

# Immunostimulatory and anti-obesity activities of *Adenocaulon himalaicum* leaves in RAW264.7 and 3T3-L1 cells

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**Abstract.** The present study investigated the immunostimulatory activity and anti-obesity activity of *Adenocaulon himalaicum* leaf extracts (AHL) in RAW264.7 cells and 3T3-L1 cells. AHL increased the production of immunostimulatory factors, such as NO, inducible nitric oxide synthase (iNOS), IL-1 $\beta$ , IL-6 and TNF- $\alpha$  and activated the phagocytotic activity in RAW264.7 cells. Inhibition of Toll-like receptor 4 (TLR4) attenuated the AHL-mediated production of immunostimulatory factors and activation of phagocytic activity in RAW264.7 cells. Inhibition of p38 and JNK blocked the AHL-mediated production of immunostimulatory factors, whereas inhibition of TLR4 suppressed the AHL-mediated phosphorylation of p38 and JNK. Additionally, AHL blocked the lipid accumulation in 3T3-L1 cells. AHL downregulated proliferator-activated receptor  $\gamma$ , CCAAT enhancer binding protein  $\alpha$  and perilipin-1 levels, while upregulating adipose triglyceride lipase, phosphorylated (p-)hormone-sensitive lipase, p-adenosine monophosphate activated protein kinase, uncoupling protein 1, peroxisome-proliferator-activated receptor- $\gamma$  coactivator-1  $\alpha$  and PR domain containing 16 levels in 3T3-L1 cells. These findings suggested that AHL may exert immunostimulatory activity through macrophages via TLR4-mediated activation of p38 and JNK and anti-obesity activity by blocking lipid accumulation via the inhibition of adipogenesis and induction of lipolysis and browning of white adipocytes.

## Introduction

Pandemics such as COVID-19 have drawn attention to human immunity. The immune system protects the human body

from disease by detecting invasion and removing foreign pathogens (1). Thus, maintenance of a well-functioning immune system is essential for human survival (1). The immune system employs both innate and adaptive immunity to fight foreign pathogens (2). Innate immunity is the first rapid defense response against these pathogens, whereas adaptive immunity is an antigen-specific immune response that is important when these pathogens are not eliminated by the innate immune system (3,4). Strong interactions between the innate and adaptive immune systems are crucial for an effective response to foreign pathogens and defects in either immune response result in various diseases (2). The most effective way to strengthen these immune systems is to activate the functions of various immune cells that constitute the immune system.

Obesity increases infection and mortality rates in infectious diseases such as COVID-19 (5). There exists a close connection between obesity and infectious diseases (6). Obesity increases susceptibility to infectious diseases by weakening the immune response of innate immune cells when they are confronted with invading foreign pathogens (6). In addition, obesity leads to impairment of the adaptive immune system (7). Therefore, obesity management is considered a good strategy for strengthening the body's immune system.

*Adenocaulon himalaicum* Edgew. (*A. himalaicum*) is a plant distributed across Korea, China, Japan, Russia and Southeast Asia. In Korea, the leaves of *A. himalaicum* have long been used as a wild vegetable (8). *A. himalaicum* has been traditionally used as an herbal medicine to treat abscesses, bleeding and inflammation (9). *A. himalaicum* has been reported to have antioxidant (8), anticancer (10) and skin damage-protection activities (8). However, other pharmacological activities of *A. himalaicum* remain to be elucidated. Specifically, there is currently no background on the immunostimulatory and anti-obesity activity of *A. himalaicum*. Thus, the present study investigated the immunostimulatory activity of *A. himalaicum* leaf extracts in RAW264.7 cells and the anti-obesity activity of *A. himalaicum* leaf extracts in 3T3-L1 cells.

## Materials and methods

**Chemical reagents.** 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Griess reagent and Neutral Red were purchased from MilliporeSigma.

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Inhibitors, such as C29 [Toll-like receptor (TLR)2 inhibitor], TAK-242 (TLR4 inhibitor), PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor) were purchased from MilliporeSigma. The primers for inducible nitric oxide synthase (iNOS), IL-1 $\beta$ , IL-6 and GAPDH were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). The primary antibodies individually raised against phosphorylated (p-)ERK1/2 (cat. no. 4377), ERK1/2 (cat. no. 9102), p-p38 (cat. no. 4511), p38 (cat. no. 9212), p-JNK (cat. no. 9251), JNK (cat. no. 9252), CCAAT enhancer binding protein  $\alpha$  (CEBPA; cat. no. 8178), proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ; cat. no. 2430), adipose triglyceride lipase (ATGL; cat. no. 2138), p-hormone-sensitive lipase (HSL; cat. no. 4137), HSL (cat. no. 4107), perilipin-1 (cat. no. 9349), p-adenosine monophosphate activated protein kinase (AMPK; cat. no. 2535), AMPK (AMPK, cat. no. 5831), uncoupling protein 1 (UCP-1; cat. no. 14670) and  $\beta$ -actin (cat. no. 5125) were purchased from Cell Signaling Technology, Inc. Other primary antibodies, such as anti-peroxisome proliferator-activated receptor- $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ; cat. no. sc-518025) and anti-PR domain containing 16 (PRDM16; cat. no. ab106410), were purchased from Santa Cruz Biotechnology, Inc. and Abcam. Secondary antibodies, such as anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody (cat. no. 7074) and anti-mouse IgG HRP-linked antibody (cat. no. 7076), were purchased from Cell Signaling Technology, Inc.

