

## Laminarin Promotes Immune Responses and Reduces Lactate Dehydrogenase But Increases Glutamic Pyruvic Transaminase in Normal Mice *In Vivo*

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**Abstract.** *Background/Aim:* Laminarin, a typical component of fungal cell walls, has been shown to induce immune responses in both adult and larval locusts. We investigated the effects of laminarin on immune response and glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and lactate dehydrogenase (LDH) levels in normal mice. *Materials and Methods:* Thirty-six normal BALB/c mice were randomly divided into four groups and treatments were provided by gavage. Group I mice acted as normal control; mice of groups II-IV received laminarin at different doses (100  $\mu$ l at 1, 2.5 and 5.0 mg/mouse in double-distilled water, respectively). All animals were treated for 14

days and were weighed, blood was collected for determination of cell markers, liver and spleen samples were weighed. Spleens were used for phagocytosis and determination of natural killer (NK) cell activity and cell proliferation by flow cytometric assay. *Results:* Laminarin reduced the body weights and weights of liver and spleen. Laminarin increased CD3, CD19 and Mac-3 cell populations at 2.5 and 5 mg/mouse, however, these did not affect CD11b marker levels. Laminarin (1 and 5 mg/mouse) reduced macrophage phagocytosis from peripheral blood mononuclear cells, but did not affect phagocytosis by macrophages from the peritoneal cavity. At an effector:target ratio of 50:1, laminarin reduced NK cell cytotoxic activity at all levels, but at a ratio of 25:1, only at 1 mg treatment. Laminarin did not affect T-cell and B-cell proliferation. Laminarin increased the level of GPT and reduced that of LDH at all doses, indicating laminarin can protect against liver injury. Laminarin is worthy of investigation in future experiments on improving immune responses.

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It is well documented that the immune system involves sophisticated networks, and is not only responsible for protecting the host against diseases including foreign materials but also protects against toxic substances (1). Therefore, there has been focus on developing immune responses in order to protect against toxic substances entering the human body (2). In particular, many studies have paid attention to improving immune responses in humans from natural products.

Laminarin (beta-1,3-glucan), a typical component of fungal cell walls (3) and from the algae *Laminaria digitate*, elicits defense-related events in tobacco and grapevine (4, 5) such as in partial resistance to tobacco mosaic virus or *Botrytis cinerea* and *Plasmopara viticola*, respectively (4, 6). In both adult and larval locusts, the injection of laminarin has been shown to induce immune responses (7, 8). It was reported that laminarin activates human intestinal epithelial cells to secrete pro-inflammatory chemokines such as interleukin-8 (IL8) and chemokine (C-C motif) ligand 2 (CCL2) in adectin-1 and spleen tyrosine kinase (SYK)-dependent manner (9).

Although several studies have reported that laminarin affects immune responses in invertebrate *in vivo*, to our knowledge there is no available information to show whether laminarin affects immune responses in normal mice. In the present study, we evaluated the effects of laminarin on immune responses in BALB/c mice *in vivo*. We analyzed cell markers, the function of macrophages, natural killer (NK) cell activity and glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), lactate dehydrogenase (LDH) levels in BALB/c mice after oral treatment with laminarin.

## Materials and Methods

**Materials and reagents.** Laminarin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). RPMI-1640 medium, L-glutamine, penicillin-streptomycin and fetal bovine serum (FBS), were purchased from Gibco Life Technologies (Carlsbad, CA, USA). Antibodies against CD3, CD11b, CD19 and Mac-3 were purchased from BD Biosciences Pharmingen Inc. (San Diego, CA, USA). Laminarin was dissolved in double-distilled water and stored at room temperature for 1 h before use.

**Male BALB/c mice and laminarin treatment.** Thirty-six male BALB/c mice at 4 weeks old at around 20-23 g each were purchased from the National Laboratory Animal Center (Taipei, Taiwan, ROC). Animals were kept with 12 h light/dark cycles at 25°C in the animal center of China Medical University (Taichung, Taiwan). Before performing experiments, the animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of China Medical University (approval ID: 104-11-B) and were cared for following the institutional animal ethical guidelines of the China Medical University as described previously (10). Thirty-six mice were randomly separated into four groups of nine mice. Group I mice received with 100 µl double-distilled water as control. Mice of group II-IV received with 100 µl laminarin of difference dose (1, 2.5 and 5.0 mg/mouse in double-distilled water, respectively). Laminarin was administered orally every 2 days for 14 days (total of seven times) *via* a steel gavage tube and the body weight was recorded. After completion of treatment, mice from each group were weighed and sacrificed as described previously (10).

