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Research Article

Enzymatic bioconversion of ginseng powder increases the content of minor ginsenosides and potentiates immunostimulatory activity

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ABSTRACT

Background: Ginsenosides are biologically active components of ginseng and have various functions. In this study, we investigated the immunomodulatory activity of a ginseng product generated from ginseng powder (GP) via enzymatic bioconversion. This product, General Bio compound K-10 mg solution (GBCK10S), exhibited increased levels of minor ginsenosides, including ginsenoside-F1, compound K, and compound Y.

Methods: The immunomodulatory properties of GBCK10S were confirmed using mice and a human natural killer (NK) cell line. We monitored the expression of molecules involved in immune responses via enzyme-linked immunosorbent assay, flow cytometry, NK cell-targeted cell destruction, quantitative reverse-transcription real-time polymerase chain reaction, and Western blot analyses.

Results: Oral administration of GBCK10S significantly increased serum immunoglobulin M levels and primed splenocytes to express pro-inflammatory cytokines such as interleukin-6, tumor necrosis factor-α, and interferon-γ. Oral administration of GBCK10S also activated NK cells in mice. Furthermore, GBCK10S treatment stimulated a human NK cell line *in vitro*, thereby increasing granzyme B gene expression and activating STAT5.

Conclusion: GBCK10S may have potent immunostimulatory properties and can activate immune responses mediated by B cells, Th1-type T cells, and NK cells.

1. Introduction

Ginseng, the root of plants in the genus *Panax*, is considered a panacea that promotes longevity; there is a particular value associated with ginseng from South Korea and China [1]. Ginseng is especially popular in Korea, China, Canada, and the United States; it had a total worth of approximately $2.084 billion in 2013 and an estimated worth of $5.9 billion in 2020 [2]. The biologically active ingredients of ginseng include saponins, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids [3]. Saponins are particularly active components of ginseng; they consist of polycyclic aglycones attached to one or more hydrophilic sugar side chains [4]. Sapogenins, the hydrophobic aglycone moieties, can be categorized as steroidal or triterpenoid saponins. Steroidal saponins are predominantly found in monocotyledons, such as *Liliaceae*, *Smilax*, and *Dioscorea*. In contrast, triterpenoid saponins are components of common metabolites in dicotyledons, such as *Leguminosae* and *Panax*. Ginsenosides are triterpenoid saponins with a four-ring structure and are only found in ginseng species within the genus *Panax* [5]. Ginsenosides identified thus far include protopanaxadiols, protopanaxatriols, and oleananes [5]. Ginsenosides,

Abbreviations: Ab, antibody; APC, allophycocyanin; CK, compound K; CO, compound O; Con A, concanavalin A; CY, compound Y; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; GBCK10S, General Bio compound-K 10 mg solution; GP, ginseng powder; GZMB, granzyme B; HPLC, high performance liquid chromatography; IFN, interferon; Ig, immunoglobulin; IL, interleukin; NK, natural killer; OD, optical density; PBS, phosphate-buffered saline; PE, phycoerythrin; RT-PCR, reverse-transcription polymerase chain reaction; SD, standard deviation; SE, standard error; STAT, signal transducer and activator of transcription; Th, helper T; TNF, tumor necrosis factor.

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the major constituents of ginseng, have been studied because of their uses in food, as well as their potential pharmacological properties.

Recently, there has been an increase in basic and clinical research regarding the pharmacological potential of ginsenosides [6]. Ginsenosides (e.g., ginsenoside-Rb1, -Rb2, -Rd, -Re, -Rg1, -Rg3, -Rg5, -Rh1, -Rh2, -Rh3, -Rk1, -Rp1, -Rc, and compound K (CK)) exhibit anti-inflammatory activity *in vitro* and *in vivo* by inhibiting pro-inflammatory cytokine expression and nitric oxide production; they also suppress signaling pathways involved in inflammatory responses. Cancer cells can be killed and cancer growth can be suppressed by ginsenoside-Rb1, -Rg1, -Rg3, -Rg5, -Rh2, -Rk1, -F2, and CK [6,7]. Additionally, ginsenosides may have antioxidant, antimicrobial, anti-diabetic, and neuroprotective properties [6]. Although ginsenosides have various potentially beneficial functions, their human pharmacological efficacy may depend on efficient absorption [8,9]. Following oral ingestion, hydrophilic ginsenosides encounter gut microbiota in the gastrointestinal tract and are metabolized to hydrophobic metabolites, which are more easily absorbed into the blood [9]. For example, ginsenoside-Rb1 is converted into CK by the human gut microbiota after oral administration of ginseng [10]. Consequently, the absorption efficiency and pharmacological effects of ginsenosides in raw ginseng depend on the composition of human intestinal microbes [9]. Therefore, recent research has focused on increasing the content of minor ginsenosides, such as CK, which are easily absorbed from the intestine.

Various methods have been developed to improve the absorption of dietary ginseng. These include acid transformation, heat transformation, fermentation, and bioconversion [9,11,12]. In particular, bioconversion of ginseng by enzymes derived from microorganisms can selectively transform the sugar side chains of ginsenosides. This procedure is also less expensive than acid- or heat-based transformation methods [13]. In this study, we used a mouse model to compare immunomodulatory properties between ginseng powder (GP) and enzymatically transformed GP.

2. Materials and methods

2.1. Experimental materials and animal experiments

Unless otherwise specified, the chemical reagents and laboratory equipment used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and SPL Life Sciences (Pocheon, Korea), respectively. General Bio compound K-10 mg solution (GBCK10S) was generated by General Bio Co., Ltd. (Wanju, Korea) via enzymatic bioconversion. Reference ginsenosides including ginsenoside-Rb1, -Rb2, -Rc, -Rd, -Re, -F1, -F2, -Rg3 (20S), compound O (CO), compound Y (CY), and CK were purchased from the Ambo Institute (Daejeon, Korea).

We purchased 5-week-old female C57BL/6 mice from the Koatech Laboratory Animal Center (Pyeongtaek, Korea). These mice were maintained under specific pathogen-free conditions with food and water provided *ad libitum*. Each experimental group of 10 mice was administered a 0 mg, 100 mg, or 200 mg sample/kg body weight/day for 4 weeks. Animal experiments were approved by the Institutional Animal Care and Use Committee of Jeonbuk National University (Approval No. JBNU 2020-0161) and the committee's guidelines were followed throughout the protocol.

2.2. Enzymatic bioconversion of GP to produce GBCK10S and analysis of ginsenoside composition

GBCK10S was produced via pectinase bioconversion from ginseng cultivated in Korea. Briefly, a suspension of GP (5%) and 5% pectinase (w/v) was prepared and pH was adjusted to 4.5. The suspension was incubated for 64–68 h at 55 °C in a shaking incubator. After centrifugation, supernatants were concentrated to prepare the sample with about 35 brix. Pellets were spray dried to prepare a powder. The ginsenoside content of supernatant and powder was analyzed by high-performance liquid chromatography (HPLC). Briefly, each sample was sonicated for 30 min in 70% MeOH, then filtered before application to the column. The ginsenosides were quantified by an Agilent 1260 Infinity II HPLC system (Agilent Scientific Instruments, Santa Clara, CA, USA) using a variable wavelength detector and a C_{18} column (3 μm, 4.6 × 150 mm; Osaka Soda, Osaka, Japan). The mobile phase was a gradient of water (A) and acetonitrile (B), and the following gradient was used: 27–30% (0–10 min), 30–51% (10–25 min), 51–70% (25–40 min), 70–90% (40–41 min), 90–95% (41–42 min), and 95% B (42–47 min). The flow rate of mobile phase was 1.0 mL/min, and the detection wavelength was 203 nm. Finally, GBCK10S was produced by combining supernatant and powder in a 99:1 ratio.

