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Yam Storage Protein Dioscorins from *Dioscorea alata* and *Dioscorea japonica* Exhibit Distinct Immunomodulatory Activities in Mice

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The aim of this study was to elucidate the effect of the major storage protein dioscorin isolated from two different yam species, Tainong No. 1 (TN1-dioscorins) and Japanese yam (Dj-dioscorins), on the immune activities of mice. Dj-dioscorins, like TN1-dioscorins, could induce expression of the pro-inflammatory cytokines and stimulate phagocytosis of RAW 264.7. Intraperitoneal injection of the TN1-dioscorins into mice stimulated phagocytosis of bone marrow, spleen, and thymic cells. In contrast, the T and B cells in bone marrow, spleen, and thymus isolated from mice injected with Dj-dioscorins had higher proliferative responses to mitogens. Furthermore, Dj-dioscorins enhanced proliferation of $CD4^+$, $CD8^+$, and $Tim3^+$ (Th1) cells in spleen and $CD19^+$ cells in both spleen and thymus. Supplement of Dj-dioscorins in the lymphoid cells isolated from Dj-dioscorins primed mice induced cell proliferation of both spleen and thymic cells. These findings indicated that TN1-dioscorins have a higher ability to stimulate the phagocytic activity of the lymphoid cells than Dj-dioscorins, whereas Dj-dioscorins possess more abilities than TN1-dioscorins to enhance the proliferation of the lymphoid cells.

KEYWORDS: Yam storage protein; dioscorins; cytokine; TLR-4

INTRODUCTION

Fresh yam tubers (*Dioscorea* spp.) are widely consumed in Asia and Africa. The dried slices of yam tubers have been used in Chinese herbal medicine since ancient times. Chinese yam tubers are highly nutritional and possess functional components, such as mucin, dioscin, diosgenin, allantoin, choline, polyphenol oxidases, and proteins (*1*). Yam mucilage exhibits antioxidant and antihypertensive activities (*2*, *3*). It can also increase the number of T helper cells in the peripheral blood and enhance the phagocytic activity of granulocytes, monocytes, and macrophages (*4*).

Yam tubers contain about 1−3% proteins on a dry weight basis (*5*). Two proteins have been identified in yam tubers, dioscorins and phytoglycoproteins (*6*, *7*). The phytoglycoproteins have been identified recently and account for only 0.02% of the tuber total dry weight (*6*). They have a molecular mass of 30 kDa and consist of 61% carbohydrate and 39% protein contents. The phytoglycoproteins from *Dioscorea batatas* tubers contain antioxidation activity, antiproliferation activity in Chang liver cells, and anti-inflammatory activity via the p38 MAP kinase signal transduction pathway in murine macrophage RAW 264.7 (*7*, *8*).

Dioscorins are the major storage proteins in the yam tubers and contribute approximately 85% of the total soluble protein content of the tubers (*5*). Dioscorins are distribute over the tubers except in the leaves, and they are aggregated within the vacuoles and cytoplasm of mature tubers (*5*, *9*). Dioscorins have been shown to possess carbonic anhydrase, free radical scavenger, dehydroascorbate reductase, and trypsin inhibitor activities (*10*−*12*). Dioscorins from yam tubers of *D. batatas*, *D. alata*, and *D. pseudojaponica* are reported to contain both carbonic anyhydrase and trypsin inhibitor activities (*11*). However, Gaidamashvili et al. (*13*) subsequently showed that dioscorin proteins DB2, DB3S, and DB3L from yam tubers of *D. batatas* do not contain carbonic anhydrase or trypsin inhibitor activity. Instead, DB3L exhibits unique maltose-binding lectin activity. Dioscorins from *D. cayenensis* have been shown to contain a carbonic anhydrase motif with active site alteration that renders them unlikely to have classical α-carbonic anhydrase activity (*14*). Dioscorins from yam tubers of *D. alata* cv. Tainong No. 1 and their peptic hydrolysates can inhibit angiotensin converting enzymes in a dose-dependent manner, suggesting that dioscorins and their peptic hydrolysates exhibit antihypertensive activity (*15*). Oral administration of the purified dioscorins and their peptic hydrolysates can effectively lower the blood pressure of spontaneously hypertensive rats, further proving their antihypertensive activity (*16*). Purified dioscorins also contain both dehydroascorbate reductase and monodehydroascorbate reductase activities in the presence of glutathione and NADH, respectively, suggesting that dioscorins have antioxidation ability (*10*).

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Dioscorins from yam tubers of *D. batatas* are reported to contain 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity in a pH-dependent manner (*12*).

In addition to the biochemical functions of dioscorins, recent research has shown that dioscorins exhibit immunomodulatory activity. Dioscorins isolated from *D. alata* activate MAP kinases (ERK, p38, and JNK) and NF-κB via the Toll-like receptor 4 (TLR-4) signal transduction pathway and stimulate pro-inflammatory cytokine expression, such as TNF-α, IL-1β, and IL-6, in RAW 264.7, murine bone marrow cells, and human monocytes ex vivo (*17*, *18*). The TLR-4 signaling pathway is crucial for immunological activity against microbes. TLR-4 recognizes lipopolysaccharides (LPS) from Gram-negative bacteria, triggers phosphorylation of ERK, p38, and JNK, and subsequently activates NF-κB, which leads to pro-inflammatory responses (*19*). Dioscorins from *D. alata* enhance the phagocytic activity of RAW 264.7 against *Escherichia coli* and also stimulate the proliferation of murine spleen cells (*18*). Moreover, dioscorins induce the expression of the inducible nitric oxide synthase gene and the accumulation of nitric oxide and increase oxidative burst in RAW 264.7 (*18*). However, the immunomodulatory activity of dioscorins in vivo remains unclear. The aim of this study was to evaluate the direct effects of various purified dioscorin proteins from different yam species on immune responses of mice.

MATERIALS AND METHODS

Plant Materials. Fresh tubers of Japanese yam (*Dioscorea japonica* Thunb.) were purchased from a local supermarket. The tubers of Tainong No. 1 (*Dioscorea alata* cv. Tainong No. 1) were routinely purchased from a farmers' association (Mingchien Shiang, Nantou County, Taiwan). All yam tubers were stored in a cooler controlled at 16 °C for subsequent experiments.

Experimental Animals and Cell Culture. Six- or seven-week-old male BALB/c mice (approximately 20−25 g) were purchased from BioLASCO (Taipei, Taiwan). Mice were allowed to acclimatize for 7−10 days upon arrival and allocated randomly into treatment groups. Mice were maintained on a 12/12 h light/dark cycle at 22 ± 2 °C and provided with food (PMI Nutrition International, St. Louis, MO) and water at the Graduate Institute of Biotechnology, National Dong Hwa University, Hualien County, Taiwan. All mice were housed and cared for according to the "Guide for the Care and Use of Laboratory Animals" of National Dong Hwa University.

The murine macrophage cell line RAW 264.7 was cultured in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator containing 5% CO_2.