**Measurement of cell populations.** After treatment, all mice were individually weighed and blood samples, liver and spleen were individually collected. Spleens were used for isolating splenocytes and for measuring NK cell activity as described previously (10). Blood from each mouse (1 ml) was lysed to destroy the red blood

cells with 1x Pharm Lyse™ lysing buffer (BD Biosciences Pharmingen Inc., San Diego, CA, USA) as per guidelines from BD Biosciences. Leukocytes from each mouse were collected and stained with phycoerythrin (PE)-labeled anti-mouse CD3 and CD19, 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 were analyzed by flow cytometry to measure the percentage of cell marker (population) as previously described (10).

**Measurement of macrophage phagocytosis.** After treatment, all macrophages were isolated from peripheral blood mononuclear cells (PBMCs) and peritoneum of each mouse from each group as described previously (11). Based on the PHAGOTEST® kit manufacturer's instructions (Orpegen Pharma, Heidelberg, Germany), all isolated macrophages were placed in plates containing 10 µl of target *Escherichia coli*-FITC and were individually mixed and then were analyzed for phagocytosis using flow cytometry. Quantification of phagocytosis was performed by CellQuest software as described previously (10).

**Measurement of NK cell cytotoxic activity.** Isolated splenocytes from each mouse were placed in 96-well plate (2.5-5×10<sup>5</sup> cells/well) with 100 µl of RPMI 1640 medium. Target YAC-1 cells (1×10<sup>4</sup> cells) (Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC) and PKH-67/Dil.C buffer (Sigma-Aldrich Corp.) were individually added to each well and were mixed thoroughly 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 a further 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 (10).

**Measurement of T- and B-cell proliferation.** RPMI-1640 medium (100 µl) was added to each well of a 96-well plate and 100 µl of isolated splenocytes (1×10<sup>5</sup> cells/well) from each mouse were added to the well. For measuring T-cell proliferation, concanavalin A (Con A, 1 µg/ml) (Sigma Chemical Co., St. Louis, MO, USA) was added to the cells for stimulation for 3 days. For measuring B-cell proliferation, lipopolysaccharide (LPS, 1 µg/ml) (Sigma Chemical Co.) was added to the cells for stimulation for 5 days. All samples were then measured for cell proliferation by using CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) as previously described (10).

**Measurement of blood GOT, GPT and LDH of BALB/c mice after treatment with laminarin.** After treatment, blood was collected and the levels of GOT, GPT and LDH were measured using quantitative kits. LiquiUV Test (aspartate aminotransferase) for GOT, liquiUV Test (alanine aminotransferase) for GPT and liquiUV Test (lactate dehydrogenase) for LDH from Human Gesellschaft für Biochemica und Diagnostica mbH (Wiesbaden, Germany) (12, 13).

**Statistical analysis.** Data are expressed as the mean±standard error (SD) of three independent experiments. For statistical comparison between laminarin-treated and control groups, differences between groups were analyzed by one-way analysis of variance and Dunnett test for multiple comparisons (SigmaPlot for Windows version 12.0; Systat Software, Inc., San Jose, CA, USA). A value of *p*<0.05 was considered to indicate a statistically significant difference.

