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Full-length article

Soluble components of *Hericium erinaceum* induce NK cell activation via production of interleukin-12 in mice splenocytes[1]

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Key words

Hericium erinaceum; NK cells; interleukin-12

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Abstract

Aim: To investigate the immunoregulatory functions of water extracts of *Hericium erinaceum* (WEHE) focusing on natural killer (NK) cell-based anticancer activities. **Methods:** Mouse splenocytes or purely isolated NK cells were stimulated with 1–100 mg/L WEHE for 24 h followed by co-culture with ^{51}Cr-labled Yac-1 cells for 4 h, then NK cell-derived cytolytic activity was measured using a radio-release assay. Neutralizing antibodies against mouse interleukin-12 (IL-12) were added into the WEHE-stimulated splenocytes, thereafter, cytotoxicity was measured to examine the involvement of IL-12. RT-PCR and ELISA analyses were performed to confirm the induction of transcription and the translation of IL-12 and interferon-gamma (IFN-gamma) in the WEHE-treated splenocytes. **Results:** WEHE enhanced the cytolytic activity of total splenocytes towards Yac-1 cells in a dose-dependent manner. However, this activation was not observed when the NK cells isolated from the splenocytes were treated with WEHE. Furthermore, the treatment with antibodies against IL-12 abolished the effect of WEHE on splenocyte-derived cytolytic activity. RT-PCR and ELISA analyses showed the induction of IL-12 and IFN-gamma in the WEHE-treated splenocytes. **Conclusion:** WEHE indirectly activates the cytolytic ability of NK cells via the induction of IL-12 in total splenocytes, and possibly via other immuno-mediators or cellular components.

Introduction

Among the numerous antitumor cellular components in mammals, natural killer (NK) cells play important roles in inhibiting tumor development, growth, and metastasis, as well as having a significant role in tumor treatment[1–3]. Tumor cells are able to evade the adaptive immune system by downregulating the expression of major histocompatibility complex (MHC) molecules; however, NK cells are cytotoxic against MHC class I-deficient tumor cells[4,5]. Besides killing cancer cells directly through cell-mediated cytolysis, NK cells produce cytokines, such as interferon-gamma (IFN-gamma), which stimulate adaptive immunity and restrict tumor angiogenesis[6–8]. Therefore, maintaining or enhancing NK cell-based immune responses is very important for cancer treatment[9]. Accordingly, many cancer immunotherapies that enhance the activities of NK cells have been proven to be effective in both experimental animal models and cancer patients[10,11].

Natural resource-derived compounds such as ginseng extract or echinacea root extract have been investigated as potential immunomodulators for the treatment of cancer patients[12,13]. Many of these studies have focused on the activation of NK cells and demonstrated positive results[14,15]. Furthermore, polysaccharides found in various mushrooms have recently attracted attention due to their NK cell-related immune-activating properties[16–19].

Hericium erinaceum is a widely cultivated edible mushroom and has been used in several Asian countries to treat various human diseases. Recently, *H erinaceum* was reported to have cytotoxic effects on cancer cell lines, as well as

nematicidal and antimicrobial activities[20,21]. We previously reported that a water extract of *H erinaceum* (WEHE) induced the production of NO and interleukin (IL)-1-beta in rat macrophages and a macrophage-like cell line, RAW 264.7[22,23].

In the present study, we examined the immunomodulatory effects of WEHE on the cytolytic function of NK cells against an MHC class I-deficient cell line, Yac-1. Our results indicated that WEHE indirectly facilitated the cytolytic ability of mouse splenocytes by amplifying the NK cell activity induced by IL-12.

Materials and methods

Reagents and chemicals ^{51}Cr-source was obtained from PerkinElmer (Boston, MA, USA), and lipopolysaccharide (LPS, *Salmonella typhosa*) was obtained from Sigma–Aldrich (St Louis, MO, USA). All RT-PCR reagents were purchased from Bio-Rad (Hercules, CA, USA). Mouse IL-12 and the anti-mouse IL-12 rat antibody were purchased from eBioscience (San Diego, CA, USA). β-*D*-(1,3)-(1,6)-glucan was obtained from VP GmbH (Hergestellt, Germany).

