Immunostimulatory effects of a subcritical water extract of *Ganoderma*

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Abstract. *Ganoderma*, a medicinal mushroom with various physiological activities, has been extensively investigated regarding its effectiveness. The aim of the present study was to examine the effects of a subcritical water extract of *Ganoderma* (SWEG) on the immune system. The use of subcritical water with a higher temperature and pressure than hot water allows efficient elution of components from natural products. As an evaluation of the effectiveness of SWEG, a cell proliferation and a cell differentiation test were carried out using A-6 cells, a model of hematopoietic stem cells. Furthermore, an oral administration test in mice was conducted to examine the effects of SWEG on the number and function of immune cells. As a result, SWEG was revealed to promote both self-renewal and differentiation into immune cells such as T cells and natural killer (NK) cells in experiments with A-6 cells. These results were not obtained in experiments using hot water extract of *Ganoderma lucidum* and *Ganoderma sinense*. The oral administration test in mice demonstrated that SWEG increased hematopoietic precursor cells, immature B cells, and NK cells in the bone marrow, and T cells in the thymus. In addition, SWEG enhanced the immune functions in the spleen by promoting granzyme B expression and NK cell activity. SWEG was demonstrated to be a food material that acts on HSCs and regulates immunity *in vivo*.

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Abbreviations: SWEG, subcritical water extract of *Ganoderma*; HWEG, hot water extract of *Ganoderma*; GL, *Ganoderma lucidum*; GS, *Ganoderma sinense*

Key words: *Ganoderma*, subcritical water, granzyme B, hematopoietic stem cells, immune functions

Introduction

Immunodeficiency leads to the onset of cancer and the development of infectious diseases, and increased mortality from these diseases has become a medical and social problem. It is considered that there is a positive correlation between aging and the incidence of cancer, which may be in part due to prolonged exposure periods to cancerous substances, genetic changes with aging, and the decrease in immunity of the aging population. Various immune cells, such as T cells, B cells, and natural killer (NK) cells, are involved in immunity. These immune cells originate from hematopoietic stem cells (HSCs), which sparsely exist in the bone marrow. HSCs with pluripotency and self-renewal ability differentiate into all types of immune cells, thereby supplying immune cells continuously for a lifetime (1). However, both proliferative and differentiating abilities of HSCs decrease with age, contributing to the decline of immunity with age (2-4).

Ganoderma, one of the most well-known medicinal mushrooms, has various physiological functions such as immunomodulatory, antitumor, hypolipidemic, antidiabetic, and antiarteriosclerotic effects (5-9). *Ganoderma* is generally extracted with hot water, and the efficacy of *Ganoderma* is mostly confirmed with hot water extracts. Furthermore, the use of subcritical water with a higher temperature and pressure than hot water has recently attracted attention as an extraction technique for natural products. Subcritical water with a high temperature (100-374˚C) and high pressure allows efficient elution of components from natural products and extraction of relatively low-polarity components and low-molecular-weight peptides generated by hydrolysis (10-12).

The immunomodulatory effects of the hot water extract of *Ganoderma* have already been reported (13,14). However, the effects of subcritical water extracts on the immune system have not been investigated in detail. Particularly, few studies have been published on the effects of subcritical water extracts on HSCs. Thus, the present study investigated the effects of subcritical water extract of *Ganoderma* (SWEG) on immunity.

Materials and methods

Antibodies. Antibodies against CD34-FITC (cat. no. 11-0341-82), Sca1-PE (cat. no. 12-5981-82), CD117-APC (cat. no. 17-1171-82), CD11b-biotin (cat. no. 13-0112-82), Gr1-biotin (cat. no. 13-5931-82), B220-biotin (cat. no. 13-0452-82), TER119-biotin (cat. no. 13-5921-82), and streptavidin-PE-Cy7 (cat. no. 25-4317-82) were purchased from Thermo Fisher Scientific, Inc. Biotinylated antibodies were detected using streptavidin-PE-Cy7. Antibodies against CD19-FITC (cat. no. 115505), NKp46-PE (cat. no. 137603), and CD3e-AF488 (cat. no. 300319) were obtained from BioLegend, Inc.

Preparation and molecular weight analysis of SWEG. *Ganoderma* [mixture of fruiting bodies of *Ganoderma lucidum* (GL) and *Ganoderma sinense* (GS); Nikkei Co., Ltd.] was subjected to extraction in a container for subcritical water treatment at 140-180˚C. Following filtration of the extract, the filtrate was concentrated and lyophilized to prepare SWEG. *Ganoderma* was then extracted with hot water at 95-100˚C, and filtered, concentrated, and lyophilized to prepare a hot water extract of *Ganoderma* (HWEG) as a reference sample for comparison of chemical properties with SWEG. As reference samples for comparison of the efficacy tests using cultured cells, hot water extracts of GL and GS were also prepared as aforementioned.

The molecular weights of SWEG and HWEG were measured using gel filtration HPLC. Develosil 100-Diol-5 (particle diameter 5 μm, 8.0 mmϕ x 500 mm, Nomura Chemical Co., Ltd.) was used as a column, and Shimadzu RID-10A as a detector (Shimadzu Corporation). The system conditions were as follows: The mobile phase consisted of 0.1 M phosphate buffer solution (pH 6.8); the required pH of the solution was prepared by mixing 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 solutions; the injected volume was 100 μl at a flow rate of 1.0 ml/min; and the column temperature was set at 25˚C. Pullulan (Molecular Weight Markers for Gel Filtration Chromatography; cat. no. 53168; Sigma-Aldrich; Merck KGaA) was employed as a standard sample for the molecular weight.

Measurement of β-glucan contents. α-Amylase (cat. no. 635-53982; FUJIFILM Wako Pure Chemical Corporation), protease (cat. no. P5380; Sigma-Aldrich; Merck KGaA), and amyloglucosidase (cat. no. A9913; Sigma-Aldrich; Merck KGaA) were added to SWEG and HWEG, dissolved in 50 mM phosphate buffer, for the enzymatic decomposition of macromolecules other than β-glucan. Subsequently, ethanol was added at 4-fold the amount of the reaction solution to precipitate β-glucan. To this precipitate, sulfuric acid was added for acid-hydrolysis of β-glucan. Glucose contained in this solution was quantified by Glucose Assay Kit-WST (cat. no. 346-09411; Dojindo Laboratories, Inc.) to calculate the β-glucan contents (%) in SWEG and HWEG (15).