**Sample preparation.** *A. himalaicum* leaves (AHL) used in this study were provided as dry powder by the National Institute of Forest Science, Korea. *A. himalaicum* is not listed in the IUCN Red List of Threatened Species, so it is neither an endangered nor a protected species. Water extraction from *A. himalaicum* leaves was performed by adding 20 times the weight of distilled water to 10 g of *A. himalaicum* leaves and extracting them in a water bath at 40°C for 24 h. After 24 h, the extract was lyophilized, dissolved in distilled water and added to the cells.

**Cell culture.** The murine macrophage cell line RAW264.7 and mouse pre-adipocyte 3T3-L1 cells used in this study were purchased from the American Type Culture Collection. RAW264.7 cells were cultured in a CO<sub>2</sub> incubator (37°C, 5% CO<sub>2</sub>) using a DMEM/F-12 medium (Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 unit/100  $\mu$ g/ml). Subculture of RAW264.7 cells was performed once every 2 days. 3T3-L1 cells were cultured in a CO<sub>2</sub> incubator (37°C; 5% CO<sub>2</sub>) using DMEM/F-12 medium containing 10% bovine calf serum and penicillin/streptomycin (100 unit/100  $\mu$ g/ml). The 3T3-L1 cells were subcultured once every 3 days.

**Measurement of cell viability in RAW264.7 cells.** Toxicity to cells was measured using an MTT assay. RAW264.7 cells were treated with AHL and cultured for 24 h at 37°C in a 96-well plate. MTT solution (1 mg/ml) was then added and incubated for an additional 4 h. After 4 h, the DMSO solution was added and incubated for 10 min. Absorbance was measured at 570 nm using a UV/visible spectrophotometer (Human Cop., Xma-3000PC, Seoul, Korea).

**Treatment of RAW264.7 cells with the chemical inhibitors.** The chemical inhibitors, such as C29, TAK-242, PD98059, SB203580 and SP600125, were used to pre-treat RAW264.7 cells for 2 h at 37°C, 2 h prior to AHL treatment.

**Measurement of NO in RAW264.7 cells.** RAW264.7 cells were cultured in a 96-well plate using DMEM/F-12 medium supplemented with 10% FBS and treated with AHL for 24 h. To measure the amount of NO, the cell culture medium and Griess solution were mixed 1:1 and allowed to react for 15 min. The absorbance was then measured at 540 nm using a UV/Visible spectrophotometer (Humancorp; Xma-3000PC). Values were calculated using NaNO<sub>2</sub> as a standard.

**Measurement of phagocytotic activity in RAW264.7 cells.** Phagocytosis of RAW264.7 cells was performed using Neutral Red assay (11). RAW264.7 cells were cultured in a 96-well plate using DMEM/F-12 medium supplemented with 10% FBS and treated with AHL for 24 h. To measure the phagocytosis in RAW264.7 cell line, cells were stained with 0.01% Neutral Red solution for 2 h and then Neutral Red was eluted with lysis buffer containing 50% ethanol and 1% acetic acid at a ratio of 1:1. The absorbance of the eluted Neutral Red was measured at 540 nm using a UV/visible spectrophotometer (Humancorp; Xma-3000PC).

**Differentiation from preadipocytes to mature adipocytes in 3T3-L1 cells.** Two days (D0) after culturing 3T3-L1 cells in a 6-well plate to 100% confluency, the 3T3-L1 cells were differentiated for 2 days in a DMEM/F-12 medium containing DMI (0.05 mM IBMX + 1  $\mu$ M dexamethasone + 10  $\mu$ g/ml insulin) (D2). Then, the 3T3-L1 cells were cultured for 2 days in a DMEM/F-12 medium containing 10  $\mu$ g/ml insulin (D4). 3T3-L1 cells were further cultured for 4 days, with the DMEM/F-12 medium replaced once every 2 days (D6-D8). AHL were administered from D0 to D8 and experimental analysis was performed on D8.

**Oil-Red O (ORO) staining in 3T3-L1 cells.** Lipid accumulation in 3T3-L1 cells was confirmed using ORO staining. The 3T3-L1 cells were washed with PBS, fixed with 10% formalin and stained with the ORO staining reagent for 20 min at room temperature. After staining, the staining solution was removed, the cells were washed with distilled water and the degree of lipid accumulation was observed under a light microscope (Olympus Corporation). After microscopic observation, the stained ORO was eluted with 100% isopropanol and the absorbance was measured at 500 nm using a UV/visible spectrophotometer (Humancorp; Xma-3000PC). Lipid accumulation in ORO staining was calculated by measuring the absorbance of released ORO dye in cells.

**Reverse transcription-polymerase chain reaction (RT-PCR) analysis.** To obtain RNA from RAW264.7 cells, total RNA was extracted from RAW264.7 cells using a RNeasy Mini kit (Qiagen GmbH). The extracted RNA was synthesized into cDNA using 1  $\mu$ g of total RNA through quantitative analysis and Verso cDNA kit (Thermo Fisher Scientific, Inc.). RT-PCR of iNOS, IL-1 $\beta$ , IL-6, TNF- $\alpha$  and GAPDH genes was performed using the synthesized cDNA and PCR Master

Table I. Sequences of primers used in the amplification of cDNA.

Primers	Sequences
iNOS	Forward 5'-ttgtgcatgcacctaggctggaa-3' Reverse 5'-gaccttcgcattagcatggaagc-3'
IL-1 $\beta$	Forward 5'-ggcaggcagtagtactcatt-3' Reverse 5'-cccaaggccacaggtattt-3'
IL-6	Forward 5'-gaggataccactcccaacagacc-3' Reverse 5'-aagtgcacatcggtgttcataca-3'
TNF- $\alpha$	Forward 5'-tggaactggcagaagaggca-3' Reverse 5'-tgctctccacttggtggtt-3'
GAPDH	Forward 5'-ggactgtggtcatgagccctcca-3' Reverse 5'-actcacggcaattcaacggcac-3'

iNOS, inducible nitric oxide synthase.