Extraction and Purification of Dioscorins. The dioscorins of both the Tainong No. 1 yam tubers and Japanese yam tubers were extracted and purified according to the procedure of Chen and Lin (*20*). Approximately 500 g of yam tubers was peeled, homogenized with 4 volumes (w/v) of 50 mM Tris-HCl (pH 8.3), and centrifuged at 12000*g* for 30 min. The supernatant was regarded as crude extract. Total proteins of the crude extract were differentially precipitated with 45−75% ammonium sulfate, dissolved in 10 volumes (v/v) of 50 mM Tris-HCl buffer (pH 8.3), and dialyzed overnight with Cellu·Sep T4 (Regenerated Cellulose Tubular Membrane, MW 12000−14000, Seguin, TX) against the same buffer. Approximately 500 mL of the dialyzed protein sample was mixed with 5 g of ddH_2O-hydrated DEAE Sephadex A-25 ion exchange (Amersham Pharmacia Biotech, Uppsala, Sweden) and shaken at 4 °C for 1 h. The precipitate was collected after centrifugation at 10000*g* for 10 min and redissolved in 50 mL of 50 mM Tris-HCl (pH 8.3) buffer containing 150 mM NaCl. After 1 h of shaking and centrifugation at 10000*g* for 10 min, the supernatant was obtained for gel permeation. The purified protein solution was subsequently loaded onto a Sephadex G-75 column (C16/70, Amersham Pharmacia Biotech) and eluted with 100 mM Tris-HCl (pH 7.9) containing 100 mM NaCl. The eluate was dialyzed overnight with ddH_2O at 4 °C and then lyophilized. For the experiment, the lyophilized dioscorins were dissolved in phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 12000*g* for 20 min at 4 °C. The supernatant was collected and filtered with a 0.45 μm filter membrane (Millipore, Bedford, MA). The filtrate was used for experiments. Concentrations of the dioscorins were determined by Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA) and were further confirmed by SDS-PAGE loaded with standard concentrations of bovine serum albumin (Sigma). The purity of the dioscorins was further determined by scanning the SDS-PAGE with luminescent image analyzer (Fujifilm LAS-3000, Tokyo, Japan). Approximately 95−97 and 89−92% purities were obtained for TN1-dioscorins and Dj-dioscorins, respectively.

Injection of Mice and Isolation of Thymic Cells, Spleen Cells, and Bone Marrow Cells. To test the direct effect of dioscorins, mice were administered 0.5, 1, and 2 mg/kg of body weight (bwt) TN1-dioscorins (in 0.1 mL of PBS), 0.5 and 2 mg/kg of bwt of Dj-dioscorins (in 0.1 mL of PBS), or 0.1 mL of PBS (control) via intraperitoneal injection every other day for 3 times and were sacrificed by cervical dislocation at 48 h after the last injection. After dislocation, the thymus, spleen, and thigh bone were immediately removed, cleaned of fat and connective tissue, and placed in PBS. Tissues (thymus, spleen, and bone marrow) were then individually cut into small pieces and forced through a nylon mesh (80 mesh) to obtain cell suspension. The cells were collected, centrifuged at 420*g* for 10 min, and washed with 3 mL of PBS. After another wash with 3 mL of PBS, the harvested cells were suspended with 1 mL of RPMI-1640 medium containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cell numbers were counted by hemacytometer.

Detection of Immunological Activity of Dioscorins by RT-PCR. The experiments were performed following the method of Fu et al. (*17*) with modification. The macrophage RAW 264.7 (5×10^5 cells in 0.5 mL/well) were cultured in 24-well flat-bottom plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) for 24 h and then treated with 100 ng/mL lipopolysaccharide (LPS, from *E. coli* 0111:B4; Sigma), 50 μg/mL TN1-dioscorins, or 50 μg/mL Dj-dioscorins for 4 h at 37 °C. To eliminate any possible LPS contamination in the dioscorin extracts, the dioscorins were predisposed to LPS inhibitor polymyxin B (10 μg/mL; Sigma) for 20 min. The cells were collected and total RNAs were isolated by REzol according to the manufacturer's instructions (PROtech technology, Taipei, Taiwan). Approximately 1×10^6 cells were lysed with 1 mL of REzol reagent. Cell lysate was extracted with phenol/chloroform, and the total RNAs were precipitated with an equal volume of isopropanol. The RNA pellets were washed with 70% ethanol, air-dried, and resuspended with 25 μL of DEPC-treated ddH_2O. The concentration of RNA was determined by spectrophotometer. For RT-PCR, reverse transcription was performed in a 50 μL reaction volume containing 5 μg of the total RNA, 30 units of MMLV reverse transcriptase (Epicentre Biotechnologies, Madison, WI), 1× MMLV reaction buffer, 10 mM DTT, 0.7 mM each of dNTPs (GeneCraft GmbH, Munster, Germany), and 1.5 μg of oligo dT_{18}. The reaction was carried out at 37 °C for 1 h. Primer sets corresponding to TNF-α, IL-1β, IL-6, and GAPDH genes were used for PCR amplification. The PCR products were electrophoresed, and the expression of GAPDH was used as an internal control. PCR amplification of the cDNA clones were performed in a 50 μL reaction volume containing 2 μL cDNA, 10 units YEA*taq* DNA polymerase (Yeastern Biotech, Taipei, Taiwan), 1× reaction buffer, 0.2 mM each of dNTPs, and 0.2 μM of each primer. Thermocycling conditions were 94 °C for 3 min followed by 30 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 45 s, and a final 10 min at 72 °C (MJ Research, Waltham, MA). Primer sequences used in this experiment are as follows: TNF-α forward, 5′-GAACTGGCAGAAGAGGCACT-3′; reverse, 5′-CGGACTCCGCAAAGTCTAAG-3′; IL-1β forward, 5′-TGTGAAATGCCACCTTTTGA-3′; reverse, 5′-TACCAGTTGGGG AACTCTGC-3′; IL-6 forward, 5′-GCCAGAGTCCTTCAGAGAGAGATACAG-3′; reverse, 5′-CCCAACATTCATATTGTCAG-3′; GAPDH forward, 5′-ACTCCACTCACGGCAAATTC-3′; reverse, 5′-CACATTGGGGGTAGGAACAC-3′. TNF-α, IL-1β, IL-6, and GAPDH primer sets were synthesized by PROtech Technology Inc. (Taipei, Taiwan).

Phagocytic Effect of Dioscorins on Murine Macrophage RAW 264.7. The experiments were performed following the method of Liu et al. (*18*). The murine macrophages RAW 264.7 (2×10^4 cells/well) were cultured in a 96-well flat-bottom plate (100 μL/well) (Shanghai Sunub Bio-Tech Development Inc., Shanghai, China) at 37 °C for 24 h and then

treated with LPS (20 μg/mL), TN1-dioscorins (12.5, 50, 100 μg/mL), Dj-dioscorins (12.5, 50, 100 μg/mL), or 0.1 mL of PBS as a control at 37 °C for 4 h. After centrifugation at 420*g* for 10 min, the supernatant was removed, and 100 μL of PBS and 20 μL of FITC-labeled *E. coli* DH5α 2×10^6 cfu/well) were subsequently added to each well to obtain an effector/target ratio of 100:1 for phagocytosis analyses. To obtain FITC-labeled *E. coli*, an overnight culture of *E. coli* (approximately 1×10^9 cfu) was autoclaved at 120 °C for 10 min to kill the bacteria. The bacteria were washed with 1 mL of PBS and then reacted with 50 μg/mL fluorescein isothiocyanate (FITC; Sigma) in the dark at 37 °C for 40 min. Unlabeled FITC was washed out of the samples with 1 mL of PBS. The plate was incubated at 37 °C for 2 h. Two hundred microliters of trypan blue (1.25 mg/mL) was then added to each well to quench the fluorescence of FITC-labeled *E. coli* that was not ingested by RAW 264.7. The fluorescence intensity of FITC was determined by using a microplate fluorometer (Fluoroskan Ascent FL, Labsystems, Helsinki, Finland) with an excitation wavelength at 485 nm and an emission wavelength at 538 nm. Each treatment was performed in triplicate. The phagocytic effect of dioscorins on RAW 264.7 was defined as relative intensity of FITC fluorescence as compared to the control.