Cells. A-6 cells (ES derived; cell no. RCB1517) were used as a model of HSCs (16,17). YAC-1 cells (cell no. RCB1165) were used as a target for the measurement of NK cell activity. A-6 cells and YAC-1 cells were provided by the RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan.

Cell viability test. A-6 cells were suspended in DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 1% fetal bovine serum (FBS) (Sigma-Aldrich; Merck KGaA), 1% antibiotic-antimycotic solution (Gibco; Thermo Fisher Scientific, Inc.), 10 μg/ml human transferrin (Sigma-Aldrich; Merck KGaA), 10 μg/ml human insulin (Sigma-Aldrich; Merck KGaA), 100 μM 2-mercaptoethanol (Gibco; Thermo Fisher Scientific, Inc.), and 5 ng/ml fibroblast growth factor 2 (PeproTech, Inc.). The suspension ($5x10^4$ cells) was seeded into a 96-well plate and incubated at 37˚C. At 24 h after seeding, SWEG, GL, or GS were added at concentrations of 40, 80, 160, and 325 μg/ml, followed by culturing for an additional 24 h at 37˚C. The cell proliferation-promoting effects of each of the extracts was examined by cell viability assay with Cell Counting Kit-8 (CCK-8; cat. no. 343-07623; Dojindo Laboratories, Inc.). Following the addition of 10 μl CCK-8 solution into each well, the cells were incubated for 2 h at 37˚C, and then the absorbance of each well was detected at a wavelength of 450 nm.

Cell differentiation induction test. A-6 cells were suspended in each differentiation induction media for T cells, B cells, and NK cells (18-20). T cell differentiation medium consisted of DMEM/F12 medium supplemented with 20% FBS, 1% antibiotic-antimycotic solution, 10 μg/ml human transferrin, 10 μg/ml human insulin, 100 μM 2-mercaptoethanol, 30 ng/ml FMS-related tyrosine kinase 3 ligand (Flt3L) (GenScript), 30 ng/ml stem cell factor (SCF) (NKMAX Co., Ltd.), and 30 ng/ml interleukin-7 (IL-7) (GenScript). B cell differentiation medium consisted of DMEM/F12 medium supplemented with 20% FBS, 1% antibiotic-antimycotic solution, 10 μg/ml human transferrin, 10 μg/ml human insulin, 100 μM 2-mercaptoethanol, 50 ng/ml SCF, 50 ng/ml Flt3L, 10 ng/ml IL-3 (GenScript), and 20 ng/ml IL-7. NK cell differentiation medium consisted of DMEM/F12 medium supplemented with 20% FBS, 1% antibiotic-antimycotic solution, 10 μg/ml human transferrin, 10 μg/ml human insulin, 100 μM 2-mercaptoethanol, 10 ng/ml Flt3L, 20 ng/ml SCF, 10 ng/ml IL-15 (GenScript), 5 ng/ml IL-3, and 20 ng/ml IL-7. Each suspension ($7.5x10^5$ cells) was seeded into a 12 well plate and incubated for 7 days at 37˚C. During the 7-day differentiation induction, SWEG, GL, or GS were added at 325 μg/ml. Each of the media was once changed on the 3rd day. Subsequently, 7 days later, total RNA was extracted from the cells using RNAiso Plus (cat. no. 9109; TaKaRa Bio, Inc.) solution for the expression analysis of marker genes, *Cd3e*, *Ptprc*, and *Id3*, characteristic of T cells, B cells, and NK cells, respectively, by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to examine the promoting effects of differentiation induction of the each of the extracts. As positive controls, high-dose cytokines for each differentiation media were added, i.e., 50 ng/ml IL-7 for T cell differentiation medium, 20 ng/ml IL-3 and 50 ng/ml IL-7 for B cell differentiation medium, and 20 ng/ml IL-15 and 50 ng/ml IL-7 for NK cell differentiation medium, respectively.

Animal breeding. A total of 17 female, 12-week-old mice weighing 35-45 g were purchased from Japan SLC, Inc. A controlled housing environment, at 23±1˚C with humidity at 55±15%, was used to maintain the animals, with alternate 12-h light/dark cycles. A commercial pellet diet and tap water were provided *ad libitum*. The animal study protocols were

approved (approval no. HS201708) by the Animal Experiment Committee of Nippon Menard Cosmetic Co., Ltd.

Administration of SWEG and tissue removal. After 1 week of preliminary breeding, the mice were divided into control (n=9) and SWEG (n=8) groups. The SWEG group was fed *ad libitum* with MF feed (Oriental Yeast Co., Ltd.) supplemented with 2% SWEG, and the control group was fed *ad libitum* with MF feed supplemented with 2% cornstarch (FUJIFILM Wako Pure Chemical Corporation) instead of SWEG for 30 days. Regarding the breeding period, in previous studies using mice, GL was confirmed to promote NK cell activity at oral administration for 30 days (21), and *Ganoderma formosanum* was also confirmed to promote the gene expression related to immune function of the spleen at oral administration for 32 days (22). With reference to these studies, the breeding period was set to 30 days in this study. Following breeding with SWEG-supplemented feed, pentobarbital sodium (200 mg/kg body weight, ip) was used for euthanasia. When the animal ceased breathing and no heartbeat was detected, the femurs, thymus, and spleen were removed. Bone marrow cells collected from the femurs and the thymus cells were used to conduct the population analysis of immune cells. Spleen cells collected from the spleen were used to examine the immune functions. Furthermore, the spleen was partially cut into small pieces and homogenized in RNAiso Plus (TaKaRa Bio, Inc.) solution for RNA extraction to analyze gene expression relevant to NK cell activity by RT-qPCR.