Mix kit (Promega Corporation). The primer sequences used in this study are listed in Table I. PCR reaction (PCR Thermal Cycler Dice; Takara Bio, Inc.) was performed for 30 cycles under the following conditions: Denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min. After the PCR, the products were electrophoresed on a 1% agarose gel at 100 V for 15 min. The results presented are not mRNA, but rather DNA. Therefore, the brightness of the expressed DNA bands was quantified using UN-SCAN-IT (Silk Scientific, Inc.).

**Western blot analysis.** After washing the cells with PBS, RIPA buffer was added to extract proteins, the recovered cells were centrifuged at 25,200 x g for 30 min at 4°C and the protein concentration was quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc.). The quantified proteins (25  $\mu$ g/well) were separated using SDS-PAGE (10% polyacrylamide gel) and transferred onto a nitrocellulose blotting membrane (Thermo Fisher Scientific, Inc.). The transferred membranes were blocked with 5% skimmed milk for 1 h at room temperature. For the primary antibody reaction, the membrane was incubated at 4°C overnight with a 5% BSA solution containing the primary antibodies (1:1,000). Then, a 5% BSA solution containing secondary antibodies (1:1,000) was added to the membrane and allowed to react at room temperature for 1 h. To activate the horseradish peroxidase molecules bound to the secondary antibody, western blotting substrate was added to the membrane (Cytiva) and protein expression was confirmed using a LI-COR C-DiGit Blot Scanner (LI-COR Biosciences). Protein bands were quantified using UN-SCAN-IT gel software version 5.1 (Silk Scientific, Inc.). Actin was used as a loading control for normalization in western blot analysis.

**Statistical analysis.** All experiments were repeated at least three times. Statistical analyses were verified using GraphPad Prism version 5.0 (Dotmatics) and data are presented as mean  $\pm$  standard deviation. Each data point was analyzed

using one-way analysis of variance and the data was analyzed using the Bonferroni post hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Effect of AHL on macrophage activation in RAW264.7 cells.** To evaluate the effect of AHL on macrophage activation, the production of immunostimulatory factors and the activation of phagocytosis were analyzed in AHL-treated RAW264.7 cells. The production of NO was increased in AHL-treated RAW264.7 cells. DNA levels of immunostimulatory factors such as iNOS, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were increased in AHL-treated RAW264.7 cells (Fig. 1A). In addition, AHL increased the phagocytosis of RAW264.7 in a concentration-dependent manner (Fig. 1B). However, AHL had no effect on the viability of RAW264.7, indicating that AHL is not cytotoxic to RAW264.7 cells (Fig. 1C). Taken together, these results suggested that AHL activates macrophages.

**Effects of TLR2/4 on AHL-mediated activation of RAW264.7 cells.** To investigate the involvement of TLR2 and TLR4 in AHL-mediated macrophage activation, the production of immunostimulatory factors and phagocytosis was measured in AHL-treated RAW264.7 cells in the presence of C29 (TLR2 inhibitor) and TAK-242 (TLR4 inhibitor). The NO level and DNA level for iNOS, IL-1 $\beta$  and IL-6 were increased in both AHL treatment alone and AHL treatment in the presence of C29 with little difference (Fig. 2A). However, inhibition of TLR4 by TAK-242 markedly reduced the AHL-mediated increase in the NO level and DNA level for iNOS, IL-1 $\beta$  and IL-6 (Fig. 2A). Furthermore, inhibition of TLR2 by C29 did not reduce AHL-mediated phagocytosis of RAW264.7 cells, whereas inhibition of TLR4 by TAK-242 significantly blocked AHL-mediated phagocytosis (Fig. 2B). Taken together, these results indicated that AHL-mediated activation of macrophages may be dependent on TLR4.

**Effects of MAPK signaling pathway on AHL-mediated activation of RAW264.7 cells.** To analyze the effect of AHL on MAPK activation, the phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK1/2), p38 and c-JNK were investigated in AHL-treated RAW264.7 cells. AHL increased the phosphorylation of ERK1/2, p38 and JNK, indicating that AHL can activate ERK1/2, p38 and JNK signaling (Fig. 3A). To analyze the effect of ERK1/2, p38 and JNK signaling on AHL-mediated activation of RAW264.7 cells, the present study investigated the level of immunostimulatory factors in AHL-treated RAW264.7 cells in the presence of specific ERK1/2, p38 and JNK signaling inhibitors. Inhibition of p38 by SB203580 and inhibition of JNK by SP600125 significantly reduced AHL-mediated increase in the NO level and DNA level for iNOS, IL-1 $\beta$  and IL-6 (Fig. 3B). However, inhibition of ERK1/2 by PD98059 did not reduce AHL-mediated increase in the NO level and DNA level for iNOS, IL-1 $\beta$  and IL-6. These results indicate that the activation of p38 and JNK signaling may be important for AHL activation in macrophages. Finally, the effect of TLR4 on AHL-mediated activation of p38 and JNK signaling was