2.3. Cell culture

The YAC-1 mouse lymphoma cell line used in this study was kindly provided by Dr. C. Kim (Inha University, Incheon, Korea); the NK-92MI human natural killer (NK) and K562 human lymphoblast cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) [14,15]. The YAC-1 and K562 cells were cultured in RPMI-1640 medium (Welgene, Gyeongsan, Korea), supplemented with 10% fetal bovine serum (FBS) and appropriate antibiotics (Thermo Fisher Scientific, Waltham, MA, USA). NK-92MI cells were cultured in alpha-MEM (Welgene) supplemented with 10% FBS, antibiotics, 2-mercaptoethanol, and MEM vitamin solution (Thermo Fisher Scientific).

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to measure immunoglobulins (Igs) in serum samples from blood that had been collected from mice 2 days after their final oral dose [16]. Absorbance at 405 nm was measured using a SPECTROstar Nano ELISA plate reader (BMG Labtech, Ortenberg, Germany), and the results were expressed as optical densities (ODs).

Table 1
Sequences of the primers used for quantitative real-time RT-PCR. Primers used to measure the expression levels of genes involved in NK-cell activity. Human β-actin was used as an endogenous control.

Gene	Primer sequences
Human β-actin	F: 5′-GAG CTA CGA GCT GCC TGA CG-3′ R: 5′-GTA GTT TCG TGG ATG CCA CAG-3′
Human IFN-γ	F: 5′-TGG CTT TTC AGC TCT GCA TC-3′ R: 5′-CCG CTA CAT CTG AAT GAC CTG-3′
Human FasL	F: 5′-CCT GTG TCT CCT TGT GAT GTT-3′ R: 5′-CTG TAG GTG GAA GAG CTG AAA C-3′
Human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)	F: 5′-ACC AGA GGA AGA AGC AAC AC-3′ R: 5′-GAA TGC CCA CTC CTT GAT GA-3′
Human granzyme B (GZMB)	F: 5′-ACA CTC ACA CAC ACT ACA AGA G-3′ R: 5′-ACG CAC AAC TCA ATG GTA CT-3′

F and R, sequences of the forward and reverse primers, respectively.

2.5. Preparation of cells

At 2 days after the final oral dose, spleens were collected from the mice and dissociated using a cell strainer. Splenocytes were isolated, washed, and resuspended in 40% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Then, the cell mixture was floated on 75% Percoll and centrifuged for 20 min at 800×*g*. Mononuclear cells were collected from the interface between the 75% and 40% Percoll layers, then washed with serum-free medium. NK cells were enriched from the splenocytes using magnetic beads in an NK cell isolation kit II (Miltenyi Biotec, Inc., Bergisch Gladbach, Germany). To stimulate the splenocytes with T-cell mitogen, 2×10^5 splenocytes were cultured with 2 μg/mL concanavalin A (Con A) for 48 h. Cell proliferation was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (MTT assay; Roche, Mannheim, Germany), as described previously [15].

2.6. Antibodies (Abs) and flow cytometry

For flow cytometric analyses, the following Abs against mouse molecules were purchased from Miltenyi Biotec: anti-CD3 conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), anti-CD4-FITC or -PE, anti-CD8-allophycocyanin (APC), anti-CD107a-FITC or -APC, anti-interferon (IFN)-γ-APC, anti-NK1.1-PerCP-Vio 700, anti-CD11b-APC-Vio 770, anti-CD27-PE-Vio 770, and anti-KLRG1-PE. Flow cytometric analyses were performed using a Cytoflex flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) and the data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

2.7. NK cell activity

NK-cell activity was determined by measuring NK cell-mediated cytotoxicity against YAC-1 and K562 target cells, as described previously [14,15]. Briefly, YAC-1 and K562 target cells were labeled with Paul Karl Horan (PKH)-26, then mixed with splenocytes or NK-92MI effector cells at various effector to target-cell ratios. The cells were then incubated for 4 h at 37 °C in a CO_2 incubator. Finally, 7-aminoactinomycin D (7-AAD) and FITC-annexin V (both from BD Biosciences, San Jose, CA, USA) were added; the level of apoptosis induced in the target cells was measured via flow cytometry. NK-cell maturation was estimated via flow cytometry by measuring the expression levels of CD3, NK1.1, CD27, and CD11b.

2.8. Analysis of cytokine expression in splenocytes

To analyze cytokine expression, splenocytes (2×10^5) were added to each well of a 96-well plate. Splenocytes were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Merck Millipore, Danvers, MA, USA) and 0.5 μg/mL ionomycin (Thermo Fisher Scientific) for 4 h. The culture supernatants were harvested after stimulation, and the expression of various cytokines was measured using a cytometric bead-array (CBA) mouse helper T (Th) 1/Th2/Th17 cytokine kit (BD Biosciences). The results were analyzed using FCAP Array software (BD Biosciences). Cytokine concentrations were calculated using a standard curve generated from cytokine standards.

Table 2
Enzymatic bioconversion of ginseng powder (GP) into General Bio compound K-10 mg solution (GBCK10S) increased the content of minor ginsenosides. Ginsenosides in GP and GBCK10S were analyzed using high-performance liquid chromatography. ND = not detected.

Ginsenosides	Ginsenoside content (mg/g)	
	GP	GBCK10S
Ginsenoside-Rg1 + -Re	3.90 ± 0.05 (25%)	1.09 ± 0.01 (46%)
Ginsenoside-Rf	1.53 ± 0.06 (10%)	0.17 ± 0.01 (7.2%)
Ginsenoside-Rb1	4.64 ± 0.06 (30%)	ND
Ginsenoside-Rg2	0.46 ± 0.04 (2.9%)	ND
Ginsenoside-Rh1	0.67 ± 0.05 (4.3%)	0.29 ± 0.02 (12%)
Ginsenoside-Rc	1.96 ± 0.03 (12%)	ND
Ginsenoside-Rb2	1.51 ± 0.06 (9.8%)	ND
Ginsenoside-Rd	0.70 ± 0.07 (4.5%)	ND
Ginsenoside-F1	ND	0.18 ± 0.01 (7.6%)
Compound O	ND	ND
Ginsenoside-F2	ND	0.01 ± 0.00 (0.4%)
Ginsenoside-Rg3 (20S)	ND	0.02 ± 0.00 (0.8%)
Compound Y	ND	0.17 ± 0.01 (7.2%)
Compound K	ND	0.41 ± 0.01 (17%)
Total	15.36 ± 0.34	2.35 ± 0.04