Population analysis of immune cells by flow cytometer (FCM). The femurs removed from the mice were resected at both ends to collect bone marrow cells. To calculate the number of immune cells in the bone marrow, the surface antigens of the collected bone marrow cells were analyzed using FACSAria flow cytometer and FlowJo software (version 10.5.3) (Becton, Dickinson and Company) to identify the cell types. Bone marrow cells (0.5-1.0x10^7 cells) were stained with aforementioned antibodies in 0.1% BSA containing PBS buffer at 4˚C for 30 min. Each analysis was assessed with 1.0x10^6 cells on the FCM. Cells identified as $CD34^-$, $Sca1^+$, $CD117^+$, $lineage^-$ (Lin^-) were analyzed as HSCs, those identified as $Sca1^+$, $CD117^+$, Lin^- as hematopoietic precursor cells (HPCs), those identified as $B220^+$, $CD19^-$ as immature B cells, and those identified as $NKp46^+$ as NK cells. The lineage marker was defined as the combination of the following antibodies: CD3e (Clone: 145-2C11), CD11b (Clone: M1/70), Gr1 (Clone: RB6-8C5), B220 (Clone: RA3-6B2), and TER119 (Clone: TER-119).

Furthermore, the thymus removed from the mice was ground on a metal mesh and suspended in 0.1% BSA containing PBS buffer. The thymus cells (0.5-1.0x10^7 cells) were stained with CD3e antibody at 4˚C for 30 min to calculate the number of T cells by FCM as aforementioned. Cells identified as $CD3e^+$ were analyzed as T cells.

NK cell activity and cytokine expression. To prepare the spleen cells, the spleen was ground on a metal mesh and suspended in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. The spleen cells (1x10^6 cells) were seeded into a U-shaped 96-well plate, to which 2.5x10^4 target cells (YAC-1) were added, followed by culturing at 37˚C for 20 h. Subsequently, the 96-well plate was centrifuged at 250 x g for 10 min at 25˚C, and the supernatant was subjected to assessment of lactate dehydrogenase activity with an LDH Cytotoxicity Detection Kit (cat. no. MK401; TaKaRa Bio, Inc.), to determine NK cell activity, according to the manufacturer's instructions. Furthermore, granzyme B and interferon-gamma (IFN-γ) in the culture supernatant of spleen cells were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (for granzyme B, cat. no. ELM-GranzymeB-1; for IFN-γ, cat. no. ELM-IFNg-1; RayBiotech Life, Inc.). The expression levels of mRNAs relevant to NK cell activity were determined using RT-qPCR to examine the effects of SWEG on transcription. In addition, correlation analysis between granzyme B and IFN-γ expression and NK cell activity in the spleen for all mice, including both control and SWEG groups, were carried out. Measured values of granzyme B and IFN-γ expression and NK cell activity were plotted, calculating Pearson's product-moment correlation coefficients to reveal which cytokine was more closely related to NK cell activity.

RT-qPCR. First strand cDNA synthesis was performed with the RNA as the template (500 ng) using High Capacity RNA-to-cDNA Kit (cat. no. 4374967; Thermo Fisher Scientific, Inc.). Reverse transcription was performed at 37˚C for 60 min and then at 95˚C. qPCR amplification was performed using SYBR Select Master Mix (cat. no. 4472919; Thermo Fisher Scientific, Inc.). The thermocycling conditions were: Denaturation at 95˚C for 15 sec, annealing and extension at 60˚C for 60 sec for 40 cycles. qPCR was performed using Step One Plus Real-Time PCR System (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The results were calculated using the $2^{-\Delta\Delta Cq}$ method (23), normalized to GAPDH mRNA levels and reported as the relative fold change. Primers synthesized by Nippon Gene Co., Ltd. were used for mRNA amplification. The sequences of the primers are listed in Table SI.

Statistical analysis. All experimental results are expressed as the means ± standard error (SE). Statistical analyses were conducted in R (Version 3.6.0). Differences between two groups were assessed using unpaired Student's *t*-test. Differences between multiple groups were compared using one-way ANOVA with post-hoc Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference. The analysis of correlations between granzyme B and IFN-γ expression and NK cell activity was based on Pearson's product-moment correlation coefficient. Correlation coefficients were graded as follows: Low ($r<0.5$), moderate ($0.5\leq r<0.7$), and high ($r\geq0.7$) (24,25). $P<0.05$ indicated a significant correlation.

Results

Comparison of chemical properties between SWEG and HWEG. The distributions of the molecular weights of SWEG and HWEG were examined using pullulan as the standard sample of molecular weight, demonstrating an increase in the peak of ~2.2 kDa for SWEG (Fig. 1A). The β-glucan contents in SWEG and HWEG were 36.7 and 8.1%, respectively (Fig. 1B). The partial degradation and conformation changes of β-glucan