Phagocytic Effect of Dioscorins on Lymphocytes. Approximately 1×10^6 immune cells (in 100 μL) from dioscorin-injected mice were mixed with 1×10^8 cfu of FITC-labeled *E. coli* DH5α in 100 μL to obtain an effector/target ratio of 100:1. The cell samples were incubated in the dark at 37 °C for 25 min. Eight hundred microliters of ice-cold PBS was added to stop the reaction. The fluorescence intensity of FITC was determined by flow cytometry (Cytomics FC500, Beckman, Fullerton, CA). The phagocytosis ability of the immune cells was defined as the percentage of cells ingesting FITC-labeled *E. coli* with green fluorescence within a total of 1×10^4 cells detected. Each treatment was performed in triplicate, and all data were collected from five different experiments ($n = 5$).

Proliferation Assay of Immune Cells. Immune cells isolated from dioscorin-injected mice were suspended with RPMI-1640 medium. About 5×10^4 cells/well of the isolated immune cells were seeded in a 96-well flat-bottom plate (100 μL/well) and treated with 0.1, 1, 5, and 10 μg/mL concanavalin A (ConA; Sigma) or 1, 5, and 10 μg/mL LPS for 48 h at 37 °C in a humidified incubator containing 5% CO_2. After incubation, the culture media of the immune cells were replaced with PBS and proliferation of the immune cells in response to mitogens was assessed in triplicate by CellTiter 96 AQuenous Non-Radioactive Cell Proliferation Assay according to the manufacturer's recommendation (Promega, Madison, WI). The assay reagents, 333 μg/mL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (MTS; Promega) and 25 μM phenazine methosulfate (PMS; Sigma) were added to each well of the 96-well plate, and the plate was incubated at 37 °C for 2 h. Absorbance at 490 nm was recorded with an ELISA plate reader (SpectraMax 190, Nihon Molecular Devices, Tokyo, Japan). The values were calculated as relative intensity of the absorbance as compared to the vehicle control with no added mitogen. Each treatment was performed in triplicate, and all data were collected from five different experiments ($n = 5$).

Determination of Surface Phenotype by Using Immunofluorescence Labeling and Flow Cytometric Analysis. Immune cells isolated from dioscorin-injected mice were suspended with PBS. Approximately 1×10^6 immune cells (in 100 μL) were analyzed for the percentage of cells bearing a particular cell surface marker. The cells were stained for 30 min at 37 °C with rat anti-mouse monoclonal antibodies specific for CD4, CD8, CD19, Tim1, and Tim3 (BioLegend, San Diego, CA). After three washings with 1 mL of PBS, samples were incubated with 0.5 μg of FITC-labeled polyclonal goat anti-rat IgG antibodies (BioLegend) in the dark at 37 °C for 25 min. After two washings with 1 mL of PBS, samples were suspended with 1 mL of PBS. The number of cells with a particular surface marker was determined by flow cytometry (Cytomics FC500, Beckman). The percentage of subpopulation of the immune cells was determined by the number of cells recognized by the FITC-labeled antibodies within a total count of 1×10^4 cells. Each treatment was performed in triplicate, and all data were collected from five different experiments ($n = 5$).

Effect of Supplement of Dioscorins on Proliferation of the Lymphoid Cells ex Vivo. The spleen cells and thymic cells were harvested from mice injected with 0, 0.5, and 2 mg/kg TN1-dioscorins or 0, 0.5, and 2 mg/kg Dj-dioscorins. Approximately 5×10^4 cells were cultured in 100 μL of RPMI-1640 medium per well in 96-well plates. The cells were then individually treated with 0, 12.5, and 50 μg/mL of TN1-disocorins or Dj-dioscorins (in 10 μL) for 24 and 48 h. After incubation, the culture media of the immune cells were replaced with 100 μL of PBS, and proliferation of the immune cells in response to supplemental dioscorins was assessed in triplicate by CellTiter 96 AQuenous Non-Radioactive Cell Proliferation Assay as described above. All data were collected from five different experiments ($n = 5$).

Statistical Analysis. All values were expressed as mean ± standard error of experiments performed in triplicate for the in vitro studies or five mice ($n = 5$) for each group of the in vivo and ex vivo studies. The results were examined by analysis of variance followed by the Student's *t* test for significant main effects at $P < 0.05$ (*). All statistical analyses were performed by the SigmaStat statistical software of Windows version 2.03 (SPSS Inc., Chicago, IL).

RESULTS

Dj-Dioscorins Have the Same Immunological Activity as TN1-Dioscorin in Vitro. Dioscorins isolated from the yam tubers of *D. alata* cv. Tainong No. 1 have been shown to induce pro-inflammatory cytokine expressions such as TNF-α, IL-1β, and IL-6 via the TLR-4 signaling pathway (*17*, *18*). To test whether the dioscorins extracted from Japanese yam tubers (Dj-dioscorins) had immunomodulatory effects similar to those of *D. alata* cv. Tainong No. 1 (TN1-dioscorins), Dj-dioscorins and TN1-dioscorins were isolated from fresh yam tubers and were applied to macrophage RAW 264.7 as described under Materials and Methods. To eliminate any possible contamination of LPS in the dioscorin extracts, LPS inhibitor polymyxin B was added in the reaction. Expression of the TNF-α, IL-1β, and IL-6 genes in RAW 264.7 in response to dioscorin stimulation was assessed by RT-PCR. The data revealed that Dj-dioscorins stimulated the pro-inflammatory cytokine gene expression of TNF-α, IL-1β, and IL-6 similarly to TN1-dioscorins (**Figure 1**). This result indicated that dioscorins from both species had similar immunomodulatory effects for inducing the pro-inflammatory cytokine expression via the TLR-4 signaling pathway.

TN1-dioscorins are reported to possess the ability to stimulate phagocytosis of RAW 264.7 (*18*). To determine whether the Dj-dioscorins had the ability to stimulate phagocytosis as well, RAW 264.7 cells were treated with LPS, TN1-dioscorins, and Dj-dioscorins for phagocytic activities. The results revealed that Dj-dioscorins stimulated phagocytosis of RAW 264.7 and that the phagocytic activity was even higher than that of TN1-dioscorins, indicating that Dj-dioscorins had immunomodulatory activity similar to that of TN1-dioscorins in vitro (**Figure 2**).

Effects of Dioscorins on Phagocytosis of Lymphoid Cells against *E. coli*. The TN1-dioscorins have been shown to stimulate phagocytosis of macrophage RAW 264.7 in vitro (*18*). However, the effect of the TN1-dioscorins and Dj-dioscorins on phagocytosis of lymphoid cells in vivo has not been elucidated. The TN1-dioscorins and Dj-dioscorins were injected intraperitoneally

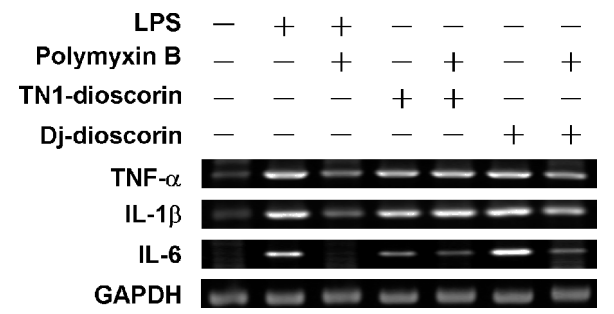

Figure 1. Detection of immunological activity of dioscorins. The expressions of TNF-α, IL-1β, and IL-6 genes were detected by RT-PCR, and the GAPDH gene is used as an internal control.

into mice as described previously under Materials and Methods. The bone marrow cells, spleen cells, and thymic cells of the injected mice were isolated and tested for phagocytosis against *E. coli*. The results showed that the administered TN1-dioscorins significantly stimulated phagocytosis of bone marrow cells and thymic cells at a level higher than 1 mg/kg of bwt (**Figure 3A**,**C**). Furthermore, phagocytosis of spleen cells was significantly stimulated by the administered TN1-doscorins at a level higher than 0.5 mg/kg of bwt (**Figure 3B**). In contrast, the Dj-dioscorins exhibited no effect on stimulation of phagocytosis of the bone marrow cells, spleen cells, and thymic cells against *E. coli* at the same concentrations used for the TN1-dioscorins (**Figure 3D**−**F**).