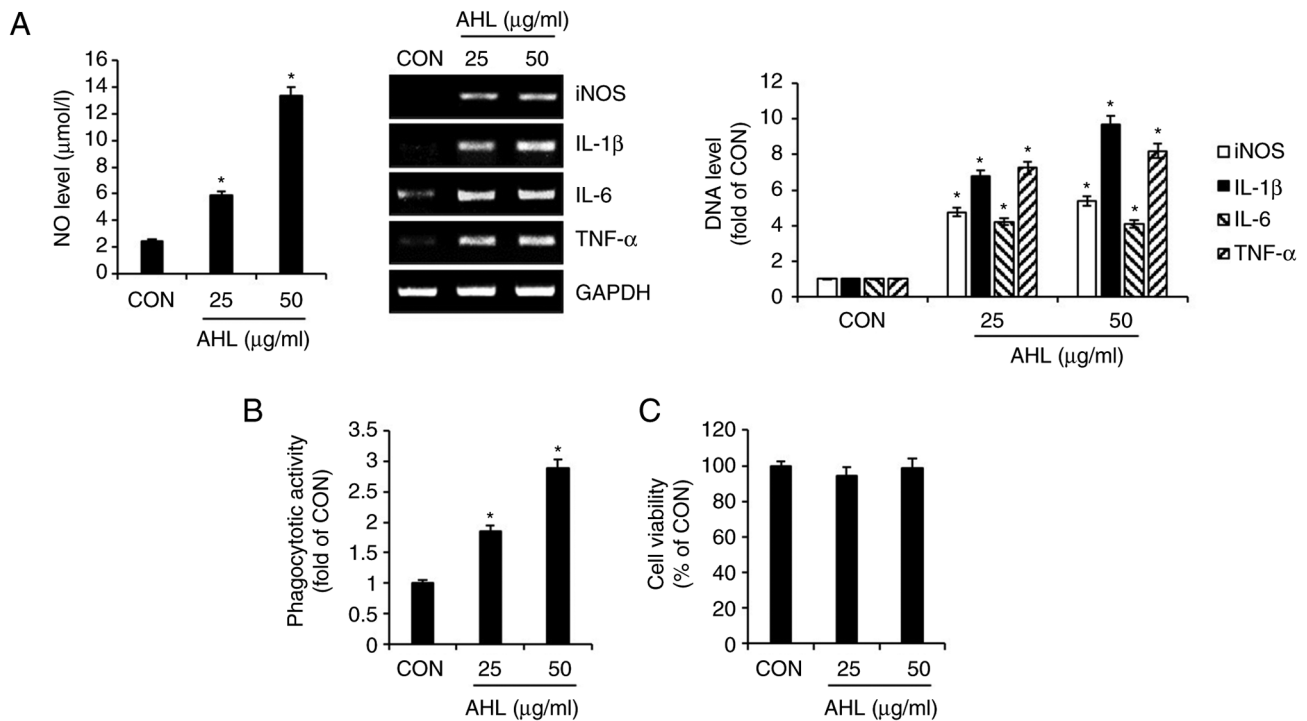


Figure 1. Effect of AHL on macrophage activation in RAW264.7 cells. RAW264.7 cells were treated with AHL for 24 h. (A) NO level (left panel) and DNA level (right panel), (B) phagocytotic activity and (C) cell viability. \* $P < 0.05$  vs. CON group. AHL, *Adenocaulon himalaicum* leaf extracts; CON, control group without AHL treatment.