Extraction, composition analysis, and fingerprinting of WEHE Dried *H erinaceum* was obtained from the Korean Mushroom Corporation (Pochon, Korea). The mushrooms (100 g) were washed 3 times with pyrogen-free water, then soaked in 1.5 L pyrogen-free water for 2 h and boiled for 2 h. Solid particles and aggregates were removed by centrifugation at 3000×*g* for 30 min, and the supernatant was lyophilized. A total of 26.35 g lyophilized water extract was obtained for use in this experiment. According to the Association of Analytical Communities[24], the general chemical composition of WEHE is crude protein (44.82%), carbohydrate (27.63%), crude ash (16.84%), moisture (9.05%), crude fiber (0.94%), and crude fat (0.72%). For additional quality control of the tested samples, high-performance-thin layer chromatography (HPTLC)-based fingerprinting was performed using the CAMAG Application System (Muttenz, Switzerland) as follows. WEHE and β-*D*-(1,3)-(1,6)-glucan were dissolved in 90% HPLC-grade methanol and applied to a pre-washed silica gel 60 F_{254} HPTLC plate (10×10 cm, 0.2 mm thick silica gel, Merck, Darmstadt, Germany) with an automated applicator (Linomat IV, CAMAG, Muttenz, Switzerland). The samples were then separated (migration distance: 60 mm) using HPLC-grade *n*-butanol/methanol/water (50:25:20). Thereafter, glucose-specific staining with aniline-diphenylamine-phosphoric acid or protein-specific staining with ninhydrin reagent was separately performed. Because the β-*D*-(1,3)-(1,6)-glucan preparation used in our experiment was likely to have proteins, bands with the same Rf value in β-*D*-(1,3)-(1,6)-glucan were stained with both reagents. The developed plate was visualized at 254 nm using a Reprostar 3 Digital Camera System (CAMAG; Figure 1A–1D).

Preparation of splenocytes and purified NK cells, and fluorescence-activated cell sorter analysis The spleens were removed aseptically from BALB/c mice and homogenized with an iron mesh and syringe plunge. The red blood cells were lysed by adding lysis buffer (0.15 mol/L ammomium chloride) to the cell pellet and washed with phosphate buffered saline (PBS). The single-cell suspension was used for NK cell isolation or cultured in RPMI-1640 medium (JBI, Daegu, Korea) containing 10% fetal bovine serum (FBS, JBI, Korea) without antibiotics for use in other assays. NK cell isolation was performed by magnetic cell separation (MACS) following the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, the splenocytes were labeled with CD49B (DX5) microbeads and then loaded onto a MACS column to capture the NK cells. The magnetically-retained NK cells were eluted as a positively-selected cell fraction. The NK cells and NK cell-depleted splenocytes were then used for ^{51}Cr release assays. To evaluate the efficacy of the NK cell selection, fluorescence-activated cell sorter (FACS) analysis was performed with anti-CD3-PE,