Effects of Dioscorins on Lymphocyte Activation. To determine whether the dioscorins exhibit a direct effect in stimulating the development of lymphoid cells in vivo, mice were treated with dioscorins via intraperitoneal injection instead of oral administration. Intraperitoneal injection of dioscorins can avoid digestion of the purified dioscorins and changes of their structures that are usually caused in the intestines through oral administration. The bone marrow cells, spleen cells, and thymic cells isolated from mice that were injected with 0.5 or 2 mg/kg of bwt of the TN1-dioscorins or Dj-dioscorins were cultured with various concentrations of T cell and B cell stimuli, ConA and LPS, and the proliferation of T cells and B cells was determined. The results revealed that bone marrow cells from mice injected with 0.5 or 2 mg/kg of bwt TN1-dioscorins were slightly proliferated in response to 0.1, 1, and 10 μg/mL ConA stimulation as compared to those of vehicle controls (mice injected with PBS), but the differences were not statistically significant (**Figure 4A**). Spleen cells from mice injected with 0.5 or 2 mg/kg of bwt

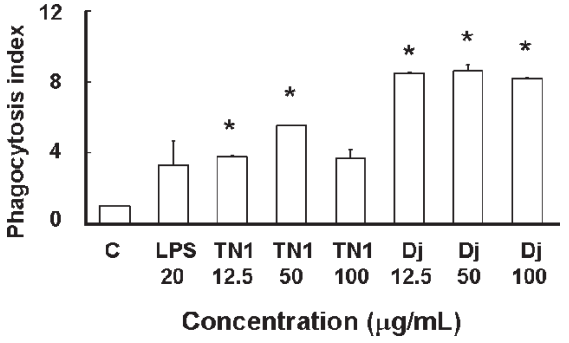

Figure 2. Phagocytosis effect of TN1-dioscorins and Dj-dioscorins on macrophage RAW 264.7 against *E. coli*.

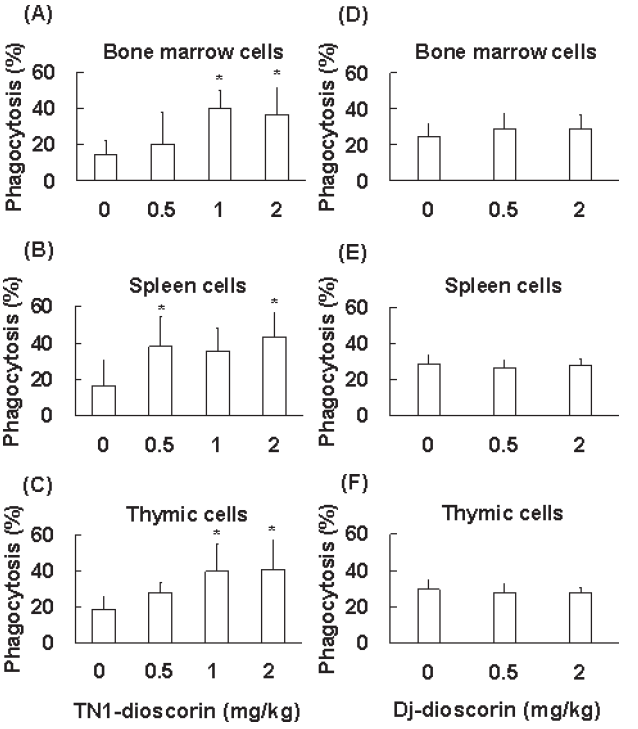

Figure 3. Effects of dioscorins on the phagocytosis of lymphocytes against *E. coli*. The lymphocytes were isolated from TN1-dioscorin injected mice (**A**−**C**) and Dj-dioscorin injected mice (**D**−**F**). The bone marrow cells (**A**, **D**), spleen cells (**B**, **E**), and thymic cells (**C**, **F**) were cultured with FITC-labeled *E. coli* to determine their phagocytosis ability.

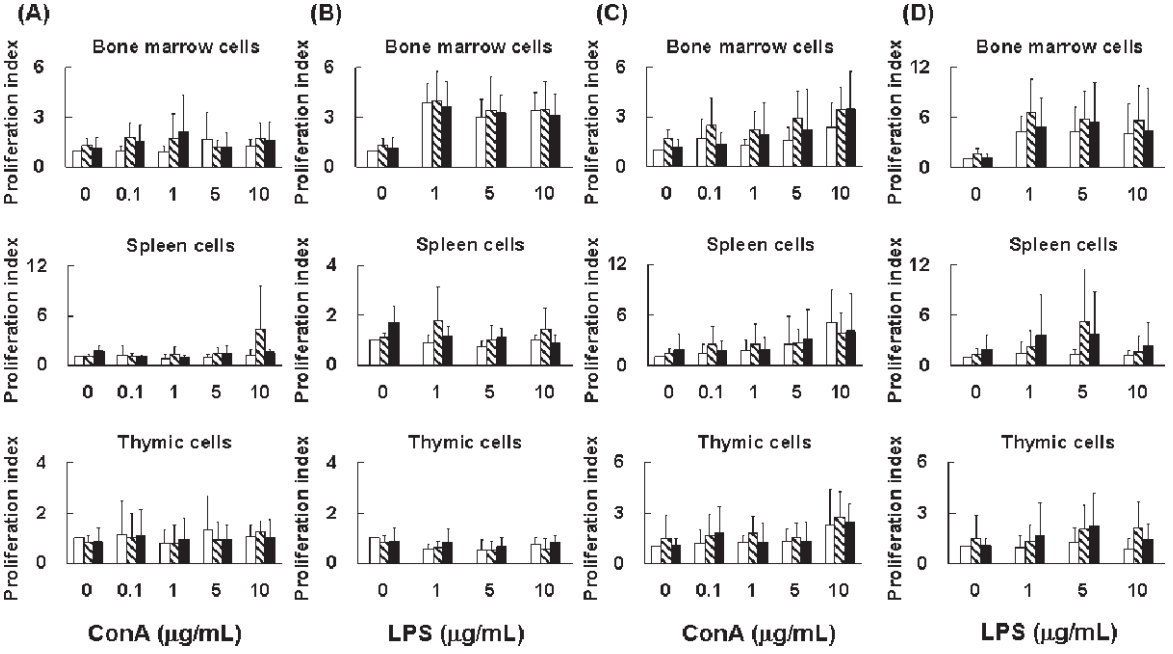

Figure 4. Effects of TN1-dioscorins and Dj-dioscorins on lymphocyte activation. The lymphocytes isolated from TN1-dioscorin injected mice (**A**, **B**) and Dj-dioscorin injected mice (**C**, **D**) were treated with ConA (**A**, **C**) or LPS (**B**, **D**) and then analyzed for cell proliferation. The white bar indicates the cells from PBS-injected mice, the slashed bar indicates the cells from mice injected with 0.5 mg/kg of bwt of dioscorins, and the black bar indicates the cells from mice injected with 2 mg/kg of bwt of dioscorins.