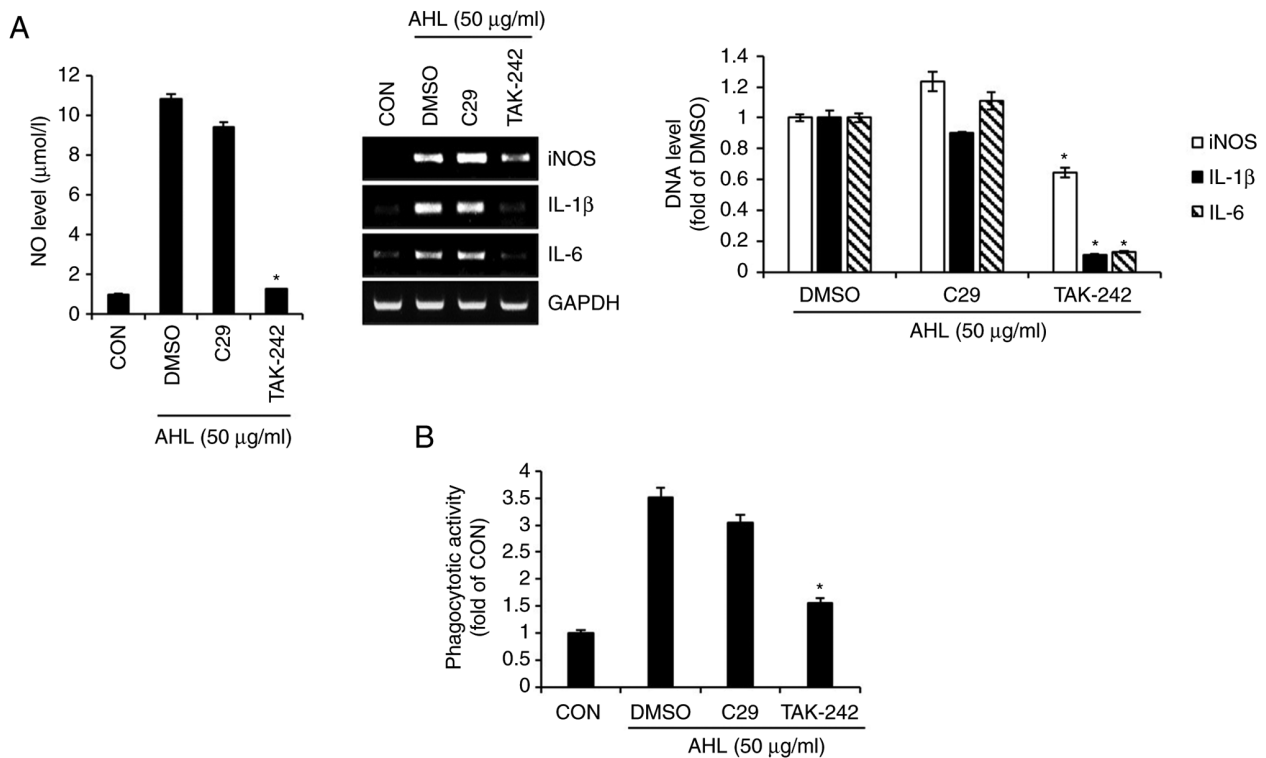


Figure 2. Effect of TLR2/4 on AHL-mediated macrophage activation in RAW264.7 cells. (A, left panel) NO level, (A, right panel) DNA level and (B) phagocytotic activity in response to AHL treatment in the presence of C29 (100 μg/ml) or TAK-242 (10 μM). \* $P < 0.05$  vs. CON group or DMSO group. TLR, Toll-like receptor; AHL, *Adenocaulon himalaicum* leaf extracts; CON, control group without AHL treatment.

investigated. Inhibition of TLR4 by TAK-242 reduced the phosphorylation of p38 and JNK by AHL (Fig. 3C), indicating that the activation of p38 and JNK signaling by AHL

may depend on TLR4. Taken together, these results suggested that AHL activates macrophages by activating p38 and JNK signaling in a TLR4-dependent manner.

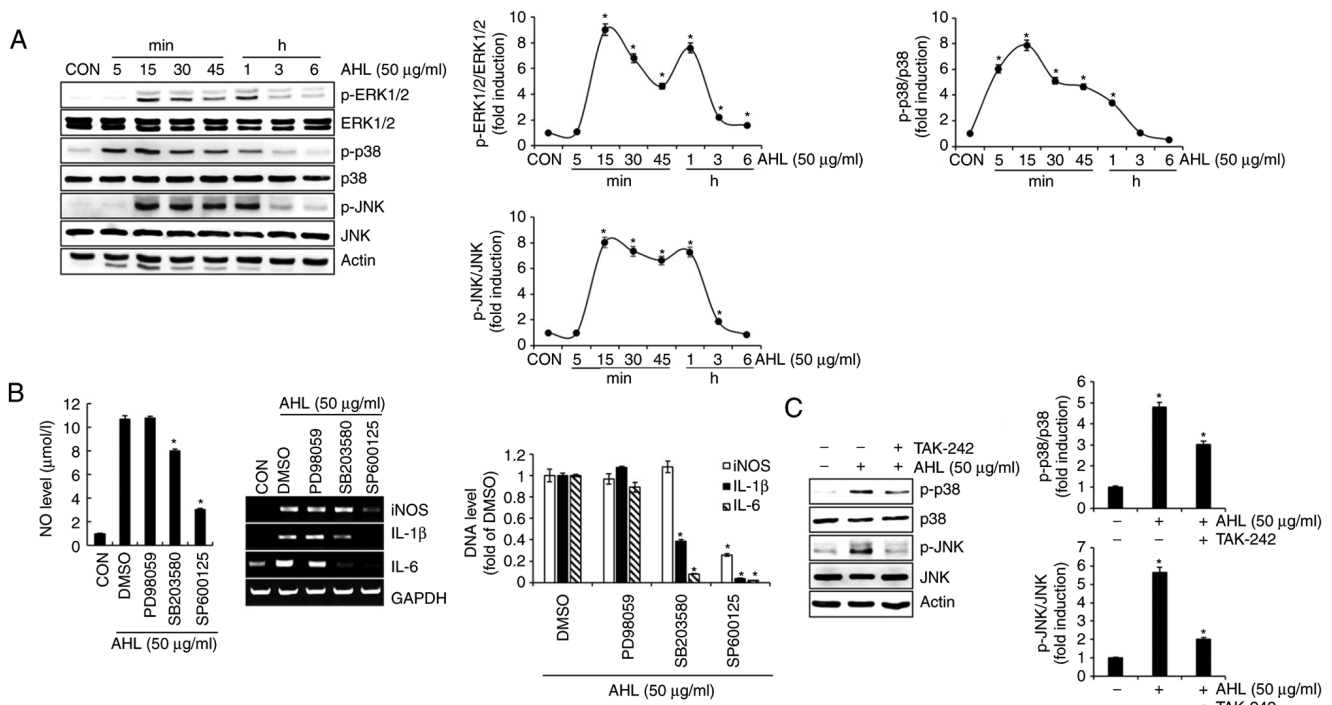


Figure 3. Effect of MAPK signaling pathway on AHL-mediated production of immunostimulatory factors in RAW264.7 cells. (A) Western blot analysis of the changes in p-ERK1/2, p-p38 and p-JNK according to AHL treatment over time. All the protein bands were derived from the same membrane. (B) NO level (left panel) and DNA level (right panel) in response to AHL treatment in the presence of D98059 (40 µM), SB203580 (40 µM), or SP600125 (40 µM) and (C) western blot analysis of the changes in p-p38 and p-JNK in response to AHL treatment in the presence of TAK-242 (10 µM). All the protein bands were derived from the same membrane. \*P<0.05 vs. CON group or DMSO group. AHL, *Adenocaulon himalaicum* leaf extracts; p-, phosphorylated; CON, control group without AHL treatment.