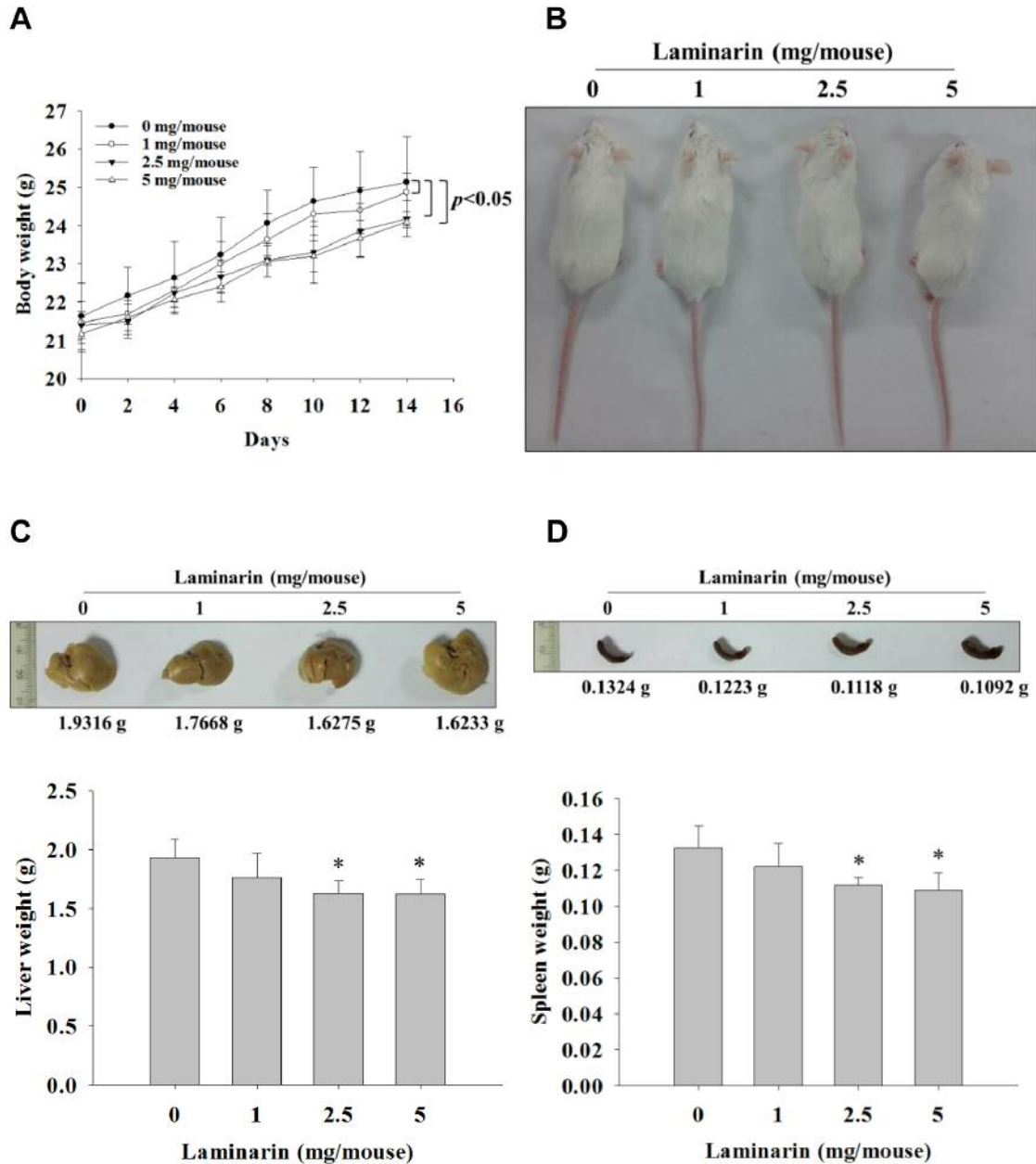


Figure 1. Effects of laminarin on body weight (A), appearance (B), liver weight (C) and spleen weight (D) of normal BALB/c mice. Group I: Normal control; group II: 1 mg/mouse of laminarin; group III: 2.5 mg/mouse of laminarin; group IV: 5 mg/mouse of laminarin. All animals were treated for 14 days. The total body weights were measured every 2 days. \*Significantly different from normal control at  $p < 0.05$ .

## Results

Laminarin reduced the body weight and weights of liver and spleen from normal BALB/c mice. Representative animal appearance, body weights, and liver and spleen weights are presented in Figure 1. These results indicate that laminarin did not significantly affect animal appearance (Figure 1B) but significantly reduced body weight (Figure 1A), and liver

(Figure 1C) and spleen (Figure 1D) weights at 2.5 and 5 mg/mouse when compared with the control group.

