga (cyanobacterium) and has a history of being used as food for over a thousand years, and has been commercially produced for more than 40 years as a food supplement (Ciferri, 1983; Gershwin and Belay, 2008). *Spirulina platensis* is prokaryote and belongs to the class Cyanophyceae, or Cyanobacteria. In its commercial use, the common name, *Spirulina*, refers to the cyanobacterium, *Arthrospira platensis*, and is a whole product of biological origin. In its taxonomic use, *Spirulina* is a name used to describe mainly two species of Cyanobacteria, *Arthrospira platensis* and *A. maxima*, which are commonly used as food, dietary supplement, and feed supplement (Vadiraja et al., 1998) . These and other *Arthrospira* species forming helical trichomes were once combined and classified into a single genus, *Spirulina* (Geitler, 1932). Before Geitler, on the basis of the presence of septa or division in the trichomes, the two genera were placed separately, that is, the *Spirulina* species being without septa and the *Arthrospira* species with septa. Recent morphological, physiological, and biochemical studies have shown that these two genera are distinctively different and that the edible forms commonly referred to as *Spirulina platensis* have little in common with other much smaller species. This distinction has been also based on results from the complete sequence of the 16S ribosomal RNA gene and the internal transcribed spacer (ITS) between the 16S and 23S rRNA genes determined for two *Arthrospira* strains and one *Spirulina* strain (Nelissen et al., 1992) showing that the two *Arthrospira* strains formed a close cluster distant from the *Spirulina* strain.

Blue green algae *Spirulina platensis* (*Arthrospira platensis*) is gaining more and more attention as a nutraceutical and source of potential pharmaceuticals. *Spirulina* is known to have nutritional advantages of high-quality protein contents and other components such as vitamins; minerals, and essential fatty acids, including γ-linolenic acid, and β-carotene (Belay et al., 1993) , and has been approved its safety in the report from United Nations International Development Organization, UNIDO (Chamorro-Cevallos, 1980). Moreover, sulfated polysaccharides, called calcium-spirulan (Ca-Sp) and isolated from a hot-water extract of *Spirulina*, exhibit immunomodulatory activity and inhibit metastasis of melanoma cells to the lungs (Mishima et al., 1998) , and can also inhibit virus entry (Hayashi et al., 1996). Immolina, a high-molecular-weight polysaccharide fraction of *Spirulina*, promotes chemokine expression in human monocytic THP-1 cells (Grzanna et al., 2006). *Spirulina* contains phycocyanin (CPC; C-phycocyanin), a blue, 270-kDa photosynthetic pigment protein, which accounts for approximately 15% of the dry weight of *Spirulina* (Ciferri, 1983).

Recently, more attention has been given to the study of the therapeutic effects of *Spirulina*. In addition to its effectiveness in reducing hyperlipidemia, diabetes, and high blood pressure in humans and animals, anti-viral and anti-cancer effects of orally administered *S. platensis* involving immune functions have also been reported (Belay, 2002).

3. Structure of phycocyanin

The biological and pharmacological properties of *Spirulina* were attributed mainly to C-phycocyanin (CPC). CPC is a major light-harvesting or photosynthetic pigment protein present in the antenna rods of *Spirulina platensis*. *S. platensis* also contains allophycocyanin (APC) as a minor component present at the core of the antenna rods. CPC and APC including phycoerythrin (PE) are the principal classes of phycobiliproteins which form supramolecular complexes known as phycobilisomes assemblies in cyanobacteria (Figure. 2a). In phycobiliproteins, a linear tetrapyrrole (bilin) as the chromophore is covalently attached to the apoprotein by thioether bonds to cysteine residues. CPC molecule is composed of two kinds of subunits, α- and β-subunits to form trimeric aggregation $\alpha 3\beta 3$ (Figures. 2b and 2c). Padyana et al. (2001) solved the crystal structure of CPC by molecular replacement technique (Figures. 2d and 2e). The α- and β-subunit polypeptides exhibit high affinity for one another and associate into ($\alpha\beta$)-monomers, which in turn aggregate into ($\alpha\beta$)3-trimers and ($\alpha\beta$)6-hexamers (Figure. 2f).

The medicinal and pharmacological properties of CPC have been reported earlier (Romay et al., 1998). Recent studies have demonstrated the antioxidant (Vadiraja et al., 1998; Wu and Annie Ho, 2008), anti-inflammatory (Reddy et al., 2000), and hepatoprotective properties (Vadiraja et al., 1998), in addition to anticancer, anti-allergic, immune-enhancing (Hayashi et al., 2008), blood-vessel-relaxing and blood-lipid-lowering effects of CPC (Gershwin and Belay, 2008).
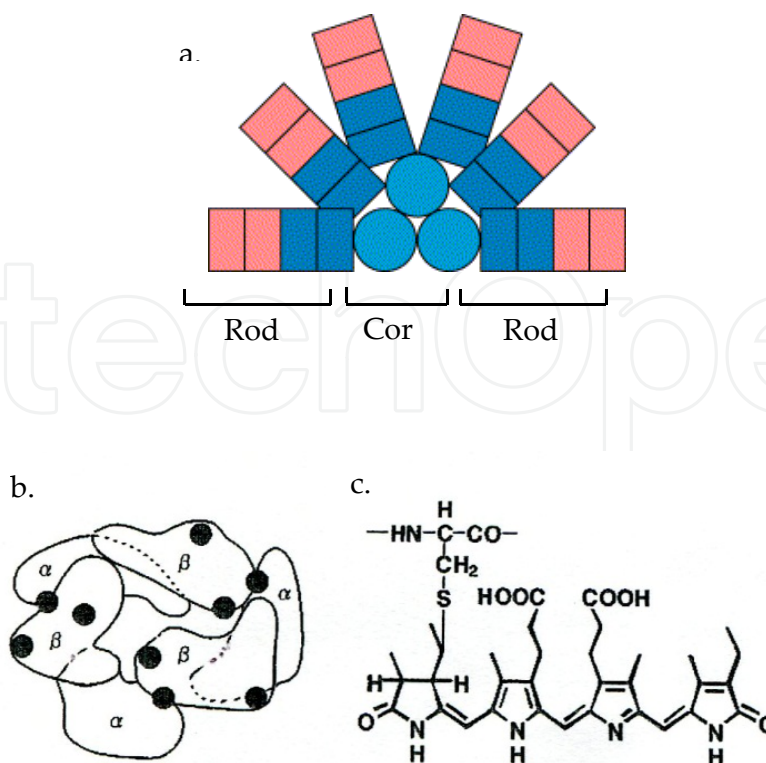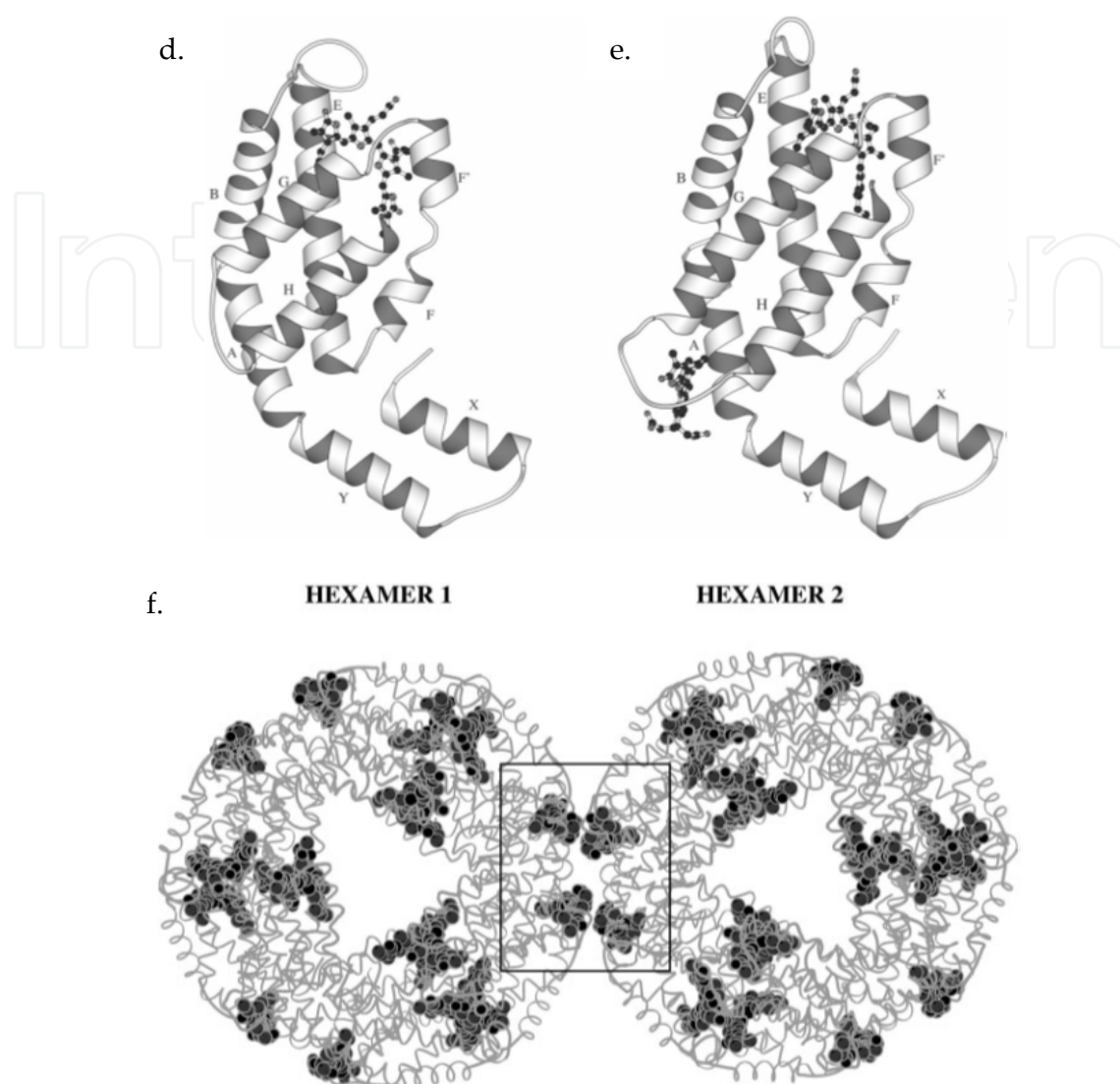
A phycobilisome (a) has six antenna rods with a three-cylinder core of allophycocyanin, APC (circles), two of the core cylinders lie on the thylakoid membrane, while the third one does not. Each rod has four hexameric disk-like structures, two of phycoerythrin, PE (red), and two of phycocyanin, CPC (blue) (MacColl, 2004). CPC consists of α- and β-subunit polypeptides to form trimeric aggregation $\alpha_3\beta_3$ and nine phycocyanobilin moieties as a chromophore shown in closed circles (b). c shows chemical structure of phycocyanobilin (Li et al., 2006). d and e show ribbon representation of CPC α-subunit and CPC β-subunit, respectively, with chromophores shown in ball and stick representation (Padyana et al., 2001). f shows coil representation of the two $(\alpha\beta)_6$-hexamers in the crystal asymmetric unit, and the box drawn at the center highlights the close proximity of phycocyanobilins at the position 155 on each β-subunit in the region between the adjacent hexamers (Padyana et al., 2001).