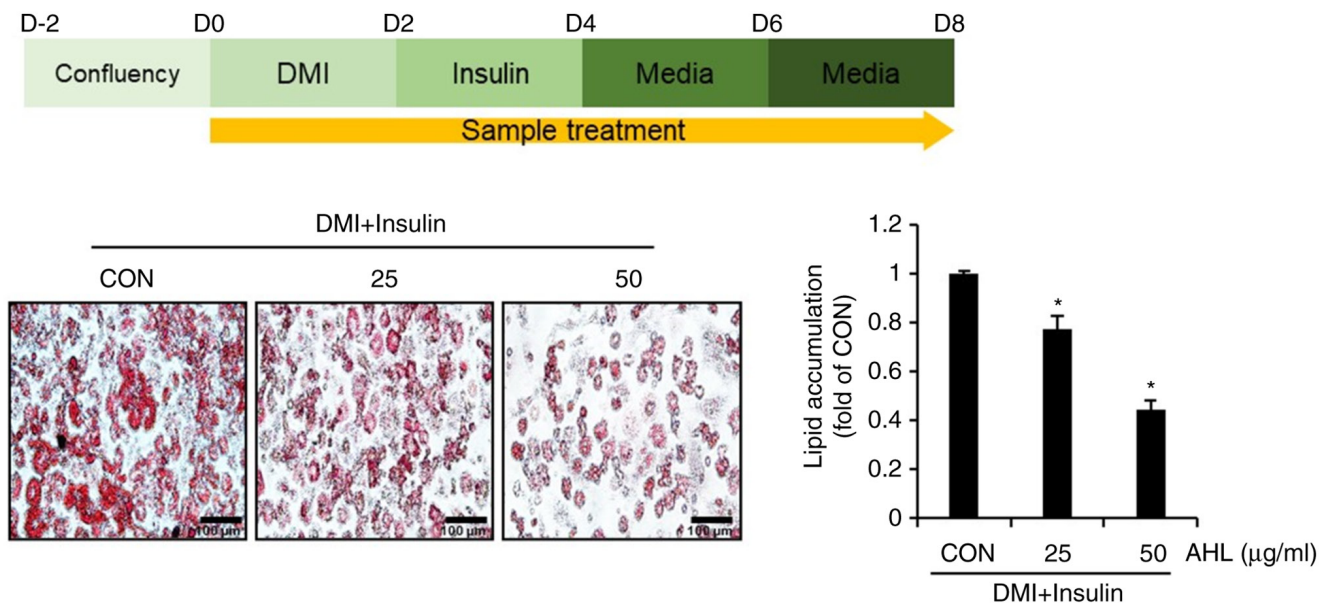


Figure 4. Inhibitory effect of AHL on the excessive accumulation of lipid droplets in 3T3-L1 cells. \*P<0.05 vs. CON group. AHL, *Adenocaulon himalaicum* leaf extracts; DMI, mixture of 0.05 mM IBMX, 1 µM dexamethasone and 10 µg/ml insulin; p-, phosphorylated; CON, control group without AHL treatment.

**Effect of AHL on lipid accumulation in 3T3-L1 cells.** To analyze whether AHL has anti-obesity activity, 3T3-L1 cells were treated with AHL during the differentiation of 3T3-L1 cells with DMI and insulin and the degree of lipid accumulation was examined by ORO staining. Intracellular lipid accumulation was reduced in AHL-treated cells compared to

that in untreated cells (Fig. 4). These results suggested that AHL may have anti-obesity effects.

**Effect of AHL on the level of protein related to lipid accumulation in 3T3-L1 cells.** The present study investigated whether the inhibitory effect of AHL on lipid accumulation could be