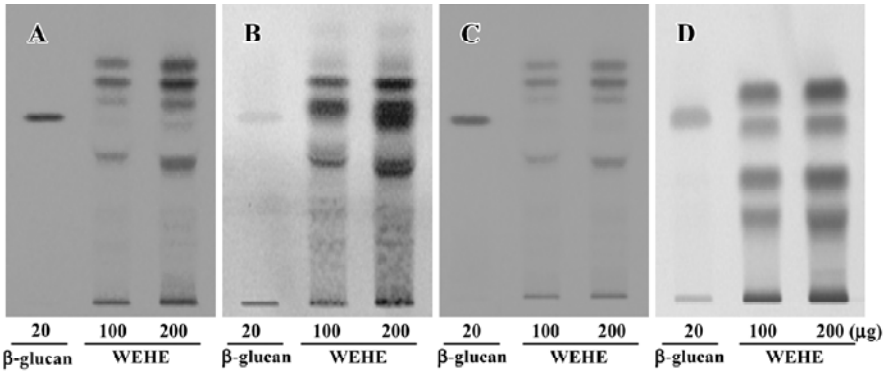

Figure 1. Fingerprint of WEHE. WEHE and β-*D*-(1,3)-(1,6)-glucan were dissolved in 90% HPLC-grade methanol and applied to a prewashed silica gel 60 F_{254} HPTLC plate. WEHE (2 or 4 mL of a 50 mg/mL solution) and 2 μL β-*D*-(1,3)-(1,6)-glucan (10 mg/mL) were separated (migration distance 60 mm) using HPLC-grade *n*-butanol/methanol/water (50:25:20). Images were visualized at UV 254 nm (A, C) or under white light after staining with aniline-diphenylamine-phosphoric acid (B) or ninhydrin reagent (D).

B220-PE, and anti-DX5 FITC antibody labeling (BD Pharmingen, San Diego, CA, USA).

Culture of Yac-1 cells and radio-labeling Yac-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS without antibiotics at 37 ºC in a humidified incubator with 5% CO_2. To test for NK cell-derived cytolytic activity, 4×10^6 Yac-1 cells were labeled with 100 μCi ^{51}Cr by incubation for 2 h at 37 ºC in a humidified incubator set with 5% CO_2. After washing twice with RPMI-1640 medium containing 10% FBS, the cells were resuspended in 10 mL RPMI-1640 medium supplemented with 10% FBS for use as target cells.

^{51}Cr release assay with splenocytes or NK cells The ^{51}Cr release assay was performed as previously described with slight modifications[25]. Briefly, the total splenocytes, isolated NK cells, or NK cell-depleted splenocytes were prepared in RPMI-1640 medium as effector cells. Aliquots (100 μL) of each cell suspension were plated onto round-bottom 96-well plates (3 wells per group), with 50 μL WEHE at various concentrations (0, 1, 10, or 100 mg/L) or LPS (0.1 mg/L), with or without IL-2 (300 U/mL), and incubated for 20 h at 37 ºC in a humidified incubator with 5% CO_2. Thereafter, 50 μL target cells (1×10^4 cells) were mixed with the effector cells (5×10^5 or 1×10^6 spleen cells or NK cell-depleted splenocytes, and 5×10^4 or 1×10^5 isolated NK cells) and incubated for an additional 4 h. The maximum-release groups were induced by adding 50 μL 2% NP-40, and the spontaneous-release groups were induced by adding 150 μL complete medium. Gamma irradiation from each well was then assessed using a scintillation counter (Packard Instruments, Meriden, CT, USA). Cytotoxic activity was defined as the percentage of specific ^{51}Cr released using the following equation:

Specific lysis (%)=

$$(RA_{experimental}-RA_{spontaneous})/(RA_{maximal}-RA_{spontaneous})\times100.$$

RA =radioactivity

In addition to the above procedure, 2000 pg/mL anti-IL-12 (p40) neutralizing antibody was added to the splenocytes to confirm the effect of WEHE on IL-12-mediated NK cell activation.

RT-PCR for the analysis of IL-12 The total RNA was extracted from splenocytes treated for 12 h with various concentrations of WEHE (0, 1, 10, or 100 mg/L) or LPS (0.1 mg/L) using Trizol (Invitrogen, Carlsbad, CA, USA) and RNeasy columns (Qiagen, Valencia, CA, USA). cDNA was synthesized using 10 pmol oligo dT and 10 pmol random hexamer (Bioneer, Daejeon, Korea). After the cDNA synthesis, RT-PCR was performed using the following primers (forward and reverse, respectively). β-actin: 5'-GTGGGGCGCCCCAG-GCACCA-3'and5'-CTCCTTAATGTCACGCACGATTTC-3'; IL-12 (p40): 5'-TCA GGG GAA CTG CTA CTG CT-3' and 5'-TGA CAC GCC TGA AGA AGATG-3'. The reactions were performed with 0.2 μL Go *Taq* DNA polymerase (5 U/mL; Promera, Madison, Wisconsin, USA), 1 μL 10 pmol primer pairs, 3 μL 10 mmol dNTP, 6 μL 5x reaction buffer, 19.8 μL distilled water, and 1 μL cDNA. PCR was performed for 27 and 40 amplification cycles for β-actin and IL-12 (p40), respectively, under the following conditions: initial denaturation at 95 ºC for 5 min, denaturation for each cycle at 95 ºC for 1 min, annealing at 58 ºC for 40 s, and elongation at 72 ºC for 40 s.