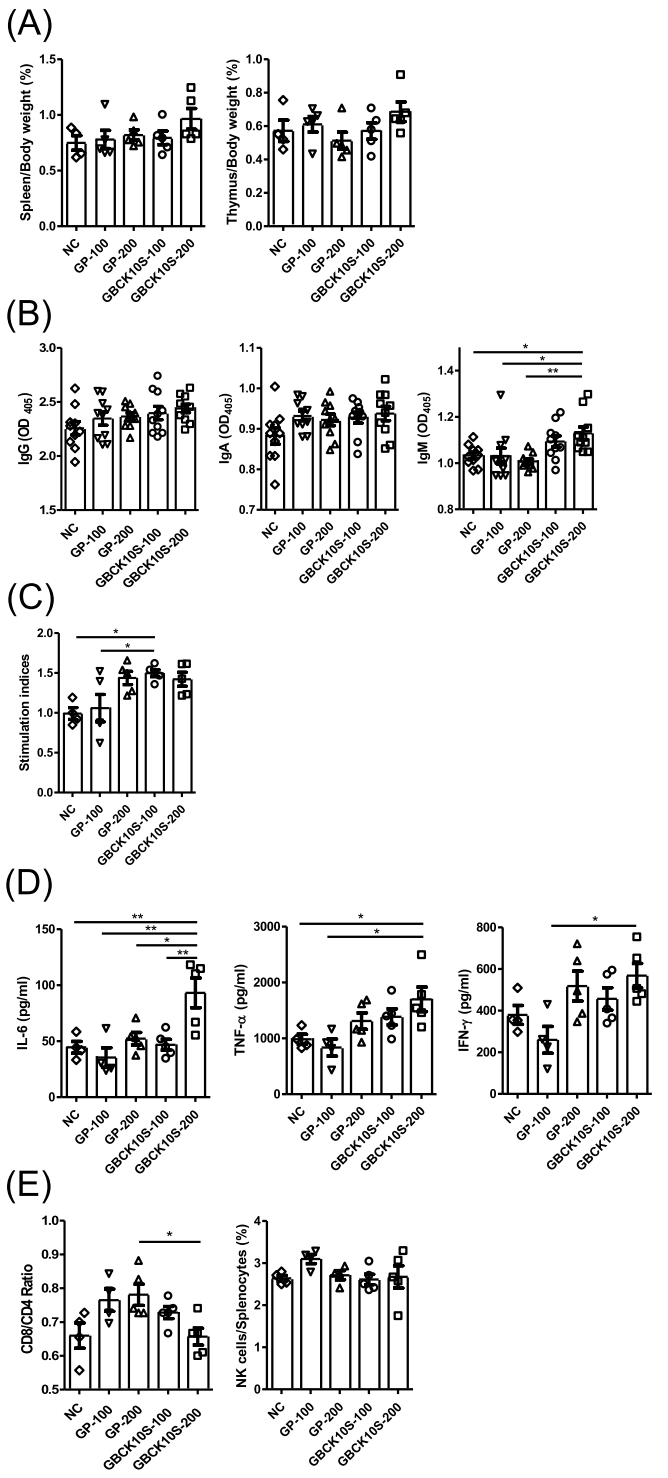


Fig. 1. Oral administration of ginseng powder (GP) and General Bio compound K-10 mg solution (GBCK10S) stimulates various components involved in immune responses in mice. (A) Spleens (left) and thymuses (right) were weighed from mice that had been administered oral doses of the following: PBS (negative control, NC), GP-100 (100 mg GP/kg body weight/day), GP-200 (200 mg GP/kg body weight/day), GBCK10S-100 (100 mg GBCK10 S/kg body weight/day), and GBCK10S-200 (200 mg GBCK10 S/kg body weight/day). Means ± standard error (SE; $n = 10$ per group) are presented. (B) Total immunoglobulin (Ig) G (left), IgA (center), and IgM (right) levels in the sera from 10 mice that had been administered oral doses as indicated were measured by enzyme-linked immunosorbent assays. Means ± SE ($n = 10$ per group) are presented as OD_{405}. (C) The levels of proliferation were measured in splenocytes prepared from five mice that had been administered oral doses as indicated. Proliferation was quantified using an MTT assay after *in vitro* stimulation with Con A, as described in the Methods section. Means ± SE ($n = 4$ or 5 per group) are presented as stimulation indices. (D) The levels of IL-6 (left), TNF-α (center), and IFN-γ (right) were measured from supernatants of cultured splenocytes prepared from five mice, following stimulation with phorbol 12-myristate 13-acetate and ionomycin, as described in the Methods section. Means ± SE ($n = 4$ or 5 per group) are presented. (E) The CD8/CD4 ratios in $CD3^+$ splenocytes (left) and the proportion of natural killer (NK) cells (right) were determined from splenocytes prepared from five mice that had been administered oral doses as indicated. Means ± SE ($n = 4$ or 5 per group) are presented. Representative results from two independent experiments are shown. One-way analysis of variance (ANOVA) and Tukey's test were used. $^*p < 0.05$ and $^{**}p < 0.01$ indicate significant differences.

2.9. RNA extraction and quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis

Gene transcript levels were determined by quantitative real-time RT-PCR. RNA was extracted using the Easy-BLUE Total RNA extraction kit (Intron Biotechnology, Sungnam, Korea) and converted into cDNA using a reverse-transcription reaction system (Promega, Fitchburg, WI, USA). Quantitative real-time PCR analyses were performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) with the CFX Connect Real-Time PCR Detection System (Bio-Rad), as described previously [17]. Gene expression was normalized against a β-actin internal control gene. The primer sets used to amplify the target genes are listed in Table 1.

2.10. Western blot analyses

Western blotting was used to analyze protein expression levels in cells that had been treated with GP or GBCK10S. Briefly, cells (4×10^6) were treated with 100 μg/mL of test sample, then harvested. Next, the cells were lysed in mammalian protein extraction reagent (Thermo Fisher Scientific) supplemented with a protease inhibitor cocktail (Thermo Fisher Scientific) and a phosphatase inhibitor (Roche). The lysate was resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Protein detection was performed as described previously [18] using Abs against Akt, phosphorylated (p)-Akt (Ser), signal transducer and activator of transcription (STAT)3, p-STAT3, STAT5, p-STAT5, and β-actin (Cell Signaling Technology, Danvers, MA, USA). Band densities were quantified using ImageJ software, as described previously [19].

2.11. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (ver. 5.0; GraphPad Software, San Diego, CA, USA). Data are expressed as means ± standard errors (SEs) or standard deviations (SDs) for repeated experiments. Differences among measurements from more than two independent variables were evaluated by comparing the control and treatment groups using unpaired two-tailed *t*-tests or one-way analysis of variance (ANOVA). For differences between/among groups, the following *p*-values were considered statistically significant: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. Enzymatic bioconversion of GP into GBCK10S increases the content of minor ginsenosides

Ginseng mostly comprises major ginsenosides (90%), such as ginsenoside-Rb1, -Rb2, -Rc, -Rd, -Re, -Rf, -Rg1, and -Rg2 [20]. Minor ginsenosides, which are more easily absorbed in the intestine