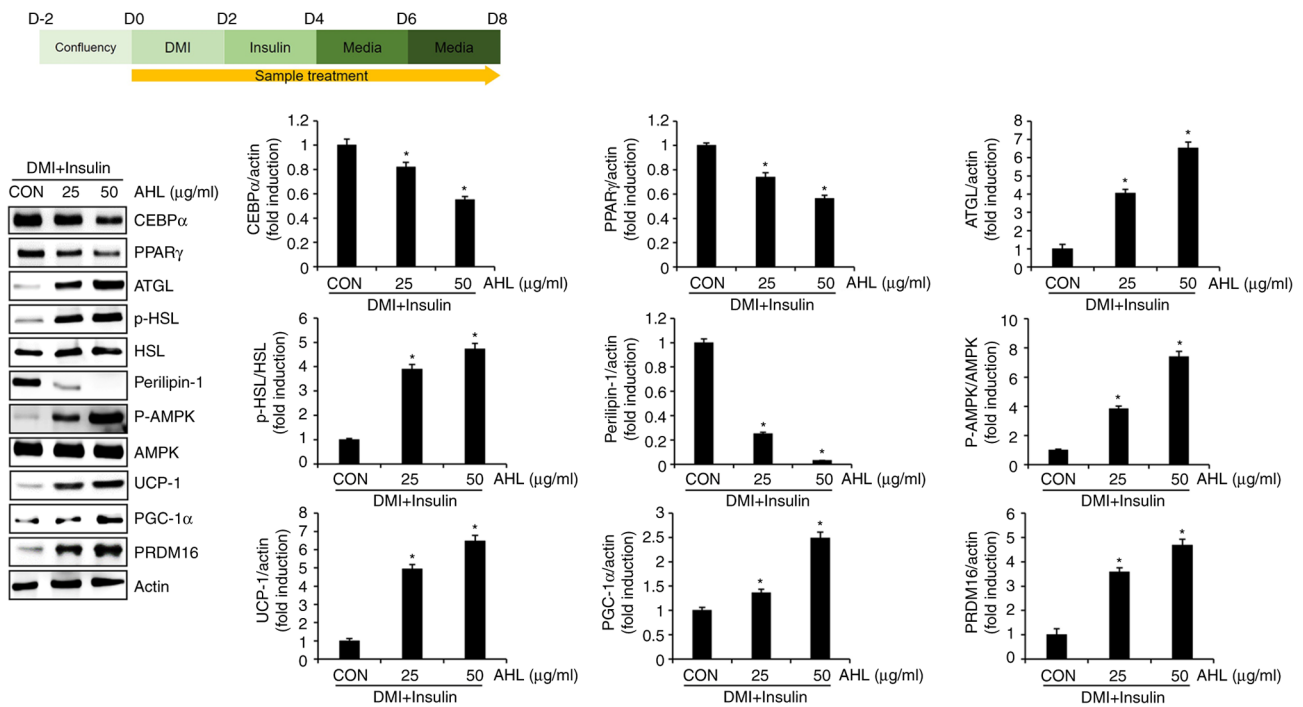


Figure 5. Effect of AHL on the protein levels associated with excessive accumulation of lipid droplets in 3T3-L1 cells. \* $P < 0.05$  vs. CON group. AHL, *Adenocaulon himalaicum* leaf extracts; DMI, mixture of 0.05 mM IBMX, 1  $\mu$ M dexamethasone and 10  $\mu$ g/ml insulin; CON, control group without AHL treatment; CEBP $\alpha$ , CCAAT enhancer binding protein  $\alpha$ ; PPAR $\gamma$ , proliferator-activated receptor  $\gamma$ ; ATGL, adipose triglyceride lipase; p-, phosphorylated; HSL, hormone-sensitive lipase; AMPK, adenosine monophosphate activated protein kinase; UCP-1, uncoupling protein 1; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor- $\gamma$  coactivator 1 $\alpha$ ; PRDM16, PR domain containing 16; p-, phosphorylated.

attributed to the regulation of proteins related to adipogenesis, lipolysis and browning of white adipocytes. AHL reduced the protein levels of CEBP $\alpha$  and PPAR $\gamma$ , which induce adipogenesis (Fig. 5). By contrast, AHL increased ATGL protein and the phosphorylation level of HSL and decreased perilipin-1 protein, which is essential for lipolysis. Additionally, AHL increased the phosphorylation level of AMPK and increased UCP-1, PGC-1 $\alpha$  and PRDM16 proteins, which induce browning of white adipocytes. Taken together, these results suggested that AHL inhibits lipid accumulation in mature adipocytes by inhibiting adipogenesis and inducing lipolysis and browning of white adipocytes.

## Discussion

The innate immune response is triggered by immune cells, such as monocytes, macrophages, neutrophils and dendritic cells (12). Macrophages eliminate invading foreign pathogens by phagocytosis and secreting various immunostimulatory factors (13). Macrophages present information regarding antigens to T cells, which are adaptive immune cells and induce T cell differentiation for cell-mediated immunity (13). Thus, because macrophages are involved in both innate and adaptive immunity, their activation is important for maintaining homeostasis of the human body against foreign pathogens and for strengthening the immune system of the human body (13). The present study showed that AHL increases the NO levels and DNA level of iNOS, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  and activates phagocytosis in RAW264.7 cells. Although the lack of improved quantitative RT-PCR data for assessing gene expression levels is

a limitation of the present study, the results indicated that AHL may have immunostimulatory activity and that AHL can be used as a potential agent to strengthen the immune system of the human body. Foreign pathogens possess pathogen-associated molecular patterns (PAMPs) that initiate an immune response by recognizing PAMPs via pattern recognition receptors (PRRs) (14). Among the PRRs of macrophages, TLR2 and TLR4 are the most specialized receptors that recognize foreign pathogens and are essential for innate and adaptive immune responses (15-17). The present study observed that AHL increased the levels of immunostimulatory factors and activated phagocytosis in a TLR4-dependent manner in RAW264.7 cells. These findings suggested that AHL activates macrophages by stimulating TLR4 expression. However, as various PRRs exist in macrophages, additional PRRs related to macrophage activation by AHL need to be identified. In addition, the present study confirmed that inhibition of p38 and JNK downregulated the AHL-mediated production of immunostimulatory factors and inhibition of TLR4 blocked AHL-mediated phosphorylation of p38 and JNK in RAW264.7 cells. MAPK signaling, involving p38, ERK1/2 and JNK, serves an important role in strengthening the human immune system by contributing to TLR4-mediated macrophage activation (18-21). Thus, the results suggested that AHL may activate macrophages through the TLR4-dependent activation of p38 and JNK signaling. AHL may have immunostimulatory activity and can be used as a potential agent to strengthen the immune system of the human body. However, to confirm that the immunostimulation caused by AHL can strengthen the immune system without causing excessive inflammatory

reaction it is necessary for *in vivo* studies to verify that this immune activation is indeed beneficial to human body.

