Research article

Nuclear factor kappa-B- and activator protein-1-mediated immunostimulatory activity of compound K in monocytes and macrophages

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ABSTRACT

Background: Compound K (CK) is a bioactive derivative of ginsenoside Rb1 in *Panax ginseng* (Korean ginseng). Its biological and pharmacological activities have been studied in various disease conditions, although its immunomodulatory role in innate immunity mediated by monocytes/macrophages has been poorly understood. In this study, we aimed to elucidate the regulatory role of CK on cellular events mediated by monocytes and macrophages in innate immune responses.
Methods: The immunomodulatory role of CK was explored by various immunoassays including cell-cell adhesion, fibronectin adhesion, cell migration, phagocytic uptake, costimulatory molecules, reactive oxygen species production, luciferase activity, and by the measurement of mRNA levels of proinflammatory genes.
Results: Compound K induced cell cluster formation through cell-cell adhesion, cell migration, and phagocytic activity, but it suppressed cell-tissue interactions in U937 and RAW264.7 cells. Compound K also upregulated the surface expression of the cell adhesion molecule cluster of differentiation (CD) 43 (CD43) and costimulatory molecules CD69, CD80, and CD86, but it downregulated the expression of monocyte differentiation marker CD82 in RAW264.7 cells. Moreover, CK induced the release of reactive oxygen species and induced messenger RNA expression of proinflammatory genes, inducible nitric oxide synthase, and tumor necrosis factor-alpha by enhancing the nuclear translocation and transcriptional activities of nuclear factor kappa-B and activator protein-1.
Conclusion: Our results suggest that CK has an immunomodulatory role in innate immune responses through regulating various cellular events mediated by monocytes and macrophages.

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1. Introduction

The innate immune response is the first-line defense mechanism against infection by pathogens such as bacteria, viruses, and fungi [1–6]. In innate immunity, macrophages and monocytes are representative cells governing various cellular functions such as cell adhesion to target cells or to tissues *via* functional activation of cell adhesion molecules [e.g., cluster of differentiation (CD) 29

(CD29), CD43, and CD44]; cell migration to infected sites; phagocytic uptake of infected pathogens; release of cytotoxic radical molecules [e.g., reactive oxygen/nitrogen species (ROS/RNS)]; the production of proinflammatory cytokines/mediators [e.g., tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, prostaglandin E$_2$, and nitric oxide]; and the stimulation of other adaptive immune cells through the expression of costimulatory molecules (e.g., CD69, CD80, and CD86) by mediating redox-sensitive transcription factors such as nuclear factor-kappa-B (NF-κB) and activator protein 1 (AP-1) [4,5,7,8]. Therefore, macrophages and monocytes have critical roles in antibacterial, antiviral, and antifungal responses in innate immunity [1−5,9−11].

Ginseng grows in North America and eastern Asia and is a perennial plant with flesh roots. It belongs to the genus *Panax* of the family Araliaceae. It is a valuable ethnopharmacological herb used in traditional medicine for supporting vitality, a long and healthy life, and supplementing spirits [12−14]. In many countries, different species of the genus *Panax* have been widely used for pharmacological and phytochemical purposes, and various types of active components isolated from *Panax ginseng* (i.e., Korean ginseng) such as ginsenosides and acid polysaccharides are critical ingredients that show regulatory activity in various disease conditions such as cardiovascular disease, diabetes mellitus, cancer, stress, and immunostimulation [15−19]. Recent studies have successfully identified different types of derivatives and metabolites isolated from ginseng such as 20S-dihydroprotopanaxatriol (2H-PPT), 20S-dihydroprotopanaxadiol (2H-PPD), ginsenoside F1, and compound K (CK), and have examined their potential uses as herbal remedies for treating human diseases [20−22].

