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Immuno-regulatory and anti-inflammatory actions of phycocyanin on Caco-2/U937 cells co-culture as a model of the intestinal barrier

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ABSTRACT

Background: Phycocyanin, a photosynthetic pigment protein of cyanobacteria *Spirulina platensis*, has been used as a health or functional food because of its health promoting and medicinal effects such as its antioxidant, anti-inflammatory, immunomodulatory, and anticancer effects. This aim of this study is to investigate the immuno-regulatory and anti-inflammatory actions of phycocyanin using a developed *in vitro* co-culture model.

Methods: Under the co-culture system using a human colorectal cancer cell line, Caco-2 cells, and differentiated U937 macrophages in the presence of sodium butyrate (SB) and/or lipopolysaccharide (LPS), immune-regulatory and anti-inflammatory activities of phycocyanin were examined.

Results: Phycocyanin attenuated a certain damage in the tight junction of co-cultured Caco-2 cells induced by LPS in the presence of SB, and suppressed IL-6 and IL-8 and conversely enhanced TGF-β1 productions. Phagocytic and bactericidal abilities of the differentiated U937

cells were also regulated under the co-cultivation.

Conclusions: Phycocyanin contributes to protecting against inflammation and to regulating macrophages in the mucosal immune responses partly through release of cytokines in the presence of butyrate.

Keywords: *Spirulina*, phycocyanin, Caco-2 cells, U937 cells, IL-6, IL-8, TGF-β1

INTRODUCTION

Intestinal microflora maintains the intestinal epithelial barrier and defends against invasion from pathogenic microbes. Butyrate, one of the major short chain fatty acids (SCFAs) influences proliferation, and apoptosis of the colonic epithelial cells. It also regulates intestinal inflammation and differentiation of the immune cells such as regulatory T cells, together with the intestinal microflora [1, 2]. Disruption of the normal microbiome, or "dysbiosis," adversely affects barrier function of the gastrointestinal epithelia. It has been recognized in the last two decades that a combination of nutrition, intestinal dysbiosis, and abnormal immune response is responsible for the inflammatory process in IBD [3].

Spirulina (*Arthrospira*) *platensis*, a helicoidal filamentous blue-green alga of the class cyanobacteria, which was used as a food during the Aztec civilization in the 16th century, has been commercially produced for about a half century as a food supplement [4, 5]. *Spirulina* is rich in high-quality proteins, vitamins and minerals, and is known to promote various therapeutic effects, against hyperlipidemia, diabetes, and hypertension, as well as immune modulatory activity, as reviewed elsewhere [6, 7]. Phycocyanin (phyco) is a photosynthetic pigment protein of *Spirulina platensis*. Its health promoting and medicinal effects such as its antioxidant, anti-inflammatory, anticancer, hepatoprotective, neuroprotective, and immunomodulatory effects have also been reported [8-10].

Co-culture *in vitro* models of the intestine have been used to study the pharmacological and toxicological effects, as well as the bio-availability of substances and materials [11]. Co-cultivation of more than one type of cell, for example, epithelial and immune cells, gives more valuable information as to understanding basic biology than mono-cultivation of cells, such as cell-cell contact and communication with soluble factors. The human epithelial cell line, Caco-2 cells, has been widely used as a model of the intestinal epithelial barrier, due to their easy accessibility, handling, and maintenance [12]. Tanoue et al. [13] evaluated anti-inflammatory actions of fucoidan by using the Caco-2/LPS-induced macrophage Raw264.7 cells co-culture. Although *in vivo* experiments are generally more reliable than *in vitro* for evaluation of pharmacological activity and therapeutic effect, co-culture system of various kinds of cells to mimic the environment close to the living body has been given attention as experimental system replacing animal experiment.

In this study we developed an *in vitro* co-culture model using a human colorectal cancer cell line, Caco-2 cells, and a monocytic leukemia cell line, U937 cells, which is known to

differentiate to macrophages by phorbol 12-myristate 13-acetate (PMA) to mimic the human intestine [11, 14], and examined immune-regulatory and anti-inflammatory activities of phycocyanin under the co-culture system in the presence of sodium butyrate and/or LPS.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS; F7524-500ML), Sodium butyrate (303410-5G), Latex beads polystyrene (LB11-1ML) were purchased from SIGMA-ALDRICH. RPMI (Roswell Park Memorial Institute) -1640 medium (189-02025), E-MEM (Eagle's minimal essential medium, 051-07615), Penicillin-streptomycin-amphotericin B (161-23181), Trypsin/EDTA 4Na Solution (202-16931), phorbol-12-myristate-13-acetate (PMA; 168-23593), Nitro Blue Tetrazolium Chloride (NBT; 148-01996) were purchased from FUJIFILM Wako Pure Chemical Corporation. Non-Essential Amino Acids (MEM NEAA 100x; 11140-050 100 ml), LPS (Standard lipopolysaccharide from *E. coli* K12 strain TLR4 ligand; tlrl-eklps), Accutase (AT-104-100 mL), normal human serum (S1-100ML) were purchased from GIBCO, InvivoGen, Innovative cell technologies, and MILLIPORE, respectively. Phycocyanin was supplied by DIC LIFETEC Co., Ltd.

Cells

Caco-2 cells RCB0988 (Riken BioResource Research Center, Japan) were cultured in E-MEM substituted with 20% heat-inactivated FBS, 1% MEM NEAA, and 1% penicillin-streptomycin-amphotericin B (20%FBS/MEM) at humidified 37 °C, 5% CO_2. The cells were seeded on 24-well transwell inserts, ThinCert™ (0.4 μm pore size and 0.336 cm^2 culture surface; Greiner Bio-One, Germany) at a density of 2.0 x 10^5 cells/cm^2 and maintained for 14 days (Fig. 1A). The transepithelial electrical resistance (TEER) to assess the barrier development of the cells was measured with Millicell®ERS-2 (MERCK MILLIPORE, Germany) and the medium was changed to fresh 20%FBS/MEM every 2 – 3 days during the culturing.

U937 cells JCRB9021 (Japanese Collection of Research Bioresources Cell Bank, Japan) were cultured in RPMI-1640 substituted with 10% heat-inactivated FBS and 1% penicillin-streptomycin-amphotericin B (10%FBS/RPMI). The U937 cells were seeded 2.5 x 10^6 cells/10 mL in 25-cm^2 flask and differentiated with PMA (100 nM) for 2 days. Subsequently, the cells were detached with Accutase, plated on each well of the transwell-suitable 24-well culture plates (Greiner Bio-One, 662120) at a density of 0.5 x 10^6 cells/mL/well, and allowed to re-attach for 1.5 - 2 h (Fig. 1A).

Co-culture

On day 14, the culture medium of the Caco-2 cells was substituted to the 20%FBS/MEM-based

medium supplemented with sodium butyrate (SB FBS/MEM) and cultured for more 2 days. For starting co-cultivation on day 16, the Caco-2 cells on the apical (Api) side of transwell insert were cultured with 100μg of phyco in 0.3 mL of SB FBS/MEM per insert, whereas 10%FBS/RPMI was used for the differentiated U937 cells in the basolateral (Baso) compartment of the culture plate. One day later, 100 ng LPS were further added to the Baso compartment of the differentiated U937 cells to mimic inflammation (Fig. 1A). One day after adding LPS, the culture conditioned medium of each Baso compartment was collected as specimen for measurements of IL-6, IL-8, and TGF-β1. Phagocytic activity and NBT reduction ability of the U937 cells in the Baso compartment were also examined.