Figure 2. Schematic representation of one type of phycobilisome (a), and various representations of C-phycocyanin (b - f).

Preparation of phycocyanin solution in the experiments Phycocyanin was extracted from spray-dried *Spirulina platensis* with 50 mM sodium-phosphate buffer (pH 6.0). The crude extract was partially purified by DE-52 ion-exchange chromatography. The eluate was dialyzed against distilled water and lyophilized. Phycocyanin contents of the resultant powder were over 80%, and the recovery from the crude extract was approximately 6%. The

phycocyanin powder was dissolved in distilled water to a concentration of 0.05%, centrifuged in a refrigerated machine for 10 min at 1,500 g, and the supernatant was sterilized by filtration through a 0.20-μm-pore filter (Hayashi et al., 2006).
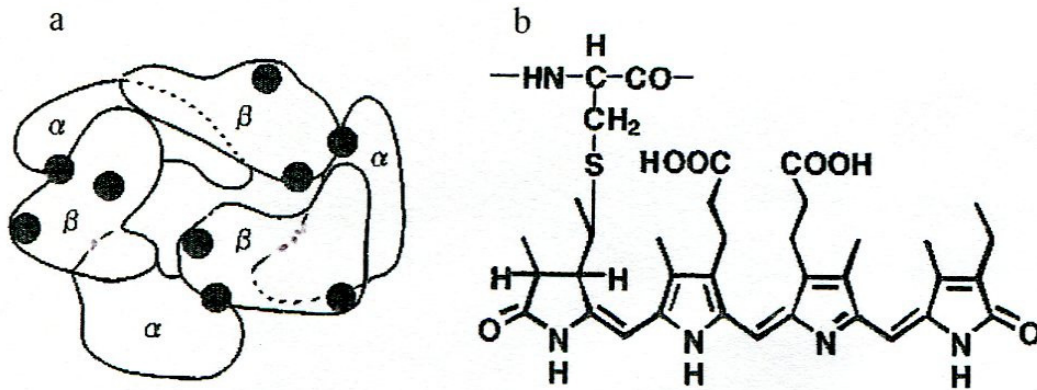

a. CPC molecule composed of two kinds of subunits(α- and β-subunits) to form α₃β₃ and nine phycocyanobilin moieties as a chromophore (closed circles). b. Chemical structure of phycocyanobilin. Phycocyanobilin was covalently bound to polypeptide chain of CPC by ways of thioetherlinkage to cysteine residues, one on the α-chain and two on the β-chain (Li et al., 2006).

Figure 3. Structure of CPC (αβ)₃-trimer and phycocyanobilin
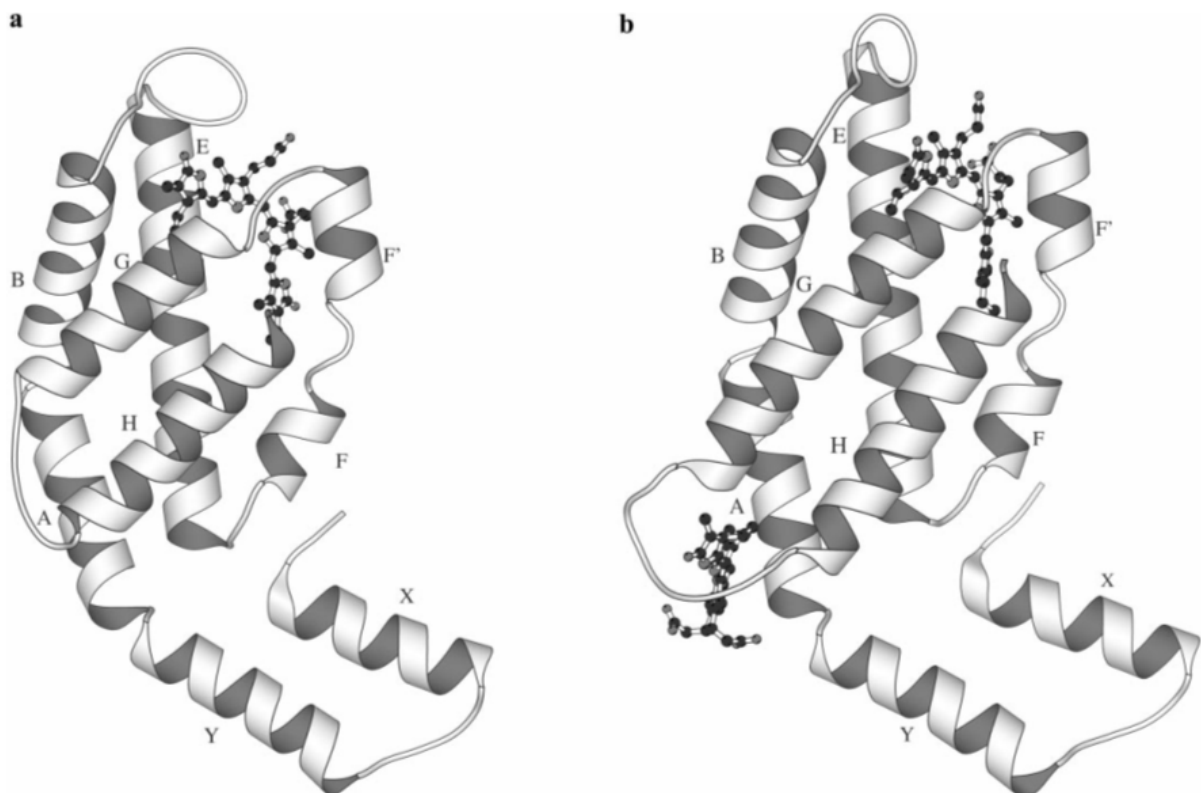
Figure 4. Ribbon representation of (a) CPC α-subunit (b) CPC β-subunit. Chromophores are shown in ball and stick representation (Padyana et al., 2001).
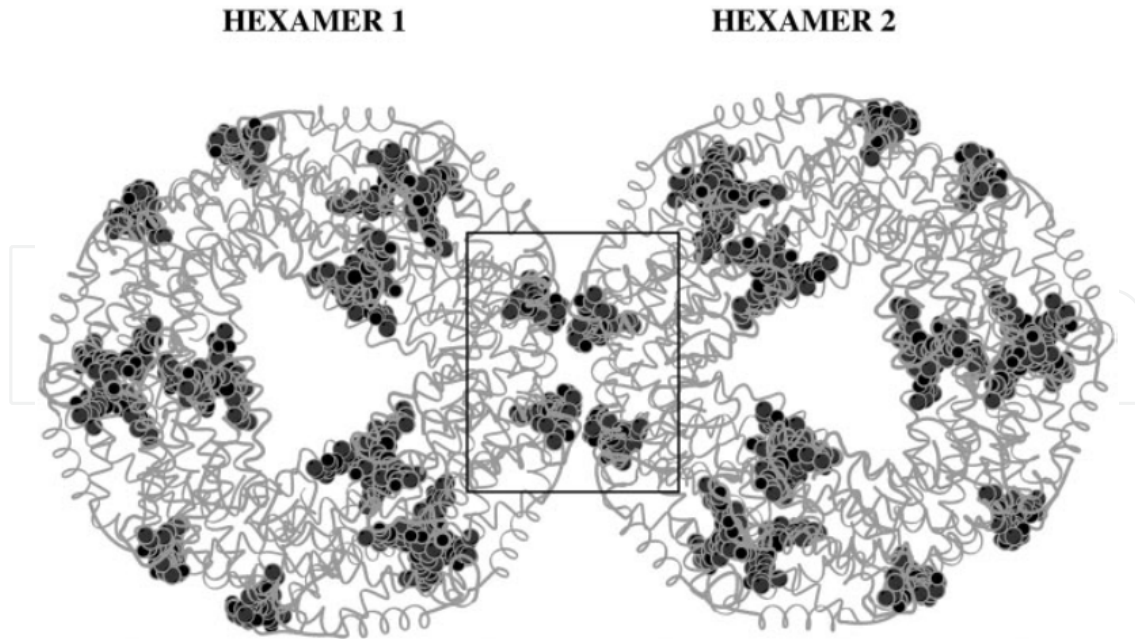
HEXAMER 1　　　　　　　　　**HEXAMER 2**

The figure illustrates the arrangement of chromophores at various locations within the hexamers. The box drawn at the center highlights the close proximity of phycocyanobilins at the position 155 on each β-subunit in the region between the adjacent hexamers (Padyana et al., 2001).