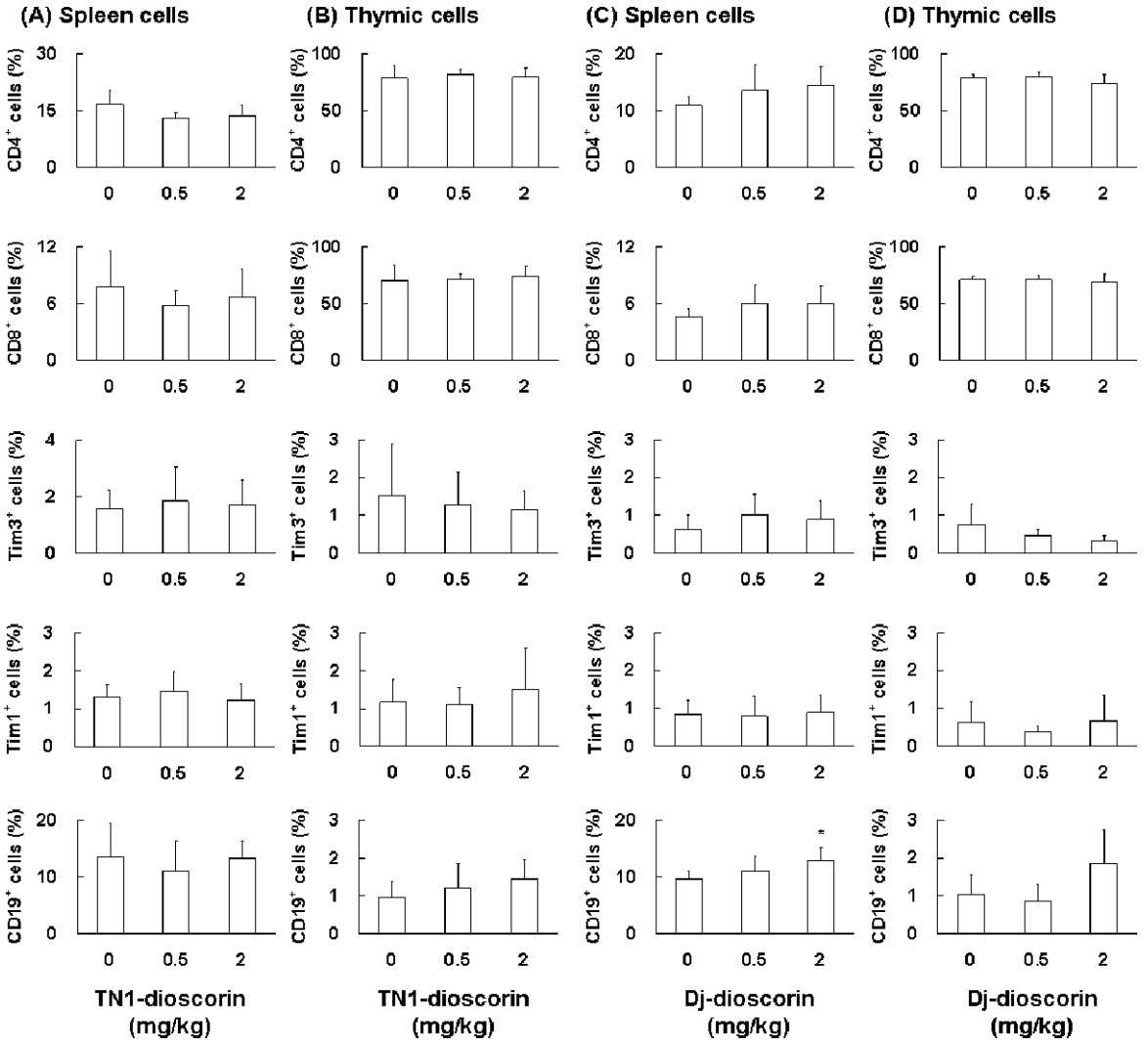

Figure 5. Effect of dioscorins on proliferation of cell subsets of lymphoid cells. The spleen and thymic cells were harvested from TN1-dioscorin injected mice (**A**, **B**) or Dj-dioscorin injected mice (**C**, **D**). The percentage of cells bearing a particular surface marker was determined by flow cytometry.

TN1-dioscorins were proliferated in response to 5 and 10 μg/mL ConA stimulation (**Figure 4A**). However, the thymic cells showed no observable proliferation in response to ConA stimulation (**Figure 4A**). Bone marrow cells and thymic cells showed no proliferative response to LPS stimulation (**Figure 4B**). Nevertheless, spleen cells from mice injected with 0.5 or 2 mg/kg of bwt TN1-dioscorins were slightly proliferated in response to LPS stimulation (**Figure 4B**). Overall, the results suggested that TN1-dioscorins could slightly stimulate the activation of T cells in bone marrow and spleen and of B cells in spleen. In contrast, bone marrow cells from mice injected with 0.5 or 2 mg/kg of bwt Dj-dioscorins had a higher proliferative response to various concentration of ConA stimulation (**Figure 4C**). Spleen cells and thymic cells from mice treated with 0.5 or 2 mg/kg of bwt Dj-dioscorins showed increasing tendency of proliferative response to lower concentration of ConA (**Figure 4C**). Bone marrow cells, spleen cells, and thymic cells from mice injected with 0.5 or 2 mg/kg of bwt Dj-dioscorins all had higher proliferative responses to the B cell stimulus LPS stimulation, although the differences were not statistically significant (**Figure 4D**). Overall, the T cells and B cells in bone marrow, spleen, and thymus have higher proliferative response to Dj-dioscorins, and the proliferation responses to Dj-dioscorins are higher than those to TN1-dioscorins.

Detection of Specific Cell Subsets Proliferated in Lymphoids. To detect the changes in lymphocyte subset numbers in response to dioscorin treatment, mice were injected intraperitoneally with 0.5 or 2 mg/kg of bwt TN1-dioscorins and Dj-dioscorins as described under Materials and Methods. Spleen cells and thymic cells isolated from the mice were detected with antibody against the surface markers of $CD4^+$ (labeled on T helper cells), $CD8^+$ (labeled on cytotoxic T cells), $Tim3^+$ (labeled on T helper 1 cells), $Tim1^+$ (labeled on T helper 2 cells), and $CD19^+$ (labeled on B cells). The results demonstrated that the percentages of $CD4^+$, $CD8^+$, $Tim3^+$, $Tim1^+$, and $CD19^+$ cells in spleen cells were not increased in response to TN1-dioscorins (**Figure 5A**). Similarly, the percentages of $CD4^+$ and $CD8^+$ in thymic cells were not increased following TN1-dioscorin treatment (**Figure 5B**), and the percentage of $Tim3^+$ cells in thymic cells even decreased in response to TN1-dioscorins. In contrast, the percentage of $Tim1^+$ cells in thymic cells was increased in response to TN1-dioscorins at an injection dose of 2 mg/kg (**Figure 5B**). Furthermore, an ascending trend in cell counts was noted for $CD19^+$ subsets at elevated concentrations of TN1-dioscorins. Taken together, the above results suggested that TN-1 dioscorins could only slightly stimulate the proliferation of $Tim1^+$ and $CD19^+$ cells in thymus.