Assignment of groups in the co-cultivation experiments described above or obtained specimens of the culture medium of each Baso compartment were as follows; C/U and SB C/U represent the groups as control co-cultured Caco-2/U937 cells in which neither phyco nor LPS were added. "SB" means that the culture medium of the Caco-2 cells was substituted to the SB FBS/MEM described above, and groups without "SB" means that the culture medium of the Caco-2 cells was not substituted, that is, Caco-2 cells were cultured continuously in the MEM-based medium (20%FBS/MEM). The co-cultured Caco-2/U937 cells in which only LPS was added but phyco was not added were designated as C/U-LPS and SB C/U-LPS, respectively, and conversely the co-cultured Caco-2/U937 cells in which only phyco was added but LPS was not added were designated as C/U-phyco and SB C/U-phyco. Further, the co-cultured Caco-2/U937 cells in which phyco was added followed by adding LPS were designated as C/U-phyco+LPS and SB C/U-phyco+LPS, respectively. Each single cultivation of Caco-2 and U937 cells was also prepared, and designated as C and U, respectively, and depending on whether the culture was with or without phyco and/or LPS as mentioned above, the groups were set up as C-phyco, C-LPS, C-phyco+LPS, U-phyco, U-LPS, and U-phyco+LPS (Figs. 1B and C).

Hematoxylin-Eosin (HE) staining

Caco-2 cells cultured on the transwell inserts were HE stained according to the instruction manuals of Muto Pure Chemicals Co. Ltd. Briefly, the cells were washed with PBS (-) 3 or 4 times and fixed with cold 3% glutaraldehyde/PBS(-) for 15 min. The cells were treated with 0.5% TritonX-100/H_2O for 15 min. to make cell membrane easy to permeate staining solutions and were stained with the Carrazzi's hematoxylin solution (Muto Pure Chemicals Co. Ltd., 3002-1) for 20 min. at the room temperature. After washing with purified water and decolorizing once with 0.5%HCl in 70% ethanol, the cells were treated with diluted ammonium hydrogen oxide solution to make the color of the membrane uniform. Then the cells were stained with the pure eosin (Muto Pure Chemicals Co. Ltd., 3204-1) diluted 3 times with 95%

ethanol followed by washing with purified water. The stained cells on the membrane were mounted with Aqua-Poly/Mount (Polysciences, Inc. 18606-20 20 mL), and observed with the optical microscope in 100 to 400 times magnifications.

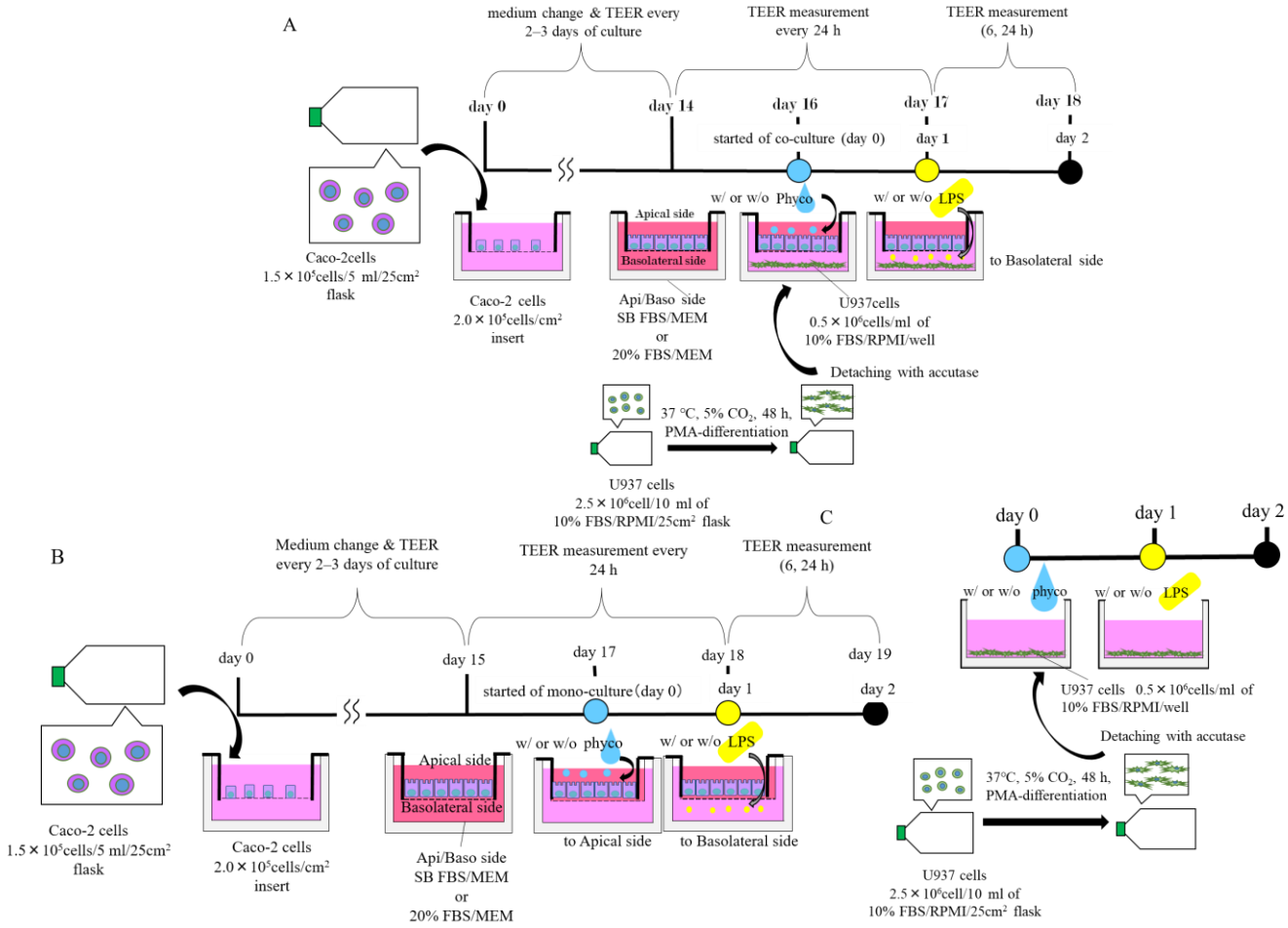


Figure 1. Outline of the co-culture experiments. (A) Caco-2 cells were cultured on 24-well transwell inserts in 20%FBS/MEM for 14 days, substituted to the SB FBS/MEM, and cultured for more 2 days. On day 16, the Caco-2 cells on the inserts were set up to the wells in which the PMA-differentiated U937 cells cultured, pyhco was added in the apical compartment of the Caco-2 cells, and co-cultivation was started. One day later, LPS was added in the basolateral compartment of the U937 cells, cultured for more 24 h. (B) Caco-2 cells mono cultured on 24-well transwell inserts were treated the same as (A) except for absence of U937 cells. (C) PMA differentiated U937 cells was single-cultured with or without phyco and LPS.