Figure 5. Coil representation of the two hexamers in the crystal asymmetric unit, a view through the approximate central axis of hexamers.

4. Enhancement of proliferation and differentiation of bone marrow cells stimulated with *Spirulina* and its extracts

Immunomodulation properties of *Spirulina* have been widely studied in chickens, prawns and fish, other animals, and humans. Generally, *Spirulina* and its extracts, such as hot-water extracts and phycocyanin, tended to enhance immune functions including mucosal or innate immunity through macrophage and secretions of the related cytokines (Belay, 2002; Hirahashi et al., 2002; Nemoto-Kawamura et al., 2004). Mao et al. (Mao et al., 2000) demonstrated that *Spirulina* stimulated the secretion of IL-1ß and IFN-γ in human peripheral blood mononuclear cells (PBMC) examined to nearly 2.0 and 3.3 times basal levels, respectively, and suggested that *Spirulina* helped balance the production of Th1 and Th2 cytokine stimulation. Phycocyanin, a characteristic photosynthesis pigment protein and an antioxidant in *Spirulina,* has been known to promote the growth of a human myeloid cell line, RPMI 8226 (Shinohara et al., 1988). Liu et al. (2000) reported that phycocyanin inhibited growth of human leukemia K562 cells and enhanced the arrest of the cell growth at G1 phase, suggesting enhancement of differentiation of the cells.

We have reported that *Spirulina* and its extracts enhanced immune responses in mice, mainly through increased production of interleukin-1 (IL-1) in macrophages (Hayashi et al., 1994; Hayashi et al., 1998). In the mice which ingested phycocyanin for 6 weeks, a marked increase of OVA antigen-specific IgA, as well as total IgA level was observed in the Peyer's patches, mesenteric lymph nodes and intestinal mucosa, as well as in the spleen cells

(Nemoto-Kawamura et al., 2004). These findings suggest that *Spirulina* or its components such as phycocyanin, affects immune functions by promoting immune competent-cell proliferation or differentiation in lymphoid organs.

We first investigated the effects of *Spirulina* and its extracts on proliferation of hematopoietic cells of mice and induction of colony-forming activity.

## Colony-formation of bone marrow cells in *in-vitro* study

Spirulina extracts such as a hot-water extract (SpHW), phycocyanin (Phyc), and cell-wall fraction (SpCW) recovered from *Spirulina* treated with 0.1 % sodium dodecyl sulfate to remove cytoplasmic material were used in this study. Culture supernatants of spleen (SP), Peyer's patch (PP), and peritoneal-exudated (PE) cells cultured with 20 μg/mL of the *Spirulina* extracts significantly enhanced proliferation of bone marrow cells (Figure 3). Each of the *Spirulina* extracts, SpHW, Phyc, and SpCW, itself, also directly enhanced proliferation of bone marrow cells in the concentration of 100 μg/mL of culture medium. In addition to that, colony- and cluster-formations of the bone marrow cells supplemented with culture supernatants of the spleen cells stimulated with *Spirulina* extracts, 50–400 μg/mL, were measured by soft agar method. The supernatants of cells cultured with Phyc and SpCW significantly increased the colony- and cluster-formations of the bone marrow cells in comparison to that of control or of the smallest concentration of each extract (Figure 4a). Culture supernatants of PE cells, which consisted of macrophages and lymphocytes in a ratio of about 50 % each and a small ratio of mast cells and neutrophils, also enhanced colony- and cluster-formations (Figure 4b). The numbers of these colonies, however, were almost the same as that by each other culture supernatant. Furthermore, Both granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin 3 (IL-3) contents in the culture supernatant or the serum as colony-forming activities were measured by commercially supplied ELISA assay kits. High amounts of GM-CSF or IL-3 were detected in the culture supernatants of the spleen and peritoneal-exudates cells stimulated with the *Spirulina* extracts, especially those with SpCW (Table 1). The amounts of IL-3 in the culture supernatants of the cells stimulated with SpHW and Phyc were relatively high, although colony formation by the supernatant was not so high. Culture supernatant of the cells stimulated with SpCW contained high amounts of GM-CSF but not of IL-3.

stimulated with	Colonies/well		GM-CSF pg/mL of CS		IL-3 pg/mL of CS	
	SP	PE	SP	PE	SP	PE
Control	0.5 ± 0.7	2.0 ± 2.3	<4	<4	47.3 ± 4.0	<3
SpHW	2.8 ± 2.6	33.0 ± 7.1	<4	7.1	76.7 ± 8.0	10.7
Phycocyanin	14.0 ± 5.9	37.3 ± 9.3	9.2 ± 0.7	4.3	94.7 ± 10.8	11.0
SpCW	28.2 ± 5.5	32.2 ±4.6	1,206 ± 333	104.7	481.7 ± 144.4	<3

Table 1. GM-CSF and IL-3 contents in the culture supernatants (SC) of the spleen (SP)and the peritoneal-exudates (PE) cells stimulated with *Spirulina* extracts (values are mean ± SD, N = 3)
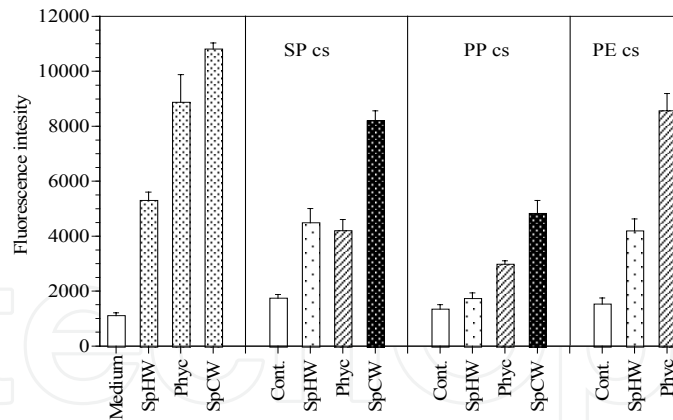
Figure 6. Bone marrow-cell proliferation by *Spirulina* extracts, SpHW, Phy, and SpCW, and by culture supernatant (CS) of lymphoid organ, spleen (SP) and Peyer's patch (PP), and peritoneal-exudates (PE) cells stimulated with the *Spirulina* extracts (Hayashi et al., 2006) (values are mean ± SD, n = 6)
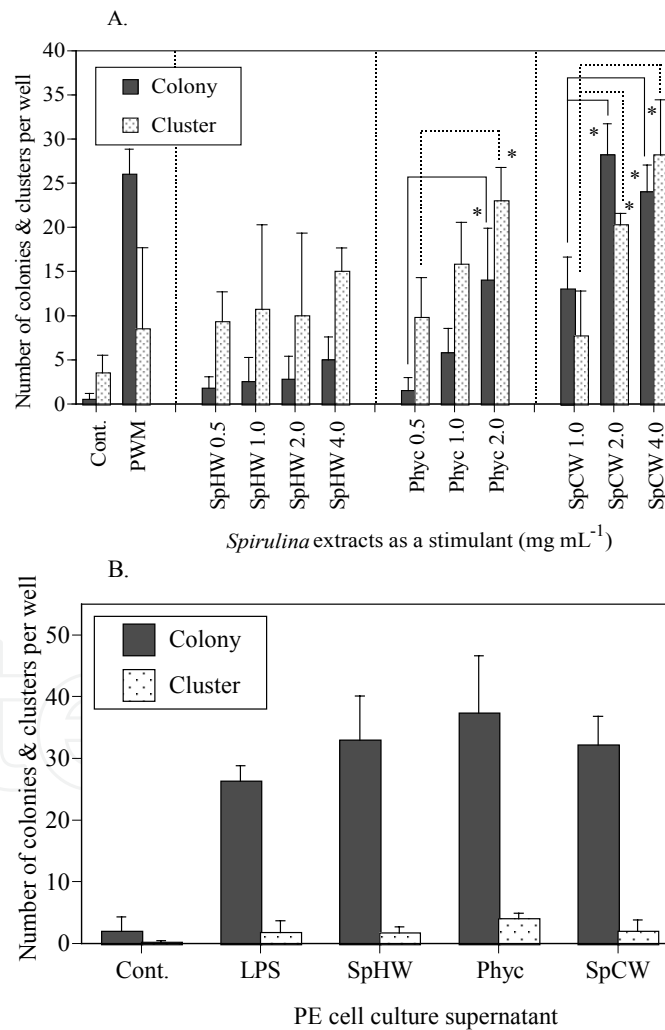
Figure 7. Bone marrow-cell colony and cluster formation in soft agar assay with the culture supernatant of the spleen cells (a) and peritoneal-exudates cells (b) stimulated with Spirulina extracts. Spleen cells were stimulated with 0.5, 1.0, 2.0 and 4.0 mg Spirulina extract/mL. Peritoneal-exudates cells were stimulated with 2.0 mg Spirulina extract /mL (Hayashi et al., 2006) (values are mean ± SD, n = 3).