Laminarin affected cell markers of white blood cells from normal BALB/c mice. Blood samples were assayed for the levels of cell markers CD3, CD19, CD11b and Mac-3 and results are shown in Figure 2. Laminarin promoted CD3 (Figure 2A) and Mac-3 (Figure 2D) expression at 5 mg/mouse

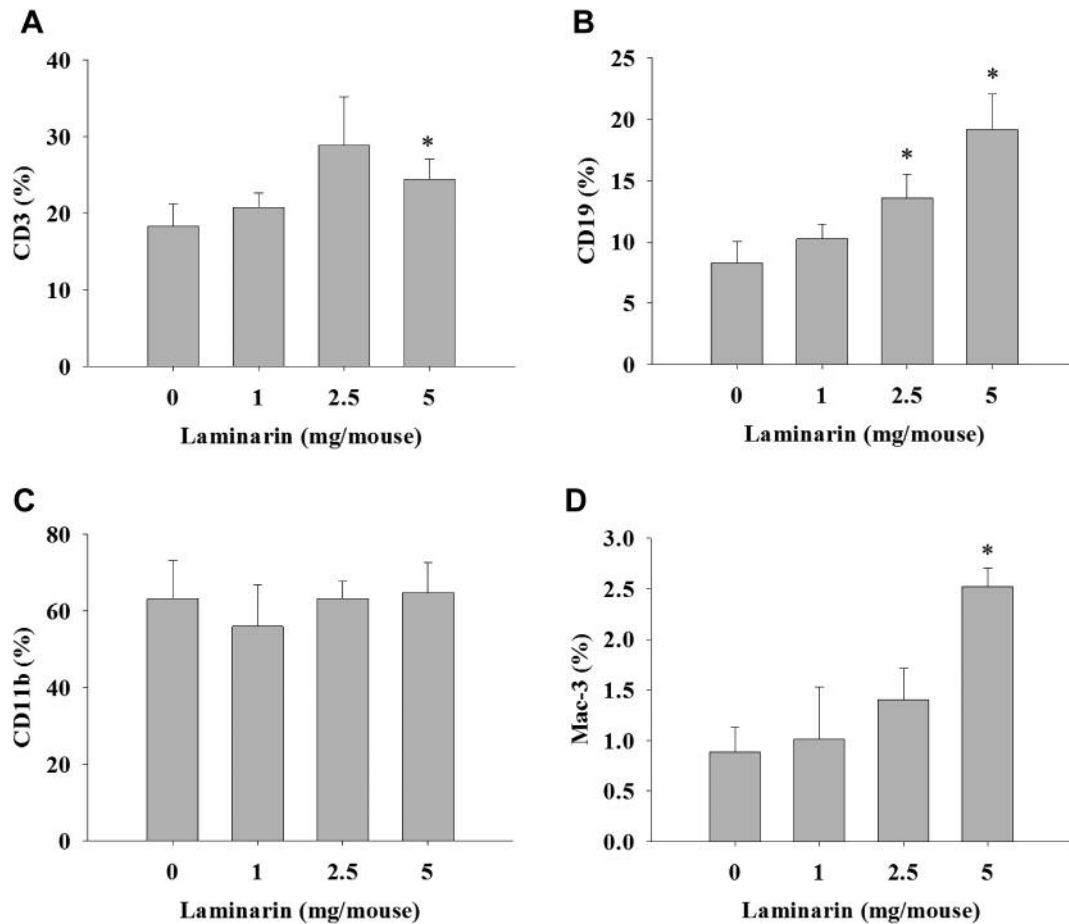


Figure 2. Effects of laminarin on cell markers in white blood cells from normal BALB/c mice. Blood was collected from all mice and were analyzed for cell markers by flow cytometry as described in the Materials and Methods. A: CD3; B: CD19; C: CD11b and D: Mac-3. \*Significantly different from normal control at  $p < 0.05$ .