Proliferation of the cell subsets in spleen and thymus from Dj-dioscorins injected mice revealed different results from those with TN1-dioscorins. Except for the $Tim1^+$ subsets, the percentages of $CD4^+$, $CD8^+$, $Tim3^+$, and $CD19^+$ cells were higher in

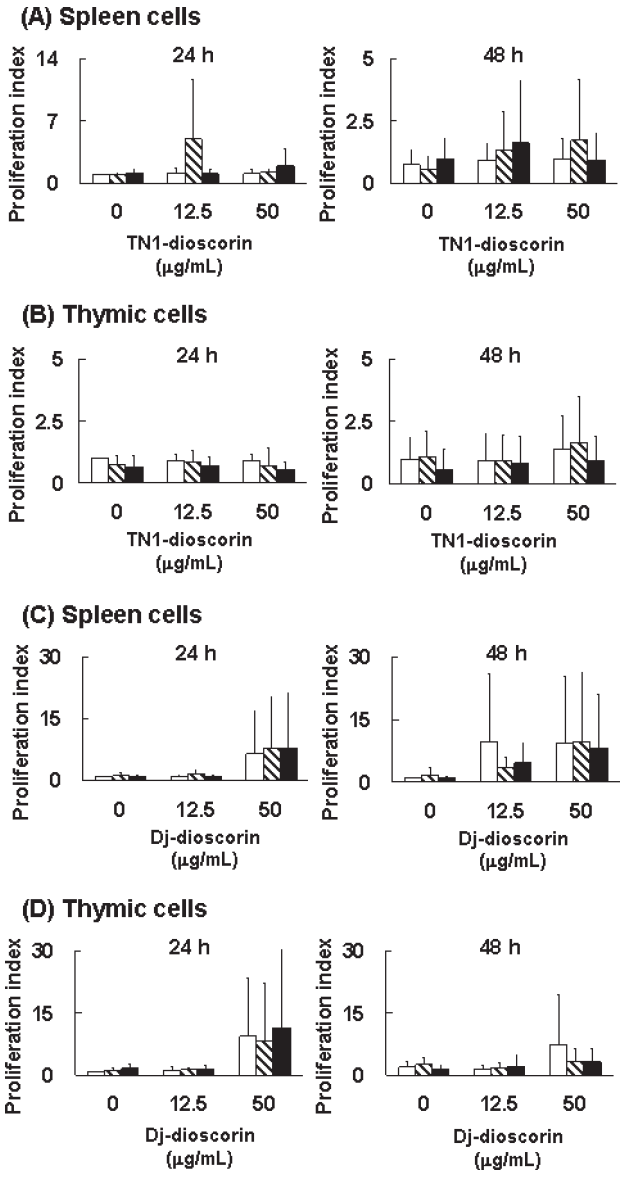

Figure 6. Effect of supplement of dioscorins on proliferation of the lymphoid cells ex vivo. The spleen cells and thymic cells were harvested from mice injected with TN1-dioscorins (**A**, **B**) or Dj-dioscorins (**C**, **D**). The cells were individually treated with 0, 12.5, and 50 μg/mL of TN1-disocorins (**A**, **B**) or Dj-dioscorins (**C**, **D**) followed by cell proliferation assay. The white bar indicates the cells from PBS-injected mice, the slashed bar indicates the cells from mice injected with 0.5 mg/kg of bwt of dioscorins, and the black bar indicates the cells from mice injected with 2 mg/kg of bwt of dioscorins.

spleen cells isolated from mice injected with 0.5 or 2 mg/kg of bwt Dj-dioscorins as compared to those of vehicle controls (mice injected with PBS, **Figure 5C**). Although the differences were not significant, an ascending trend in cell counts was noted in these cell subsets at elevated concentrations of Dj-dioscorins. No difference in the ratio of $CD4^+$ to $CD8^+$ cells was observed in spleen cells or thymic cells. The percentages of $CD4^+$, $CD8^+$, $Tim3^+$, and $Tim1^+$ cells in thymic cells isolated from mice injected with either 0.5 or 2 mg/kg of bwt Dj-dioscorins were not increased or even decreased (**Figure 5D**). However, the percentage of $CD19^+$ cells was increased in thymic cells isolated from mice injected with 2 mg/kg of bwt but not with 0.5 mg/kg of bwt Dj-dioscorin. Overall, the above results demonstrated that T cells are mainly proliferated in spleen cells but not in thymic cells when treated with Dj-dioscorins, whereas B cells are proliferated in both spleen cells and thymic cells in response to Dj-dioscorin treatment.

In Vitro Treatment of Dioscorins. To understand the effect of supplement of dioscorins ex vivo on lymphoid cells harvested from mice that were predisposed to dioscorins, the lymphoid cells isolated from mice intraperitoneally injected with 0.5 or 2 mg/kg of bwt TN1-dioscorins or Dj-dioscorins were treated with 12.5 and 50 μg/mL TN1-dioscorins or Dj-dioscorins for 2 days in a 96-well ELISA plate, and proliferation of the lymphoid cells was monitored. The results revealed that supplement of 12.5 μg/mL TN1-dioscorins for 24 h induced proliferation 5-fold of spleen cells isolated from mice predisposed to 0.5 mg/kg injection as compared to the control (cells isolated from mice injected with PBS and supplemented with no dioscorins). Other than that, supplement of 12.5 or 50 μg/mL TN1-dioscorins did not significantly induce proliferation of spleen cells or thymic cells at 24 or 48 h after treatment, no matter what the origin of the lymphoid cells was (**Figure 6A**,**B**). However, supplement of 50 μg/mL Dj-dioscorin induced proliferation of spleen cells at about 6−8-fold at 24 h, regardless of predisposition of mice to Dj-dioscorins or not (**Figure 6C**). When treated for 48 h, supplement of 12.5 μg/mL Dj-dioscorins induced proliferation of spleen cells at about 3−10-fold higher than from non-predisposed mice. Moreover, supplement of 50 μg/mL Dj-dioscorins induced proliferation of spleen cells at about 8−10-fold, regardless of the source of the cells. Like spleen cells, supplement with 50 μg/mL Dj-dioscorins for 24 h induced thymic cell proliferation approximately 8−12-fold higher than lower dose treatments, regardless of the source of the cells (**Figure 6D**). When treated for 48 h, supplement of 50 μg/mL Dj-dioscorins induced proliferation of thymic cells at about 3−7-fold with higher proliferation for thymic cells isolated from mice that were not predisposed to Dj-dioscorins. Taken together, these data revealed that proliferation of the lymphoid cells from untreated mice or mice predisposed with Dj-dioscorins could be induced in response to the addition of Dj-dioscorins. The proliferation ratios of lymphoid cells from untreated mice were similar to or higher than those from Dj-dioscorin-treated mice. In contrast, the TN1-dioscorins did not possess the ability to stimulate proliferation of the lymphoid cells.

DISCUSSION

In this study, the results showed that Dj-dioscorins, like TN1-dioscorins, could induce expression of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α and stimulated phagocytosis of RAW 264.7, indicating that Dj-dioscorins had the same immunomodulatory activity as TN1-dioscorins (**Figures 1** and **2**) (*17*, *18*). However, the immunomodulatory activities of Dj-dioscorins in vivo differed from those of TN1-dioscorins. Intraperitoneal injection of the TN1-dioscorins in mice stimulated the phagocytic effect of bone marrow cells, spleen cells, and thymic cells against *E. coli* (**Figure 3A**). In contrast, Dj-dioscorins had no effect on stimulation of phagocytosis of the lymphoid cells, including bone marrow cells, spleen cells, and thymic cells (**Figure 3B**). These results suggested that TN1-dioscorins presented different immunological properties from Dj-dioscorins with regard to stimulation of phagocytosis of lymphoid cells in vivo.