CK is a very biologically and pharmacologically active protopanaxadiol-type ginsenoside metabolite [23]. Ginseng researchers have recently focused on CK because of its antidisease activity such as hepatoprotective activity [24], inhibition of tumor invasion [20], induction of tumor cell apoptosis [25], and protection from wrinkle and skin damage [26,27]. Despite these previous studies focusing on the regulatory activities of CK in various diseases, its immunomodulatory role in innate immunity mediated by monocytes and macrophages has been poorly explored. Therefore, in this study, we investigated the immunomodulatory activity of CK on the cellular responses of monocytes and macrophages, which have critical roles in innate immunity.

2. Materials and methods

2.1. Materials

Compound K (Fig. 1A) was obtained from Ambo Institute (Daejeon, Korea); its purity was higher than 97%, based on high-performance liquid chromatography analysis. RAW264.7 and U937 cells were purchased from American Type Culture Collection (Manassas, VA, USA). Roswell Park Memorial Institute 1640 (RPMI 1640) medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, phosphate-buffered saline (PBS), streptomycin, penicillin, and L-glutamine were purchased from Gibco (Grand Island, NY, USA). Fibronectin and antibodies specific for CD18, CD29, CD43, CD69, CD80, CD82, and CD86 were purchased from BD Bioscience (San Diego, CA, USA). Antibodies specific for p65, c-Fos, and γ-tubulin were purchased from Cell Signaling Technology (Beverly, MA, USA). Lipopolysaccharide (LPS), sodium nitroprusside (SNP), dihydrorhodamine 123 (DHR 123), crystal violet, phorbol 12-myristate 13-acetate (PMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), polyethylenimine, and fluorescein isothiocyanate (FITC)-dextran were purchased from Sigma (St Louis, MO, USA). The AP-1 luciferase construct was a gift from Professor Hae Young Chung (Pusan National University, Pusan,

	R1	R2
Ginsenoside Rb1	-O-Glc-Glc	-O-Glc-Glc
CK	-OH	-O-Glc
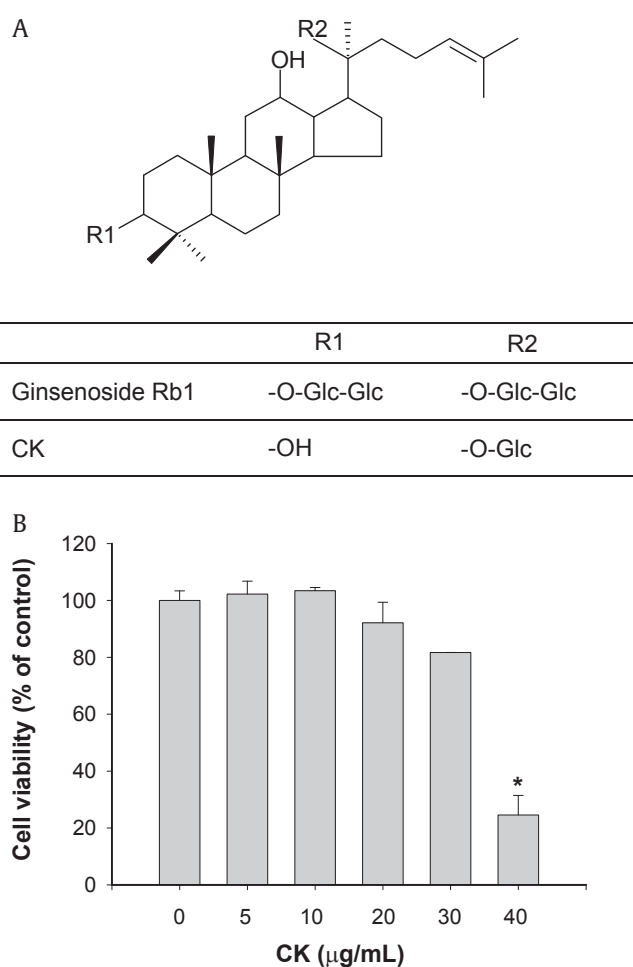
Figure 1. The effect of compound K (CK) on cell viability. (A) The chemical structure of ginsenoside Rb1 and its derivative, CK. (B) RAW264.7 cells were treated with the indicated concentration of CK for 24 h. Cell viability was determined by the MTT assay. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. * $p < 0.01$, compared with the control.