ELISA analysis for IL-12 and IFN-gamma The splenocytes (3×10^7 cells) were plated on 24-well plates and pre-cultured for 4 h before being incubated with various concentrations of WEHE (0, 1, 10, or 100 mg/L) or LPS (0.1 mg/L) for 12 or 24 h in RPMI-1640 medium containing 10% FBS. The cell-free supernatant was collected and used for measuring the concentration of released IL-12 (p40) and IFN-gamma using an ELISA assay kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Briefly, a 96-well microplate was coated with a capture antibody by incubating overnight at room temperature, then washed, blocked, and rewashed. The samples and standards were added to the plate and incubated for 2 h at room temperature. After adding streptavidin-horseradish peroxidase and mixing the substrate solution, the plate was read at 450 nm and 560 nm (ie, the reference wavelengths) using a microplate reader (Molecular Devices, Union City, CA, USA).

Results

WEHE activates NK cells among splenocytes to lyse Yac-1 cells Yac-1, a NK cell-sensitive target cell line[26], was labeled with ^{51}Cr to examine the effect of WEHE on NK cell cytotoxicity using BALB/c splenocytes. The splenocytes treated for 20 h with 1, 10, or 100 mg/L WEHE or 0.1 mg/L LPS as a positive control were co-cultured with ^{51}Cr-labeled Yac-1 cells for 4 h. Upon exposure of the cells to WEHE, the cytolytic activity of the splenocytes was significantly increased in a dose-dependent manner ($P<0.01$, Figure 2A). A synergic effect was observed following co-treatment with WEHE and IL-2 as expected ($P<0.05$ or 0.01, Figure 2B). We then investigated whether the WEHE-driven cytolytic effect was caused by NK cells and not by other cellular components within the spleen. Thus, we repeated the assay with NK cell-depleted splenocytes, and found no enhancement of cytotoxicity following treatment with WEHE (Figure 2C). Those results showed that WEHE-activated NK cells among the splenocytes were responsible for lysis of the Yac-1 cells.