Cytokine quantification

Releases of cytokines, IL-6, IL-8, and TGF-β1, in the culture conditioned medium of the Baso side were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits, Human IL-6 Uncoated ELISA (Invitrogen, 88-7066), Human IL-8 Uncoated ELISA (Invitrogen, 88-8086), Human/Mouse TGF beta 1 Uncoated ELISA (Invitrogen, 88-8350), respectively, according to each manufacturer's instruction. Absorbance (A450-A570) was measured with a microplate reader SH-1000 (CORONA Electrical Co. Ltd., Japan) and

the cytokine contents were calculated with the attached application software, SF60.

Phagocytic activity

Phagocytic activity was measured by using the method modified by Ishii et al. [15] To prepare 0.02 % opsonized LB solution, equal volumes of 0.04 % Latex beads polystyrene suspended in RPMI and 10 % normal human serum in RPMI were mixed. Each 0.5 mL of U937 cell suspension (0.5 x 10^6 cells/mL) were mixed to 0.5 mL of 0.02 % opsonized LB solution, and seeded on 48-well plate (Corning, Costar® Multiple Well Cell Culture Plates, 3548). After incubating at 37 °C for 2 hours, it was washed once with RPMI. The cells were collected to slides with Cytospin 3 (Thermo Fisher Scientific, USA), and treated with May-Grünwald-Giemsa staining solution. The cells which contained more than one LB particles were counted as positive under light microscopy, and the percentage of phagocytic cells out of 100 was determined.

Nitro blue tetrazolium (NBT) reduction activity

NBT reduction activity was measured by using the method modified by Ishii et al. [15] Each 0.5 mL of U937 cell suspension (0.5 x 10^6 cells/mL) was seeded on 48-well plate. After removal of the supernatant by centrifugation, 200 μL of 10 % FBS/RPMI and each 100 μL of reagents, 1 % NBT/PBS (-), 500 ng PMA/mL PBS(-), and 0.1% DMSO/ethanol, were added. After incubating at 37 °C for 30 min., the cells were washed once with PBS (-). The cells were collected to slides with Cytospin 3. The cells which contained intracellular blue black formazan deposits were counted as positive under light microscopy, and the percentage of positive cells out of 100 was determined.

Statistical analysis

The data analysis was performed with Microsoft Excel. Data were represented as mean ± standard deviation (S.D.). Data of multiple groups were compared for each treatment with control by two-way ANOVA and Bonferroni's test for interaction. Statistical significant in each case was defined as $p < 0.05$. The experimental data were compared to the controls obtained from co-cultured Caco-2/U937 cells without SB or LPS.

RESULTS

Cell growth and measurement of TEER in the co-cultivation

Caco-2 cells on trans-well insert membrane increased along with the growth for 14 days. After the medium was substituted to the MEM-based medium supplemented with SB (SB FBS/MEM), the TEER also increased and reached 3,300 Ω・cm^2 on day 16 (Fig. 2A). Under the co-cultivation in the presence of SB, phyco added in advance to Apical side of Caco-2 cells attenuated the decrease of TEER level which was caused by adding LPS (SB C/U-phyco+LPS in Fig. 2B). TEER levels of the co-cultured Caco-2 cells with U937 cells in the presence of SB (SB C/U, SB C/U-phyco, SB C/U-LPS, and SB C/U-phyco+LPS in Figs. 2C-5, C-6, C-7, and C-8) were significantly higher than those of mono-cultured Caco-2 cells in the medium with SB (SB C, SB C-phyco, SB C-LPS, and SB C-phyco+LPS), respectively. Such co-

culture effect on TEER was not clear in the medium without SB (Figs. 2C-1, C-2, C-3, and C-4). High density of cells was also partly observed in the co-cultured Caco-2 cell-layer in the SB FBS/MEM with phyco treatment in hematoxylin and eosin (HE) staining (Fig. 2 D).

