in vivo *32*: 783-790 (2018)
doi:10.21873/invivo.11308

Laminarin Promotes Immune Responses and Normalizes Glutamic Oxaloacetic Transaminase and Glutamic Pyruvic Transaminase Levels in Leukemic Mice *In Vivo*

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Abstract. *Background/Aim: Laminarin, mainly found in the fronds of Laminaria, has antimicrobial characteristics and induces immune responses. However, there are no available information to show the laminarin effect on glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels in mice with leukemia in vivo. Materials and Methods: Fifty normal BALB/c mice were separated randomly into five groups. Group I mice received normal diet as control. Leukemia was generated in groups II-V using WEHI-3 cells: Group II mice received normal diet as positive control; group III, IV and V mice received laminarin at 1, 2.5 and 5 mg/ml with ddH_2O, respectively, by oral gavage every 2 days for 14 days (total of seven times). All mice were weighed during the treatment. After treatment, mice were sacrificed, blood was collected for determination of cell markers, liver and spleen samples were weighed, and spleens were used for phagocytosis and natural killer (NK) cell activity and cell proliferation using flow cytometric assay. Results: Laminarin did not affect animal appearances, but increased the body weight at all doses. It reduced the weight of liver at 2.5 and 5 mg/ml and of spleen at 5 mg/ml. Laminarin increased CD3 (2.5 mg/ml) and CD19 (1 and 5 mg/ml) cell populations but reduced CD11b (5 mg/ml) cell populations, however, these did not affect Mac-3 marker level. Laminarin at 1 mg/ml increased phagocytosis by macrophages from peripheral blood mononuclear cell, but did not affect those from the peritoneal cavity. Laminarin increased NK cell cytotoxic activity at all doses and at a target ratio of 25:1 and 50:1. Laminarin did not affect B-cell proliferation, but at 5 mg/ml significantly reduced T-cell proliferation. Laminarin restored glutamate oxaloacetate transaminase (2.5 and 5 mg/ml) and glutamate pyruvate transaminase (2.5 mg/ml) levels. Based on these results, we suggest that laminarin can promote immune responses and protect against liver injury.*

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Key Words: Laminarin, leukemia BALB/c mice, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, immune response.

Acute myeloid leukemia (AML) is an aggressive form of cancer of the bone marrow and blood which is characterized by an accumulation of immature myeloid blasts in the bone marrow (1) and is the most common acute leukemia in adults (2). However, progress in understanding AML and novel treatment concepts are inadequate (3). The two standard treatments for patients with AML are chemotherapy and hematopoietic stem cell transplantation, however, the 5-year survival rate remains below 50% due to chemoresistance or toxicity from these treatments (4-6). Numerous studies have focused on finding or developing novel therapeutic strategies or agents for such patients. Improving immune responses to mediate protection against leukemia (7, 8) is needed. There is also focus on finding and improving immune responses from natural products.

Laminarin, beta-1,3-glucan, a typical component of fungal cell walls (9), found mainly in the fronds of *Laminaria*, has antimicrobial properties (10). It also induces defense-related events against Tobacco mosaic virus in tobacco and grapevine (11-13). Injections of laminarin to both adult and larval locusts lead to stimulate the immune responses (14, 15). Laminarin has also been shown to inhibit heparanase activity and tumor metastasis (16), and can boost the immune system, reduce cholesterol level and lower systolic blood pressure (17). It was reported that the pro-inflammatory chemokines such as interleukin-8 (IL8) and monocyte chemoattractant protein-1 (CCL2) are secreted in human intestinal epithelial cells after exposure to laminarin (18). Laminarin influenced the adherence and the translocation of bacteria across the epithelial wall (in jejunum, ileum, caecum and colon) of Wistar rat (*Rattus norvegicus*). (19). In fish, laminarin increased the expression of immune response genes *IL1β, IL8,* and toll-like receptor 2 (*TLR2*), therefore, it was suggested that laminarin modulates the immune response and stimulates growth of fish (20). Based on these observations, laminarin extracts have great potential as a supplement in functional food.