## Colony-formation activity in the mice fed *Spirulina* in *in-vivo* study

As a preliminary experiment for *in-vivo* study, we measured next colony forming activity in the mice fed with the *Spirulina* extracts, SpHW, Phyc and SpCW, for 5 consecutive days or in the mice treated with an intra-peritoneal single injection of the extracts.

The sera from the mice which ingested Phyc or SpCW (1 mg/0.2 mL) for 5 consecutive days with feeding catheter enhanced colony formation of bone marrow cells (Figure 5). The serum from Phyc-feeding group significantly increased it in comparison to controls in which normal serum was added. All of the sera obtained from the mice which were treated with intra-peritoneal single injection of the *Spirulina* extracts (10 mg/0.5 mL) also showed significantly high colony formation in comparison to control of normal serum, although levels of the activities of the sera were almost the same each other (data were not shown). Colony-stimulating factors, GM-CSF and IL-3, in the sera from the mice which were either fed or intra-peritoneally injected with the extracts, however, were under detection limit (<4 and 3 pg/mL serum, respectively). Concentration of GM-CSF in the LPS serum obtained by *i.p.* injection was 50.1 pg/mL.

For longer-period experiment in *in-vivo* study, colony- and cluster-formation in the bone marrow cells with culture supernatants of the spleen (SP), Peyer's patch (PP), and peritoneal-exudated (PE) cells from the mice, which ingested the *Spirulina* extracts, SpHW, Phyc, and SpCW, for 5 weeks were then measured to confirm the former results. Culture supernatants of each lymphoid-organ, SP, PP, and PE cells from the groups were prepared under stimulation with or without phycocyanin. Colony formation by the culture supernatant of SP cells from the mice of SpHW group, as well as by that of PE cells from Phyc or SpCW group, under stimulation with phycocyanin, was significantly higher than that by each culture supernatant of cells from control group, and thus colony-forming activity was also significantly induced in the blood, spleen, and Peyer's patch cells in the mice which ingested *Spirulina* extracts for 5 weeks although neither significant amount of GM-CSF nor IL-3 was detected in the blood (data not shown). On the other hand, ratios of neutrophils in the SpHW-ingesting group and of lymphocytes in the SpCW-ingesting group were significantly higher than in controls, while ratios of lymphocytes, neutrophils, and monocytes in the peripheral blood of control group were in the normal range. A significant increase in ratio of lymphocytes was also observed in bone marrow cells in Phyc-ingesting group, although the number of cells was small. In addition, increased ratio of reticulocytes was observed in the bone marrow of the mice fed with SpHW.

In the mice ingested 0.05% phycocyanin solution for 6 weeks, a marked increase in the antigen-specific IgA antibody level as well as the total IgA antibody level was observed in the intestinal mucosa, the Peyer's patches and mesenteric lymph nodes, which comprise a major part of the gut-associated lymphoid tissues (GALT), whereas neither IgG1 nor IgE was affected in the spleen cells (Nemoto-Kawamura et al., 2004). Phycocyanin ingestion for 8 weeks, on the other hand, suppressed the production of antigen-specific IgG1 and IgE antibody in the serum. Further, we investigated the effect of *Spirulina* on salivary IgA antibody level of the subjects who customarily ingested the *Spirulina* tablets as health food

in various period of usage in their daily life, and measured correlation between the salivary IgA level and the amount of *Spirulina* ingested (Ishii et al., 1999). Total S-IgA level of the group ingesting *Spirulina* for more than one year was significantly increased ($p < 0.01$) in comparison to the group ingesting *Spirulina* for less than half a year, and statistically significant correlation between S-IgA levels in the saliva and total amount of *Spirulina* ingested by the subjects was observed (correlation coefficient $R = 0.288$, $n = 72$, $p < 0.05$).
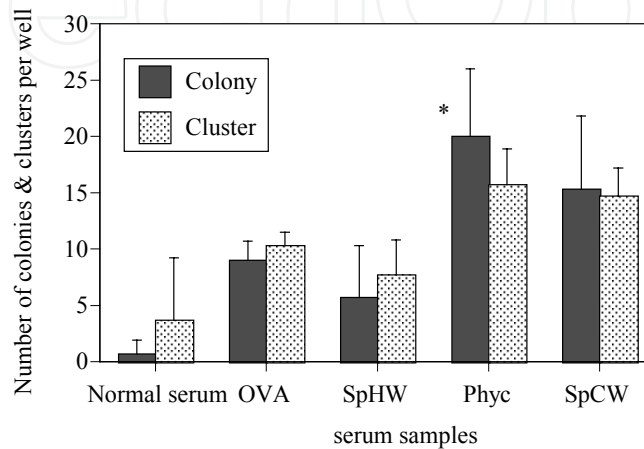
Figure 8. Bone marrow-cell colony and cluster formation in soft agar assay with the serum from the mice fed with *Spirulina* extracts for 5 consecutive days (Hayashi et al., 2006) (values are mean ± SD, $n = 3$). *; $p<0.05$ compared to Normal

It is known that multi-potent colony-stimulating factors such as G- and GM-CSF and IL-3, which are produced by a variety of cells including monocytes and lymphocytes can support proliferation of immature hematopoietic cells (Ihle, 1992). Liu et al. (2000) reported that phycocyanin from *Spirulina platensis* inhibited growth of human leukemia K562 cells in a dose-dependent manner, arresting them at the G1 phase with increased level of c-*myc* expression, suggesting that phycocyanin may enhance differentiation of the leukemia cells. Seya et al. (Hirahashi et al., 2002; Akao et al., 2009) reported that hot-water extract of *Spirulina* when taken orally in adult human enhances NK activation through the MyD88 pathway via Toll-like receptor (TLR) 2 and TLR4 on myeloid dendritic cells. From these findings, it appeared that *Spirulina*, including its components such as phycocyanin can affect enhancing proliferation or differentiation of immune competent-cells including bone marrow cell, which may cause normally sustaining or enhancing immune functions. Colony-stimulating activity other than IL-3 or GM-CSF, for example arginase and G-CSF, in the serum may contribute to the cell differentiation, although this is still not clear.

Zhang et al. (Zhang, 1994) found that C-phycocyanin and polysaccharide isolated from *Spirulina* increased leukocyte and bone marrow nucleated cell counts as well as colony formation of colony forming unit-granulocyte and macrophage (CFU-GM) in the gamma-ray irradiated mice, and also found that C-phycocyanin possessed high erythropoietin activity. Some institution facilities have reported the potential radiation protection effects of *Spirulina* against radiation-induced membrane damage and cellular dysfunction by reactive

oxygen species in mice and against reduced levels of the leukocytes in the blood and nucleated cells in the bone marrow in dog (Zhang et al., 2001; Verma et al., 2006). Doctors in Belarus reported that ingestion of 5 g of *Spirulina* a day resulted in the reduction of Cesium-137 in urine by 50%, in children subjected to low level of radiation over a long period of time (Loseva and Dardynskaya, 1993). Rahadiya and Patel in India (Rabadiya and Patel, 2010) also reported radiation-effect-reducing activity of *Spirulina* in their review. Anti-oxidant and anti-inflammatory effects as well as proliferation and differentiation activity of *Spirulina* possibly contribute to the radiation protection effects.

5. Effect of phycocyanin on differentiation of human myeloid leukemia cell lines

A study of aerosolized GM-CSF demonstrated tolerance and possible efficacy in patients with malignant metastases to the lungs, possibly through upregulation of antigen-specific cytotoxic T-cells (Rao et al., 2003). It is known that various food compounds and the metabolites involving phycocyanin can influence the processes in cellular differentiation, apoptosis, and proliferative potential, and there is considerable evidence that vitamins and micronutrients are able to regulate gene expression of cancer cells, resulting in influence on the carcinogenic process (Sacha et al., 2005). All-trans-retinoic acid and vitamin D3 are known as one of the physiologic agents which can modulate the proliferation and differentiation of hematopoietic cells (Collins, 2002). The vitamin plus interferon-γ (IFNγ) treatment and enrichment with polyunsaturated fatty acids such as arachidonic acid, eicosapentaenoic acid or docosahexaenoic acid also significantly enhanced immunoregulatory effects, or enhanced the expression of monocytic surface antigens CD11b and CD14 on human premonocytic U937 cells (Obermeier et al., 1995). In this section, we investigated the effects of phycocyanin on differentiation and morphological and cytochemical changes of human myeloid leukemia cell lines, U937 and HL-60 cells, generally used for the studies of cell differentiation.