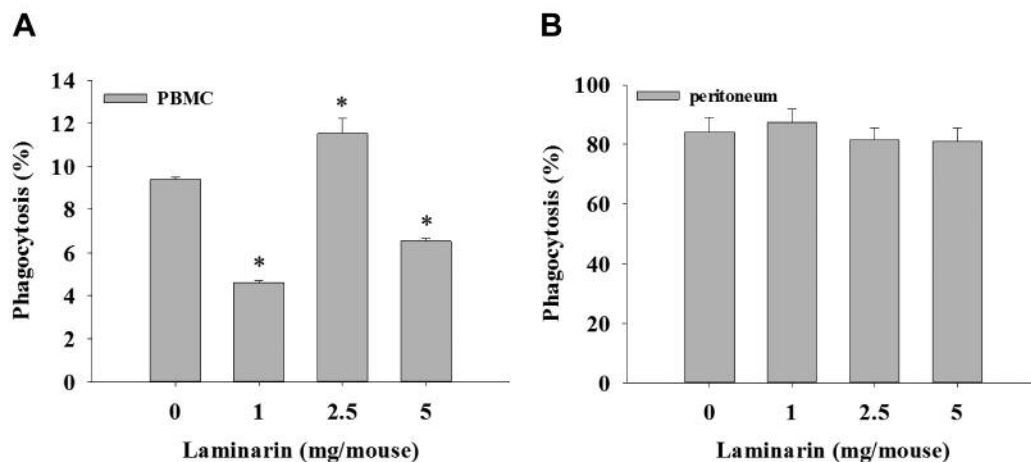


Figure 3. Effects of laminarin on macrophage phagocytosis from peripheral blood mononuclear cells (PBMCs) and peritoneal cavity of normal BALB/c mice. Blood samples were collected from mice then macrophages were isolated from PBMCs (A) and peritoneum (B) of each mouse. Phagocytosis of macrophage were measured by flow cytometry and quantified by CellQuest as described in the Materials and Methods. \*Significantly different from normal control at  $p < 0.05$ .

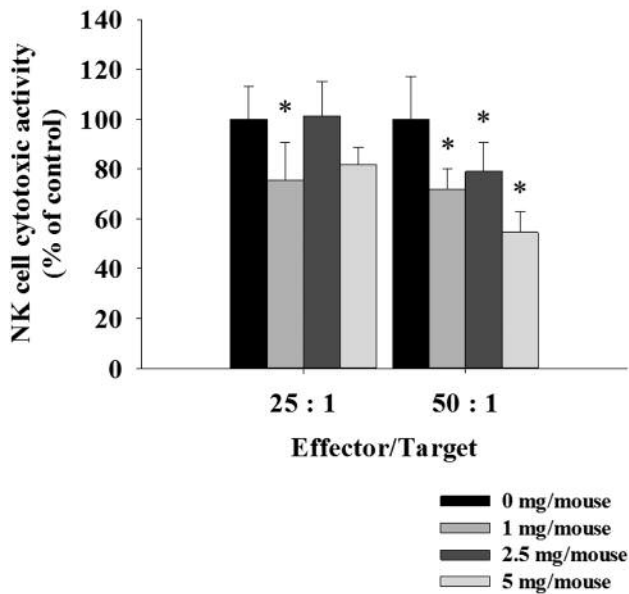


Figure 4. Effects of laminarin on cytotoxic activity of natural killer (NK) cells in normal BALB/c mice. Isolated splenocytes were placed in 1 ml of RPMI 1640 medium in 96-well plates. Target YAC-1 cells with serum-free RPMI 1640 medium and PKH-67/Dil.C buffer was added to the cells and then NK cell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

and CD19 at 2.5 and 5 mg/mouse (Figure 2B), but did not significantly affect CD11b (Figure 2C) when compared to the control group.

**Laminarin effects on phagocytosis by macrophages from PBMCs and peritoneal cavity of normal BALB/c mice.** After treatment, macrophages were isolated from PBMCs and the peritoneal cavity for measuring the percentage of phagocytosis. Laminarin treatment at 1 and 5 mg/mouse significantly reduced phagocytosis by macrophages from PBMCs but significantly increased at 2.5 mg/mouse treatment (Figure 3A), however, laminarin did not significantly affect phagocytosis by macrophages from the peritoneal cavity at any dose (Figure 3B).

**Laminarin reduced cytotoxic activity of NK cells from normal BALB/c mice.** For measuring the NK cell activity, YAC-1 cells were used as targets and were assayed by flow cytometry. Results indicated that laminarin significantly reduced NK cell cytotoxic activity, at all doses at a target ratio of 50:1 but only at 1 mg/mouse at a ratio at 25:1 when compared to control treated groups (Figure 4).