Herein, we investigated the effects of laminarin on immune responses in leukemic BALB/c mice *in vivo*.

Materials and Methods

Materials and reagents. Laminarin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Iscove's modified Dulbecco's medium (IMDM), Roswell Park Memorial Institute (RPMI)-1640 media, L-glutamine, penicillin-streptomycin and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Carlsbad, CA, USA). Antibodies against CD3, CD11b, CD19 and lysosomal-associated membrane protein 2 (Mac-3) were purchased from BD Biosciences Pharmingen Inc. (San Diego, CA, USA). Laminarin was dissolved in double-distilled water (ddH_2O) and stored at room temperature for 1 h before use.

WEHI-3 cells. Murine acute myelomonocytic leukemia WEHI-3 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). Cells were cultured with IMDM supplemented with 10% FBS, 2 mM L-glutamine and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) in 75 cm^2 tissue culture flasks and placed at 37˚C in a humidified atmosphere of 5% CO_2 (21).

Male BALB/c mice. Fifty male BALB/c mice around 20-23 g at 4 weeks old were obtained from the National Laboratory Animal Center (Taipei, Taiwan, ROC). All mice were kept in stainless steel mesh-bottomed cages and were maintained with specified pathogen-free conditions at the animal center of China Medical University (Taichung, Taiwan, ROC). For all mice, the institutional guidelines for animal welfare of China Medical University were followed and the study was approved by the Institutional Animal Care and Use Committee of China Medical University (Taichung, Taiwan) (approval ID: 104-11-B).

Treatment of animals with laminarin. Fifty BALB/c mice were randomly separated into five groups (N=10): Group I were normal animals with normal diet as control; groups II-V were given a peritoneal injection with 8×10^4 WEHI-3 leukemia cells. Group II mice received normal diet as positive control; group III, IV and V mice received laminarin at 1, 2.5 and 5 mg/ml with ddH_2O, respectively, by oral gavage every 2 days for 14 days (total of seven times). All animals were individually weighed during the oral treatment, and at the end of treatment, all mice were weighed and sacrificed as described previously (21).

Measurements of cell populations. At the end of treatment, all mice were individually weighed, and the blood sample, liver and spleen organs were individually collected. A sample of 1 ml blood/mouse was lysed with 1X Pharm Lyse™ lysing buffer (BD Biosciences Pharmingen Inc., San Diego, CA, USA) for destroying the red blood cells as per the guideline from BD Biosciences. Leukocytes were collected and stained with phycoerythrin (PE)-labeled anti-mouse CD3 and CD19, and fluorescein isothiocyanate (FITC)-labeled anti-mouse CD11b and Mac-3 antibodies (BD Biosciences Pharmingen Inc.) for 30 min. All samples were washed with phosphate-buffered saline (PBS) and cell markers (populations) were analyzed by flow cytometry as previously described (21, 22).

Measurements of macrophage phagocytosis. All macrophages were isolated from peripheral blood mononuclear cells (PBMCs) and peritoneum as described previously (21, 22). All macrophages were placed in plates containing 50 μl of target FITC-labelled *Escherichia coli* and were mixed and then were analyzed for phagocytosis by using flow cytometery following the PHAGOTEST® kit manufacturer's instructions (ORPEGEN Pharma Gesellschaft für biotechnologische, Heidelberg, Germany). Quantifying phagocytosis was performed by CellQuest software (Becton Dickinson) as described previously (22).

Measurements of natural killer (NK) cell cytotoxic activity. Isolated splenocytes were maintained in 96-well plate ($2.5\text{-}5\times10^5$ cells/well) with 100 μl of RPMI-1640 medium. Target YAC-1 cells (1×10^4 cells) and PKH-67/Dil.C buffer were added to each well (Sigma-Aldrich Corp.) for 2 min at 25˚C. Two milliliters of PBS was added to each well for 1 min, then 4 ml medium was also added to the well and plates were incubated for 10 min. After incubation, all samples were centrifuged at 290 × g for 2 min. NK cell cytotoxic activity was measured by flow cytometry as described elsewhere (22).