A human hematopoietic cell line, U-937, was derived from a patient with generalized histiocytic lymphoma. The histiocytic origin of the cell line was shown by its capacity of lysozyme production and the strong esterase activity (naphtol AS-D acetate esterase inhibited by NaF) of the cells (Sundstrom and Nillson, 1976). The cell line was morphologically identical to that of the tumor cells in the pleural effusion, and is known to be functionally differentiated to phagocytic macrophage by cytokines from lymphocytes (Koren et al, 1979).

A continuous human myeloid cell line, HL-60, was derived from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia and established. The predominant cell type is a neutrophilic promyelocyte with prominent nuclear/cytoplasmic asynchrony (Gallangher et al., 1979). HL-60 cells lack specific markers for lymphoid cells, but express surface receptors for Fc fragment and complement (C3), which have been associated with differentiated granulocytes. They exhibit phagocytic activity and responsiveness to a chemotactic stimulus commensurate with the proportion of mature cells.

Preparation of Conditioned Medium (CM) of peripheral blood mononuclear cells cultured with phycocyanin

Human peripheral blood mononuclear cells (PBMCs) from 7 healthy volunteers were separated by density centrifugation (800 g, 30 mins) using a Lymphoprep™ (Density 1.077 g/mL, NYCOMED) under approving by the Institutional Review Board of our University. Cells from each subject were suspended in RPMI-FBS medium and adjusted to 1 x 10^6 cells/mL and were cultured with and without 2 mg/mL of phycocyanin (Phyc) using 24-well cultured plates. The conditioned mediums (CMs), both with and without Phyc (Phyco-CM and Cont-CM, respectively), were harvested on the 7th day and filtered through an 0.45 μmφ filter to remove cell debris.

Cell growth of U937 as well as HL-60 cells supplemented with Phyco-CM resulted in significant inhibition during the 7-day culture in comparison with those of control without supplementation, while supplementation with 2 mg/mL Phyc itself had no effect on growth in these cells. Both U937 and HL-60 cells stimulated with Phyc and Phyco-CM were more than 80% viable, as were those cells stimulated with phorbol-12-myristate-13-acetate (PMA) 0.02 μg/mL as a positive control of differentiation.

Morphology and flow cytometric assay of cell surface antigens on U937 and HL-60 Cells

U937 cells cultured in the medium supplemented with Phyco-CM morphologically changed into the cells with large cytoplasm with vacuoles, non-condensed nuclear chromatin, and a persistence of nucleolus that resembled to those stimulated with PMA as a positive control (Figure 6a) while control U937 cells, without stimulation, were promonocyte-like with variable nuclear shapes and regular indentations and comprised moderate cytoplasm containing numerous small eosinophilic granules and a few vacuoles. Cont-CM or conditioned medium of lymphocytes cultured without phycocyanin, only partially changed U937 cells into monocytic cells comprising moderate cytoplasm with large indented nucleus (Figure 6a). U937 cells stimulated with Phyco-CM and Cont-CM consisted of monocytes/macrophages in the ratio of 57% and 21%, respectively, and each ratio was significantly higher than that of control (1.4%) without stimulation. Stimulation by Phyc changed U937 cells partially to promonocytes with indents on the nuclei. The ratio of monocytes/macrophages was only 3.4 %.

Control HL-60 cells, without stimulation, was predominantly promyelocytes with azurophilic granules, large round nuclei, and prominent nucleoli. Morphological classification of the cells, especially those stimulated with Phyco-CM, was relatively difficult because various features of promyelocytes coexisted. The Phyco-CM-stimulated HL-60 cells showed a morphologically matured monocytic cell lineage (about 15.4%), that is, with decreased nuclear/cytoplasmic ratio and a paler cytoplasm with vacuoles (Figure 6b). The cells (about 80%) other than monocytic cells consisted of granulocytes, including promyelocytes and myelocytes, with large nuclei, less prominent cytoplasmic granules and a marked decrease or complete disappearance of nucleoli. Almost of all HL-60 cells

stimulated with Phyc and Cont-CM were promyelocyte-like, while all-*trans* retinoic acid (ATRA) induced cells to differentiate into granulocytes (Figure 6b).
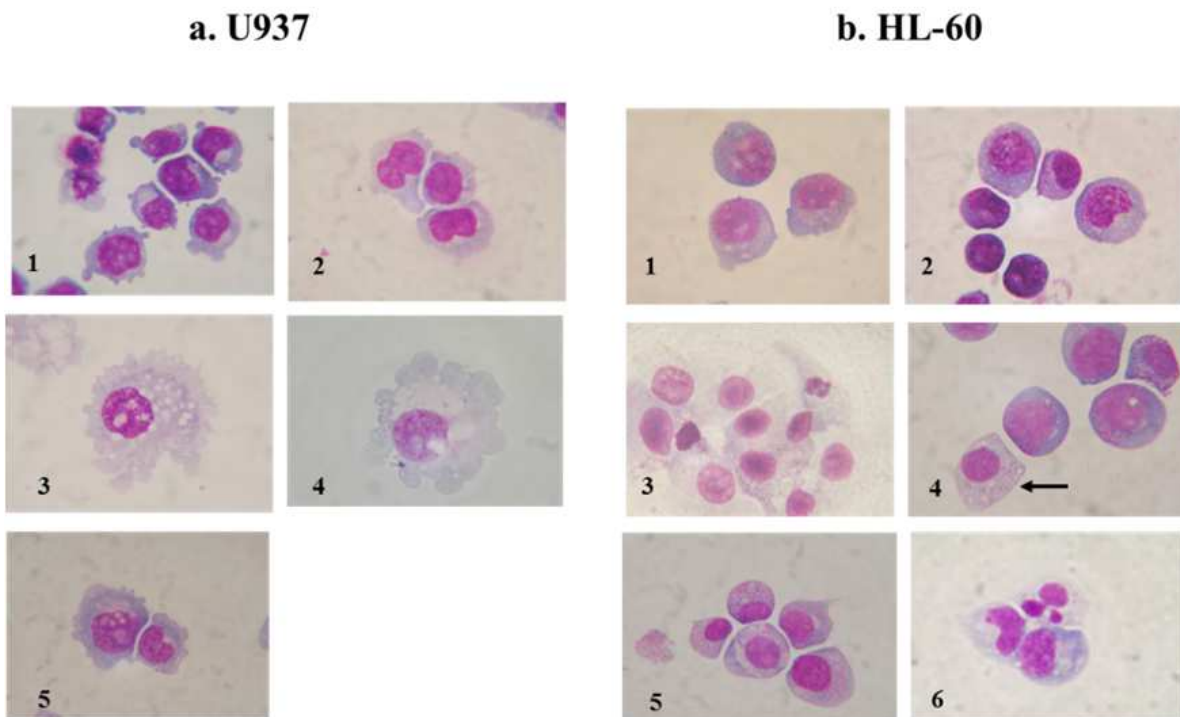
Cell morphology was measured under light microscopy. U937 cells (a) and HL-60 cells (b), 0.5 x 10⁶ cells/mL of medium, were cultured for 3 days with RPMI-FBS (Cont.)
(1); with Phyc (2); with PMA (3); with Phyco-CM (4); with Cont-CM (5); with ATRA (6) (Ishii et al., 2009). (Original magnification x 1000)

**Figure 9.** Morphology of U937 and HL-60 cells stimulated with Phyco-CM and others.

Flow cytometric assay was carried out using a Flow Cytometer (FCM; EPICS® ALTRA™, Beckman Coulter, Inc., Fullerton, CA). The expression of cell surface antigens, CD14, CD11b, CD66b and CD15, on U937 and HL-60 cells stimulated with various CMs, as described above, were determined by direct immunofluorescence method using appropriate fluorescent labeled monoclonal antibodies. Typical patterns of FCM analysis for CD14 antigen in both U937 and HL-60 cells stimulated with Phyco-CM were shown in Figure 7.