The immunomodulatory effects in response to mitogen stimulations by TN1-dioscorins or Dj-dioscorins pretreated lymphocytes from various tissues also exhibited distinct activation patterns. The lymphocytes isolated from mice treated with Dj-dioscorins responded to the mitogen, ConA and LPS, stimulation (**Figure 4C**,**D**). In contrast, the lymphocytes isolated from mice treated with TN1-dioscorins had no response to ConA and LPS

stimulation (**Figure 4A**,**B**). The different immunomodulatory activities between Dj-dioscorins and TN1-dioscorins were further demonstrated by their ability to stimulate proliferation of the T and B cell subsets of the different lymphoid cells in the injected mice (**Figure 5**). Intraperitoneal injection of Dj-dioscorins in mice stimulated proliferation of $CD4^+$, $CD8^+$, and Th1 cells in spleen but not in thymus and of $CD19^+$ cells in both spleen and thymus (**Figure 5C**,**D**), whereas TN1-dioscorins in vivo only slightly stimulated proliferation of the $CD19^+$ cells in thymus (**Figure 5B**). Taken together, our studies indicate that Dj-dioscorins have an immunomodulatory activity that is different from that of TN1-dioscorins. This difference is further supported by testing the effect of adding dioscorins on proliferation of the predisposed lymphoid cells ex vivo (**Figure 6**). Different lymphoid cells isolated from TN1-dioscorin and Dj-dioscorin primed mice were added with dioscorins. No cell proliferation was observed from the lymphoid cells added with TN1-dioscorins (**Figure 6A**,**B**), whereas cell proliferation was observed in spleen cells and thymic cells isolated from PBS-treated and Dj-dioscorin-treated mice and subsequently incubated with 12.5 or 50 μg/mL Dj-dioscorins for 24 or 48 h (**Figure 6C**,**D**). No stimulating effect of the TN1-dioscorins on the lymphoid cells suggested a tolerance (hyporesponsiveness) or an anergy effect toward TN1-dioscorins. The immunological hyporesponsiveness has been broadly discussed in LPS pretreatment. The phenomenon known as endotoxin (LPS) tolerance is a state of altered responsiveness to bacterial endotoxin in the host immune cells, which has been described both in experimental animals and in humans (*21*, *22*). Endotoxin tolerance can be induced both in vitro and in vivo (*21*−*23*). Moreover, LPS pretreatment decreases of TLR-2 and TLR-4 proteins in the spleens of mice during endotoxin tolerance could be due to the induction of cross-tolerance of TLR-2 to LPS challenge in vivo (*24*). In contrast, the stimulatory effect of Dj-dioscorins on the lymphoid cells suggests that Dj-dioscorins have a hypersensitive effect on cell proliferation of mice. This result supports the idea that the ability for stimulating cell proliferation by Dj-dioscorin is higher than that by TN1-dioscorins.

Although the cell types proliferated in bone marrow were not determined, the T helper cells with $CD4^+$ and $Tim3^+$ markers and cytotoxic cells with the $CD8^+$ marker were proliferated in splenocytes in response to Dj-dioscorin treatment (**Figure 5C**). However, the $CD4^+$, $CD8^+$, $Tim3^+$, and $Tim1^+$ cells were not proliferated in thymic cells in response to Dj-dioscorin treatment (**Figure 5D**). This result indicates that the Dj-dioscorins stimulate different immune responses in different lymphoid tissues. The TN1-dioscorins could not stimulate the $CD4^+$, $CD8^+$, $Tim3^+$, and $Tim1^+$ cells, further suggesting that TN1-dioscorins have an immunomodulatory activity that differs from that of Dj-dioscorins (**Figure 5A**). Proliferation of the B cells was stimulated in bone marrow, spleen cells, and thymic cells in response to Dj-dioscorins followed by the induction of mitogen LPS (**Figure 4D**). This result coincided with proliferation of the B cells with $CD19^+$ surface marker stimulated by Dj-dioscorins in the spleen cells and thymic cells (**Figure 5C**,**D**), suggesting possible activation of the adaptive immune response in mice by Dj-dioscorins.

In the in vivo study, TN1-dioscorin primed mice showed enhanced phagocytosis of lymphoid cells and depletion of the B and T cell (hyperreactive). By contrast, Dj-dioscorin primed mice presented hyporesponsiveness of phagocytosis on lymphoid cells and hyporeactive proliferation of B and T cells in vivo. Various immunomodulatory effects between two proteins could be attributed to their physical and biochemical properties. Differences in the primary structure, secondary structure, and glycosylation between the two proteins may contribute to the different immunomodulatory activities between TN1-dioscroins and Dj-dioscorins. Comparison of the primary structures between TN1-dioscorin and Dj-dioscorin isoforms showed 88−91% similarity. The results of Fourier transform Raman spectroscopy indicated that the secondary structures of different species yam dioscorins were quite different (*25*). The secondary structures of dioscorins from *D. alata* L. are predominantly α-helices, whereas the dioscorins from *D. alata* L. var. *purpurea* contain mostly antiparallel β-sheets. In contrast, the secondary structures of dioscorins from *D. japonica* exhibit a mixed form of α-helices and antiparallel β-sheets. Furthermore, dioscorins of different yam species or cultivars may have different levels of glycosylation. Dioscorins extracted from yam tubers of *D. ratundata* are not glycoproteins (*5*), whereas the dioscorins from yam tubers of *D. cayenensis* and *D. batatas* are glycoproteins (*11*). Therefore, the level of glycosylation of the dioscorins might be different among different species or cultivars. All of the above studies suggest that the tertiary structures of the dioscorins among different species or cultivars may be quite different from each other. In essence, this proposal needs to be further explored. In addition, the dioscorins extracted from yam tubers are a mixture of dioscorin proteins with different p*I* values, and it is unclear which dioscorin isoform is in the majority in the yam tubers.

It is unclear why the Dj-dioscorins could stimulate phagocytosis of RAW 264.7 in vitro but not phagocytosis of lymphoid cells in vivo (**Figures 2** and **3**). This might be due to the different model systems used. Herb medicines extracted from the same species have been shown to exhibit opposing effects when used in different model systems. For example, milk thistle (*Silybum marianum*), a commonly used herbal product, is known to inhibit growth of certain tumors. Murine lymphocytes treated with milk thistle extract in vitro show an increase in IL-4 expression (*26*). The IL-4 expression, however, is decreased in mice intraperitoneally injected with milk thistle extract (*27*). The Indian herb *Withania somnifera* (ashwagandha) has been used in the treatment of wasting syndromes, general debility, and certain tumors (*28*). Administration of the extract of *W. somnifera* in mice has been shown to increase the level of TNF-α (*29*). However, the extract from *W. somnifera* reduces the TNF-α production in macrophages isolated from mice (*30*). Therefore, the evaluation of natural products in biological functions needs to consider (1) administration regimen (oral or injection), (2) in vivo or in vitro assay, and (3) sources of the natural products (cultivar or variety) for obtaining more precise information and interpretation. Overall, our results suggest for the first time that dioscorins, the major storage proteins of different yam species, may have different immunomodulatory activities. Our results also imply that consumption of a small amount of Tainong No. 1 and Japanese yam raw extracts could be helpful in stimulating macrophage function and immunomodulatory effect on the mucosal-associated lymphocyte tissues, respectively, whereas consumption of a large amount of Japanese yam raw extracts might cause inflammation.

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LITERATURE CITED

(1) Hooker, E. Final report of the amended safety assessment of *Dioscorea villosa* (wild yam) root extract. *Int. J. Toxicol.* **2004**, *23*, 49–54.