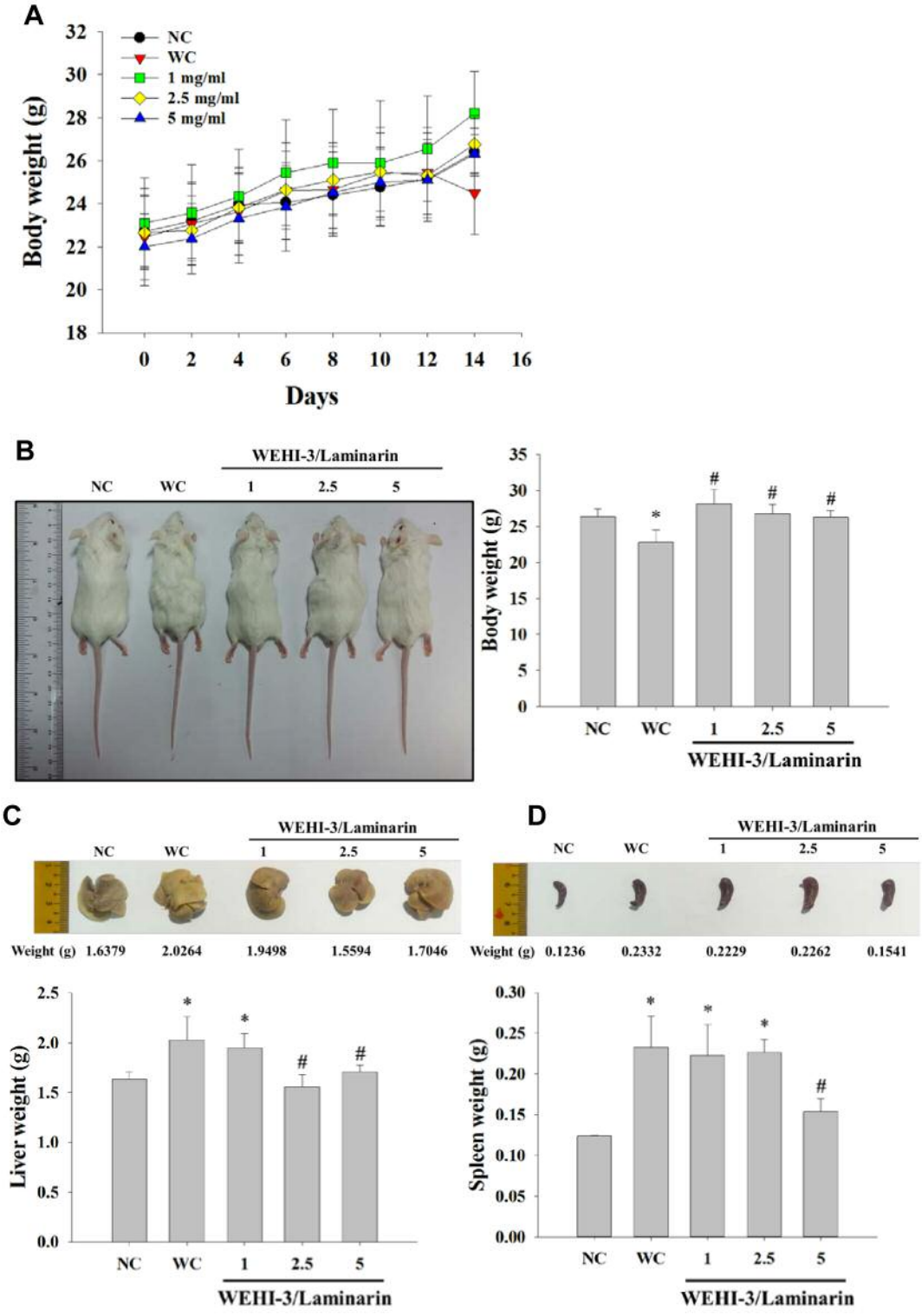

Figure 1. *Laminarin affected the body, liver and spleen weights of leukemic BALB/c mice. Fifty mice were randomly separated into five groups (N=10): Group I were normal animals with normal diet as control; groups II-V were given a peritoneal injection with 8×10^4 WEHI-3 leukemia cells. Group II mice received normal diet as positive control; group III, IV and V mice received laminarin at 1, 2.5 and 5 mg/ml with ddH_2O, respectively, by oral gavage every 2 days for 14 days (total of seven times). A: Body weight during the experiment. B: Final animal appearance and weight. C: Representative examples of liver and weight. D: Representative examples of spleen and weight. Significantly different at $p<0.05$ vs. *normal control (NC) group and $^{\#}$leukemic control (WC) group.*

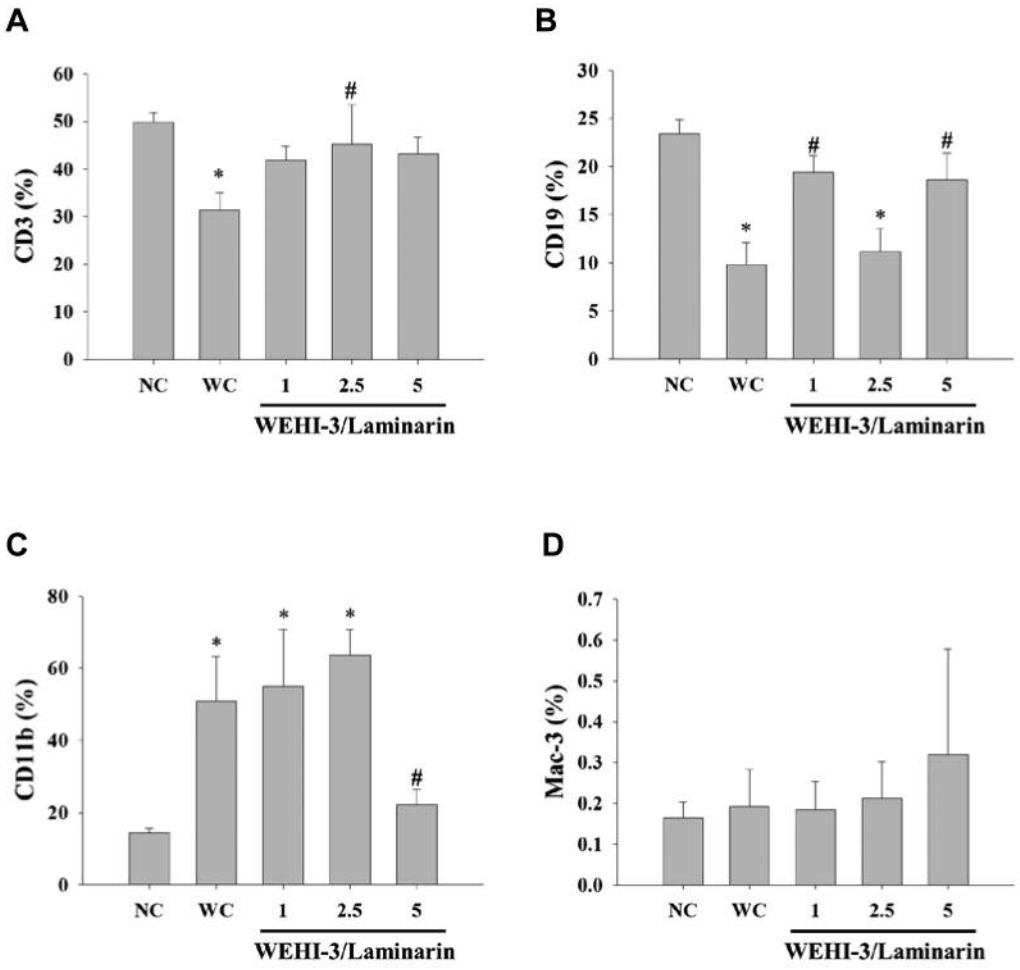

Figure 2. *Laminarin affected the levels of cell markers in white blood cells from leukemic BALB/c mice. Blood was collected from all mice and was analyzed for cell markers by flow cytometry as described in the Materials and Methods. A: CD3. B: CD19. C: CD11b and D: Mac-3. Significantly different at p<0.05 vs. *normal control (NC) group and #leukemic control (WC) group.*