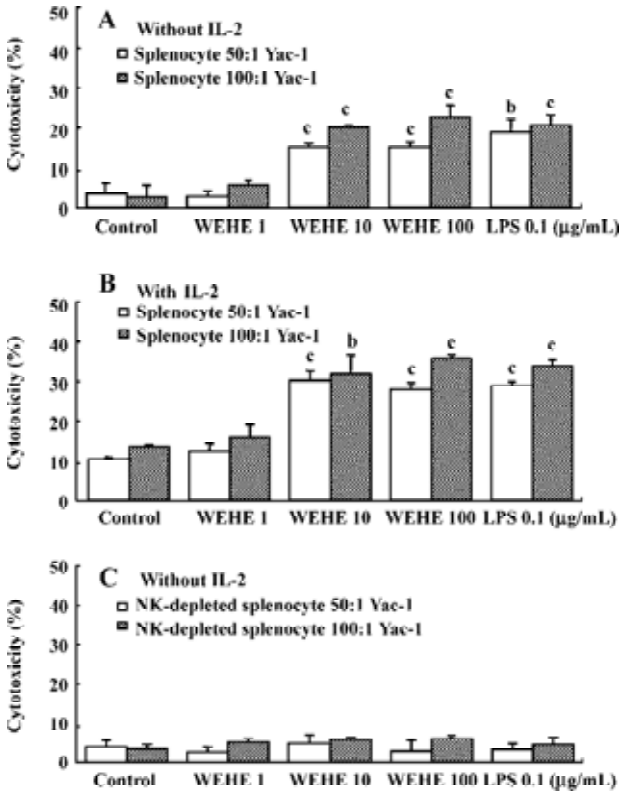

Figure 2. NK cell-specific cytotoxicity induced by WEHE. Total splenocytes (A, B) or NK cell-depleted splenocytes (C) were treated with various concentrations of WEHE (0, 1, 10, or 100 mg/L) or LPS (0.1 mg/L) with (B) or without (A, C) 300 U/mL IL-2 for 20 h. Yac-1 cells (1×10^4 cells) were mixed with effector cells (5×10^5 or 1×10^6 cells) and incubated for additional 4 h. Cell-free supernatant was analyzed for gamma irradiation. n=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control group.

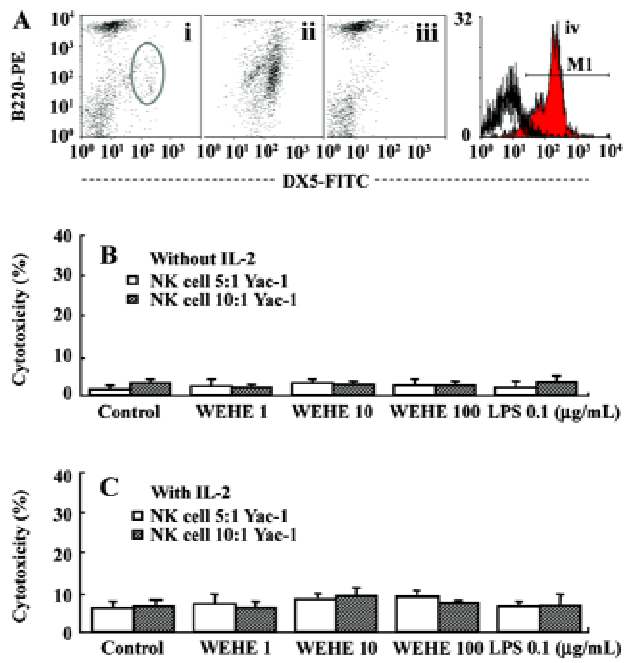

Figure 3. FACS analysis of isolated NK cells and the cytolytic activity of WEHE-treated NK cells. (A) total splenocytes (i), NK cell-depleted splenocytes (ii), and purified NK cells (iii) isolated using MACS were analyzed by FACS after labeling with anti-CD3-PE, B220-PE, and anti-DX5 FITC antibodies. NK cell-depleted splenocytes (white) and purified NK cells (red) are comparably presented in the histogram (iv). Purified NK cells were treated with various concentrations of WEHE (0, 1, 10, and 100 mg/L) or LPS (0.1 mg/L) with (C) or without (B) 300 U/mL IL-2 for 20 h. Yac-1 cells (1×10^4 cells) were mixed with effector cells (5×10^4 or 1×10^5 cells) and incubated for additional 4 h. Cell-free supernatants were analyzed for gamma irradiation.

WEHE indirectly activates NK cells to kill Yac-1 cells Since WEHE stimulated splenocyte-derived NK cells to lyse Yac-1 cells, we next examined whether WEHE could directly activate NK cells using NK cells purified from splenocytes by MACS (Figure 3A). Interestingly, regardless of whether the NK cells were treated with WEHE alone or in combination with IL-2, no enhancement of NK cell cytolytic activity was observed (Figure 3B, 3C). This result indicated that WEHE activated NK cells indirectly via the induction of other immuno-mediators or cellular components.