**Laminarin did not affect B- and T-cell proliferation in normal BALB/c mice.** Isolated splenocytes were assayed for B- and

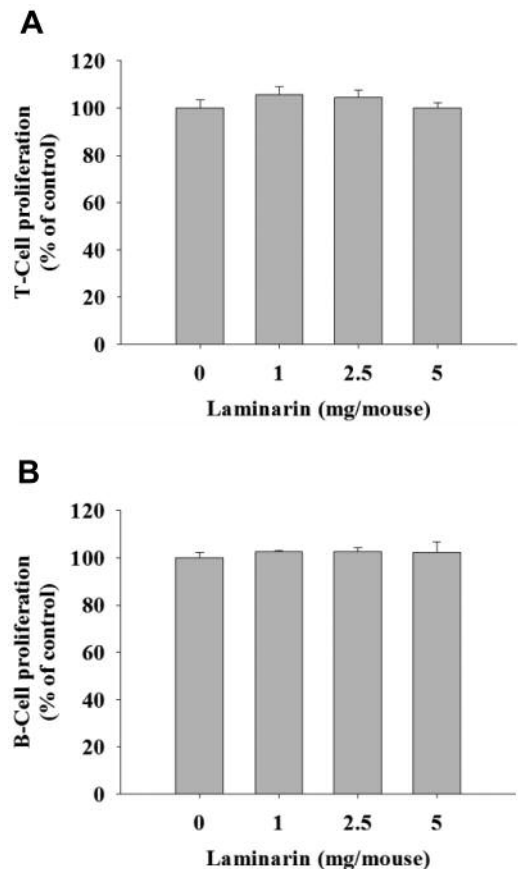


Figure 5. Effects of laminarin on B- and T-cell proliferation in normal BALB/c mice. Isolated B- and T-cells were pretreated with concanavalin A for proliferation of T-cells (A) and with lipopolysaccharide for B-cells (B), and then were analyzed by flow cytometry as described in the Materials and Methods. \*Significantly different from normal control at  $p < 0.05$ .

T-cell proliferation. Figure 5A and B indicates that laminarin did not significantly affect B- or T-cell proliferation at any dose when compared to the control group.

**Laminarin effects on GOT, GPT and LDH levels of BALB/c mice.** Blood sample were collected for measurement of GOT, GPT and LDH. The results indicate that laminarin increased the GOT level at 1 mg/mouse but reduced it at 2.5 and 5 mg/mouse (Figure 6A). All doses increased the level of GPT (Figure 6B) and reduced those of LDH (Figure 6C).

## Discussion

It is interesting that an injection of laminarin can induce immune responses in locusts (7, 8). It was also reported that laminarin activates human intestinal epithelial cells to secrete pro-inflammatory chemokines (IL8 and CCL2) (9). Furthermore, *Drosophila* gram-negative bacteria binding