Measurements of T-and B-cell proliferation. Isolated splenocytes (100 μl, 1×10^5 cells/well) were added to a plate with 96 wells which each contained 100 μl of RPMI-1640 medium. Concanavalin A (5 μg/ml) was added to stimulate the cells for 5 days in order to measure T-cell proliferation. Lipopolysaccharide (LPS, 5 μg/ml) was added to stimulate the cells for 3 days in order to measure B-cell proliferation. All samples were measured for cell proliferation using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) as previously described (22).

Measurement of blood glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). Blood samples were used for measurement of the levels of GOT and GTP by using liquiUV Test (aspartate aminotransferase) for GOT, and liquiUV Test (alanine aminotransferase) for GPT from Human Gesellschaft fur Biochemica und Diagnosica mbH (Wiesbaden, Germany) (22-24).

Statistical analysis. Data are expressed as mean±standard deviation (SD). Comparisons differences between groups were analyzed by one-way analysis of variance and Tukey test for multiple comparisons (SigmaPlot for Windows version 12.0; Systat Software, Inc., San Jose, CA, USA). Values of $p<0.05$ were considered to indicate a statistically significant difference.

Results

Laminarin affected the weights of body, liver and spleen from leukemic BALB/c mice. Representative animal body weights, liver and spleen samples and weights are present in Figure 1. An increase in weight gained by laminarin-treated groups when compared with positive control group was observed (Figure 1A). Laminarin did not significantly affect animal appearance but increased final body weight when compared with the positive control group (Figure 1B). When compared with the positive control group, laminarin significantly reduced liver weight at 2.5 and 5 mg/ml treatment (Figure 1C) and spleen weight (Figure 1D) at 5 mg/ml treatment.

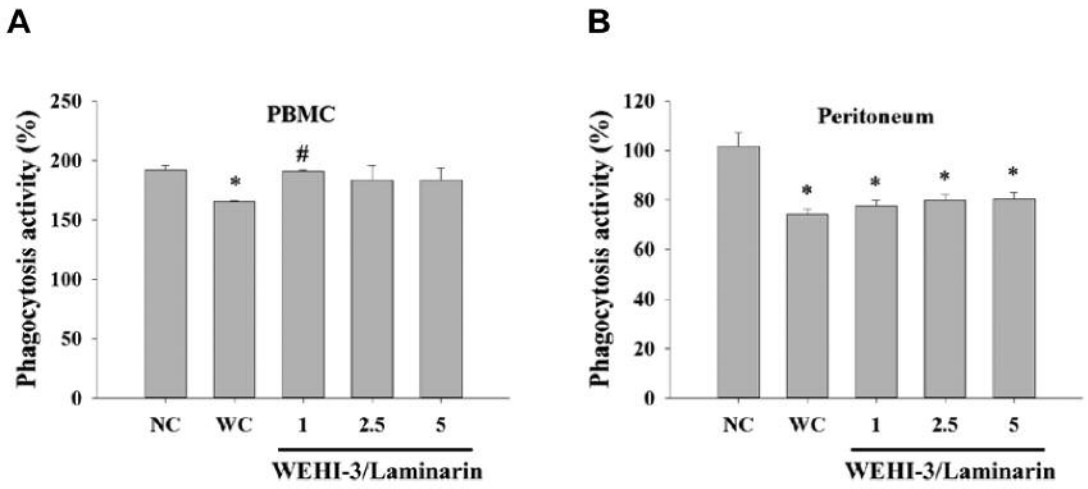

Figure 3. *Laminarin affected phagocytosis by macrophages from peripheral blood mononuclear cells (PBMCs) and peritoneal cavity of leukemic BALB/c mice. Blood samples were collected from mice and macrophages were isolated from PBMCs (A) and peritoneum (B) of each mouse. Phagocytosis was measured by flow cytometery and quantified by CellQuest as described in the Materials and Methods. Significantly different at p<0.05 vs. *normal control (NC) group and #leukemic control (WC) group.*