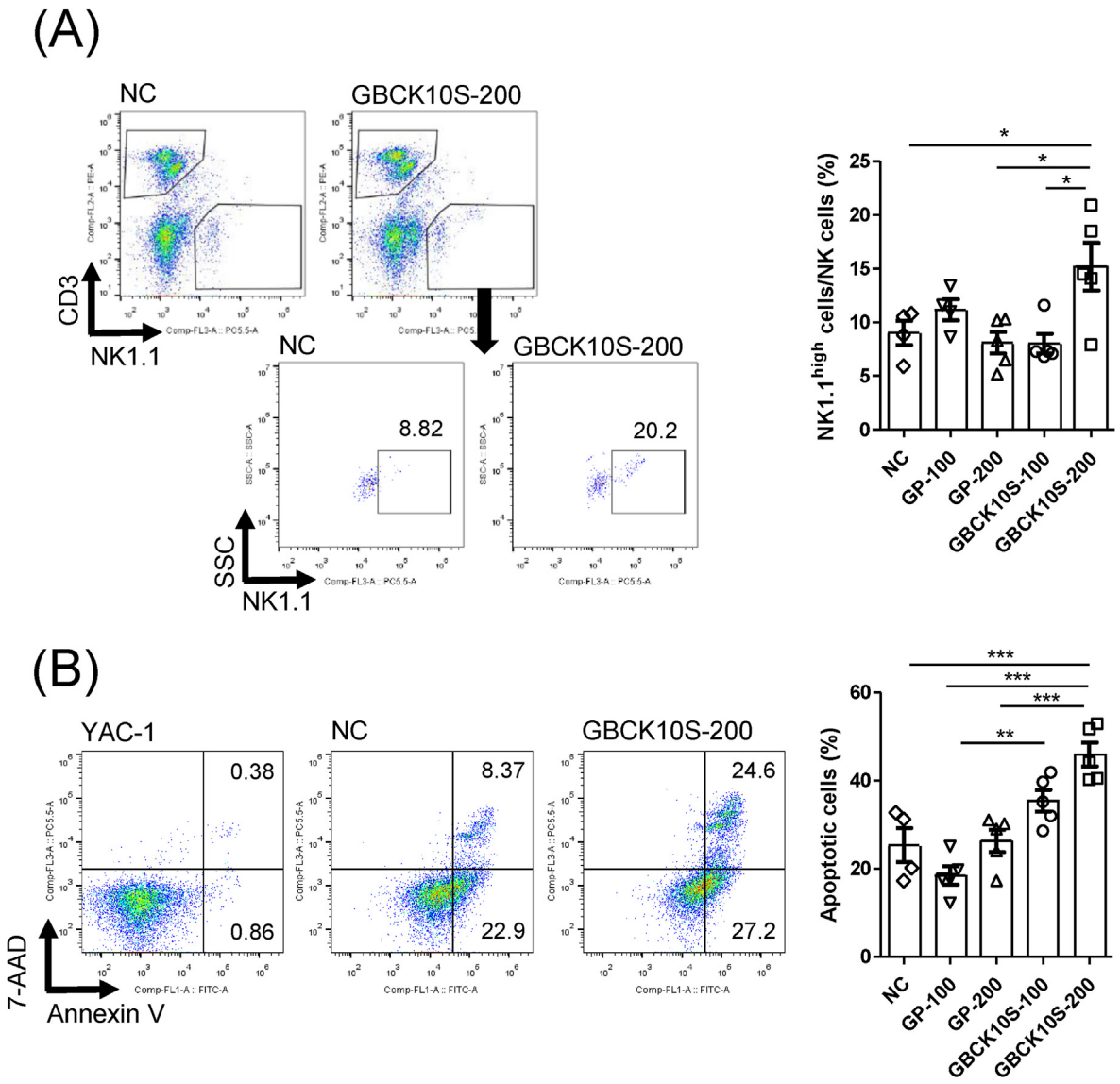
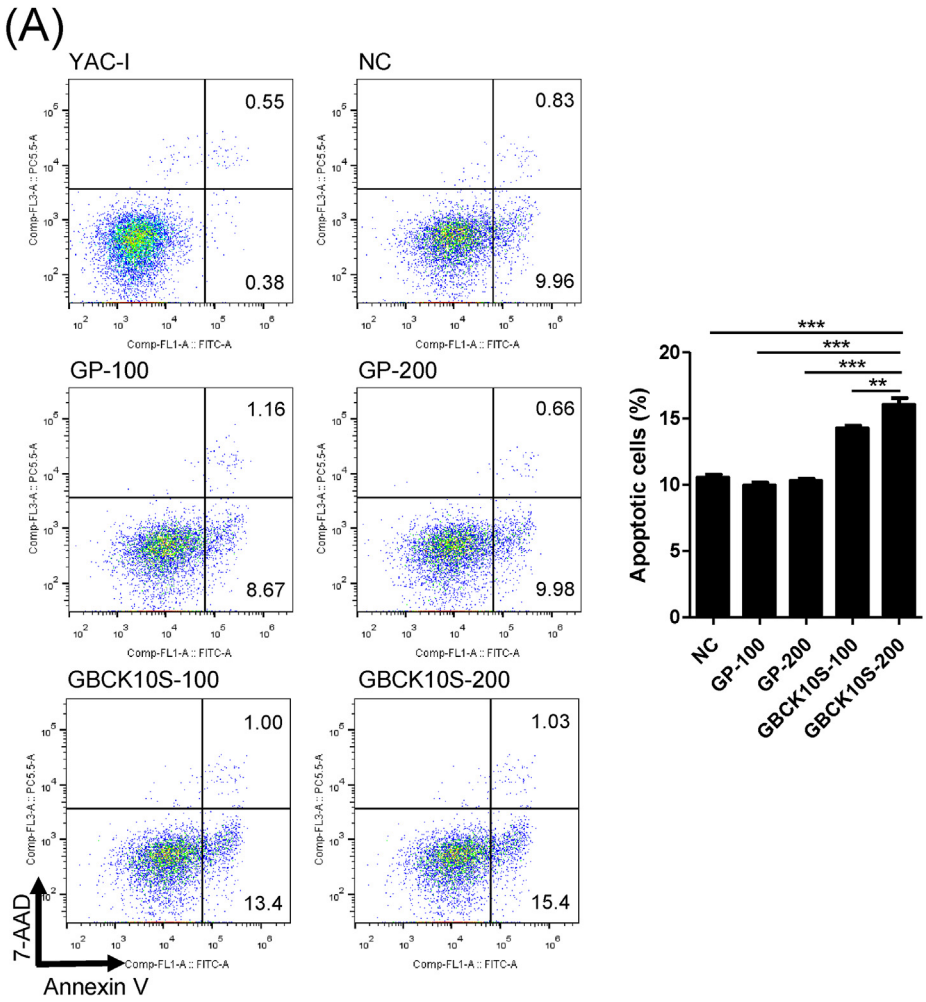
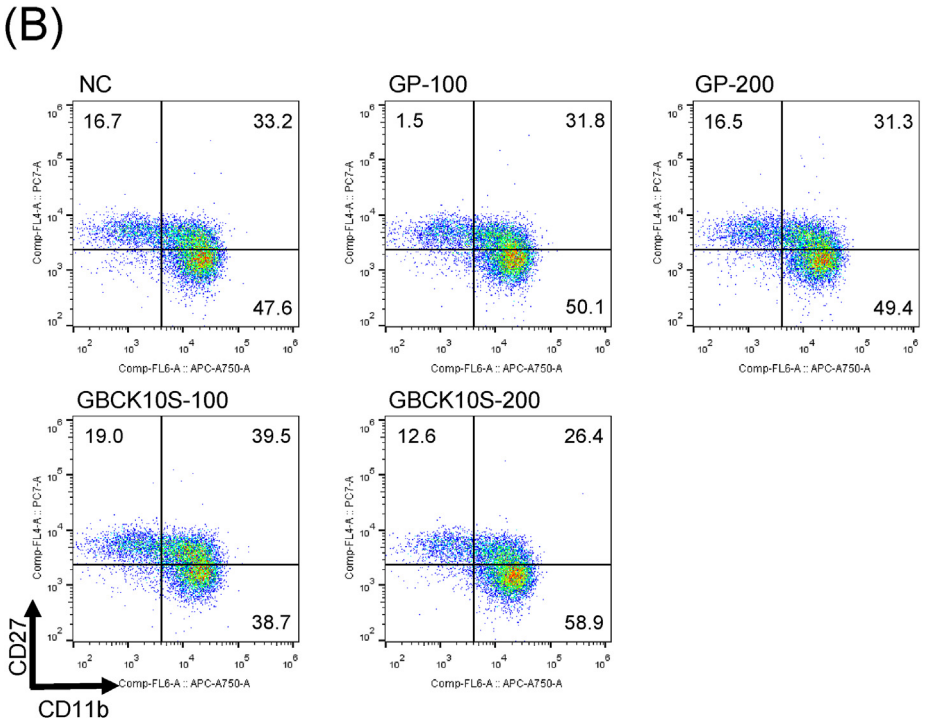