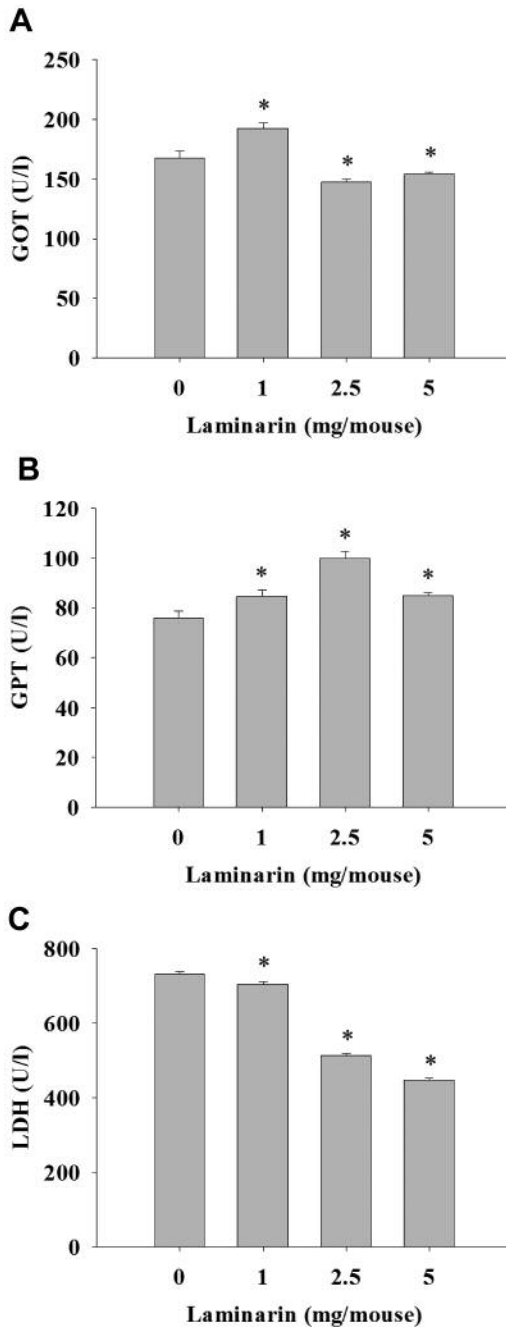


Figure 6. Effects of laminarin on serum biochemical values in normal BALB/c mice. Measurement of glutamic oxaloacetate transaminase (GOT) (A), glutamic pyruvate transaminase (GPT) (B) and lactate dehydrogenase (LDH) (C) in the blood of BALB/c mice following exposure to laminarin. \*Significantly different from normal control at  $p < 0.05$ .

protein-1 (DGNBP-1) can bind to LPS and laminarin to enhance immune-related gene expression (11).

In particular, B- and T-cells, monocytes and macrophages play an important role for immune responses to invading

foreign antigens (14). NK cells are important immune cells in innate immunity, which is related to cancer cell survival, migration and even proliferation. Results indicated that laminarin reduced NK cell cytotoxic activity at a target ratio of 50:1. Use of Yac-1 cells as a target cells for examining NK cell cytotoxic activity measurement is well documented (15).

It is well known immune responses involve multiple cell interactions such as of macrophages, B- and T-cells. Although it has been reported that laminarin induces immune responses (7, 8), although not in a vertebrate model, herein, we show that laminarin did not significantly affect B- and T-cell proliferation when compared to the control group of BALB/c mice *in vivo*. It is well documented that concanavalin A is a mitogen for T-cells and LPS is for B-cells (16). Although in this study cells were pre-stimulated with these two agents, treatment with laminarin did not elicit B- or T-cell response. Apparently, further investigations are needed.

Our results indicate that laminarin did not significantly affect phagocytosis by macrophages from the peritoneal cavity (Figure 3B), while it significantly reduced that by macrophages from PBMCs (Figure 3A). Therefore, the exact effects of laminarin on Mac-3 marker associated with macrophage function need further studies.

The GPT level was increased at all doses of laminarin, while GOT was reduced at the two higher doses. Serum levels of GPT and GOT higher than normal are indicative of hepatic cell destruction (17). Thus, our finding may indicate that laminarin may protect against hepatic cell death in this mouse model. However, laminarin may also induce hepatotoxicity in mice based on increased GPT that is not clear. Further studies are needed. In patients with abdominal trauma, abnormal hepatic transaminase and LDH levels are associated with liver injury (18). Herein, we found that laminarin reduced the level of LDH at all doses, thus, laminarin may protect against this type of injury.

In conclusion, based on these results, we suggest that laminarin mediates immune responses through increasing B-, T-, and macrophage-cell population in normal mice *in vivo*. However, whether or not laminarin can protect or induce hepatotoxicity still needs further investigation.

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## References

- Chaplin DD: Overview of the immune response. *J Allergy Clin Immunol* 125: S3-23, 2010.