Laminarin affected white blood cell markers from leukemic BALB/c mice. Blood samples were assayed for the levels of cell markers CD3, CD19, CD11b and Mac-3 by flow cytometry. The results indicate that laminarin promoted expression of CD3 at 2.5 mg/ml (Figure 2A) and CD19 at 1 and 5 mg/ml (Figure 2B), and reduced that of CD11b at 5 mg/ml treatment (Figure 2C) but did not significantly affect that of Mac-3 (Figure 2D) when compared with the positive control group.

Laminarin affected phagocytosis by macrophages from PBMCs and peritoneal cavity of leukemic BALB/c mice. Macrophages were isolated from PBMCs and peritoneal cavity to measure the percentage of phagocytosis. Laminarin treatment at 1 mg/ml significantly increased phagocytosis from macrophages from PBMCs (Figure 3A), however, none of the three doses of laminarin affected phagocytosis from macrophages of the peritoneal cavity when compared with the positive control group (Figure 3B).

Laminarin affected the cytotoxic activity of NK cells from leukemic BALB/c mice. For measuring the NK cell activity, YAC-1 cells were used as targets and were assayed by flow cytometry. The results indicate that laminarin significantly increased NK cell cytotoxic activity at all laminarin doses and both target ratios (25:1 and 50:1) when compared to the positive control group (Figure 4).

Laminarin affected proliferation of T-and B-cells from leukemic BALB/c mice. Isolated splenocytes were assayed for

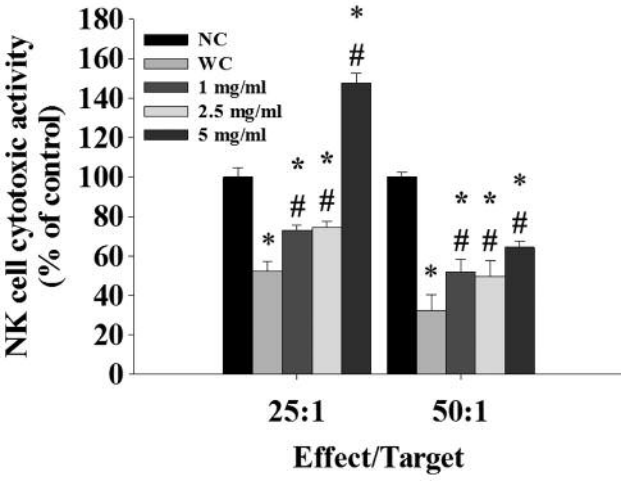

Figure 4. *Laminarin affected the cytotoxic activity of natural killer (NK) cells in leukemic BALB/c mice. Isolated splenocytes were placed in 1 ml of RPMI 1640 medium in 96-well plates. Target YAC-1 cells with serum-free RPMI 1640 medium and PKH-67/Dil.C buffer was added to the cells and NK cell cytotoxic activity was measured by flow cytometry as described in the Materials and Methods. Significantly different at p<0.05 vs. *normal control (NC) group and #leukemic control (WC) group.*

T-and B-cell proliferation. The results indicate that laminarin at 5 mg/ml significantly reduced T-cell proliferation (Figure 5A), however, none of the treatments significantly affected B-cell proliferation (Figure 5B) when compared with the positive control group.

Laminarin affect the blood GOT and GPT levels of leukemic BALB/c mice. Blood sample were collected for measuring the levels of GOT and GTP. GOT and GTP levels were significantly elevated in untreated leukemic mice (positive control) compared with the healthy mice of the negative control group. Treatment with laminarin at 2.5 and 5 mg/ml restored GOT to a relatively normal level (Figure 6A) and at 2.5 mg/ml restored the GPT level (Figure 6B) when compared with the positive control.