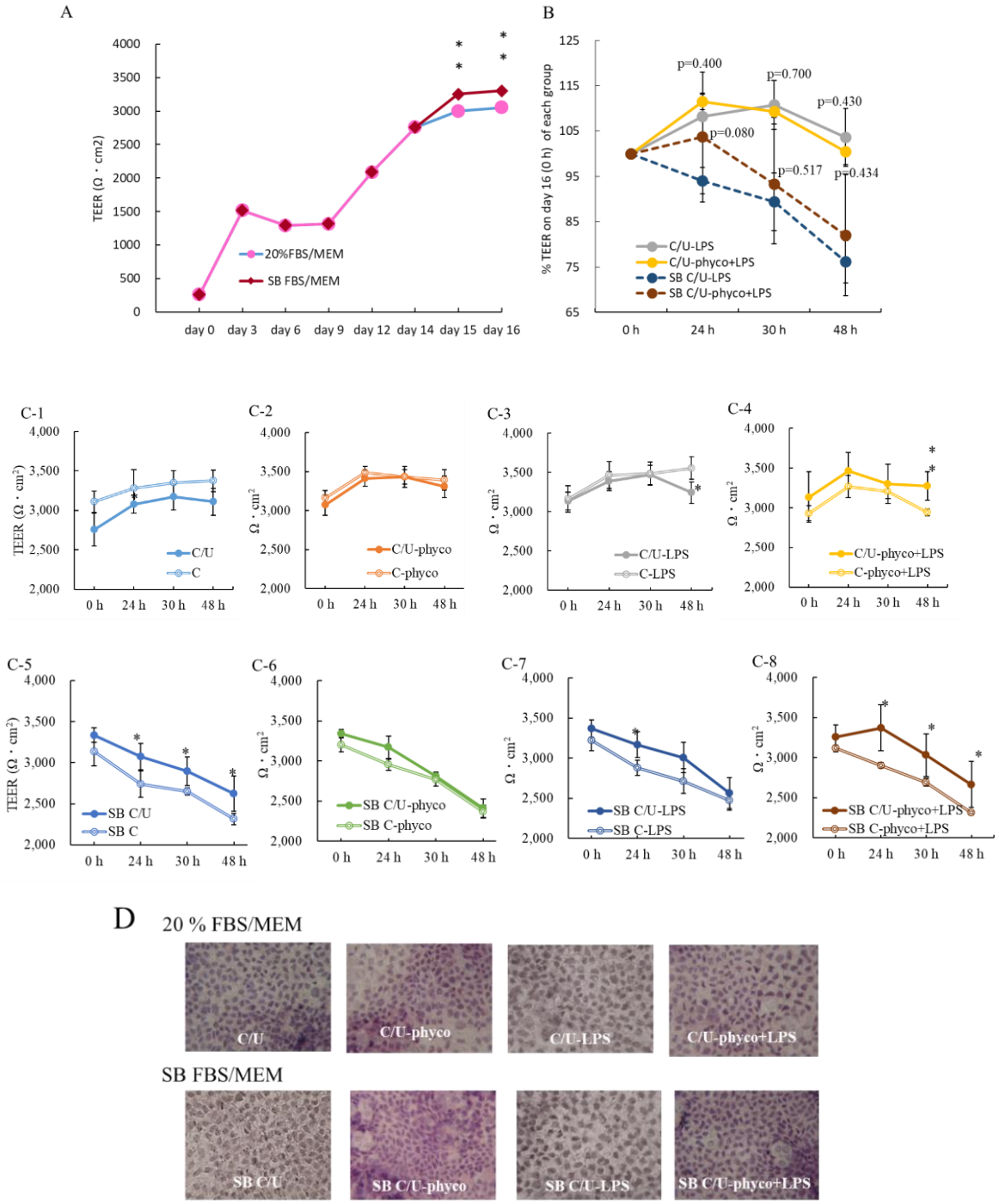


Figure 2. TEER and HE staining of the mono- and co-cultured Caco-2 cells. (A) TEER development of Caco-2 cells during 16 days of culture. Values are expressed as mean ± S.D. (n=12 in 20 % FBS/MEM and n=10 in SB FBS/MEM), **; $p < 0.01$ compared to 20 % FBS/MEM in Student's two-tailed t test). (B) TEER ratios of the co-cultured Cacco-2 cells of each group with or without phyco treatment and LPS stimulation in the presence or absence of SB. Each ratio of TEER of Caco-2 cells cultured in the presence or absence of SB on day 16 was stated as 100%. Each value represents mean ± S.D. (n=6). (C) Comparison of TEERs between mono- and co-cultured Caco-2 cells. Each value represents mean ± S.D. (n=6 in the co-

cultured Caco-2 and n=3 in the mono-cultured Caco-2 cells, *; $p < 0.05$, **; $p < 0.01$ compared to the mono-cultured Caco-2 cells; Student's two-tailed t test. (D) HE staining of the co-cultured Caco-2 cells was done on the last day of cultures.

Cytokine productions

IL-6 production in the medium of basolateral compartment of co-cultured Caco-2/U937 cells in the 20%FBS/MEM was increased by LPS stimulation (400 pg/mL) approximately 200 times higher than that without LPS as shown in C/U and C/U-phyco in Fig. 3A and C/U-LPS and C/U-phyco+LPS in Fig. 3B, respectively. IL-6 levels both in the C/U-phyco and SB C/U-phyco co-cultured with phyco treatment were significantly lower than that in the single-cultured U937 with phyco treatment (U-phyco in Figs. 3C-2 and C-4). IL-6 in mono-cultured Caco-2 cells was under the minimum detectable level of the Human Cytokines ELISA kit used in the present assay. The increase of IL-6 in the co-cultured U937 cells was attenuated in the SB-supplemented 20%FBS/MEM (SB FBS/MEM) and reached only to 238 pg/mL even if the cells were stimulated with LPS (SB C/U-LPS in Fig. 3B). The lowered level of IL-6 was moreover significantly reduced to 132 pg/mL by pretreatment of phyco (SB C/U-phyco+LPS in Fig. 3B).

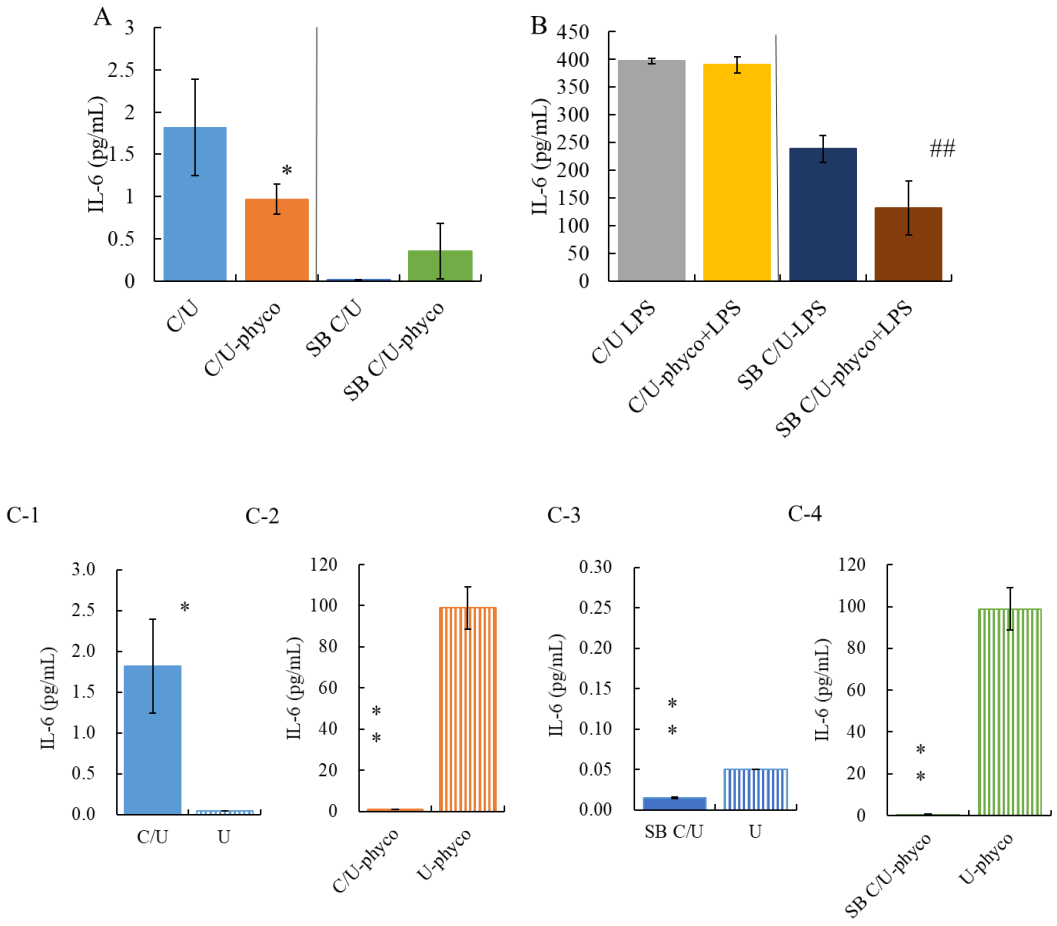
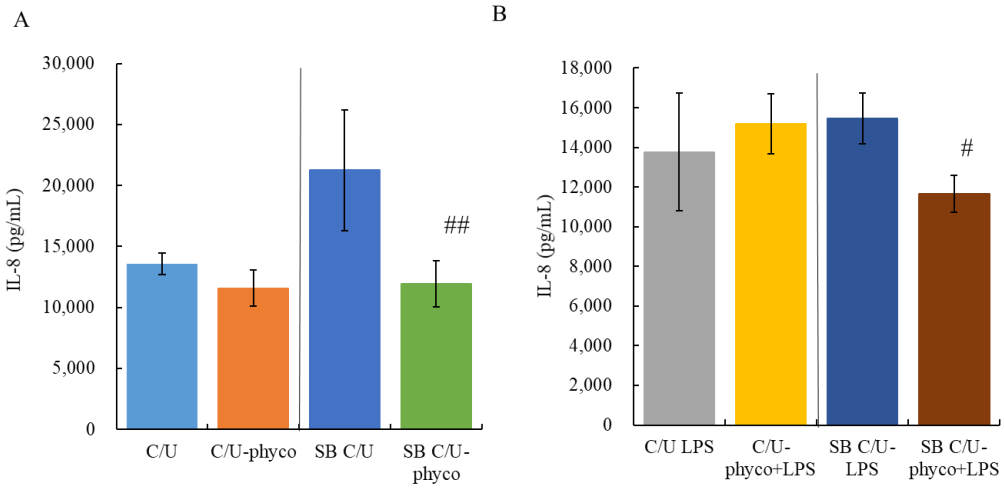


Figure 3. IL-6 in the Basolateral compartment of the co-cultured U937 cells in the presence or

absence of SB. (A) phyco was added but LPS was not added, and (B) LPS was added following adding of phyco. Each value represents mean ± S.D. (n=3). *; $p < 0.05$ compared to C/U and ##; $p < 0.01$ compared to SB C/U-LPS. (C) Comparison of release of IL-6 between co-cultured Caco-2/U937 and single-cultured U937 cells. Each value represents mean ± S.D. (n=3). *; $p < 0.05$, **; $p < 0.01$ compared to single-cultured U937 cells in Student's two-tailed t test.