Treatment with anti-IL-12 antibody reduces WEHE-induced cytolytic activity towards Yac-1 cells The above results led us to hypothesize that WEHE generates the cytolytic activity of NK cells by inducing IL-12 production, which is the main activator of NK cells[27]. We found that IL-12-treated splenocytes were strongly activated to lyse Yac-1 cells, but this activity was abolished by co-treatment with a neutralizing antibody against IL-12 ($P<0.01$, Figure 4A). We also observed a significant decrease of WEHE-derived cytolytic activity in splenocytes by treatment with an anti-IL-12 antibody as expected ($P<0.05$, Figure 4B). These results strongly suggest the involvement of IL-12 in the WEHE-derived augmentation of NK cell activity.

WEHE induces the transcription and translation of IL-12 and IFN-gamma in splenocytes Based on the above results indicating that IL-12 mediates the activation of NK cells by WEHE, we examined the changes in the expression of IL-12 (p40) and protein production in splenocytes treated with WEHE for 12 h. In addition, we measured the production of IFN-gamma in WEHE-treated splenocytes. WEHE treatment increased the expression of the IL-12 (p40) in splenocytes in a dose-dependent manner (Figure 5A). In addition, the production of the IL-12 (p40) and IFN-gamma proteins increased in a dose-dependent manner at 12 and

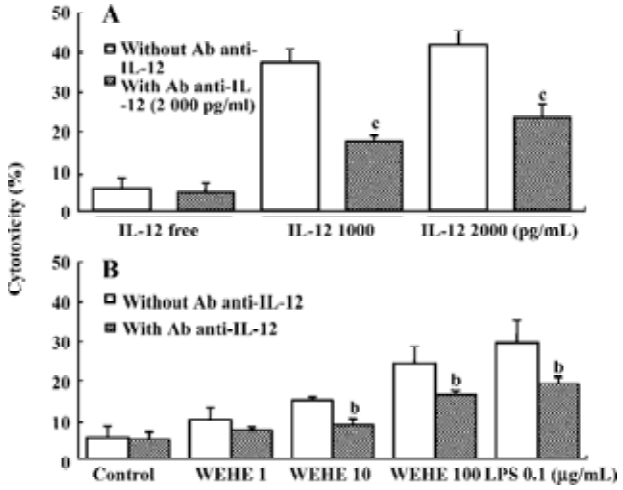

Figure 4. NK-specific cytotoxicity mediated by IL-12 from WEHE-stimulated splenocytes. Total splenocytes (1×10^6 cells) treated with IL-12 (1000 or 2000 ng/L) (A) or various concentrations of WEHE (0, 1, 10, or 100 mg/L) or LPS (0.1 mg/L) (B) were cultured with or without a neutralizing antibody against IL-12 (2000 pg/mL) for 20 h. Yac-1 cells (1×10^4 cells) were mixed with effector cells and incubated for additional 4 h. Cell-free supernatants were analyzed for gamma irradiation. n=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* IL-12 antibody-free group.