Fig. 2. Oral administration of GBCK10S increases the proportion of NK1.1^{high} cells and NK cell-mediated cytotoxicity in splenocytes. (A) The proportion of NK1.1^{high} cells was analyzed in splenocytes prepared from mice that had been administered oral doses of GP and GBCK10S as indicated. The flow cytometry plot shows a representative result and the data shown in the bar graph are means ± SE ($n = 4$ or 5) from flow cytometry plots. (B) Paul Karl Horan (PKH)-26-labeled YAC-I target cells and effector splenocytes from GP- or GBCK10S-treated mice were co-cultured at a ratio of 1:20 for 4 h. For flow cytometric analyses, PKH-26-labeled YAC-I cells were stained with Annexin V and 7-aminoactinomycin D (7-AAD). The flow cytometry plot shows a representative result and the data shown in the bar graph are means ± SE ($n = 4$ or 5) from flow cytometry plots. Representative results from two independent experiments are shown. One-way analysis of variance (ANOVA) and Tukey's test were used. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate significant differences.

(compared with major ginsenosides), are mainly generated by intestinal microflora [9]. In this study, we attempted to enhance the efficacy of ginseng by increasing its minor ginsenoside content via enzymatic bioconversion. We analyzed the ginsenoside compositions of supernatant and pellet separately using HPLC (Supplementary Fig. 1), and the ginsenoside compositions of GBCK10S were calculated according to the ratio of supernatant and pellet to prepare GBCK10S. The ginsenoside compositions of GP and its bioconverted product, GBCK10S, are compared in Table 2. GP mostly consists of major ginsenosides, including ginsenoside-Rg1 + -Re (25%), -Rf (10%), -Rb1 (30%), -Rg2 (2.9%), -Rh1 (4.3%), -Rc (12%), -Rb2 (9.8%), and -Rd (4.5%). Some minor ginsenosides (e.g., ginsenoside-F1, -F2, -Rg3 (20S), CO, CY, and CK) were not detected in GP. However, GBCK10S showed increases in the contents of minor ginsenosides, including ginsenoside-F1 (7.6%), -F2 (0.4%), -Rg3 (20S) (0.8%), CY (7.2%), and CK (17%). In particular, CK content was


(A)
YAC-I
0.55
0.38
NC
0.83
9.96
GP-100
1.16
8.67
GP-200
0.66
9.98
GBCK10S-100
1.00
13.4
GBCK10S-200
1.03
15.4
Comp-FL3-A :: PC5.5-A
Comp-FL1-A :: FITC-A
7-AAD
Annexin V
Apoptotic cells (%)
***
***
***
**
NC
GP-100
GP-200
GBCK10S-100
GBCK10S-200


(B)
NC
16.7
33.2
47.6
GP-100
1.5
31.8
50.1
GP-200
16.5
31.3
49.4
GBCK10S-100
19.0
39.5
38.7
GBCK10S-200
12.6
26.4
58.9
Comp-FL4-A :: PC7-A
Comp-FL6-A :: APC-A750-A
CD27
CD11b

greatly increased in GBCK10S. Although the major ginsenoside content of GBCK10S remained relatively high, our results show that the major ginsenoside content had decreased and the minor ginsenoside content had increased, suggesting that major ginsenosides in GP had been converted into minor ginsenosides in GBCK10S.

3.2. Oral administration of GBCK10S stimulates various components involved in immune responses in mice

Enzymatic bioconversion of GP into GBCK10S increased the content of minor ginsenosides, some of which have beneficial effects on human health. For example, CK reportedly exhibits anti-inflammatory, anticancer, anti-diabetic, anti-aging, neuroprotectant, and anti-atherosclerotic properties [7,9,10]. Ginsenoside-F1 also exhibits anticancer, anti-aging, and antioxidant properties; recent reports have shown that it can activate NK cells [21,22]. Therefore, we compared the effects of GP and GBCK10S on the mouse immune system (Fig. 1). We weighed some of the organs involved in immune responses (e.g., spleen and thymus) after daily oral administration of 100 or 200 mg/kg body weight of GP (GP-100 or GP-200) or GBCK10S (GBCK10S-100 or GBCK10S-200) for a period of 4 weeks. We found that the spleens and thymuses from the GBCK10S-200 group of mice were heaviest among the tested groups, although the differences were not statistically significant (Fig. 1A). In addition, because saponin has been used as an adjuvant to induce antigen-specific immune responses, we quantified serum Abs in the mice to monitor B lymphocyte activation (Fig. 1B) [23]. Among the tested groups, the levels of total IgG and IgA were highest in the GBCK10S-200 group of mice, although the differences were not statistically significant. However, total IgM levels were significantly higher in the GBCK10S-200 group than in the other groups ($p < 0.05$ and $p < 0.01$). This is an important finding because total IgM levels are a marker for stimulation of the innate immune response.

Next, we measured T-cell activation in response to oral administration of the GP and GBCK10S products after splenocytes had been stimulated with Con A, which also stimulates T cells (Fig. 1C). T-cell proliferation increased more in the GP- and GBCK10S-treated groups than in the control group; the increase observed in the GBSK10S-100 group was statistically significant ($p < 0.05$). We also measured cytokine levels to quantify the activation of T cell-mediated immune responses in splenocytes that had been stimulated with PMA and ionomycin; we found that interleukin (IL)-6 levels were significantly increased in the GBCK10S-200 group ($p < 0.05$ and $p < 0.01$). Among the groups tested, we also found that the levels of tumor necrosis factor (TNF)-α and IFN-γ were highest in the GBSK10S-200 group (Fig. 1D). Because $CD8^+$ cytotoxic T cells, NK cells, NKT cells, and $CD4^+$ Th1 cells all produce IFN-γ, we noted any changes in CD8/CD4 cell ratios and NK-cell frequencies that were induced by oral administration of GP or GBCK10S (Fig. 1E). We found that the CD8/CD4 ratio was highest in the GP-200 group and lowest (similar to the negative control) in the GBCK10S-200 group. In addition, we found no obvious changes in the NK-cell populations from mice treated with the different products. These results suggest that oral administration of GBCK10S induced T-cell priming and immunostimulation in mice, although CD8/CD4 T-cell ratios and NK-cell frequencies were not markedly altered.