Korea) and NF-κB was used, as previously reported [28]. The luciferase assay system was purchased from Promega (Madison, WI, USA). TRI reagent was purchased from Molecular Research Center, Inc. (Cincinnati, OH, USA). murine leukemia virus reverse transcriptase was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The enhanced chemiluminescence system was purchased from AbFrontier (Seoul, Korea). The primers used for quantitative real-time polymerase chain reaction (PCR) were synthesized and PCR premix was purchased from Bioneer, Inc. (Daejeon, Korea).

2.2. Cell culture

RAW264.7 and U937 cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/mL streptomycin, 100 U/mL penicillin, and 2 mM L-glutamine at 37°C in a humidified 5% carbon dioxide incubator. HEK293 cells were cultured in DMEM under the same conditions. The cells were freshly maintained by splitting once weekly and refreshing the medium three times weekly.

2.3. Cell viability assay

RAW264.7 cells (1×10^6 cells/mL) plated on 96-well cell culture plates were treated with the indicated concentrations of CK for

24 h. The cytotoxicity of CK was determined by an MTT assay, as previously described [29]. In brief, 10 μL of MTT solution (10 mg/mL) dissolved in PBS (pH, 7.4) was added to 100 μL of the supernatant of the cultured cells and incubated for 4 h. After incubation, 15% sodium dodecyl sulfate (SDS) was added to the incubation mixture to stop the reaction and the mixture was further incubated for 24 h. The optical density (OD) was determined at 490 nm using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Cell-cell and cell-extracellular matrix protein (fibronectin) adhesion assay

The U937 cell-cell adhesion assay was performed as previously described [9,30]. In brief, U937 cells pretreated with the indicated concentration of CK at 37°C for 1 h were incubated with function-activating (i.e., agonistic) anti-CD29 or CD43 antibodies (1 μg/mL each) at 37°C for 2 h. After incubation, the cell-cell adhesions were measured via a homotypic cell-cell adhesion assay using a hemocytometer [9,10]. The cell images were photographed using an inverted light microscope equipped with a high-performance charge-coupled device video camera (Nikon, Tokyo, Japan). Adhesion between cells and extracellular matrix protein were determined by the cell-fibronectin adhesion assay. U937 cells preincubated with the indicated concentration of CK were plated onto fibronectin-coated (50 μg/mL) cell culture plates and further cultured at 37°C for 3 h [31]. Unbound cells were washed away using PBS, and the attached cells were incubated with 0.1% crystal violet for 15 min. The OD values were measured at 570 nm using a Spectramax 250 microplate reader (Molecular Devices).

2.5. Flow cytometric analysis

The surface expression of cell adhesion molecules (CD18, CD29, and CD43), costimulatory molecules (CD69, CD80, and CD86), and the monocyte differentiation marker CD82 was determined by flow cytometric analysis [9,10]. Cells were treated with the indicated concentration of CK in the absence or presence of PMA for 12 h and washed with a staining buffer (2% rabbit serum and 1% sodium azide in PBS). The cells were then incubated for 45 min on ice with antibodies specific for each molecule. After washing the cells three times with staining buffer, the surface expression levels of the molecules were analyzed by a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

2.6. Wound healing assay

RAW264.7 cells were grown to a confluent monolayer in a cell culture plate and a wound was generated by scratching the confluent cells with a pipette tip, as previously described [29]. Forty-eight h after generating the wound, cells that had migrated to the wound were visualized using an inverted phase microscope (Olympus, Tokyo, Japan) and quantified via a homotypic cell-cell adhesion assay using a hemocytometer.

2.7. Cell cluster formation assay

RAW264.7 cells were treated with the indicated concentration of CK for 72 h, and the cluster formation of the cells was analyzed by imaging the cells with an inverted light microscope equipped with a high-performance charge-coupled device video camera (Nikon, Tokyo, Japan).