Discussion

It has been reported that the binding of laminarin to an amino-terminal β-1,3-glucan binding domain (N-βGRP) from *Plodia interpunctella* (*Pi*-N-βGRP) can induce the formation of a protein–carbohydrate macrocomplex containing multiple *Pi*-N-βGRP molecules; it was suggested that this complex is an initiation signal for activation of serine protease cascades that promote immune responses (10). Our earlier studies, we showed that laminarin promotes immune responses and reduces lactate dehydrogenase but increases GPT in hormal mice *in vivo* (25). However, the exact immune response to laminarin treatment in leukemic mice was not clear. To our knowledge, this is the first study evaluating the effect of laminarin on immune responses in leukemic mice *in vivo*.

Cell population assay from blood samples of treated mice indicated that laminarin elevated the expression of CD3 (Figure 2A) and CD19 (Figure 2B), but reduced that of CD11b (Figure 2C). It is well known that T-cells (CD3+), B-cells (CD19+) and monocytes (CD11b+) play critical roles in immune responses (26). Immune responses can be divided into innate and adaptive immune responses; during viral infection, viral replication is initially controlled by innate immunity before adaptive immune responses (T-cell and B-cell) for host recovery (27). Mac-3 is a marker of macrophages (28). Herein, results did not show that laminarin significantly increased the Mac-3 level when compared to control groups. Laminarin reduced the $CD11b^+$ number, thus reducing the population of monocytes. In order to further investigate the effects of laminarin on the activities of macrophage, we used the *E. coli* target cells. These cells were added to the macrophages from PBMCs and peritoneum. Laminarin increased phagocytosis from macrophages from PBMCs (Figure 3A). It is well known that one of the factors for pathogen clearance by macrophages is a high level of ROS production when they are in contact with pathogen (29-32).

Laminarin significantly increased NK cell cytotoxic effect in leukemic mice (Figure 4), reduced T-cell proliferation at 5 mg/ml treatment after stimulation (Figure 5A), but did not significantly affect B-cell proliferation after stimulation (Figure 5B). B-Cells play an important role in producing antibody against antigens. NK cells are also important immune cells in innate immunity, herein, we used targeting of YAC-1 cells for measuring NK cell cytotoxic activity which is a well-documented protocol.