3.3. Oral administration of GBCK10S increases the frequency of $NK1.1^{high}$ cells and the NK cell-mediated cytotoxicity of splenocytes

Because we observed no change in the frequency of NK cells in splenocytes from mice that had been administered GP or GBCK10S, we analyzed the activation of NK cells. We monitored the expression level of NK1.1, an NK-cell marker linked to cytotoxic activity in NK cells (Fig. 2) [24,25]. Notably, the frequency of $NK1.1^{high}$ cells among the $CD3^-NK1.1^+$ cell population was significantly altered ($p < 0.05$); it was 1.5- to 2-fold higher in the GBCK10S-200 group than in the other groups (Fig. 2A). This increase in $NK1.1^{high}$ cells suggested that splenocytes from GBCK10S-treated mice may exhibit efficient cytotoxicity, regardless of changes in NK-cell numbers. Consequently, we co-cultured splenocytes from mice that had been administered the different products with YAC-1 target cells to monitor NK cell-mediated cytotoxicity (Fig. 2B). Importantly, the proportion of apoptotic cells was significantly increased ($p < 0.001$) and approximately 2-fold higher in the GBCK10S-200 group than in the other groups. Furthermore, the GBCK10S-100 group showed a significant increase in apoptotic activity, compared with the GP-100 group ($p < 0.01$), although this increase was not significantly higher than in the GP-200 group. These results suggest that oral administration of GBCK10S increased the cytotoxic potential of splenocytes regardless of changes in NK-cell frequencies.

3.4. Oral administration of GBCK10S enhanced the cytotoxicity and maturation of NK cells purified from splenocytes

To determine whether activation of NK cells was responsible for the increased cytotoxic potential of splenocytes from mice that had been administered GBCK10S, we purified NK cells from splenocytes taken from mice that had been administered the different products and analyzed their cytotoxic activity against target cells (Fig. 3). Similar to the results from the cytotoxicity assay using splenocytes (Fig. 2), the proportion of apoptotic cells significantly increased ($p < 0.001$ and 0.01) and was approximately 1.5-fold higher in the GBCK10S-200 group than in the other groups (Fig. 3A). Next, we investigated whether NK-cell maturation was affected by oral administration of the different products (Fig. 3B). Based on the expression levels of CD11b and CD27 on the cell surface, mouse NK cells were divided into four subsets as follows (ordered sequentially from naïve to mature phenotypes): $CD11b^{low}CD27^{low}$, $CD11b^{low}CD27^{high}$, $CD11b^{high}CD27^{high}$, and $CD11b^{high}CD27^{low}$ [15,26]. In the GBCK10A-200 group, the proportion of mature NK cells ($CD11b^{high}CD27^{low}$) was 58.9%, which was approximately 10% higher than in the other groups. In addition, the proportions of $CD11b^{low}CD27^{high}$ and $CD11b^{high}CD27^{high}$ cells were approximately 4% lower in the GBCK10A-200 group than in the other groups. Collectively, these results suggest that mature cytotoxically active NK cells were more enriched in the splenocytes of GBCK10S-treated mice than in the other groups.

Fig. 3. Oral administration of GBCK10S enhanced the cytotoxicity and maturation of NK cells in splenocytes. NK cells were purified from splenocytes prepared from five mice per group. (A) PKH-26-labeled YAC-I target cells and purified NK effector cells from GP- or GBCK10S-treated mice were co-cultured at a ratio of 1:4 for 4 h. For flow cytometric analysis, PKH-26-labeled YAC-I cells were stained with Annexin V and 7-AAD. The flow cytometry plot shows a representative result and the bar graph are means ± standard deviation (SD) of triplicate experiments. Unpaired two-tailed *t*-tests were used. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences. (B) Flow cytometry plots showing the expression of CD11b and CD27 in a $CD3^-NK1.1^+$ NK-cell population purified from splenocytes prepared from mice that had been administered oral doses as indicated. One representative result from two independent experiments is shown.

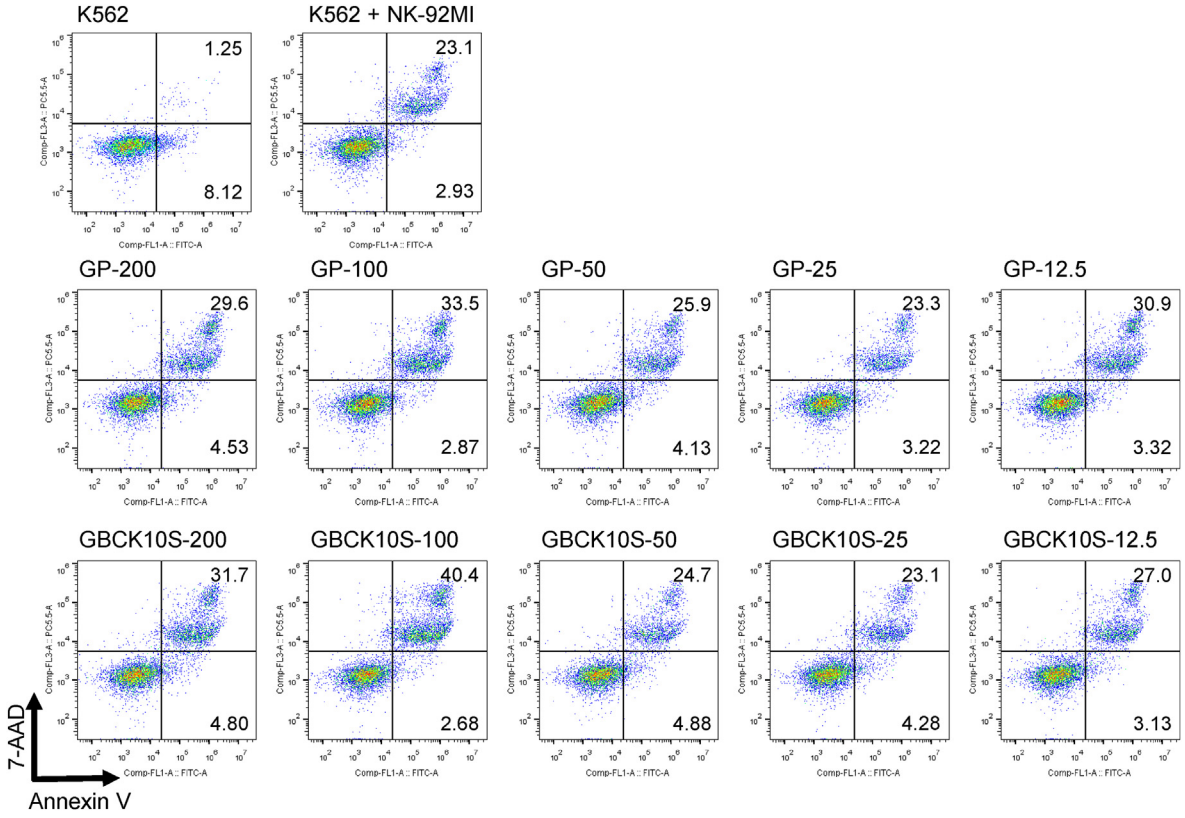


Fig. 4. GBCK10S enhanced cytotoxic activity in a human NK cell line. NK-92MI cells were treated with various doses of GP or GBCK10S (12.5–200 μg/mL) for 24 h. After stimulation, PKH-26-labeled K562 target cells and NK-92MI effector cells were co-cultured at a ratio of 1:8 for 4 h. The PKH-26-labeled K562 cells were stained with Annexin V and 7-AAD for analysis using flow cytometry. Representative results from three independent experiments are shown.