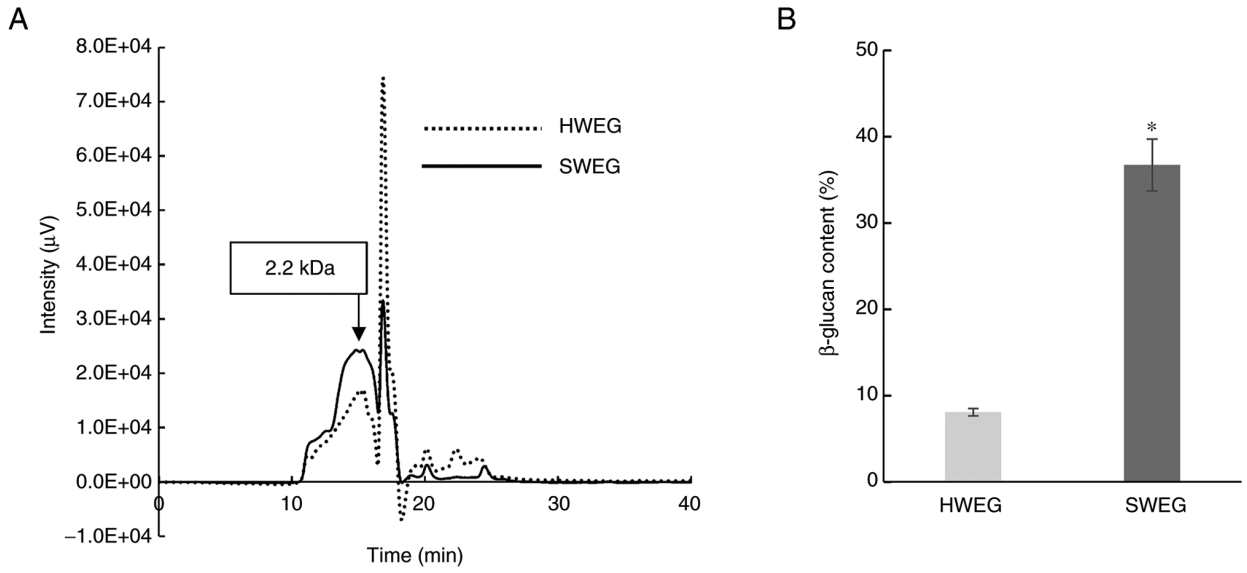

Figure 1. Comparison of the chemical characteristics between SWEG and HWEG. (A) Distribution of the molecular weights of SWEG and HWEG was analyzed using Develosil 100-Diol-5 as a column and Shimadzu RID-10A as a detector. (B) Comparison of β-glucan contents of SWEG and HWEG. Following acid-hydrolysis of β-glucan of SWEG and HWEG, the amount of glucose was quantified using the glucose oxidase method. Data are presented as the mean ± SE (n=3). *P<0.05 compared to HWEG. SWEG, subcritical water extract of *Ganoderma*; HWEG, hot water extract of *Ganoderma*.

have been reported after heat treatment (26), hence these differences in SWEG and HWEG appear to have occurred due to the thermal energy of the extraction temperature.

The ~18-min HWEG signal below zero in Fig. 1A (dotted line) may be caused by bubbles dissolved in the mobile phase solution or sample solution used for HPLC analysis. This may be caused due to insufficient deaeration in the mobile phase solution or sample solution.

Effects on the self-renewal and differentiation abilities of HSCs. A-6 cells cultured for 1 day after the addition of SWEG exhibited enhanced cell viability in a concentration-dependent manner, as compared with the control group (Fig. 2A). Since the addition of 325 μg/ml SWEG exhibited a clear effect on the cell viability, this concentration (325 μg/ml) was applied to the subsequent cell differentiation test. As a result, the addition of SWEG to A-6 cells during differentiation induction promoted the expression of marker genes, *Cd3e* and *Id3*, characteristic of T cells and NK cells, respectively (Fig. 2B). Thus, SWEG promoted both self-renewal and differentiation into immune cells in the A-6 cells, whereas GL and GS had little effect. Regarding the gene expression analysis conducted on differentiation induction test in the A-6 cells, high-dose cytokines as positive controls also promoted the differentiation into immune cells (Fig. S1).

Increases in immune cells in vivo. The bone marrow cells removed from femurs and the thymus cells were examined for the population analysis of immune cells by FCM. Since the cell surface antigens CD3e, CD11b, Gr1, B220, and TER119 are expressed in immune cells such as NK cells, T cells, monocytes, macrophages, and dendritic cells, and also, these antigens are considered not to be expressed in immature cells, the immature cell population in bone marrow was first separated as Lin^- ($CD3e^-$, $CD11b^-$, $Gr1^-$, $B220^-$, and $TER119^-$) cells (27,28). To detect HSCs and HPCs, CD117, Sca1, and CD34 antibodies on the subpopulation of Lin^- cells were used and the Lin^- $Sca1^+$ $CD117^+$ cells (HPCs) and Lin^- $Sca1^+$ $CD117^+$ $CD34^-$ cells (HSCs) (Fig. 3A and B) were quantified. As a result, FCM analysis demonstrated significant increases in HPCs, immature B cells, and NK cells from femurs, and T cells from the thymus after the administration of SWEG, as compared with the control group (Fig. 3C).

Enhancing effects on immune functions. Immune functions were examined with the spleen cells prepared from the spleens of the reared mice. The NK cell activity in the SWEG group was significantly higher than that in the control group (Fig. 4A). The expression of granzyme B and IFN-γ in the spleen cells of the SWEG group were significantly higher than those of the control group (Fig. 4B and C). To reveal the relevant mechanisms, the gene expression levels relevant to NK cell activity were examined in the removed spleens, demonstrating the enhanced gene expression levels of multiple factors critical for NK cell activity, especially *Gzmb* (granzyme B) (Fig. 5).

Correlation between granzyme B and IFN-γ expression and NK cell activity. There were significant correlations both between granzyme B expression and NK cell activity ($P<0.05$), and IFN-γ expression and NK cell activity ($P<0.05$). The correlation coefficients were 0.844 (graded as high) for granzyme B and 0.683 (graded as moderate) for IFN-γ, respectively (Fig. 6A and B).

Discussion

Subcritical water may enhance the potentials of natural products because it allows extraction of components that cannot be easily obtained by routine hot water extraction (29,30). In the present study, *Ganoderma*, a type of medicinal mushroom, was subjected to subcritical water extraction to examine its