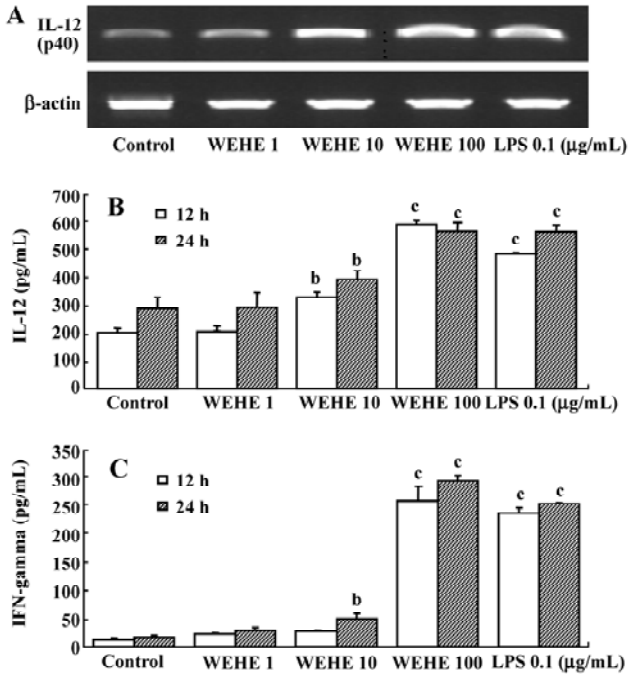

Figure 5. Transcription and expression of the IL-12 (p40) and IFN-gamma proteins in splenocytes. Total splenocytes were treated with WEHE (0, 1, 10, or 100 mg/L) or LPS (0.1 mg/L) for 12 h. Total RNA was isolated and then analyzed for IL-12 (p40) gene expression using RT-PCR (A). Cell-free supernatant from splenocytes treated with WEHE for 12 or 24 h were analyzed for IL-12 (p40) and IFN-gamma production using ELISA (B and C). n=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control group.

24 h (P<0.05 and 0.01, respectively, Figure 5B, 5C). No increase in IL-12 p70 production was observed under the same experimental condition (data not shown).

Discussion

NK cells are an important part of the innate immune system and efficiently eliminate cancer cells in the body. Thus, the development of NK cell-based immunomodulating agents with minimal side effects could be very important for treating cancer patients[28–30]. Here, we report that WEHE enhances NK cell activity in mouse splenocytes. In addition, WEHE induces the expression of IL-12, which efficiently activates the cytolytic ability of NK cells.

The increase in NK cell cytolytic activity appears to be an indirect effect of WEHE. Indeed, isolated NK cells treated with WEHE do not exhibit increased cytolytic activities towards Yac-1 cells, although a significant increase in NK cell activity by WEHE was observed in splenocytes containing mixed immune cells. It is possible that mediators positively influence NK cells. For example, the cytokine IL-12 may mediate the WEHE-derived activation of NK cells, since IL-12 remarkably increased the cytotoxic activities of NK cells. In addition, when WEHE plus an anti-IL-12 neutralizing antibody were co-administered to splenocytes, Yac-1 cell lysis was significantly attenuated. Moreover, IFN-gamma, whose expression is strongly induced by IL-12, was highly expressed in splenocytes cultured with WEHE. These results support previous data showing that IL-12 and IFN-gamma are important anticancer cytokines with anti-angiogenic and antimetastatic effects[31–34].

Although we found that IL-12 was involved in the activation of NK cells, we did not identify which cell types among the splenocytes preferentially respond to WEHE. Splenocytes include various cell types, including B cells, T cells, macrophages, NK cells, and dendritic cells. Previous studies have shown that IL-12 is largely produced by activated antigen presenting cells, such as dendritic cells, B cells, and macrophages[35]. Thus, future studies are needed to identify which cells respond to WEHE, leading to the production of cytokines, including IL-12, that subsequently enhance NK cell activity. It remains to be clarified how WEHE controls the expression of IL-12. Even though IL-12 is comprised with two distinct subunits, p35 and p40, we here verified the induction of IL-12 as only p40 at the gene and protein levels.

So, further study should be performed for the expression of p35 and p70).

Despite these unresolved matters, the enhanced NK cell cytotoxicity and production of IL-12 and IFN-gamma suggest that WEHE may be a valuable immunomodulating agent for cancer patients. We previously reported that approximately 28% (*w*/*w*) of WEHE was composed of polysaccharides, mainly β-glucan, that participate in the induction of NO and IL-1-beta expression via increased NF-κB binding activity in rat macrophages and RAW 264.7 cells[22,23]. Similarly, several studies have shown that mushroom-derived fractions or polysaccharides activate NK cell function and cytokine production[37,37]. One group reported an increase in the activity and number of NK cells, including a high IFN-gamma plasma concentration following a 12-week treatment with *Gano-derma lucidum* polysaccharide extract in advanced cancer patients[38].

Taken together, the current data indicate that WEHE has an inductive effect on splenocyte-derived NK cell activation leading to cytolysis of Yac-1 cells. We also verified that the underlying mechanism of the immunomodulatory effect of WEHE on splenocytes involves the stimulation of IL-12 production. This study enhances our understanding of the cancer-related biological properties of *H erinaceum* and supports its clinical applications as an immunomodulator.

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