3.5. GBCK10S enhanced the cytotoxic activity of a human NK cell line and upregulated the expression of granzyme B

To determine whether GBCK10S also increases NK cell-mediated cytotoxicity in human cells, we used the human NK cell line NK-92MI (Fig. 4). We found that up to 200 μg/mL of GP or GBCK10S alone were not cytotoxic (data not shown). When we incubated the NK-92MI cells with K562 target cells in the presence of phosphate-buffered saline (PBS) as a control, approximately 26% of the cells were apoptotic (upper panel). When the NK-92MI cells were treated with 100 μg/mL of GBCK10S, we observed a considerable increase in apoptotic cells (approximately 43%). This increase was more than 1.5-fold greater than the increase observed in a control experiment (lower panel). GP also increased the NK-92MI cell-mediated apoptosis of K562 target cells by approximately 36% (center panel). Therefore, GBCK10S activated the NK-92MI NK cell line more strongly than did GP.

To understand how cytotoxicity in the NK92-MI cells was enhanced by GBCK10S treatment, we measured the levels of gene transcripts involved in NK-cell activity, including FasL, IFN-γ, TRAIL, and GZMB (Fig. 5A) [27]. After 2 h, FasL transcript levels in GBCK10S-treated cells were similar to the levels in negative control or GP-treated cells. However, at 6 h and 24 h, FasL transcript levels in GP- and GBCK-10S-treated cells had decreased and were lower than the levels in negative control cells. IFN-γ transcript levels in GBCK10S-treated cells were also lower than the levels in negative control cells at 6 h and 24 h. However, TRAIL transcript levels were unaffected by GP or GBCK10S treatment. GZMB transcript levels in

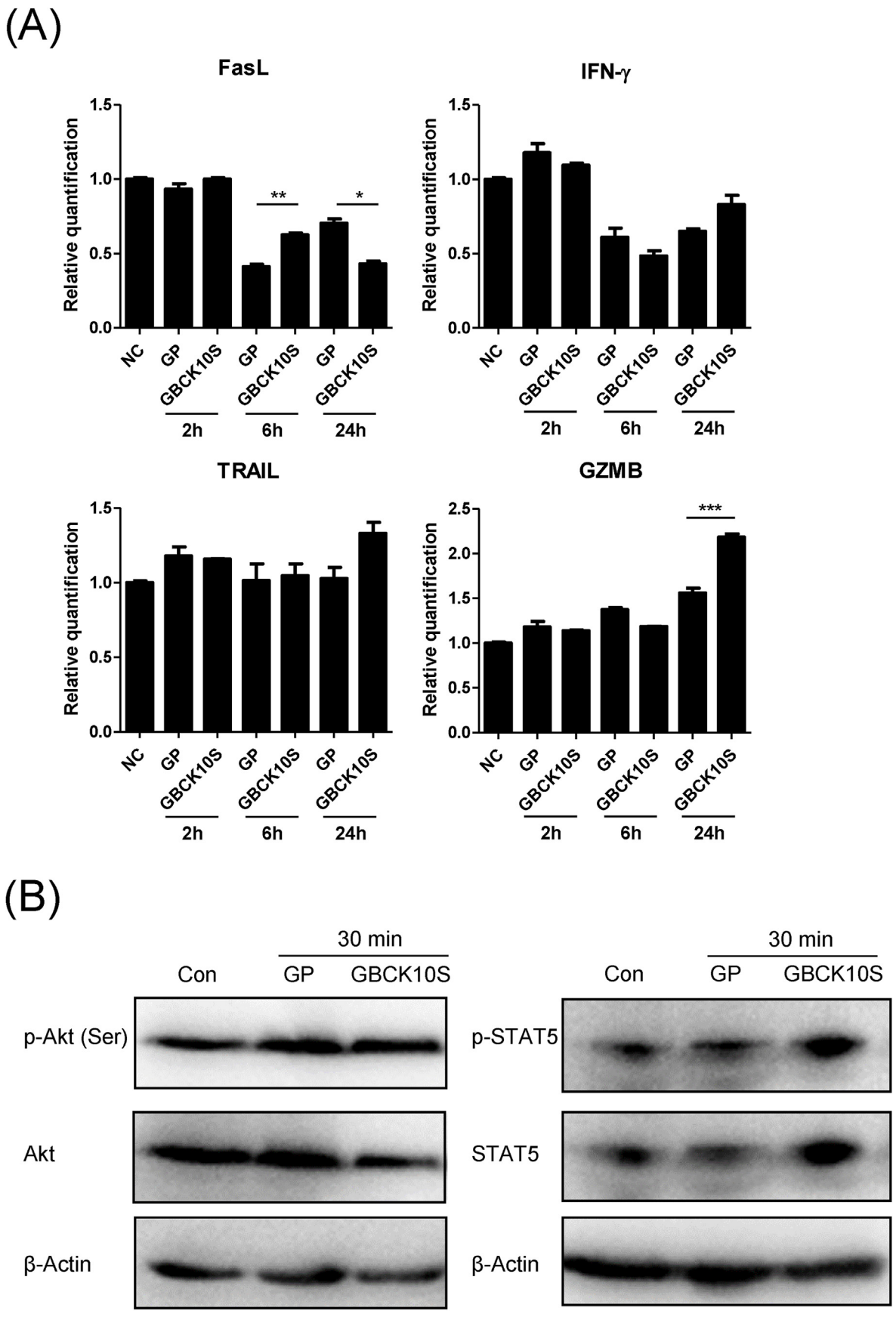


Fig. 5. GBCK10S upregulated the expression of cytotoxicity-related cell signaling molecules. (A) NK-92MI cells were treated with GP or GBCK10S (100 μg/mL) for 2 h, 6 h, and 24 h. FasL, IFN-γ, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and human granzyme B (GZMB) mRNA levels were measured by quantitative real-time reverse-

GP- or GBCK10S-treated cells were similar to the levels in negative control cells at 2 h or 6 h. However, at 24 h, GZMB transcript levels were approximately 1.5-fold higher in GBCK10S-treated cells than in GP-treated cells ($p < 0.01$).