2.8. Determination of ROS generation

Intracellular ROS levels were measured by fluorescence changes due to oxidation of the fluorescent probe, DHR 123, as previously described with slight modifications [32]. RAW264.7 cells pretreated with the indicated concentration of CK for 30 min were incubated with SNP (0.25 mM) as an inducer of ROS generation at 37°C for 6 h. The cells were further incubated with the fluorescent probe DHR 123 (20 μM) at 37°C for 1 h. The fluorescence corresponding to intracellular ROS levels was analyzed using a FACScan flow cytometer (Becton-Dickinson).

2.9. Determination of phagocytic uptake

The phagocytic activity of RAW264.7 cells was determined as previously described with slight modifications [33]. RAW264.7 cells were pretreated with the indicated concentration of CK and further incubated in 100 μL of PBS containing 1% human AB serum and FITC-dextran (1 mg/mL) at 37°C for 30 min. Two milliliters of ice-chilled PBS containing 1% human serum and 0.02% sodium azide was added to the incubation mixture to stop the reaction, and phagocytic levels were analyzed using a FACScan flow cytometer, as previously reported [34].

2.10. Luciferase reporter gene activity assay

HEK293 cells transfected with β-galactosidase constructs (0.1 μg/mL, as the transfection control) and either NF-κB-Luc (1 μg/mL) or AP-1-Luc constructs (1 μg/mL) using polyethylenimine transfection reagent for 24 h [35,36] were incubated with the indicated concentration of CK or PMA (100 nM) for another 24 h. The cells were collected and lysed to measure luciferase reporter gene activity using a luciferase assay system in accordance with the manufacturer's instructions. All luciferase activities were normalized to β-galactosidase activity.

2.11. Preparation of nuclear lysates and western blot analysis

RAW264.7 cells treated with either CK (10 μg/mL) or LPS (1 μg/mL) for the indicated times were washed three times with PBS and nuclear lysates from the cells were prepared as previously described [29]. For western blot analysis, the nuclear lysates were subjected to SDS-polyacrylamide electrophoresis and then transferred to a polyvinylidene fluoride membrane. The transferred nuclear lysates were detected by antibodies specific for p65, c-Fos, and γ-tubulin, and were visualized using an enhanced chemiluminescence system in accordance with the manufacturer's instructions.

2.12. Quantitative real-time PCR

The messenger RNA (mRNA) levels of inducible nitric oxide synthase (iNOS) and TNF-α were determined by quantitative real-time PCR analysis. Total RNA was extracted from the RAW264.7

Table 1
Primer sequences used for quantitative real-time polymerase chain reaction

| Targets		Sequences (5′ to 3′)
iNOS	Forward	GGAGCCTTTAGACCTCAACAGA
	Reverse	TGAACGAGGAGGGTGGTG
TNF-α	Forward	TGCCTATGTCTCAGCCTCTT
	Reverse	GAGGCCATTTGGGAACTTCT
GAPDH	Forward	CAATGAATACGGCTACAGCAAC
	Reverse	AGGGAGATGCTCAGTGTTGG

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-alpha

cells treated with either the indicated concentration of CK or with LPS (1 µg/mL) for 6 h using TRI reagent (Molecular Research Center, Inc.) in accordance with the manufacturer's instructions. They were then immediately stored at −80°C until use. Single stranded complementary DNA (cDNA) was synthesized from the extracted total RNA (1 µg) using murine leukemia virus reverse transcriptase in accordance with the manufacturer's instructions. Real-time PCR reactions were performed, as previously described [37]. The primer sequences used for real-time PCR reactions are listed in Table 1.