IL-8 production under the co-cultivation with Caco-2 cells either in the presence or absence of SB reached approximately 2 to 4 times higher (13,600 pg/mL in C/U and 21,200 pg/mL in SB C/U, respectively as shown in Fig. 4A) than that of the single-cultured differentiated U937 cells (5,525 pg/mL in U as shown in Fig. 4C-1). The high levels of IL-8 under the co-cultivation were suppressed especially by treatment with phyco, that is, the IL-8 level of SB C/U decreased significantly almost by half (SB C/U-phyco in Fig 4A). Further, the IL-8 level either in the C/U-phyco or SB C/U-phyco was significantly lower than that in the single-cultured differentiated U937 cells with phyco treatment (U-phyco) as shown in Figs. 4C-2 and C-4. The increased IL-8 production of co-cultured Caco-2/U937 cells in the presence of SB (21,200 pg/mL in SB C/U) was lowered to 15,500 pg/mL by LPS stimulation (SB C/U-LPS in Fig. 4B), and further significantly suppressed by pretreatment with phyco to 11,600 pg/mL (SB C/U-phyco+LPS in Fig. 4B). IL-8 in the mono-cultured Caco-2 cells was also under the minimum detectable level of the Human Cytokines ELISA kit used in the present assay.



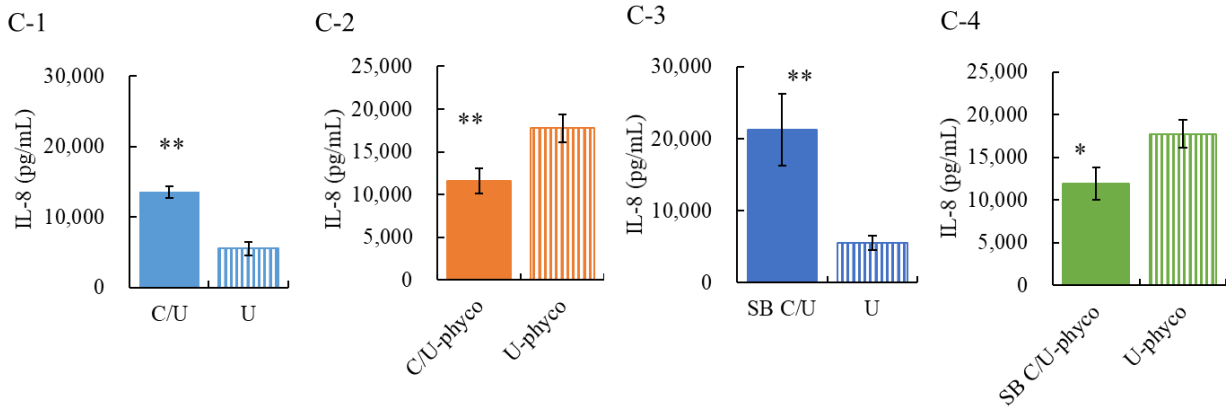


Figure 4. IL-8 in the Basolateral compartment of the co-cultured U937 cells in the presence or absence of SB. (A) phyco was added but LPS was not added, and (B) LPS was added following adding of phyco. Each value represents mean ± S.D. (n=3). ##; $p < 0.01$ compared to SB C/U and #; $p < 0.05$ compared to SB C/U-LPS. (C) Comparison of release of IL-8 between co-cultured Caco-2/U937 and single cultured U937 cells. Each value represents mean ± S.D. (n=3). *; $p < 0.05$, **; $p < 0.01$ compared to single cultured U937 cells in Student's two-tailed t test.

TGF-β1 production, 330 pg/mL, in the co-cultivation under the presence of SB (SB C/U in Fig. 5A), on the other hand, was significantly accelerated by phyco treatment to 611 pg/mL (SB C/U-phyco in Fig. 5A). Release of the TGF-β1 was slightly increased by LPS stimulation to 450-500 pg/mL and tended to be decreased by phyco pretreatment although it was not significant (SB C/U-LPS and SB C/U-phyco+LPS in Fig. 5B). TGF-β1, about 450 pg/mL, was detected in either the mono-cultured Caco-2 or single-cultured U937 cells (Fig. 5C-1). TGF-β1 level of the SB C/U was significantly lower than that of the mono-cultured Caco-2 cells in the presence of SB (SB C in Fig. 5C-3). On the other hand, TGF-β1 level in the SB C/U-phyco was significantly higher than that in the single-cultured differentiated U937 cells (U-phyco, $p<0.01$) as shown in Fig. 5C-4 and that in the co-cultured SB C/U ($p<0.01$).