(2) Hou, W. C.; Hsu, F. L.; Lee, M. H. Yam (*Dioscorea batatas*) tuber mucilage exhibited antioxidant activities in vitro. *Planta Med*. **2002**, *68*, 1072–1076.
(3) Lee, M. H.; Lin, Y. S.; Lin, Y. H.; Hsu, F. L.; Hou, W. C. The mucilage of yam (*Dioscorea batatas* Decne) tuber exhibited angiotensin converting enzyme inhibitory activities. *Bot. Bull. Acad. Sin*. **2003**, *44*, 267–273.
(4) Shang, H. F.; Cheng, H. C.; Liang, H. J.; Liu, H. Y.; Liu, S. Y.; Hou, W. C. Immunostimulatory activities of yam tuber mucilages. *Bot. Stud*. **2007**, *48*, 63–70.
(5) Harvey, P. J.; Boulter, D. Isolation and characterization of the storage protein of yam tubers (*Dioscorea ratundata*). *Phytochemistry* **1983**, *11*, 1687–1693.
(6) Oh, P. S.; Lim, K. T. HeLa cells treated with phytoglycoprotein (150 kDa) were killed by activation of caspase 3 via inhibitory activities of NF-κB and AP-1. *J. Biomed. Sci*. **2007**, *14*, 223–32.
(7) Lee, S. J.; Lim, K. T. Phytoglycoprotein inhibits interleukin-1β and interleukin-6 via p38 mitogen-activated protein kinase in lipopolysaccharide-stimulated RAW 264.7 cells. *Naunyn Schmiedebergs Arch. Pharmacol*. **2008**, *377*, 45–54.
(8) Oh, P. S.; Lim, K. T. Blocking of intracellular ROS production by phytoglycoprotein (30 kDa) causes anti-proliferation in bisphenol A-stimulated Chang liver cells. *J. Appl. Toxicol*. **2008**, *28*, 749–758.
(9) Conlan, S.; Griffiths, L. A.; Turner, M.; Fido, R.; Tatham, A.; Ainsworth, C.; Shewry, P. Characterisation of the yam tuber storage protein dioscorin. *J. Plant Physiol*. **1998**, *153*, 25–31.
(10) Hou, W. C.; Chen, H. J.; Lin, Y. H. Dioscorins, the major tuber storage proteins of yam (*Dioscorea batatas* Decne), with dehydroascorbate reductase and monodehydroascorbate reductase activities. *Plant Sci*. **1999**, *149*, 151–156.
(11) Hou, W. C.; Chen, H. J.; Lin, Y. H. Dioscorins from different *Dioscorea* species all exhibit both carbonic anhydrase and trypsin inhibitor activities. *Bot. Bull. Acad. Sin*. **2000**, *41*, 191–196.
(12) Hou, W. C.; Lee, M. H.; Chen, H. J.; Liang, W. L.; Han, C. H.; Liu, Y. W.; Lin, Y. H. Antioxidant activities of dioscorin, the storage protein of yam (*Dioscorea batatas* Decne) tuber. *J. Agric. Food Chem*. **2001**, *49*, 4956–4960.
(13) Gaidamashvili, M.; Ohizumi, Y.; Iijima, S.; Takayama, T.; Ogawa, T.; Muramoto, K. Characterization of the yam tuber storage proteins from *Dioscorea batatas* exhibiting unique lectin activities. *J. Biol. Chem*. **2004**, *279*, 26028–26035.
(14) Hewett-Emmett, D.; Tashian, R. E. Functional diversity, conservation, and convergence in the evolution of the α-, β-, and γ-carbonic anhydrase gene families. *Mol. Phylogenet. Evol*. **1996**, *5*, 50–77.
(15) Hsu, F. L.; Lin, Y. H.; Lee, M. H.; Lin, C. L.; Hou, W. C. Both dioscorin, the tuber storage protein of yam (*Dioscorea alata* cv. Tainong No. 1), and its peptic hydrolysates exhibited angiotensin converting enzyme inhibitory activities. *J. Agric. Food Chem*. **2002**, *50*, 6109–6113.
(16) Lin, C. L.; Lin, S. Y.; Lin, Y. H.; Hou, W. C. Effects of tuber storage protein of yam (*Dioscorea alata* cv. Tainong No. 1) and its peptic hydrolyzates on spontaneously hypertensive rats. *J. Sci. Food Agric*. **2006**, *86*, 1489–1494.
(17) Fu, S. L.; Hsu, Y. H.; Lee, P. Y.; Hou, W. C.; Hung, L. C.; Lin, C. H.; Chen, C. M.; Huang, Y. J. Dioscorin isolated from *Dioscorea alata* activates TLR4-signaling pathways and induces cytokine expression in macrophages. *Biochem. Biophys. Res. Commun*. **2006**, *339*, 137–144.
(18) Liu, Y. W.; Shang, H. F.; Wang, C. K.; Hsu, F. L.; Hou, W. C. Immunomodulatory activity of dioscorin, the storage protein of yam (*Dioscorea alata* cv. Tainong No. 1) tuber. *Food Chem. Toxicol*. **2007**, *45*, 2312–2318.
(19) Dauphinee, S. M.; Karsan, A. Lipopolysaccharide signaling in endothelial cells. *Lab Invest*. **2006**, *86*, 9–22.
(20) Chen, Y. T.; Lin, K. W. Effects of heating temperature on the total phenolic compound, antioxidative ability and the stability of dioscorin of various yam cultivars. *Food Chem*. **2007**, *101*, 955–963.
(21) Mathison, J. C.; Virca, G. D.; Wolfson, E.; Tobias, P. S.; Glaser, K.; Ulevitch, R. J. Adaptation to bacterial lipopolysaccharide controls lipopolysaccharide-induced tumor necrosis factor production in rabbit macrophages. *J. Clin. Invest*. **1990**, *85*, 1108–1118.
(22) Astiz, M. E.; Rackow, E. C.; Still, J. G.; Howell, S. T.; Cato, A. Von Eschen, K. B.; Ulrich, J. T.; Rudbach, J. A.; McMahon, G.; Vargas, R. Pretreatment of normal humans with monophosphoryl lipid A induces tolerance to endotoxin: a prospective, double-blind, randomized, controlled trial. *Crit. Care Med*. **1995**, *23*, 9–17.
(23) Rayhane, N.; Lortholary, O.; Fitting, C.; Callebert, J.; Huerre, M.; Dromer, F.; Cavaillon, J. M. Enhanced sensitivity of tumor necrosis factor/lymphotoxin-α-deficient mice to *Cryptococcus neoformans* infection despite increased levels of nitrite/nitrate, interferon-γ, and interleukin-12. *J. Infect. Dis*. **1999**, *18*, 1637–1647.
(24) Zhong, B.; Ma, H. Y.; Yang, Q.; Gu, F. R.; Yin, G. Q.; Xia, C. M. Decrease in toll-like receptors 2 and 4 in the spleen of mouse with endotoxic tolerance. *Inflamm. Res*. **2008**, *57*, 252–259.
(25) Liao, Y. H.; Wang, C. H.; Tseng, C. Y.; Chen, H. L.; Lin, L. L.; Chen, W. Compositional and conformational analysis of yam proteins by near infrared fourier transform Raman spectroscopy. *J. Agric. Food Chem*. **2004**, *52*, 8190–8196.
(26) Wilasrusmee, C.; Kittur, S.; Shah, G.; Siddiqui, J.; Bruch, D.; Wilasrusmee, S.; Kittur, D. S. Immunostimulatory effect of *Silybum marianum* (milk thistle) extract. *Med. Sci. Monit*. **2002**, *8*, 439–443.
(27) Johnson, V. J.; Osuchowski, M. F.; He, Q.; Sharma, R. P. Physiological responses to a natural antioxidant flavonoid mixture, silymarin, in BALB/c mice: II. Alterations in thymic differentiation correlate with changes in c-myc gene expression. *Planta Med*. **2002**, *68*, 961–965.
(28) Spelman, K.; Burns, J.; Nichols, D.; Winters, N.; Ottersberg, S.; Tenborg, M. Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators. *Altern. Med. Rev*. **2006**, *11*, 128–150.
(29) Dhuley, J. N. Effect of some Indian herbs on macrophage functions in ochratoxin A treated mice. *J. Ethnopharmacol*. **1997**, *58*, 15–20.
(30) Davis, L.; Kuttan, G. Effect of *Withania somnifera* on cytokine production in normal and cyclophosphamide treated mice. *Immunopharmacol. Immunotoxicol*. **1999**, *21*, 695–703.

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