2.13. Statistical analysis

All data acquired in this study are presented as the mean ± the standard error of the mean of at least three independent
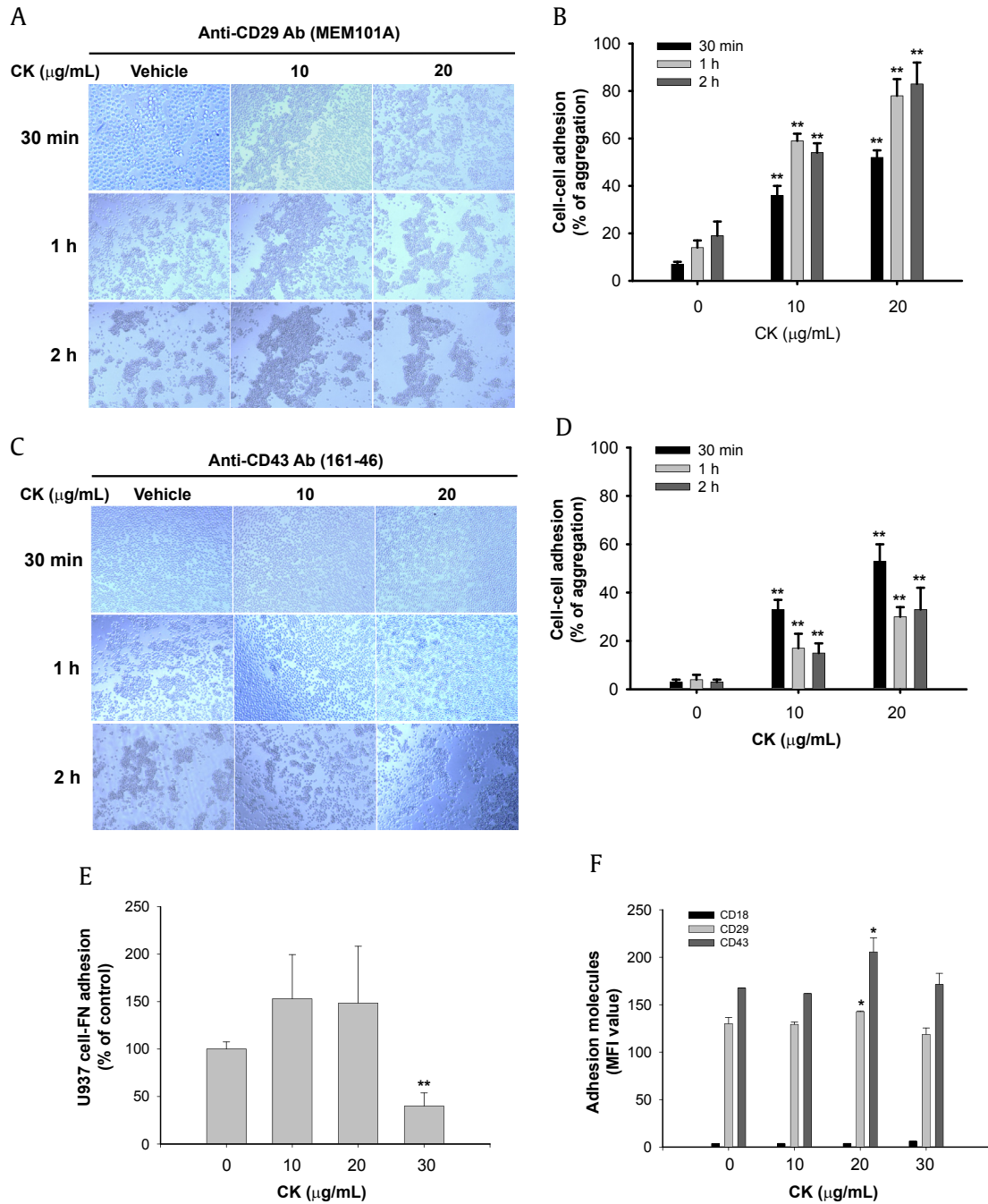
Figure 2. The effect of CK on U937 cell adhesion (A and B) U937 cells (1 × 10^6 cells/mL) pretreated with the indicated concentration of CK. The cells were incubated for the indicated times with proaggregative antibodies (1 µg/mL) specific for (A and B) CD29 (MEM101A) and (C and D) CD43 (161−46). Images of the cells were obtained by an inverted light microscope equipped with a high-performance charge-coupled device video camera (A and C). Quantitative measurements of U937 cell-cell clusters were performed by a quantitative homotypic cell-cell adhesion assay using a hemocytometer (B and D). (E) U937 cells treated with the indicated concentrations of CK were plated onto fibronectin-coated cell culture plates for 3 h. After washing away unattached cells three times with PBS, the attached cells were visualized by crystal violet assay. (F) U937 cells were treated with the indicated concentration of CK for 6 h, and the surface expression levels of CD18, CD29, and CD43 in the U937 cells were determined by flow cytometry analysis. All data are expressed as the mean ± the SEM of three independent experiments performed in triplicate. CD, cluster of differentiation; CK, compound K; MFI, mean fluorescence intensity; SEM, standard error of the mean. $^*p < 0.05$ and $^{**}p < 0.01$, compared with control.