Next, we analyzed Akt activation in GP- and GBCK10S-treated cells, because Akt activation induces the expression of GZMB in NK cells (Fig. 5B) [28]. GP or GBCK10S treatment of NK cells slightly increased the level of phosphorylated Akt (p-Akt) at Ser 473 (p-Akt (Ser) by 1.73- and 2.42-fold, respectively. We also analyzed the activation of STAT5, a transcription factor involved in NK-cell activation; we found no changes in the level of phosphorylated STAT5 (p-STAT5) in GP- or GBCK10S-treated NK cells [29]. However, the level of STAT5 protein increased by 2.21-fold in GBCK10S-treated cells compared with the control, which was higher than that of GP-treated cells (1.25-fold increase). In addition, GP or GBCK10S treatment of NK cells induced an increase in STAT3 phosphorylation that was visible after 30 min, compared with control cells (Supplementary Fig. 2). However, the level of phosphorylated STAT3 had decreased in GBCK10S-treated cells at 2 h, whereas the increased level of p-STAT3 was maintained in GP-treated cells. Collectively, these results suggest that GBCK10S treatment stimulates signaling molecules involved in NK-cell activation.

4. Discussion

The countries that produce the largest proportions of fresh ginseng worldwide include China (55%), Korea (34%), Canada (8.1%), the United States (1.3%), and others (0.4%); Korea and China are also the greatest consumers of ginseng [2]. Ginseng is produced as fresh, red, or white ginseng, and is sold as powder, capsules, extracts, or in drinks. The processing method influences the product's specific composition and the ease with which it is absorbed in the human gut [5]. CK is produced by various procedures, including fermentation and bioconversion; its pharmacological effects and absorption characteristics have been studied in detail [7]. The ginseng industry is currently developing products with a higher content of minor ginsenosides (e.g., CK). In this study, we developed GBCK10S via enzymatic bioconversion and analyzed its pharmacological characteristics. GBCK10S has increased levels of minor ginsenosides, such as CK and ginsenoside-F1 (Table 2).

GBCK10S-treated mice had heavier spleens and thymuses. In addition, the levels of IgM were significantly higher in sera from GBCK10S-treated mice than in sera from GP-treated mice (Fig. 1A and B). We also observed a significant increase in the level of IL-6, a pleiotropic pro-inflammatory cytokine that is important for inducing B-cell differentiation and generating Ab-producing plasma cells. This suggests that oral administration of GBCK10S has immunostimulatory effects on mice [30]. Although this observation does not directly support enhancement of the B-cell immune response, it suggests that GBCK10S is more effective than GP in the B-cell immune response. Additionally, oral administration of GBCK10S induces splenocyte proliferation and the production of the Th1-type pro-inflammatory cytokines TNF-α and IFN-γ (Fig. 1D). Importantly, IFN-γ is associated with the activation of $CD8^+$ T cells and NK cells [31]. Although the CD8/CD4 T-cell ratio was increased in GP-treated mice, it was decreased in GBCK10S-treated mice (Fig. 1E). We presume that GBCK10S activated Th1-type T cells but had no effect on $CD8^+$ T-cell proliferation, which resulted in a reduced CD8/CD4 T-cell ratio in GBCK10S-treated mice. A critical finding in this study was that the oral administration of GBCK10S increased the $NK1.1^{high}$ NK-cell population in mice (Fig. 2A), although the absolute frequency of NK cells in splenocytes was not significantly altered (Fig. 1E). In addition, GBCK10S increased the cytotoxic potential of NK cells against target cells (Fig. 2B). Our results suggest that oral administration of GBCK10S induced the maturation and activation of NK cells in mice without altering NK-cell frequencies.

NK cells are cytotoxic lymphocytes that have important roles in the innate immune defense. They belong to a rapidly expanding family of innate lymphoid cells that constitute 5–20% of circulatory human lymphocytes. NK cells are important for mediating antitumor and antiviral immune responses [26]. Many studies of ginseng product-mediated immune enhancement have focused on NK-cell activation. Ginsenoside-F1 reportedly enhances the cytotoxic activity of NK cells through an insulin-like growth factor-1-dependent mechanism [22]. Additionally, the expression levels of GZMB and perforin 1 were increased and Akt signaling was activated when primary human NK cells were treated with ginsenoside-F1 [22]. The phosphoinositide 3-kinase-Akt-mTOR pathway induced GZMB expression in NK cells [28], and we found that GZMB gene transcript levels and phosphorylation of Akt were increased in a GBCK10S-treated NK cell line (Fig. 5). In addition, we discovered that the frequency of $CD27^-CD11b^+$ NK cells, which exhibit upregulated expression levels of NK cell-mediated cytotoxicity genes (e.g., GZMB, perforin, and various NK-cell receptors), was increased in GBCK10S-treated mice (Fig. 3B) [32]. However, the expression of IFN-γ, a major cytokine indicating NK cell activation, was decreased in the experiment using a human cell line (Fig. 5A). Mature NK cells have low cytokine expression but produce high levels of IFN-γ when stimulated via receptor activation [33]. However, we did not treat the target cells capable of activating receptors, but only the NK cell line. Consequently, we speculate that IFN-γ expression was not increased due to the failure to stimulate receptors in NK cells. This observation suggests that increasing minor ginsenosides in GBCK10S via enzymatic bioconversion stimulates cell signaling pathways involved in NK-cell activation, although this stimulation is expected to be unrelated to the receptor activation in NK cells. One notable point is that total ginsenoside content in GBCK10S was decreased by enzymatic bioconversion of GP (Table 2), but GBCK10S showed enhanced immune-stimulating activity compare with GP. Although we did not directly confirm the absorption efficiency of the samples *in vivo*, we assume that ginsenosides in GBCK10S was efficiently absorbed into mice because it has been previously reported that the absorption rate of minor ginsenosides such as CK is higher than that of major ginsenosides [9,10]. Therefore, we believe that GBCK10S exerted a stronger immune-stimulating activity than GP despite the decrease in total amount of ginsenosides.

STATs are transcription factors that are involved in NK-cell activation. STAT1, 4, and 5 stimulate NK-cell maturation and cytolytic activity, whereas STAT3 and 6 inhibit NK-cell cytotoxicity [29]. STAT5 is involved in NK-cell survival, proliferation, and development [34]. Notably, levels of STAT5 protein increased in GBCK10S-treated cells, suggesting that GBCK10S may activate NK cells (Fig. 5B). We suspect that ginsenoside-F1 and CK, which reportedly inhibit STAT3 phosphorylation, may increase STAT5 levels [35]. Ginsenoside-F1 and CK were not detected in GP, but were present in GBCK10S. GBCK10S may increase cytotoxicity and NK-cell

transcription polymerase chain reaction and normalized against β-actin. The data are presented relative to the negative control (i.e., NC = 1). The graphs show means ± SD of triplicate experiments. Unpaired two-tailed *t*-tests were used. $*p < 0.05$, $**p < 0.01$, and $***p < 0.01$ indicate significant differences. (B) NK-92MI cells were treated with GP or GBCK10S (100 μg/mL) for 30 min. The expression and activation of Akt and signal transducer and activator of transcription (STAT)5 were analyzed using Western blotting of proteins extracted from cells treated as indicated. β-actin was used as an internal loading control for the Western blot analyses. Representative results from two independent experiments are shown.

maturation via Akt and STAT5 signaling pathways. Although GBCK10S is not a purified material, it can induce B-cell activation, Th1-type T-cell activation, and NK-cell activation both *in vivo* (in mice) and *in vitro* (in NK cell studies).

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2021.12.005.

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