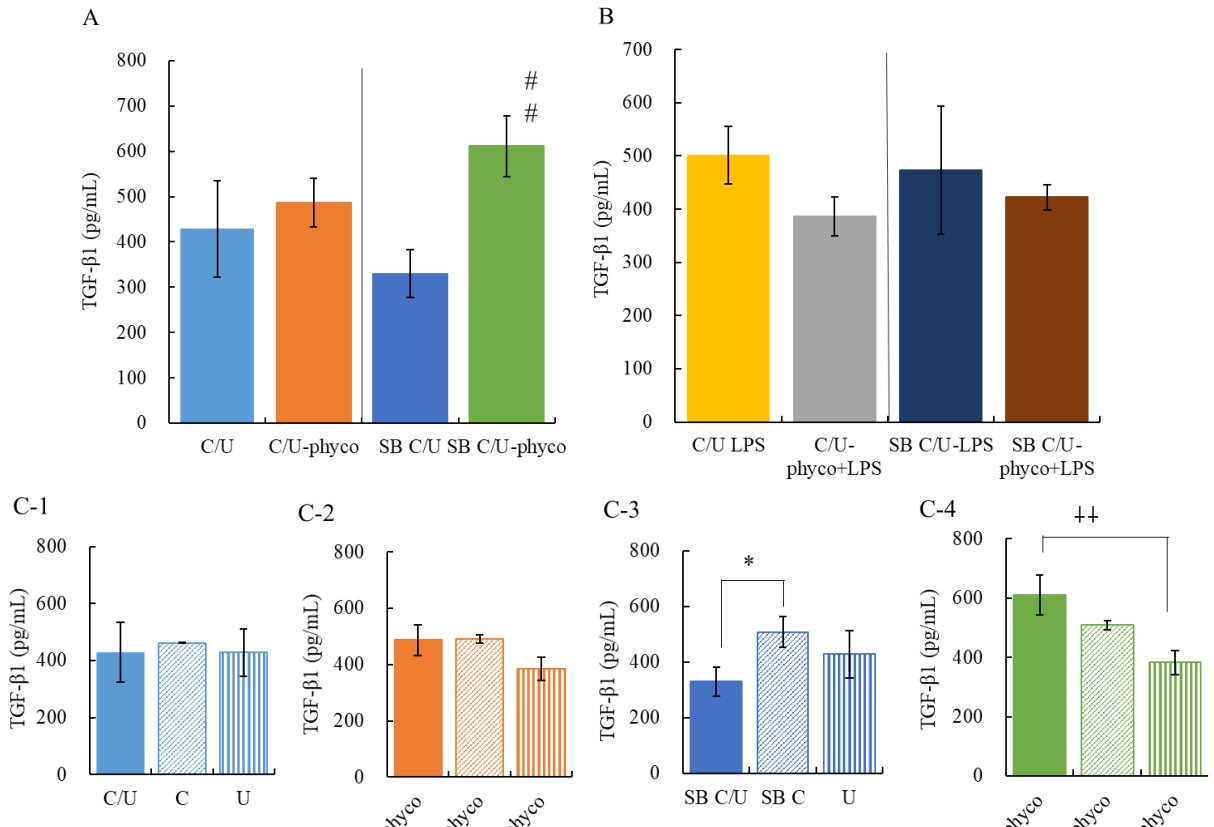
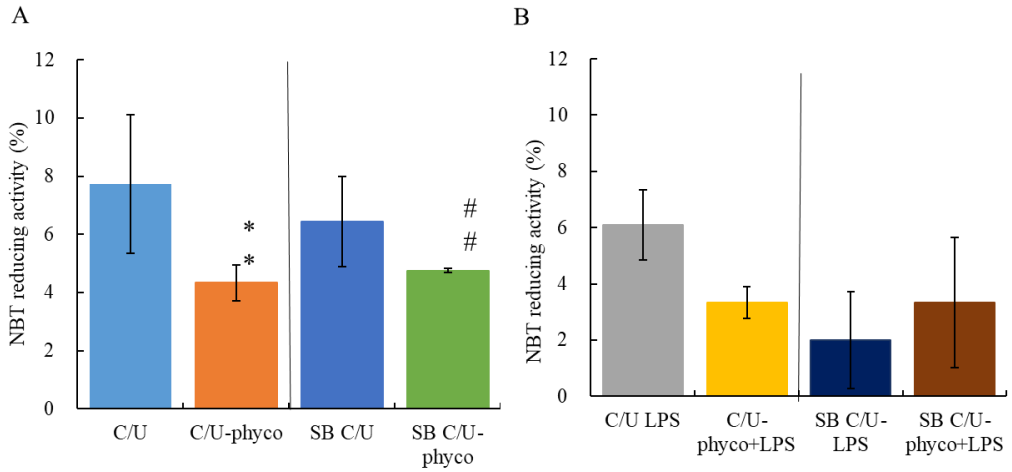


Figure 5. TGF-β1 in the Basolateral compartment of the co-cultured U937 cells in the presence or absence of SB. (A) phyco was added but LPS was not added. ##; $p < 0.01$ compared to SB C/U. (B) LPS was added following adding of phyco. Each value represents mean ± S.D. (n=3). (C) Comparison of release of TGFβ-1 between co-cultured Caco-2/U937, mono-cultured Caco-2, and single-cultured U937 cells. Each value represents mean ± S.D. (n=3). *; $p < 0.05$, ‡‡; $p < 0.01$ compared to SB C and SB U-phyco, respectively, by one-way ANOVA and Bonferroni's test.

NBT reduction and phagocytic activities

In the co-cultivation either in the presence or absence of SB, NBT reduction activity of the differentiated U937 cells was significantly decreased by phyco treatment (C/U-phyco and SB C/U-phyco in Fig. 6A) in comparison with those in the C/U and SB C/U, respectively (Fig. 6A). In the co-cultivation under the presence of SB, NBT reduction activity of the U937 cells stimulated with LPS (SB C/U-LPS) was increased by phyco treatment (SB C/U-phyco+LPS in Fig. 6B), although the NBT reduction activity of the co-cultured U937 cells stimulated with LPS (C/U-LPS) was decreased in the absence of SB (C/U-phyco+LPS in Fig. 6B). NBT reduction activity of the single-cultured U937 cells (U) was almost same as those of the co-cultured U937 cells (C/U and SB C/U in Figs. 6C-1 and C-3). Although phagocytic activity in the U937 cells treated with LPS (U-LPS in Fig. 7C-1) was as low as that in the cells without LPS treatment (U, data not shown), the low phagocytic activity in the U937 cells by LPS stimulation was significantly raised by phyco treatment in the co-cultivation in the presence of SB (SB C/U-phyco+LPS in Fig. 7B). Phagocytic activities of the co-cultured U937 cells, C/U-LPS, SB C/U-LPS, and SB C/U-phyco+LPS shown in Figs. 7C-1, C-3, and C-4, were significantly higher than those of the single-cultured U937 cells.



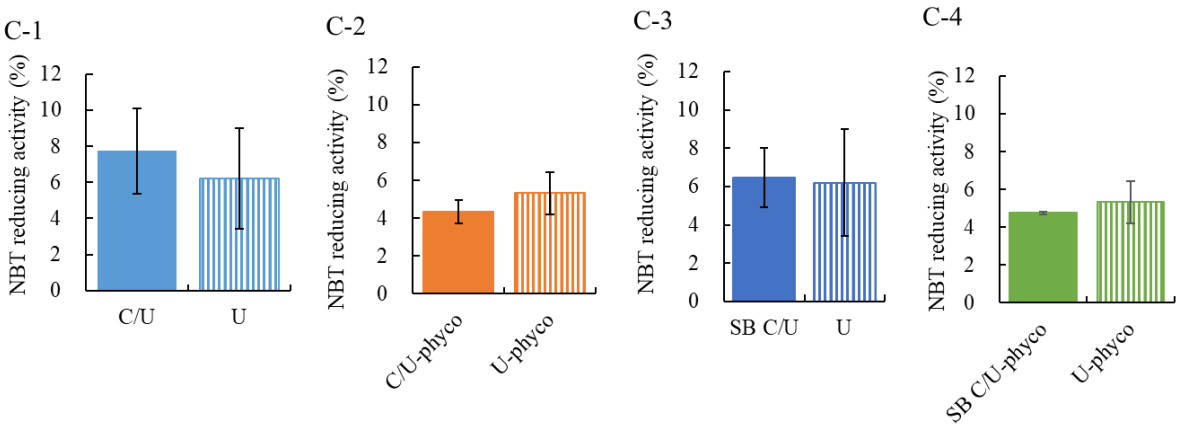


Figure 6. NBT reduction activity of the co-cultured or single-cultured differentiated U937 cells. Cells which contained intracellular blue-black formazan deposits were counted as positive, and the percentage of positive cells out of 100 was expressed as NBT reduction activity. (A) phyco was added but LPS was not added, and (B) LPS was added following adding of phyco. Each value represents mean ± S.D. (n=3). **; $p < 0.01$ compared to C/U and ##; $p < 0.01$ compared to SB C/U in Student's two-tailed t test. (C) Comparison of NBT reduction activity between the co-cultured Caco-2/U937 and single-culture U937 cells.