Ratio of CD14-antigen positive cells in U937 cells stimulated with Phyco-CM, 53%, was significantly high in comparison with those of Cont-CM and Cont without stimulation, 30% and 15%, respectively, while ratio of CD14-positive cells in HL-60 cells was originally low but was significantly increased by Phyc and Phyco-CM stimulations, about 20% (Figure 8a). Ratio of FcγR positive cells in U937 cells was originally high and those of the cells stimulated with Phyco-CM and Cont-CM were almost the same as Cont without stimulation, 65%. In HL-60, on

the other hand, Phyco-CM increased FcγR positive cells significantly, 41%, compared with Cont and Cont-CM, 24% and 20%, respectively (Figure 8b). Further, a final concentration of 0.01 mg/mL of Phyc significantly increased the population of CD11b-antigen positive cells in U937 cells compared with Cont and Cont-CM (Figure 9a). Although the fluorescence intensity of anti-CD14 antibody per cell in U937 cells stimulated with Phyc was marginally higher than that of Cont (Figure 7a), the ratio of CD14-antigen positive cells was low (Figure 8a) and morphologically comprised 3.4% of monocytes/macrophages. This suggests that phycocyanin stimulates U937 cells to some extent to differentiate or express some CD antigens such as CD11b and CD14. CD11b as well as CD66b is specific in monocytes and granulocytes. Expression of CD11b is known to be up-regulated during granulocytic and monocytic differentiation, and is used as a marker of differentiation of myelomonocytic lineage (Lubbert et al., 1991). It has been also recognized that morphological changes of differentiating U937 cells are accompanied by cellular adherence and are paralleled by an expression of the β_2 integrins, CD11a, CD11c, CD18, and particularly CD11b (Hass et al., 1989). CD11b glycoprotein represents the α-subunit of a heterodimeric association with the common β-subunit CD18 in β_2 integrin, an adhesion molecule. Their extracellular domains with the CD11b/CD18 (CR3/Mac-1) β_2 integrin contribute to adhesion to adjacent cells, for example, the regulation of leukocyte-endothelial cell interactions (Ebnet et al., 2004). In the study using stably-transfected U937 cells with a vector containing the β_2 integrin gene in antisense orientation, Otte et al. (2011) suggested that induced adherence predominantly mediated by a functional CD11b/CD18 integrin contributed to cell cycle regulation and apoptosis during monocytic maturation. Concerning apoptotic cell death, photodynamic therapy (PDT) for tumors which is based on the tumor-selective accumulation of protoporphyrin IX (PpIX), as a photosensitizer after addition of 5-aminolevulinic acid (ALA) followed by irradiation with visible light has been demonstrated by some investigators, and it was reported that ALA-based photodynamic action (PDA) induced apoptotic cell death in U937 cells through a mitochondrial pathway and that ferrochelatase inhibitors might enhanced the effect of PDT for tumors (Amo et al., 2009). Furthermore pulsating electromagnetic field (PEMF) can affect cancer cells proliferation and death (Kaszuba-Zwoinska et al., 2010). They reported that U937 cells exposed to a pulsating magnetic field 50Hz, 45±5 mT three times for each 3 h with 24 h intervals induced cells death in higher cell density and conversely prevented puromycin-induced cell death. We could take advantage of the halfway differentiated U937 cells induced by phycocyanin as a cell culture model of cell differentiation and apoptotic cell death to investigate the molecular mechanism of these various tumoricidal treatments.

Both Phyc and Phyco-CM significantly increased the ratio of CD11b-antigen positive cells in U937 cells, about 30%, in comparison with those of Cont and Cont-CM, 12% and 22%, respectively, while in HL-60 neither CD11b nor CD66b cells showed significant increases in ratio when stimulated with Phyco-CM (16%, 22%) or Cont-CM (10%, 18%) cells. In contrast, the ratio of CD15-antigen positive cells in the U937 cells was low regardless of stimulation (Figure 9a). CD15-antigen is generally characteristic of granulocytes and monocytes. The ratio of CD15-antigen positive cells in the HL-60 cells showed insignificant changes when stimulated with Phyc, Phyco-CM and other CMs (Figure 9b).
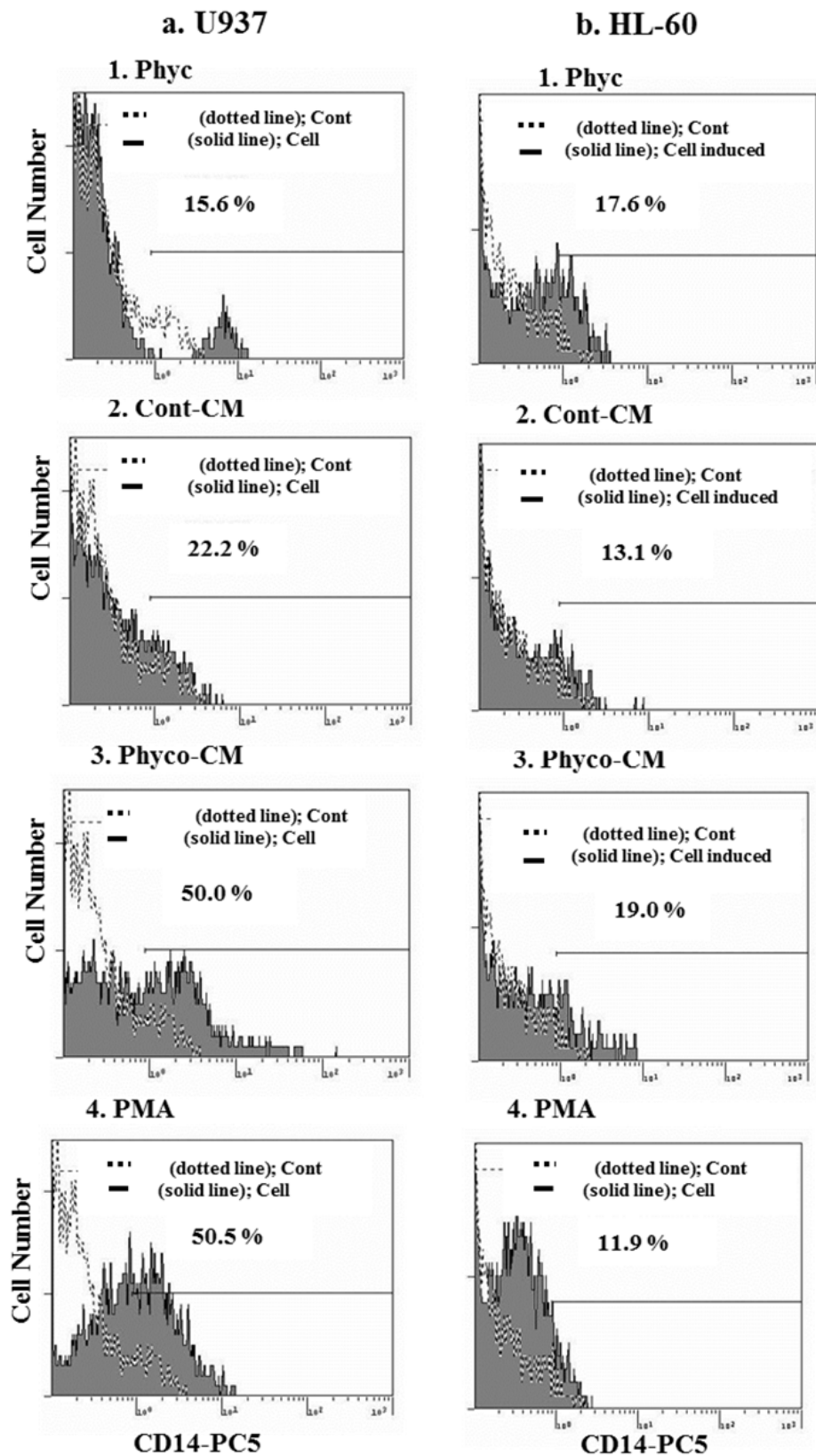
Patterns of U937 and HL-60 cells (0.5 x 10⁶ cells/mL of medium) stimulated with Phyc (1), Cont-CM (2), Phyco-CM (3) and PMA (4) were shown in solid lines and that of Cont was shown in dotted lines (Ishii et al., 2009).

Figure 10. Typical patterns of CD14-positive cells in U937 and HL-60 cells.
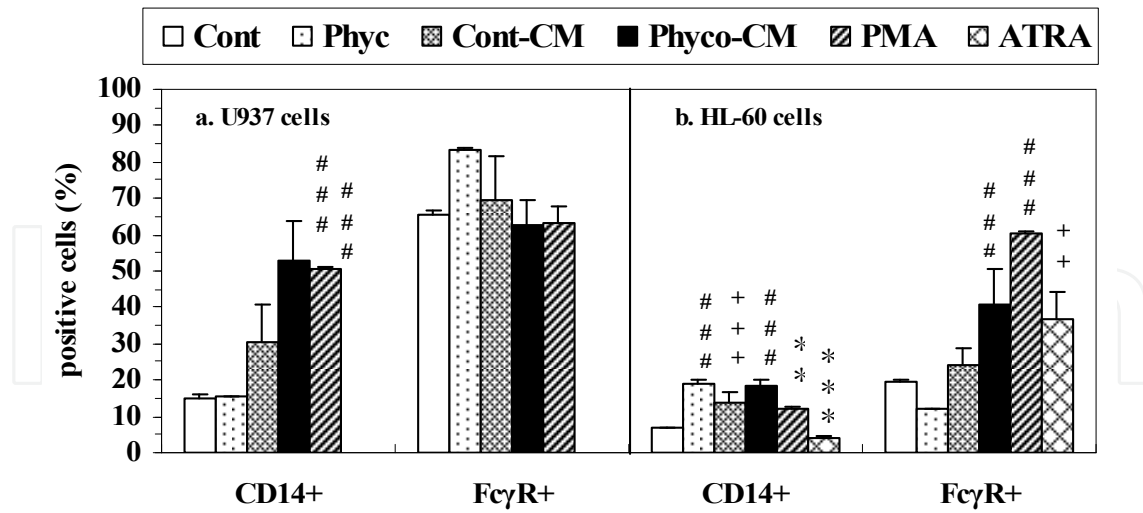
Data analysis was based on examination of 5000 cells/sample.
++; p < 0.01, +++; p < 0.001 to each Cont, **; p < 0.01, ***; p < 0.001 to each Cont-CM, ###; p < 0.001 to each Cont and Cont-CM. Each value shows mean ± SD, n=3~7 (Ishii et al., 2009)