experiments performed in triplicate. To determine the statistical significance between the experimental and control groups, all results were analyzed using the Student *t* test and one-way analysis of variance. A value of $p < 0.05$ was considered statistically significant and all statistical analyses were performed by SPSS (Version 22.0, 2013, IBM Corp., Armonk, NY, USA; SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

In this study, the regulatory role of CK (Fig. 1A), which is a bioactive compound derived from the ginsenoside Rb1 (Fig. 1A) in *Panax ginseng*, on the cellular responses of monocytes and macrophages such as cell-cell or cell-tissue (i.e., extracellular matrix) adhesion, modulation of surface adhesion molecule expression, cell migration, cell cluster formation, phagocytic uptake, ROS generation, and expression of proinflammatory genes were explored in a mouse macrophage-like cell line, RAW264.7 cells, and in a human monocyte cell line, U937 cells. To test the cytotoxicity of CK before exploring its regulatory roles, U937 cells were treated with the indicated concentration of CK for 24 h and the cell viabilities were determined by the MTT assay. As shown in Fig. 1B, CK did not exert any cytotoxicity within the concentration range of 5—30 μg/mL, but showed slight and significant cytotoxicity at 40 μg/mL.

Once the human body is infected by pathogens, monocytes and macrophages are recruited to the inflamed sites of the infected tissues. The functional activation of cell adhesion molecules such as CD29, CD43, CD98, and CD147 on the surfaces of monocytes/macrophages is rapidly induced to remove the infecting pathogens [9,10,38]. Therefore, to examine whether CK could modulate the cellular adhesion mediated by cell surface adhesion molecules (i.e., CD29 and CD43), the U937 cells were incubated with function-activating antibodies specific for CD29 and CD43 in the absence or presence of CK. The adhesion between the U937 cells was then determined. CK induced the CD29 antibody-mediated cell adhesion (Figs. 2A and 2B) and CD43 antibody-mediated cell adhesion (Figs. 2C and 2D) between U937 cells in a dose-dependent manner, while it significantly inhibited the adhesion of U937 cells to fibronectin, a representative extracellular matrix protein in tissues at a concentration of 30 μg/mL (Fig. 2E). Moreover, CK at a concentration of 20 μg/mL significantly induced the expression of CD29 on the surface of U937 cells (Fig. 2F). These results indicate that CK facilitates CD29- and CD43-mediated cell-cell interactions rather than cell-tissue interactions.