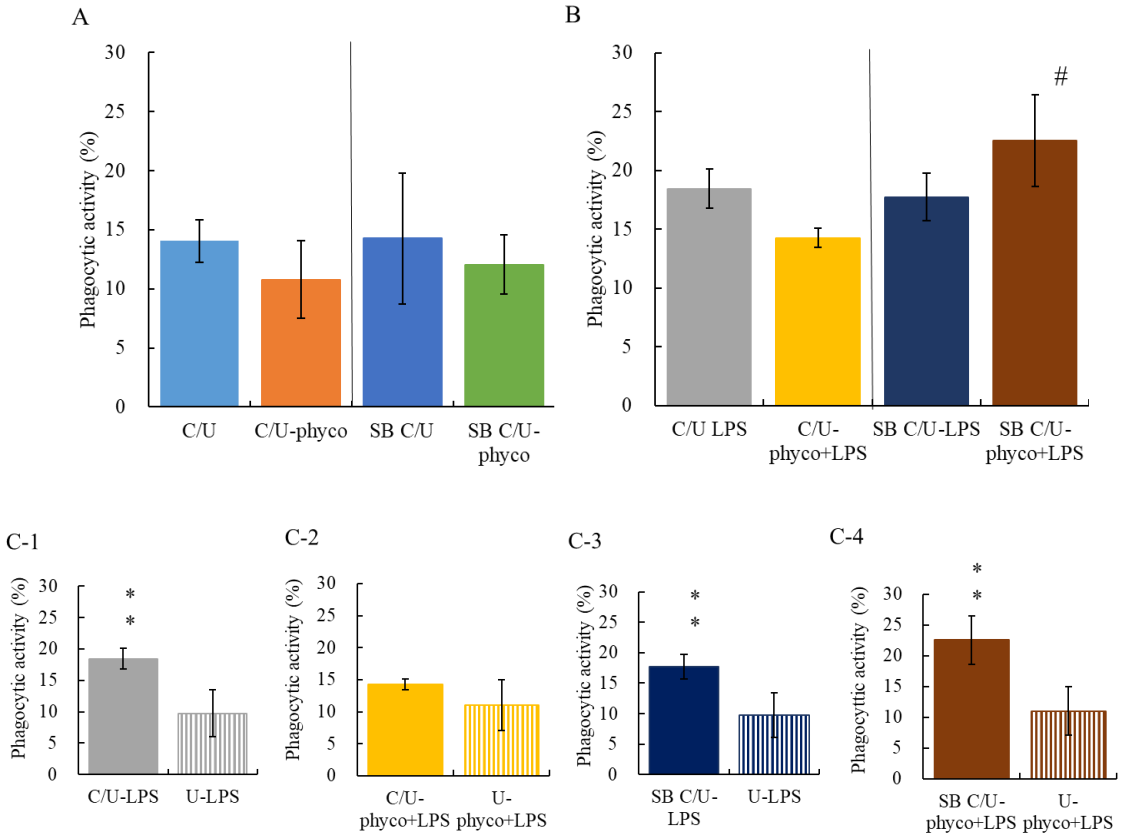


Figure 7. Phagocytic activity of the co-cultured or single-cultured differentiated U937 cells. (A) phyco was added but LPS was not added, and (B) LPS was added following adding of phyco. Each value represents mean ± S.D. (n=3). #; $p < 0.05$ compared to SB C/U-LPS in Student's two-tailed t test. (C) Comparison of phagocytic activity between the co-cultured Caco-2/U937 and single-cultured U937 cells.

DISCUSSION

Butyric acid, a metabolite of dietary fiber produced by the intestinal microflora, acts in intestinal health maintenance or suppression intestinal diseases [16]. Phycocyanin from *Spirulina platensis* has recently attracted attentions as an IBD therapeutic drug [17]. TEER value of the mono-cultured Caco-2 cells significantly increased after substitution to 20% FBS/MEM with SB (Fig. 2A). In addition to enhancing the proper function of the supplemented SB, pretreatment of phycocyanin attenuated the LPS-induced decrease of TEER (Fig. 2B). In the previous paper [18], we showed that the oral administration of phycocyanin attenuated the vascular permeability in the small-intestine caused by the allergic inflammatory in mice. The TEERs of the co-cultured Caco-2 cells in the SB-supplemented medium unexpectedly decreased with progress of time (Figs. 2B, and 2C-5 – 2C-8). Butyric-acid uptake into Caco-2 cells increased in a time-dependent manner and was stimulated by a lowering extracellular pH [19]. Since the concentration of HEPES, 10mM, applied to the SB-supplemented medium as a buffer reagent might be too low for sufficient buffer effect, the TEER value reduced in some degree by the lesioned Caco-2 cells. TEERs in the co-cultivation of Caco-2/U937 cells in the SB FBS/MEM were significantly higher than those of the mono-cultured Caco-2 cells in the same medium SB FBS/MEM (SB C/U, SB C/U-phyco, SB C/U-LPS, and SB C/U-phyco+LPS in Figs. 2C-5, C-6, C-7, and C-8). In addition to that, phycocyanin significantly attenuated the LPS-induced decrease of TEERs of the co-cultured Caco-2/U937 cells, especially in the case of the co-cultivation in the SB FBS/MEM in comparison with that of mono-cultured Caco-2 cells (Figs. 2C-4 and C-8). Phycocyanin may effectively function to Caco-2 cells in the presence of butyric acid and co-cultured U937 cells or it may prevent LPS-induced lesion in the tight junction of Caco-2 cells in the presence of both butyric acid and differentiated U937.

Releases of inflammatory cytokines, IL-6 and IL-8, from the PMA-differentiated U937 cells were decreased in the co-cultivation with Caco-2 cells with phycocyanin as shown in Figs. 3A and 4A. The cytokine levels in either C/U-phyco or SB C/U-phyco were significantly lowered in comparison to that of the single-cultured U937 cells, U-phyco (Figs. 3C-2 and C-4, and Figs. 4C-2 and C-4). Further, increased level of either IL-6 or IL-8 in the co-cultured Caco-2/U937 cells under LPS stimulation was significantly suppressed by phycocyanin treatment especially in the presence of SB (Figs. 3B and 4B). It is noted that the release of cytokine either IL-6 or IL-8 in the co-cultured Caco-2/U937 cells was reinforced near 3 times in comparison with that of single-cultured U937 cells (Figs. 3C-1 and 4C-1). Kämpfer et al. [11] established a co-culture model using Caco-2 cells and PMA-differentiated THP-1 cells, a

human monocytic leukemia cell line, which can mimic the intestine in homeostatic or inflamed states, and showed that the priming of Caco-2 cells together with the stimulation of THP-1 cells by LPS and IFN-γ provoked an inflammation-like response such as showing high concentrations of pro-inflammatory cytokines, IL-8 and TNFα. They suggested that the variability of THP-1 cells in the response to PMA, as well as the seeding density of the cells, can impact the outcomes of a co-culture system with intestinal epithelial cells (IECs), and that the influence of the THP-1 cell number on the co-culture stability could be related to the immuno-stabilizing effects of the Caco-2 cells. In our preliminary experiment, we observed that fluctuation of TEERs of the co-cultured Caco-2 cells became small and stable as the density of the differentiated U937 cells lowered, and effects of both butyric acid and phycocyanin on the cytokine production were most prominent at the density 0.5 x 10^6 cells/mL used in the present study.