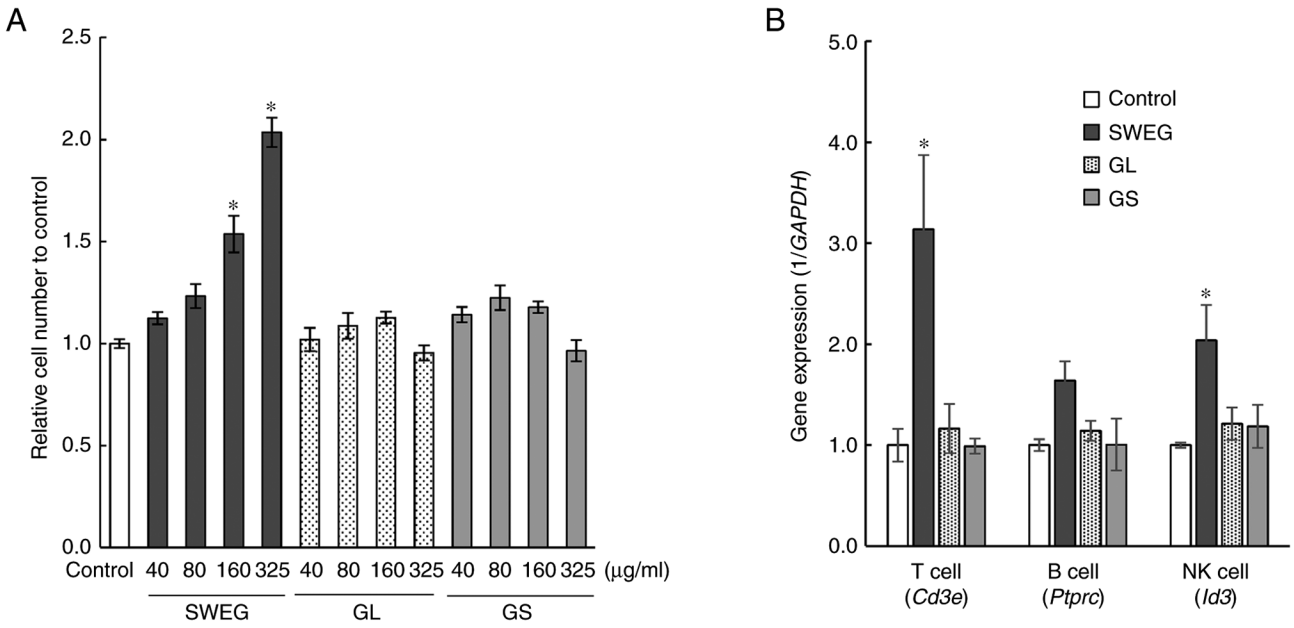

Figure 2. Cell viability and differentiation tests with A-6 cells. (A) SWEG, GL, or GS were added to the cells at concentrations of 40, 80, 160 and 325 μg/ml, followed by culturing for 24 h. The promoting effects on the cell proliferation of these extracts were examined by cell viability assay. Data are presented as the mean ± SE (n=6). *P<0.05 compared with the control. (B) A-6 cells were cultured to differentiate into T cells, B cells, and NK cells under respective differentiation-inducing conditions. During the differentiation induction, SWEG, GL, or GS were added at 325 μg/ml, followed by the analysis of the expression of marker genes, *Cd3e*, *Ptprc*, and *Id3*, characteristic of T cells, B cells, and NK cells, respectively. Data are presented as the mean ± SE (n=3). *P<0.05 compared with the control. SWEG, subcritical water extract of *Ganoderma*; GL, hot water extract of *Ganoderma lucidum*; GS, hot water extract of *Ganoderma sinense*; NK, natural killer.

effects on immunity. *Ganoderma* with immunomodulatory effects contains polysaccharides, and various polysaccharides have been separated depending on the molecular weight, constituent monosaccharides, and branched structures of polysaccharides (31-33). A purified polysaccharide with a molecular weight of >3,000 kDa has also been reported (34). SWEG and HWEG, used in the present study, contained large amounts of relatively low-molecular-weight components. However, SWEG differed from HWEG, i.e., SWEG contained larger amounts of components with a molecular weight of ~2.2 kDa than HWEG. This may be explained by the fact that larger amounts of components of ~2.2 kDa were extracted due to the hydrolysis of high-molecular-weight polysaccharides in *Ganoderma* by high-temperature and high-pressure subcritical water treatment. Furthermore, SWEG contained β-glucan at >4-fold compared with HWEG. β-glucan forms a strong triple helix structure in water, but it has been reported that the structure collapses when dissolved at temperatures >135˚C, and then adopts less organized conformations to form random coils (35,36). Since over 140˚C of extraction temperature was employed to obtain SWEG from *Ganoderma* in the present study, β-glucan extraction from the structure with reduced solidity was presumed to be more efficient. Although the binding energy of β-glucan is considered to be closely related to the ease of extraction of β-glucan, experimental information on the association between the thermal energy of the subcritical water treatment and the binding energy of β-glucan is not available at this stage. The cleavage of β-glucan during the heating process can be detected as the formation of the oxidized functional groups, i.e., carbonyl groups along the chain (37). In addition, the structural changes in β-glucan due to heat treatment can be investigated by analysis methods such as X-ray fiber diffraction (38), carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy (39), fluorescence resonance energy transfer (FRET) spectroscopy (40), and molecular dynamic simulation (41). By using such analytical techniques, it may be possible to investigate the effect of subcritical water treatment on *Ganoderma* in more detail. At present, the details of the extract from *Ganoderma* by subcritical water treatment are not clear, but at least, SWEG was confirmed to differ from HWEG in the molecular weight distributions and β-glucan contents. Therefore, the efficacy study focused on the effects of SWEG on immunity, especially on HSCs.

The effects of SWEG on the self-renewal and differentiation abilities of A-6 cells with the same properties as HSCs were examined. GL and GS, which are routine hot water extracts, were also subjected to the experiments as reference samples. As a result, among these extracts, only SWEG promoted both self-renewal and differentiation into immune cells. Recent studies have indicated that several factors may be responsible for efficacy of *Ganoderma*. With regard to extraction temperature, a previous study found that extracts of *Ganoderma* with water below 100˚C exhibit high antioxidant capacity and cytoprotective effects against oxidative damage (42). In terms of molecular weight of *Ganoderma* polysaccharides, the association between molecular weights and biological activities of the polysaccharides have been demonstrated in several studies. For instance, high-molecular weight polysaccharides exhibited better mitigation effects on ethanol-induced acute gastric injury than low-molecular weight polysaccharides in rats (43). By contrast, another study revealed that low-molecular weight polysaccharides exhibited stronger antioxidant activities than high-molecular weight polysaccharides in several *in vitro* assays (44). Thus, the extraction temperature for *Ganoderma*