Figure 11. Percentage of CD14 and FcγR positive cells in U937 and HL-60 cells stimulated with Phyco-CM and other stimulants.
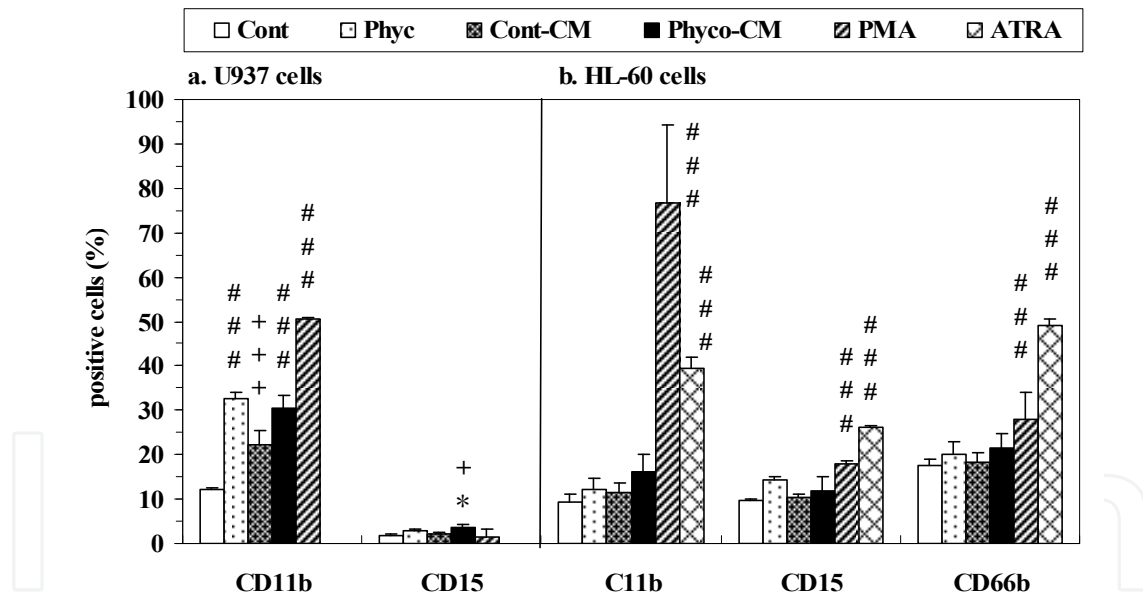
Data analysis was based on examination of 5000 cells/sample. Each value shows mean ± SD, n= 3 (Ishii et al., 2009)

Figure 12. Percentage of CD11b-, CD15- and CD66b-antigen positive cells in U937 and HL-60 cells stimulated with Phyco-CM and other stimulants.

Phagocytic activity and TNF-α production, cytochemical analysis

Differentiation of both HL-60 and U937 cells was also assessed by cytochemical analysis with specific and non-specific esterase (SE/NSE) double staining method and Nitro-blue tetrazolium (NBT) reducing activity which is characteristic of phagocytic cells (Table 2).

Although U937 cells were originally NSE positive, the Phyco-CM stimulated cells showed equally high ratio, while most HL-60 cells were SE positive under all stimulants. Phyco-CM stimulation significantly increased the ratio of NBT-positive cells in both U937 and HL-60 cells, while that of Cont-CM was almost the same as Cont. In addition to that, phagocytic activity in U937 cells stimulated with Phyco-CM was significantly higher than that of the cells stimulated with Cont-CM. In HL-60 cells, both Phyco-CM and Cont-CM increased phagocytic activity, as compared with Cont. Increased levels of TNF-α in both U937 and HL-60cells stimulated with Phyco-CM, were relatively high in comparison with Cont-CM but not significant, and were not synergistically increased by supplementation with LPS (1000 ng/mL) in the culture. Level of TNF-α in the cells stimulated with Phyc was under the detection limit.

Stimulators	Phagocytic activity (%)	TNF-α (pg/mL)	NBT reducing activity (%)
U937			
Cont	18 ± 5.2	ND	1.3 ± 0.3
Phyc	23 ± 5.1	< 15.6	5.0 ± 3.0
Cont-CM	32 ± 5.5 [++]	46 ± 31	1.3 ± 0.8
Phyco-CM	81 ± 13 [+++, ***]	66 ± 15	7.5 ± 4.1[**]
PMA	96 ± 4.6 [+++, ***]	968 ± 150 [***]	5.2 ± 2.9
HL-60			
Cont	17 ± 6.2	ND	0.7 ± 0.3
Phyc	16 ± 3.8	< 15.6	2.0 ± 1.0
Cont-CM	35 ± 12 [++]	32 ± 29	2.1 ± 0.7
Phyco-CM	36 ± 13 [++]	71 ± 18	5.3 ± 1.9[+++, **]
PMA	84 ± 13 [+++]	585 ± 39	1.7 ± 0.8
ATRA	72 ± 5.2 [+++]	ND	10.8 ± 3.3 [+++, ***]

Phagocytic activity was determined by ingestion of opsonin-treated latex beads. NBT reducing activity was measured as percentage of the positive cells, which contained intracellular blue-black formazan deposits. Positive cells out of 200 cells were counted under light microscopy. ++; $p < 0.01$, +++; $p < 0.001$ compared to Cont, **; $p < 0.01$, ***; $p < 0.001$ compared to Cont-CM, < ; under detection limit (15.6 pg/mL), ND; not detected (values are mean ± SD, n=3)

Table 2. Cytochemical analysis of U937 and HL-60 cells stimulated with Phyco-CM

Phyco-CM and Phyc quite significantly increased the population of CD14-antigen positive cells in HL-60 cells, although the level was lower than that in U937 cells (Figure 8). In addition to that, Phyco-CM increased also the NBT reducing activity of HL-60 cells. Tamagawa et al. (1998) reported that NBT reducing activity was used as a marker of HL-60 cell differentiation into granulocytes and monocytes. Fontana et al. (1981) suggested that HL-60 cells were able to commit themselves to the development of two different program of hematopoietic differentiation, that is, either myeloid or macrophage depending on cytokine or stimulant. In fact, both monocytic and granulocytic cells coexisted in HL-60 cells stimulated with Phyc and Phyco-CM as the result of differentiation in two types of directions. However, NSE and SE ratios in HL-60 cells did not necessarily correspond to morphological observation. Normally NSE has been thought to be specific for

monocyte/macrophage activity (Tanaka et al., 1983), and Chiao et al. (1981) reported that conditioned medium of normal human peripheral blood lymphocytes induced HL-60 cells into macrophage and monocyte-like cell lines, but most HL-60 cells stimulated with Phyco-CM in the present study were SE positive, and only about 15% of monocytic matured cells were found in the cells after Phyco-CM stimulation. It appeared that the effects of Phyco-CM on HL-60 cells may insufficiently induce to matured cells.

CD14 antigen has been reported to be a receptor for the complex of LPS and LPS-binding protein (LBP). It is known that LPS and Gram negative bacteria as triggers (Beutler, 2000; Lu et al., 2008) can cause TNF-α release in human monocytes through TLR4 (Tudhope et al., 2008). The U937 cells stimulated with Phyco-CM, that showed high ratio of CD14-positive cells, are expected to express TLR4 in addition to CD4 because expression of TLR4 needs CD14 and LBP in response to the binding of LPS with LBP. Phyco-CM induced TNF-α production in the culture supernatants of U937 and HL-60 cells. A high molecular weight polysaccharide, Immulina, from *Spirulina* was a potent activator of nuclear factor-kappaB (NF-κB) and induced both IL-1β and TNF-α mRNAs in THP-1 human monocytes (Pugh et al., 2001), and expression of TLR2 and CD14 probably contributed to the NF-κB activation and immune enhancing activity of the Immulina in mice (Balachandran et al., 2006). The levels of TNF-α, however, were not further increased with LPS stimulation (1000 ng/mL) in the U937 cells stimulated with phyco-CM. Phagocytic activity in the stimulated U937 cells was significantly higher than that of the cells stimulated with Cont-CM, and there was no stimulatory effect in the existence of LPS. Phyc alone did not induce TNF-α in U937 and HL-60 cells.

6. Age-related changes in intestine intraepitherial lymphocyte subsets and their functional preservation by *Spirulina* in mice

Age-related immune dysfunction has been reviewed by many researchers (Solana et al., 2006). The complex age-related changes in the immune system, collectively termed "immunosenescence," have been demonstrated in diverse species, including humans, and have been recognized as contributing to morbidity and mortality due to greater incidence of infectious diseases, autoimmune diseases, and cancer. The concept of age-related immunosenescence is in agreement with numerous data such as the change of cytokine balances, the decrease of interleukin (IL)-2 contrary to the increase of IL-6, and nutritional imbalance or malnutrition (Miquel, 2001; De la Fuente, 2002). It was reported that antigen-specific secretory immunoglobulin A titer in the intestinal lumen declined in senescent animals (Koga et al., 2000). Some studies have also reported that reduced bioavailability of key conditionally essential nutrients might limit immune response in aging (Cunningham-Rundles, 2004) and that well-nourished elderly people appear to have less significant or minimal changes in immune response (Krause et al., 1999).