The release of TGF-β1, on the other hand, was significantly increased by phycocyanin, especially in the co-cultured Caco-2/U937 cells in the presence of SB (Fig. 5A). The TGF-β1 level of the SB C/U-phyco was significantly higher than those of the co-cultured SB C/U and the single-cultured U937 cells (U-phyco in Fig. 5C-4). Release of TGF-β1 was also substantially observed both in the mono-cultured Caco-2 and U937 cells, 400 pg/mL (Fig. 5C-1). High release of TGF-β1 in the co-cultured Caco-2/U937 cells by LPS stimulation was not affected by phycocyanin treatment (Fig. 5B). Phycocyanin might not work sufficiently for suppression of inflammation with U937 cells since the concentration of LPS was too high to be neutralized by phycocyanin. Smythies et al. [20] showed that the resident intestinal macrophages prepared from the sections of human jejunum neither expressed innate response receptors such as the receptors for LPS (CD14), the integrin LFA-1 (CD11a/CD18) nor produced proinflammatory cytokines including IL-1, IL-6, and TNF-α in response to an array of inflammatory stimuli, but they retained phagocytic and bactericidal activity. They reported that the intestinal macrophages originally derived from blood monocytes were markedly distinct from the blood monocytes in phenotype and function, and TGF-β1 derived from the intestinal stromal cells, such as epithelium and lamina propria mast cells, downregulated monocyte receptor expressions for LPS and growth factor but affected neither phagocytic nor bactericidal activity of the monocytes. Thus they suggested that blood monocytes recruited to the intestinal mucosa characteristically retained avid scavenger and host defense functions but acquired profound “inflammatory anergy”. Phycocyanin increased the production of TGF-β1 in the co-cultured Caco-2/U937 cells (Fig. 5A) but reduced the productions of both IL-6 and -8 (Figs. 3A and 4A) to the levels which were still lower than those in the single-cultured U937 cells treated with phycocyanin (Figs. 3C-2 and 4C-2). The tendency, that is,

increase of TGF-β1 and suppression of IL-6 and IL-8 in the co-cultivation with phycocyanin, was emphasized in the presence of SB (Figs. 3C-4, 4C-4, 5C-4). Hung et al. [21] showed that pretreatment of Caco-2 cells with acetate or butyrate suppressed inflammatory responses such as IL-6 and IL-8 expressions induced by TNF-α through a butyrate transporter, monocarboxylate transporter-1 (MCT-1), of the intestinal cells. Chen et al. [22] showed that pre-treatment of both the mono-cultured RAW246.7 and co-cultured Caco-2/RAW246.7 cells with sodium butyrate (SB) dramatically inhibited the expressions of TNF-α and IL-6 in LPS-induced RAW246.7 macrophages in *in vitro* study, and also showed that in the *in vivo* study using wild type and *GPR109A*$^{-/-}$ mutant C57BL/6 mice orally administered SB in drinking water significantly ameliorated the inflammation and intestinal epithelium barrier dysfunction in 2,4,6-trinitorbenzene sulfonic acid (TNBS)-induced wild type mice through activating G-protein coupled receptor 109A (GPR109A) receptor which is known to contribute for reduction of inflammation in atherosclerosis and obesity. The mechanism underlying GPR109A mediation of the process by which butyric acid inhibits inflammation and maintains epithelial barrier integrity may provide a new theoretical basis for treatment of inflammatory bowel disease [22]. Zhu et al. [17] reported that administration of selenium-containing phycocyanin (Se-PC) yielded from Se-enriched *Spirulina* (Se-SP) effectively reduced the extent of dextran sodium sulfate-induced IBD colitis in mice, that is, significantly reduced the pro-inflammatory cytokines such as IL-6, TNF-α, and Monocyte Chemotactic Protein-1 (MCP-1), and increased anti-inflammatory cytokine such as IL-10. Increased production of TGF-β1 and suppressed IL-6 and IL-8 in the co-cultivation with phycocyanin and emphasized changes of them in the presence of SB in our present study supported these results.

Both phagocytic and especially NBT reduction activities were decreased by phycocyanin in the co-cultured Caco-2/U937 cells either in the presence or absence of SB (Figs. 6A and 7A). Although phycocyanin decreased both phagocytic and NBT reduction activities in the co-cultured Caco-2/U937 cells under LPS stimulus in the absence of SB (C/U-phyco+LPS), it significantly increased especially phagocytic activity in the co-cultivation of Caco-2/U937 cells under LPS stimulus in the presence of SB (SB C/U-phyco+LPS in Fig. 7B). Phycocyanin may affect Caco-2 cells to control excess phagocytic capacity to preserve homeostasis of the intestinal function. Remirez et al. [23] showed that C-phycocyanin significantly decreased Kupffer cell phagocytosis in the perfused mouse liver, and suggested that its effects might contribute to the abolition of oxidative stress-induced TNF-a response. In an inflammatory state, phycocyanin may regulate the phagocytic or bactericidal capacity of PMA-differentiated U937 macrophages in the presence of butyric acid. Although there are some points remaining to be improved, such as more appropriate incubation days of Caco-2 cells and concentrations of PMA and LPS to be used, it could be shown that phycocyanin

attenuates a certain damage in the tight junction of co-cultured Caco-2 cells induced by LPS in the presence of SB, suppresses inflammatory cytokine production but promotes anti-inflammatory cytokine production, such as TGF-β1, and adjusts phagocytic and/or bactericidal ability of macrophages under the epithelial cells.

CONCLUSIONS

Phycocyanin works to maintain tight junction of the Caco-2 cells in co-cultivation under the presence of butyrate, and probably contributes to protecting or attenuating the inflammation and to regulating the mucosal immune responses through suppression of inflammatory cytokines such as IL-6 and IL-8 and conversely enhancement of production of TGF-β1 as an anti-inflammatory and an immunomodulatory cytokine. While these results support our previous paper [18], further consideration should be necessary to develop more accurate conditions for the co-cultivation of the Caco-2/U937 cells.

List of Abbreviations: SCFAs; short chain fatty acids, phyco: phycocyanin, PMA; phorbol 12-myristate 13-acetate, LPS; lipopolysaccharide, SB; sodium butyrate, RPMI; Roswell Park Memorial Institute-1640 medium, E-MEM; Eagle's minimal essential medium, NEAA; Non-Essential Amino Acids, PBS (-); calcium-magnesium free phosphate-buffered saline, TEER; transepithelial electrical resistance, Api; apical side of transwell insert, Baso; basolateral compartment, ELISA; enzyme-linked immunosorbent assay, NBT; nitro blue tetrazolium, HE; Hematoxylin-Eosin

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