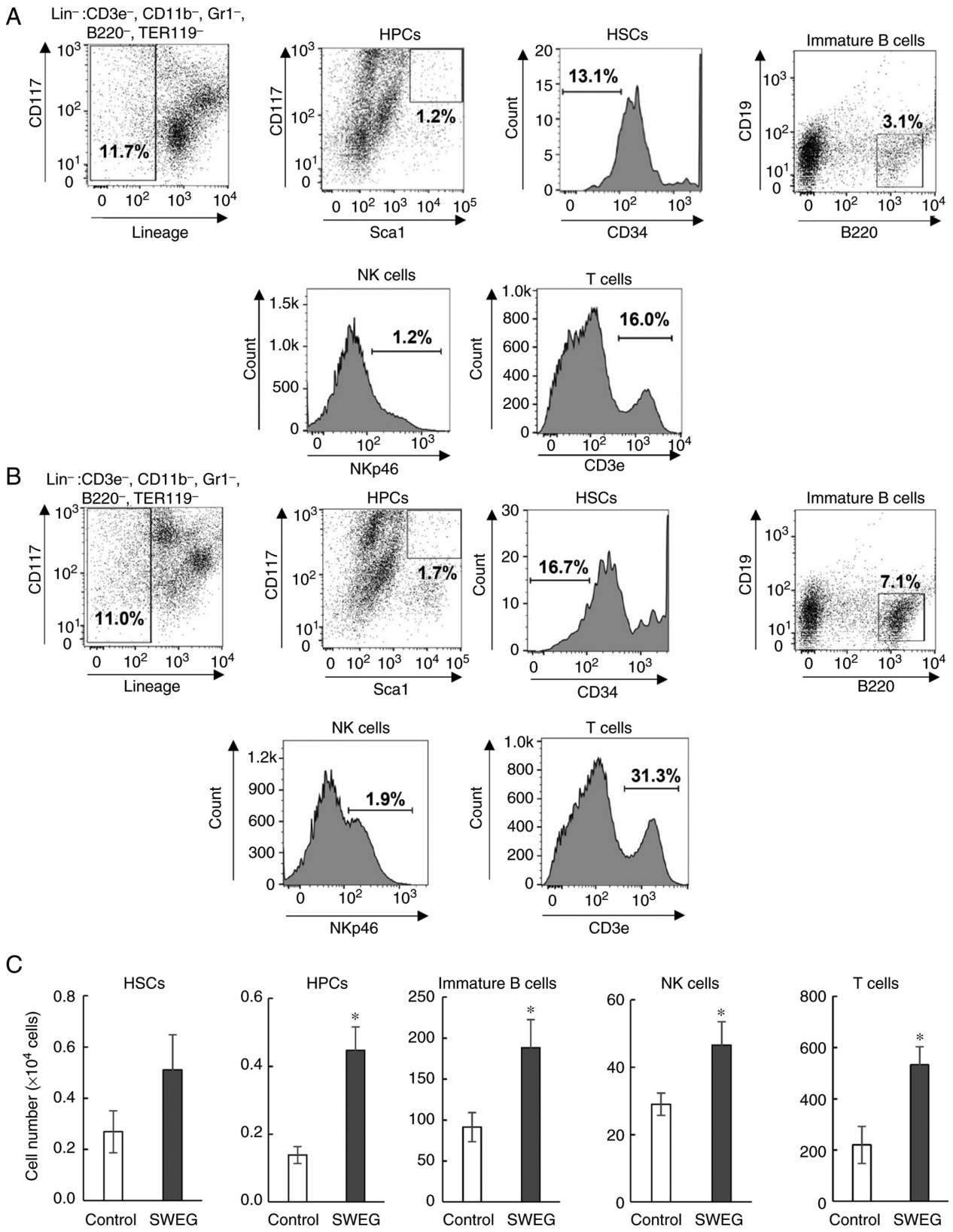

Figure 3. Representative flow cytometric dot plots and histograms of immune cells in (A) the control group and (B) the SWEG group. The far left dot plot represents Lin⁻ ($CD3e^-$, $CD11^-$, $Gr1^-$, $B220^-$, and $TER119^-$) bone marrow cells. (C) Comparison of the number of HSCs, HPCs, immature B cells, NK cells, and T cells in the control group and SWEG group. Data are presented as the mean ± SE (n=9, control group; n=8, SWEG group). *P<0.05 compared with the control. SWEG, subcritical water extract of *Ganoderma*; HSCs, hematopoietic stem cells; HPCs, hematopoietic precursor cells; NK, natural killer.

and the molecular weight of the resulting extract are closely related to bioactivity. In the present study, SWEG was confirmed to contain components with a molecular weight of ~2.2 kDa, and to have unique effects on A-6 cells. In previous studies focusing on immunomodulatory effects, GL polysaccharides with relatively high molecular weights have been