An imbalance between energy intake and consumption leads to an excessive accumulation of fat in the body, eventually leading to obesity. As it causes various metabolic diseases, obesity has been considered as a disease that must be controlled. Moreover, as obesity reduces immunity, obesity treatment is urgently needed during the COVID-19 pandemic (5-7). In the present study, AHL inhibited excessive lipid accumulation in 3T3-L1 cells, indicating that AHL could be used as a potential agent for obesity treatment. Obesity can be prevented by regulating the various molecules involved in lipid accumulation in adipocytes. The first treatment for obesity is the inhibition of adipogenesis, which is the process by which preadipocytes differentiate into mature adipocytes (22). A number of anti-obesity agents that inhibit adipogenesis have been developed (22). The present study confirmed that AHL effectively reduced the protein levels of CEBP $\alpha$  and PPAR $\gamma$  in 3T3-L1 cells. CEBP $\alpha$  and PPAR $\gamma$  are major factors involved in adipogenesis and inhibition of CEBP $\alpha$  and PPAR $\gamma$  reduces lipid accumulation in the body in animal models (23,24). Based on these preliminary reports, AHL appears to be capable of inhibiting adipogenesis through the reduction of CEBP $\alpha$  and PPAR $\gamma$  in adipocytes. In addition, although not staining the nuclei with hematoxylin was a limitation of this study, the number of cells decreased in the AHL-treated group. As adipogenesis is accompanied by an increase in the number of adipocytes, the decrease in cell number in AHL-treated cells suggested that AHL inhibited adipogenesis. Obesity can be treated by inducing the lysis of lipids accumulated in adipocytes (25). Lipolysis is the process by which triacylglycerol (TAG) in adipose tissue is converted to diacylglycerol and monoacylglycerol and finally decomposes into fatty acids and glycerol (26,27). The present study found that ATGL protein levels and HSL phosphorylation increased and perilipin-1 protein levels decreased in AHL-treated 3T3-L1 cells. ATGL and HSL are enzymes that participate in the hydrolysis of TAG and a deficiency of these enzymes inhibits lipolysis in adipose tissue, resulting in excessive lipid accumulation (27). There is evidence that a decrease in perilipin-1, which surrounds lipid droplets in adipocytes, promotes the hydrolysis of TAG (26). As the increase of ATGL and HSL and the decrease of perilipin-1 imply the induction of lipolysis induction (26,27), AHL is presumed to induce lipolysis by increasing ATGL and HSL and decreasing perilipin-1. Finally, the browning of white adipose tissue is also involved in suppressing excessive lipid accumulation (28). Brown adipose tissue plays a role in dissipating the energy stored as TAG in white adipose tissue as heat (29). The present study observed that AHL increased the phosphorylation of AMPK and the levels of UCP-1, PGC-1 $\alpha$  and PRDM16 in 3T3-L1 cells. AMPK promotes fatty acid oxidation and induces the browning of white adipose tissue (30). UCP-1 and PGC-1 $\alpha$  plays a role in releasing the energy stored in white adipose tissue as heat (29,31). PRDM16, a major factor in the development of brown adipose tissue, increases energy consumption by promoting the browning of white adipose tissue (32). Therefore, considering the results on AMPK phosphorylation, as well as the increase of UCP-1, PGC-1 and PRDM16 by AHL, along with previous research, it is hypothesized that AHL may induce browning of adipocyte.

The overall anti-obesity results of AHL derived from the present study indicated that AHL may block excessive lipid accumulation by inhibiting adipogenesis and inducing lipolysis and browning of white adipose tissue. However, as the present study was purely performed *in vitro*, a limitation is that in order to validate the clinical applicability of AHL, *in vivo* studies using animal models are necessary to investigate the anti-obesity effects of AHL.

The concentration of AHL used in the present study was 25-50  $\mu$ g/ml, which showed immunostimulatory and anti-obesity activity *in vitro*. However, it is necessary to determine the optimal concentration for *in vivo* studies using animal models. It is difficult to determine how much AHL should be administered in mouse models to reach the concentrations used in the present study in the serum and tissues at the current state. Thus, it is necessary to conduct *in vivo* studies using various concentrations within the range permitted in animal models to determine the optimal concentration that exhibits no toxicity and shows immunostimulatory and anti-obesity activity. If such studies are conducted, they will serve as a basis for the clinical application of AHL.

*A. himalaicum* contains various functional substances, such as neochlorogenic acid, chlorogenic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoyl-epi-quinic acid, isoquercitrin and 4,5-dicaffeoylquinic acid (8,10). Functional ingredients, such as chlorogenic acid, 3,5-dicaffeoyl-epi-quinic acid and isoquercitrin, inhibit lipid accumulation in 3T3-L1 cells (33-35). Ingredients such as chlorogenic acid, 3,5-dicaffeoyl-epi-quinic acid and isoquercitrin may potentially contribute to the AHL-mediated inhibition of lipid accumulation. However, the possibility that other components of AHL may also be involved in the AHL-mediated inhibition of lipid accumulation cannot be excluded. However, among the components of *A. himalaicum* identified to date, none exhibit immunostimulatory activity. In fact, most of component of *A. himalaicum* are polyphenols that inhibit excessive immune responses (8,10). However, polysaccharides present in plants have been reported to exhibit immunostimulatory activity in macrophages (36,37). The AHL used in the present study was extracted with water. Solvent extraction, such as with ethanol or methanol, is commonly used to extract compounds such as flavonoids or polyphenols, while water extraction is primarily used to extract components such as polysaccharides, although flavonoids and polyphenols are also extracted (38). Therefore, the present study hypothesized that the polysaccharides in AHL exhibit immunostimulatory activity in RAW264.7 cells. However, since the component analysis of AHL is a limitation of the present study, it is necessary to investigate which components of AHL have immunostimulatory activity and anti-obesity activity.

The present study verified that AHL activated macrophages through TLR4-mediated activation of p38 and JNK and suppressed excessive lipid accumulation by inhibiting adipogenesis and inducing lipolysis and browning of white adipocytes. These results implied that AHL exerted immunostimulatory and anti-obesity effects. Given that both macrophage activation and anti-obesity activity can have a positive effect on strengthening the body's immune system, AHL can be used as a potential food and drug agent to enhance this. However, since the lack of *in vivo* research using

animal models and precise component analysis in the present study is a limitation, these limitations for immunostimulatory activity and anti-obesity activity will be addressed in future research.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

HJC and JWC wrote the manuscript, performed the experiments and analyzed the data. SWI performed the experiments and drafted the manuscript. JBJ designed the experiments and wrote and edited the manuscript. HJC, JWC, SWI and JBJ confirm the authenticity of all the raw data. All the authors have read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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