- 2 Ermolina EV, Stadnikov AA and Smoliagin AI: Morphological features of the organs of the immune system in conditions of exposure to chromium and benzene. *Gig Sanit* 3: 69-71, 2012.
- 3 Franssens V, Simonet G, Bronckaers A, Claeys I, De Loof A and Vanden Broeck J: Eicosanoids mediate the laminarin-induced nodulation response in larvae of the flesh fly, *Neobellieria bullata*. *Arch Insect Biochem Physiol* 59: 32-41, 2005.
- 4 Aziz A, Poinssot B, Daire X, Adrian M, Bezier A, Lambert B, Joubert JM and Pugin A: Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopara viticola*. *Mol Plant Microbe Interact* 16: 1118-1128, 2003.
- 5 Klarzynski O, Plesse B, Joubert JM, Yvin JC, Kopp M, Kloareg B and Fritig B: Linear beta-1,3 glucans are elicitors of defense responses in tobacco. *Plant Physiol* 124: 1027-1038, 2000.
- 6 Menard R, Alban S, de Ruffray P, Jamois F, Franz G, Fritig B, Yvin JC and Kauffmann S: Beta-1,3 glucan sulfate, but not beta-1,3 glucan, induces the salicylic acid signaling pathway in tobacco and Arabidopsis. *Plant Cell* 16: 3020-3032, 2004.
- 7 Goldsworthy G, Opoku-Ware K and Mullen L: Adipokinetic hormone enhances laminarin and bacterial lipopolysaccharide-induced activation of the prophenoloxidase cascade in the African migratory locust, *Locusta migratoria*. *J Insect Physiol* 48: 601-608, 2002.
- 8 Mullen LM and Goldsworthy GJ: Immune responses of locusts to challenge with the pathogenic fungus *Metarhizium* or high doses of laminarin. *J Insect Physiol* 52: 389-398, 2006.
- 9 Cohen-Kedar S, Baram L, Elad H, Brazowski E, Guzner-Gur H and Dotan I: Human intestinal epithelial cells respond to beta-glucans via Dectin-1 and Syk. *Eur J Immunol* 44: 3729-3740, 2014.
- 10 Lu HF, Tung WL, Yang JS, Huang FM, Lee CS, Huang YP, Liao WY, Chen YL and Chung JG: *In vitro* suppression of growth of murine WEHI-3 leukemia cells and *in vivo* promotion of phagocytosis in a leukemia mice model by indole-3-carbinol. *J Agric Food Chem* 60: 7634-7643, 2012.
- 11 Kim YS, Ryu JH, Han SJ, Choi KH, Nam KB, Jang IH, Lemaitre B, Brey PT and Lee WJ: Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and beta-1,3-glucan that mediates the signaling for the induction of innate immune genes in *Drosophila melanogaster* cells. *J Biol Chem* 275: 32721-32727, 2000.
- 12 Recommendations of the German Society for Clinical Chemistry. Standardization of methods for the determination of enzyme activities in biological fluids. *Z Klin Chem Klin Biochem* 8: 658-660, 1970.
- 13 Nagamatsu Y, Yamamoto J, Fukuda A, Ohta M, Tsuda Y and Okada Y: Determination of leukocyte elastase concentration in plasma and serum by a simple method using a specific synthetic substrate. *Haemostasis* 21: 338-345, 1991.
- 14 Arpinati M and Curti A: Immunotherapy in acute myeloid leukemia. *Immunotherapy* 6: 95-106, 2014.
- 15 Fan MJ, Yeh PH, Lin JP, Huang AC, Lien JC, Lin HY and Chung JG: Anthocyanins from black rice (*Oryza sativa*) promote immune responses in leukemia through enhancing phagocytosis of macrophages *in vivo*. *Exp Ther Med* 14: 59-64, 2017.
- 16 Yeh MY, Shih YL, Chung HY, Chou J, Lu HF, Liu CH, Liu JY, Huang WW, Peng SF, Wu LY and Chung JG: Chitosan promotes immune responses, ameliorates glutamic oxaloacetic transaminase and glutamic pyruvic transaminase, but enhances lactate dehydrogenase levels in normal mice *in vivo*. *Exp Ther Med* 11: 1300-1306, 2016.
- 17 Yamashita T, Ohshima H, Asanuma T, Inukai N, Miyoshi I, Kasai N, Kon Y, Watanabe T, Sato F and Kuwabara M: The effects of alpha-phenyl-tert-butyl nitron (PBN) on copper-induced rat fulminant hepatitis with jaundice. *Free Radic Biol Med* 21: 755-761, 1996.
- 18 Bilgic I, Gelecek S, Akgun AE and Ozmen MM: Predictive value of liver transaminases levels in abdominal trauma. *Am J Emerg Med* 32: 705-708, 2014.

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