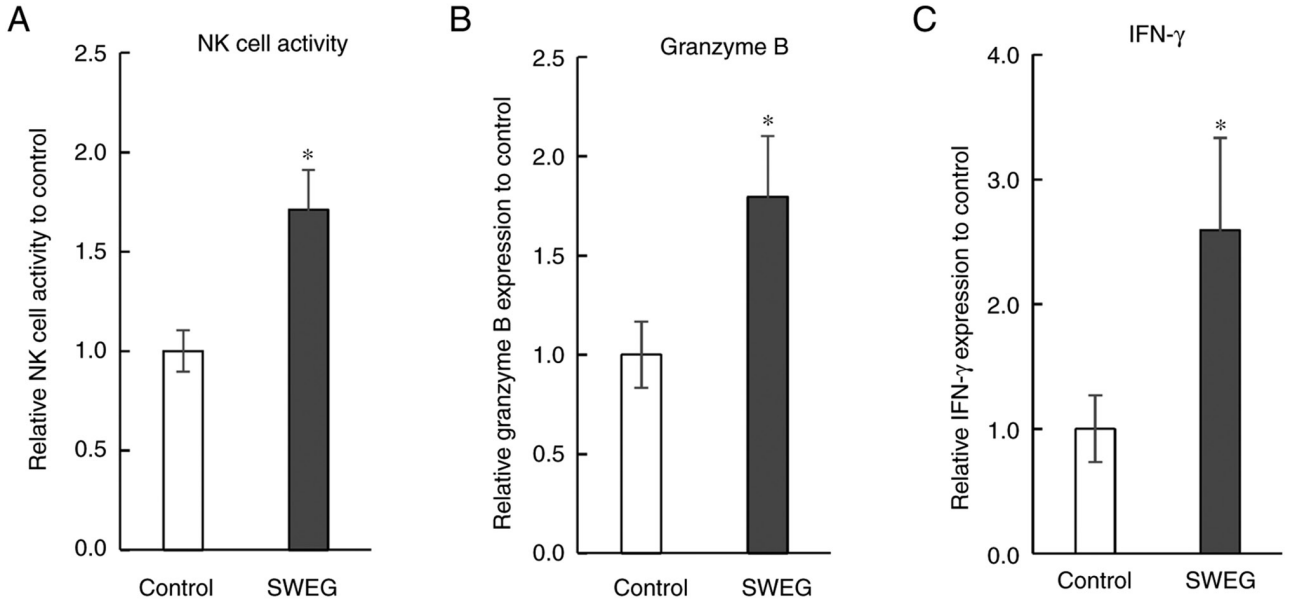

Figure 4. Effects of SWEG on NK cell activity and cytokine expression. (A) Spleen cells were seeded into a 96-well plate, to which target cells (YAC-1) were added. Subsequently, the supernatant of the culture was subjected to assessment of lactate dehydrogenase activity to determine NK cell activity. (B) Granzyme B and (C) IFN-γ expression levels in the culture supernatant of spleen cells were quantified using ELISA kits. Data are presented as the mean ± SE (n=9, control group; n=8, SWEG group). *P<0.05 compared with the control. SWEG, subcritical water extract of *Ganoderma*; NK, natural killer; IFN-γ, interferon-gamma.

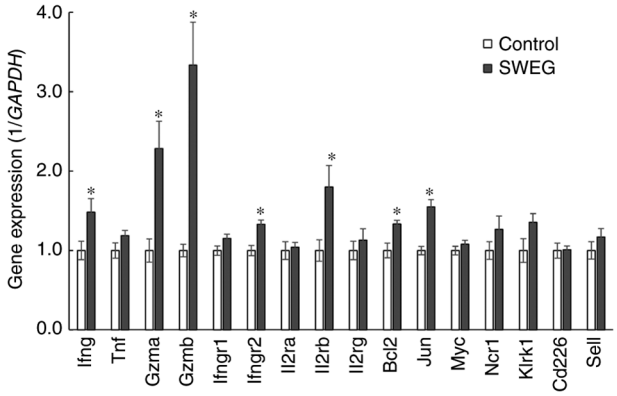

Figure 5. Gene expression analysis of the spleens of SWEG-fed mice. After 30-day breeding with SWEG-supplemented feed, the spleens of mice were removed to analyze the gene expression levels of various genes relevant to natural killer cell activity, using reverse transcription-quantitative PCR. Data are presented as the mean ± SE (n=9, control group; n=8, SWEG group). *P<0.05 compared with the control. SWEG, subcritical water extract of *Ganoderma*.

reported, e.g., inhibition of the growth of Sarcoma 180 tumor in mice (45), antitumor activity to Lewis lung cancer model (46), and the effect on stimulation of humoral immune responses in immunosuppressed mice (47). The average molecular weight of these polysaccharides was >20 kDa. SWEG, containing larger amounts of components of ~2.2 kDa, also exhibited an immunomodulatory effect, although the molecular size was small compared with the *Ganoderma* polysaccharides reported in the aforementioned studies. Thus, polysaccharides with various molecular weights derived from *Ganoderma* appear to have multiple mechanisms of action against the immune system.

The polysaccharides of fungi represented by β-glucan have been demonstrated to be recognized by receptors on the cell surface, and the signal is transmitted into the cell (48,49). Dectin-1, regarded as a key β-glucan receptor, has been reported to bind to polysaccharides with lengths longer than a decasaccharide (50). The details with regard to the components of ~2.2 kDa of SWEG are yet to be elucidated, but there is a possibility that a certain component in SWEG may bind to some type of receptor on the cell surface and has the cell proliferation- and differentiation-promoting effects on A-6 cells. In addition, it is important to examine what type of three-dimensional structure in polysaccharides is necessary for stimulation of the receptors of immune cells. For further investigation of the components with a molecular weight of ~2.2 kDa in SWEG, isolation of the polysaccharides and detailed structure studies, such as monosaccharide composition analysis and glycosidic linkage pattern analysis, are required.

Since the promoting effect of SWEG on cell viability was observed only for one day, the long-term effect has not been verified. In addition, the cell differentiation test was conducted based on the expression marker genes characteristic of T cells, B cells, and NK cells. Therefore, the effects of SWEG were verified using animals. As a result, the oral administration of SWEG in mice demonstrated that SWEG increased immune cells in the bone marrow and thymus. This effect of SWEG appears to be consistent with the *in vitro* results in A-6 cells, however it is not yet clear what type of mechanism is involved in the increase in HPCs and lymphocytes *in vivo*. For this determination, further research is required. In a previous study, mitogen-activated protein kinase (MAPK) signals were indicated to be markedly involved in the proliferation and differentiation of HSCs, which were maintained by signaling pathways such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (51). By examining whether SWEG is involved in the activation of these signaling pathways, the effect of SWEG on the immune system will be clarified.