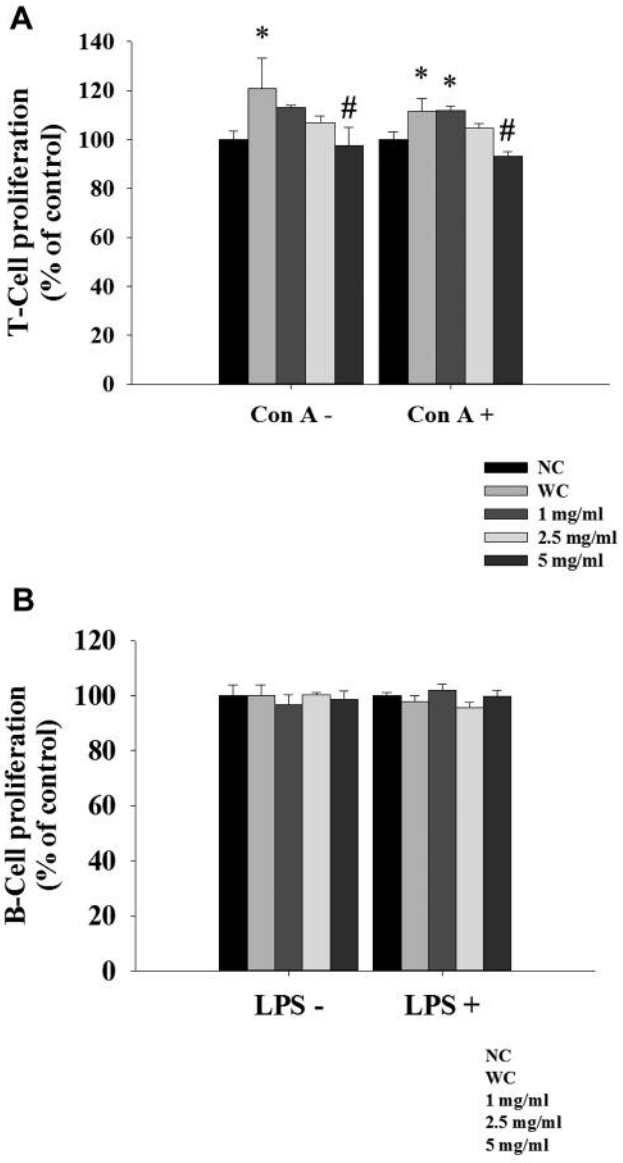

Figure 5. *Laminarin affected T- and B-cell proliferation in leukemic BALB/c mice. Isolated cells were stimulated with concanavalin A(Con A) for proliferation of T-cells (A) and with lipopolysaccharide (LPS) for B-cells (B), and then were analyzed by flow cytometry as described in the Materials and Methods. Significantly different at p<0.05 vs. *normal control (NC) group and #leukemic control (WC) group.*

Laminarin restored GOT and GPT levels although at different doses. In serum, the levels of GPT and GOT activity are higher than normal levels, that may reflect hepatic cell destruction (33).

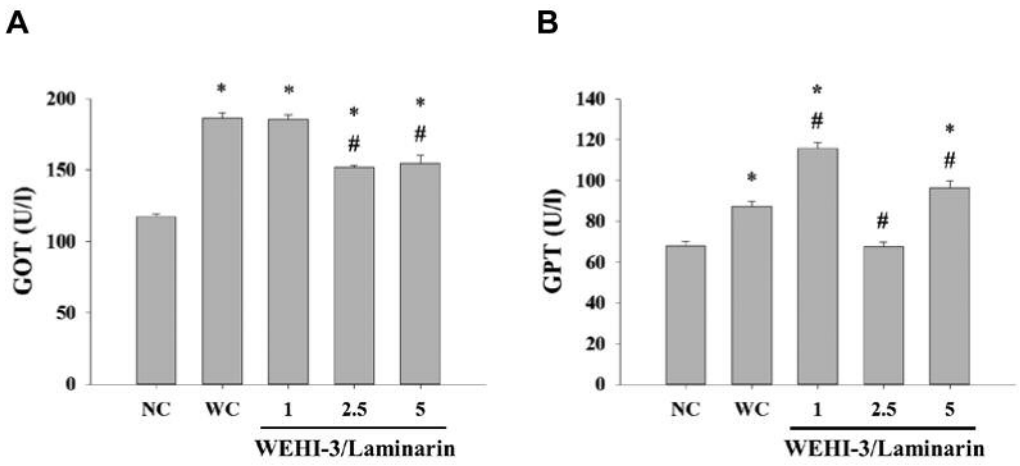

Figure 6. *Effects of laminarin on serum biochemical values in leukemia BALB/c mice. Measurement of glutamic oxaloacetate transaminase (GOT) (A) and glutamic pyruvate transaminase (GPT) (B) in the blood of leukemic BALB/c mice following exposure to laminarin. Significantly different at p<0.05 vs. *normal control (NC) group and #leukemic control (WC) group.*

Taken together, based on these findings, we suggest that laminarin may modulate immune responses through promoting T- and B-cell, and macrophage populations.

Acknowledgements

This study was supported by the grant 105-30 from Cheng Hsin General Hospital and 2018SKHAND008 from Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan. Experiments and data analysis were performed in part through the use of the Medical Research Core Facilities Center, Office of Research & Development at China medical University, Taichung, Taiwan.

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Received March 26, 2018
Revised April 30, 2018
Accepted May 2, 2018