It is generally accepted that the development of age-associated alterations occurs earlier in the mucosal immune system than in the systemic immune compartment (Schmucker et al., 2003). The mucosal immune system of the intestinal epithelia contains a functionally

specialized T-cell population known as intraepithelial lymphocytes (IELs). Because of their unique location in the mucosal epithelium, IELs are recognized as a first-line mucosal barrier against infectious diseases and food-borne allergens (Hayday et al., 2001).

We have reported that ingestion of phycocyanin enhanced the antigen-specific immunoglobulin A response in the intestinal mucosa of mice (Nemoto-Kawamura et al., 2004). In this section, we investigated age-related changes in intestine IEL subsets in mice by flow cytometric (FCM) analysis and their functional preservation after the animals were fed *Spirulina*.

Characterization of IELs of adult and aged mice

IELs possess phenotypic features distinct from those of lamina propria lymphocytes in intestine. Lamina propria lymphocytes consist of predominantly activated T cells and are mainly CD4$^+$ and CD8$^+$ single-positive T cells in proportions of about 70% and 30%, respectively. The phenotype of lamina propria lymphocytes, in general, is similar to that of the cells in the peripheral lymphoid tissues and in the circulating blood, that is, over 95% of the cells possess a surface phenotype of T-cell receptor $\alpha\beta^+$ (TCR$\alpha\beta^+$), whereas less than 5% possess TCR$\gamma\delta^+$. These cells are known to be matured in the thymus (Lydyard and Grossi, 1998). IELs, on the other hand, possess TCR$\gamma\delta^+$ in a greater percentage (30–60%) and TCR$\alpha\beta^+$ in a percentage of 40–70%, which might be related to their state of activation (Ewijk et al., 1999). In adult mice bred in a conventional environment, about half of the IELs have a phenotype of surface CD antigen similar to that of most peripheral T lymphocytes, that is, Thy-1$^+$, TCR$\alpha\beta^+$, and either CD4$^+$ or CD8$^+$, which are made up of heterodimers of CD8α and β chains (CD8$\alpha\beta^+$). These cells were matured in a thymus-dependent manner (Kaminogawa and Nanno, 2004). Another major IEL population possesses the surface phenotype TCR$\alpha\beta^+$ or TCR$\gamma\delta^+$, which expresses CD8 homodimeric α chains (CD8$\alpha\alpha^+$) but does not express CD4 or CD8 heterodimeric molecules (Rocha et al., 1994). These cells are known to be of extrathymic origin. Small percentages of the TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ but no TCR cells are CD8$^-$ CD4$^-$. TCR$\alpha\beta^+$ IELs co-expressing both CD4 and CD8 molecules are rare but bear high levels of TCR$\alpha\beta$ and CD8$\alpha\alpha$ (Lefrancois, 1991). Our preliminary experiment showed that the number of CD45$^+$ (leukocyte-common antigen-positive) cells as IELs was significantly lower in aged mice than in adult mice. Either the proportion or the number of CD8$^+$ cells in addition to CD45$^+$ cells of aged mice was significantly lower than that of adult mice, corresponding to the previous article by Komuro et al (1990). The proportion and number of CD4$^+$CD8$^+$ double-positive cells in the aged mice, on the other hand, were higher than those in adult mice. It has been reported that CD4$^+$CD8$^+$ T cells bearing TCR$\alpha\beta$ in the epithelium, which were derived from thymus-dependent populations, expanded with aging at a local site of the intestine under the influence of intestinal microflora, contributing to the first line of defensive barrier in the epithelium (Takimoto et al., 1992).

Overall, increased or decreased levels of these surface antigen-positive cells observed in the aged mice tended to be restored by the ingestion of SpHW for 5 weeks in the aged-SP group. In fact, significant decreases of CD45$^+$CD8$^+$ cells and increases of CD8$^-$CD4$^-$ and

$CD45^+TCR\alpha\beta^+$ cells were observed in the aged mice, whereas neither an increase nor a decrease was observed in the aged-SP group fed with SpHW—that is, the levels were similar to those in adult mice. In particular, the proportions of $CD45^+CD8^+$ cells and $CD45^+TCR\gamma\delta^+$ cells in the aged-SP group significantly increased in comparison to the aged group. $CD8^+$ T cells expressing $\alpha\beta$TCR ($\alpha\beta$T cells) are engaged in antigen-specific cell cytotoxicity mediated by major histocompatibility complex (MHC) molecules, whereas T cells expressing $\gamma\delta$TCR ($\gamma\delta$T cells) often manifest preliminary target cell killing without MHC restriction (Cruse and Lewis, 1995). $\gamma\delta$T cells have also been shown to be associated with regulation of the generation and differentiation of IELs (Komano et al., 1995). These results suggest that ingestion of SpHW in the aged-SP group may contribute to the functional preservation of the intestinal epithelium as a first line of mucosal barrier against infectious agents through retaining the numbers of certain IELs.

Decreased levels of RBCs, especially the level of hematocrit, Ht, in the aged group, were also restored after ingestion of SpHW in the aged-SP group. Significant decreases in WBCs in the aged-SP group, in contrast to the increase in the aged group, may be ascribed to the anti-inflammatory activity of *Spirulina* (Vila et al., 2008) and/or to the restoration of immunological function by ingesting *Spirulina*. Some reports indicated that phycocyanin and the polysaccharide isolated from *Spirulina* increased bone marrow nucleated cell and erythrocyte counts in the gamma-ray irradiated mice or dog (Zhang, 1994; Zhang et al., 2001; Verma et al., 2006). Many studies have demonstrated that *Spirulina* including phycocyanin possesses antioxidant activity, as well as an anti-inflammatory activity (Romay et al., 1998; Remirez et al., 2002), which scavenges peroxyl radicals, and also acts as an inhibitor of cyclooxygenase, like nonsteroidal anti-inflammatory drugs. In addition, a down-regulation of pro-inflammatory cytokines, such as TNF-α and -γ, was observed in the aged animals on the *Spirulina*-enriched diet (Vila et al., 2008). Overexpression of MHC class I-related chain A in the intestine of experimental transgenic mice resulted in a clonal expansion of $CD4^+CD8\alpha\alpha^+$ IELs and attenuated acute colitis in an experimental model of inflammatory bowel disease induced by dextran sodium sulfate administration (Park et al., 2003). $CD8\alpha\alpha^+$ IELs developed along an extrathymic pathway may work as anti-inflammatory regulator T cells to sustain the mucosal intranet formed by intestinal epithelial cells and IELs and to diminish the expansion of enterotoxigenic *Escherichia coli* (Kim et al., 2001). Although ingesting SpHW did not significantly increase the level of $CD4^+CD8^+$ IELs in the present study, these facts, in addition to our present results, suggest that ingestion of *Spirulina* appears to be effective for protecting immune functions or improving immune systems vulnerable to age, thereby reducing the risk of infectious and autoimmune diseases. However, additional detailed study is needed.

7. Conclusions

Spirulina and its extracts enhanced proliferation of hematopoietic cells and colony formation of bone marrow cell, as a marker for cell differentiation activity, in *in-vitro* and *in-vivo* study using mice. Phycocyanin, a light-harvesting pigment of *Spirulina*, also induced cell differentiation of human leukemia cell lines, U937 and HL-60 cells, into monocyte/macrophage

and granulocyte, respectively, to some extent directly and indirectly through enhancing cytokine production in human peripheral blood lymphocytes stimulated with phycocyanin. These distinguished activities of *Spirulina* as well as other certain functional foods can be preferably emphasized to be used, especially for elderly people. Recent intervention study showed that 6- and 12-week supplementation of *Spirulina* increased mean hemoglobin level and indoleamine 2,3-dioxygenase activity, as a sign of immune function, in the elderly subjects, suggesting that *Spirulina* may ameliorate anemia and immunosenescence in elderly people (Selmi et al., 2011). Pentón-Rol et al. (2011) demonstrated that phycocyanin triggered preventing or downgrading experimental autoimmune encephalitis (EAE) expression in rats, and that ingestion of phycocyanin induced a regulatory T cell (Treg) response in peripheral blood mononuclear cells from the patients with multiple sclerosis (MS). The authors suggested that phycocyanin may act as a neuroprotector and thereby may restore the functional damage in neurodegenerative disorders of the central nervous system (CNS). Another animal model in rats showed that *Spirulina* promoted stem cell genesis and protected against LPS-induced declines in neural stem cell proliferation, and that cytokines did appear capable of regulating several phases of the neurogenesis process, supporting their hypothesis that a diet enriched with *Spirulina* may help protect the stem/progenitor cells from insults (Bachstetter et al., 2010). These studies including reports summarized in this chapter show that *Spirulina* is useful in providing complementary nutrients for modulating or maintaining the immune system and that is also may have potential therapeutic benefits for improvement of immune dysfunctions caused by, for example, radiation, chemotherapy using anti-cancer and anti-infectious drugs, and certain microorganisms such as human immunodeficiency virus (HIV) itself, other than ageing. Further research along these lines is needed to validate these evidences.

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