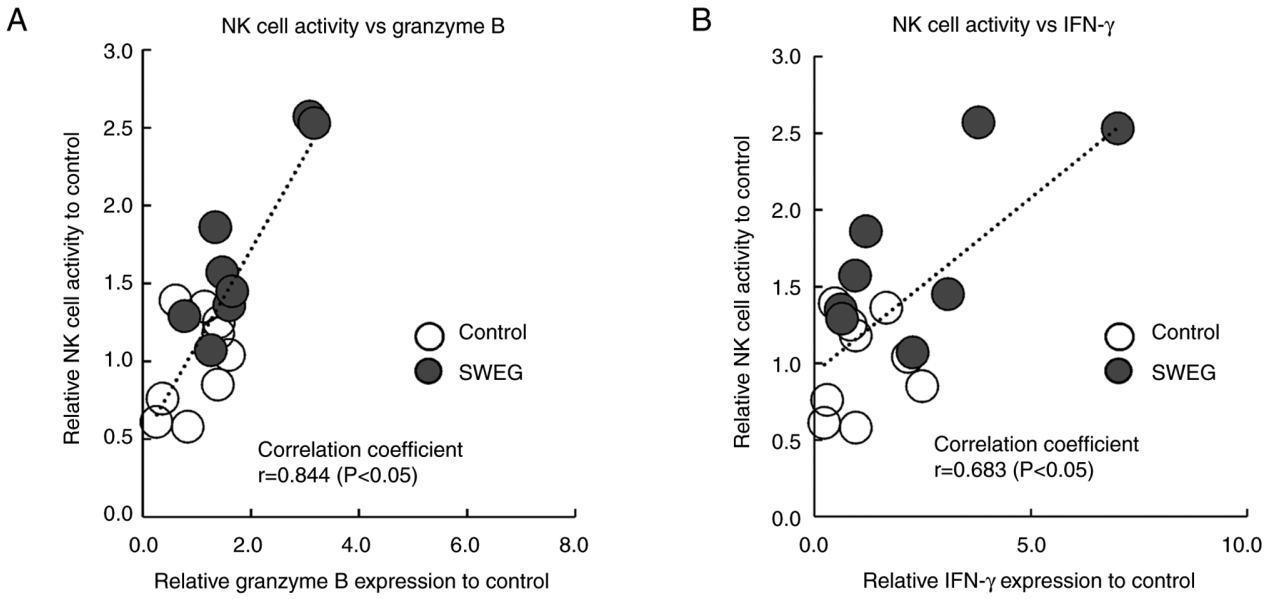

Figure 6. Correlation between granzyme B and IFN-γ expression and NK cell activity. (A) Granzyme B expression and (B) IFN-γ expression and NK cell activity in the spleens were plotted to calculate Pearson's product-moment correlation coefficients. P<0.05 indicates a statistically significant correlation. SWEG, subcritical water extract of *Ganoderma*; IFN-γ, interferon-gamma; NK, natural killer.

In addition to the increase of immune cells demonstrated in the animal study, SWEG significantly promoted NK cell activity and expression of cytokines in the spleen, compared with the control group. Therefore, gene expression analysis was conducted to investigate its mechanism, and the results revealed that SWEG promoted the gene expression of multiple factors involved in immune function, such as IFN-γ, granzyme A, granzyme B, and interleukin-2 receptor beta. Notably, SWEG markedly promoted the gene expression of granzyme B, an apoptosis-inducing factor (52,53), among the factors relevant to NK cell activity. This indicates that SWEG may have a potent effect on the transcription of the granzyme B gene.

To clarify the importance of granzyme B for NK cell activity, correlation analysis between granzyme B and IFN-γ expression and NK cell activity in the spleen were carried out. As was revealed, the correlation coefficient between granzyme B expression and NK cell activity was higher than in the case of IFN-γ, which is also known as an enhancer of NK cell activity (54). The correlation coefficients were compared, demonstrating that granzyme B was more strongly correlated with NK cell activity than IFN-γ. Thus, granzyme B was once again confirmed to be critical for NK cell activity.

Zhu *et al* reported the effect of polysaccharides with a molecular weight of >500 kDa, isolated from GL polysaccharides, in the promotion of granzyme B expression in cytokine-induced killer (CIK) cells (55). In that study, the promotion of granzyme B expression by GL polysaccharides, at the protein and mRNA level *in vitro* was demonstrated, but the *in vivo* effect of the polysaccharide was not fully elucidated. By contrast, the present study demonstrated that SWEG had an effect on A-6 cells, which are model cells of HSCs, and that SWEG promoted granzyme B expression in the spleen when taken orally *in vivo*. A previous study using the human colon cancer-derived cell line Caco-2 revealed that polysaccharides extracted from *Lycium barbarum* (>10 kDa) could be absorbed by endocytosis from the small intestine (56). For this reason, it is quite possible that components in SWEG with a molecular weight of ~2.2 kDa, which is a relatively small size among polysaccharides derived from *Ganoderma*, were absorbed from the small intestine and interacted with the immune system. Therefore, SWEG may have beneficial effects on health as an immunomodulatory food that can be orally ingested, due to its potent effect of promotion of granzyme B expression. In addition, the systemic immune functions enhanced through intestinal immunity by SWEG may have been extended to the spleen. To clarify the absorption mechanisms and immune responses of SWEG *in vivo*, further research is required.

In A-6 cells, SWEG was demonstrated to promote the cell differentiation into immune cells, but its effects on immune function, such as NK cell activity and expression of granzyme B and IFN-γ, have yet to be examined. In the future, analysis of immune function at the protein level, even in differentiated A-6 cells, is warranted. In the present study, both the differentiation-promoting effect of SWEG on A-6 cells and the immunostimulatory effect on mice were evaluated at only one dose. In order to further confirm the effectiveness of SWEG, experiments with various doses are necessary. By conducting experiments under various conditions and considering the results of both *in vitro* and *in vivo* studies combined, the understanding of the effect of SWEG on immunity would be further advanced.

In conclusion, SWEG, prepared by treating *Ganoderma* at a high temperature and high pressure, differed in the molecular weight distribution and β-glucan content from HWEG, a common hot-water extract. SWEG influenced immunity, i.e., acted on HSCs and induced highly functional immune cells. The results of the present study indicated that SWEG is a beneficial food material for immunoregulation, including enhancement of granzyme B expression and NK cell activity.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

KH, YY and SH conceived the research. KH, HTakagi, YO, TY, TS and HTanaka designed the research. KH, YO, HH and KF performed the experiments, prepared all the figures, and wrote the first draft of the manuscript. YY, SH and HTanaka supervised the research. KH, HTakagi, TY, TS, YY and SH wrote, reviewed and edited the final manuscript. HTanaka provided instructions for performing the experiments and assisted in the preparation of the manuscript. KH, HTakagi, TY and TS confirm the authenticity of all the raw data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The animal study protocols were approved (approval no. HS201708) by the Animal Experiment Committee of Nippon Menard Cosmetic Co., Ltd.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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