Leukocytes are mobile cells and morphological changes in the cells caused by the rearrangement of their cytoskeletons have a crucial role in various cellular activities such as cell migration, cell adhesion, and phagocytosis [39,40]. Migration is an essential process in myeloid lineage cells such as monocytes, macrophages, and neutrophils because of their functions in defensive and inflammatory responses [41]. Therefore, we examined whether CK is capable of modulating the migratory activities of macrophages by using a wound healing assay. For this assay, a wound was generated on the confluent RAW264.7 cells, and migration of the RAW264.7 cells to the wound area was determined. As shown in Figs. 3A and 3B, CK
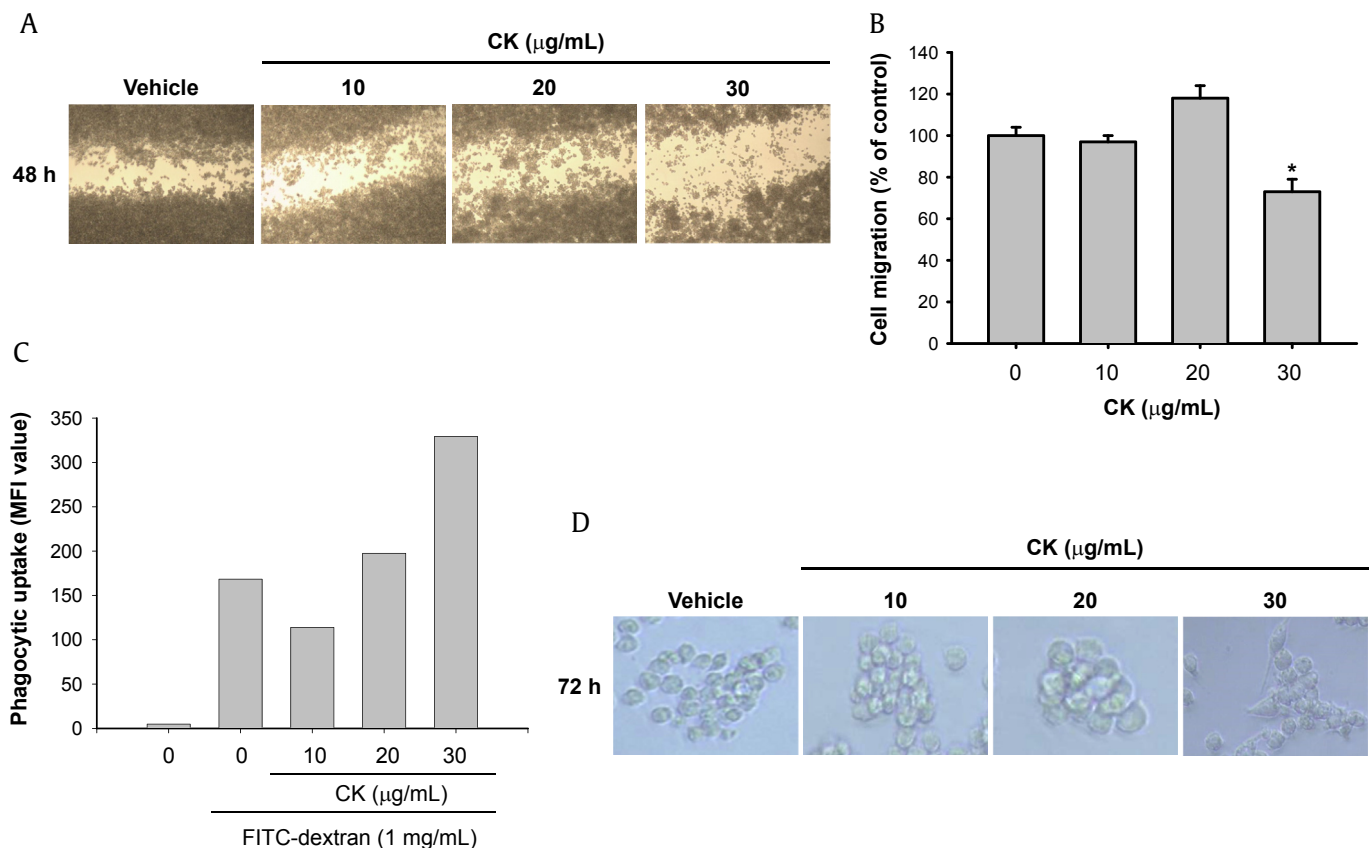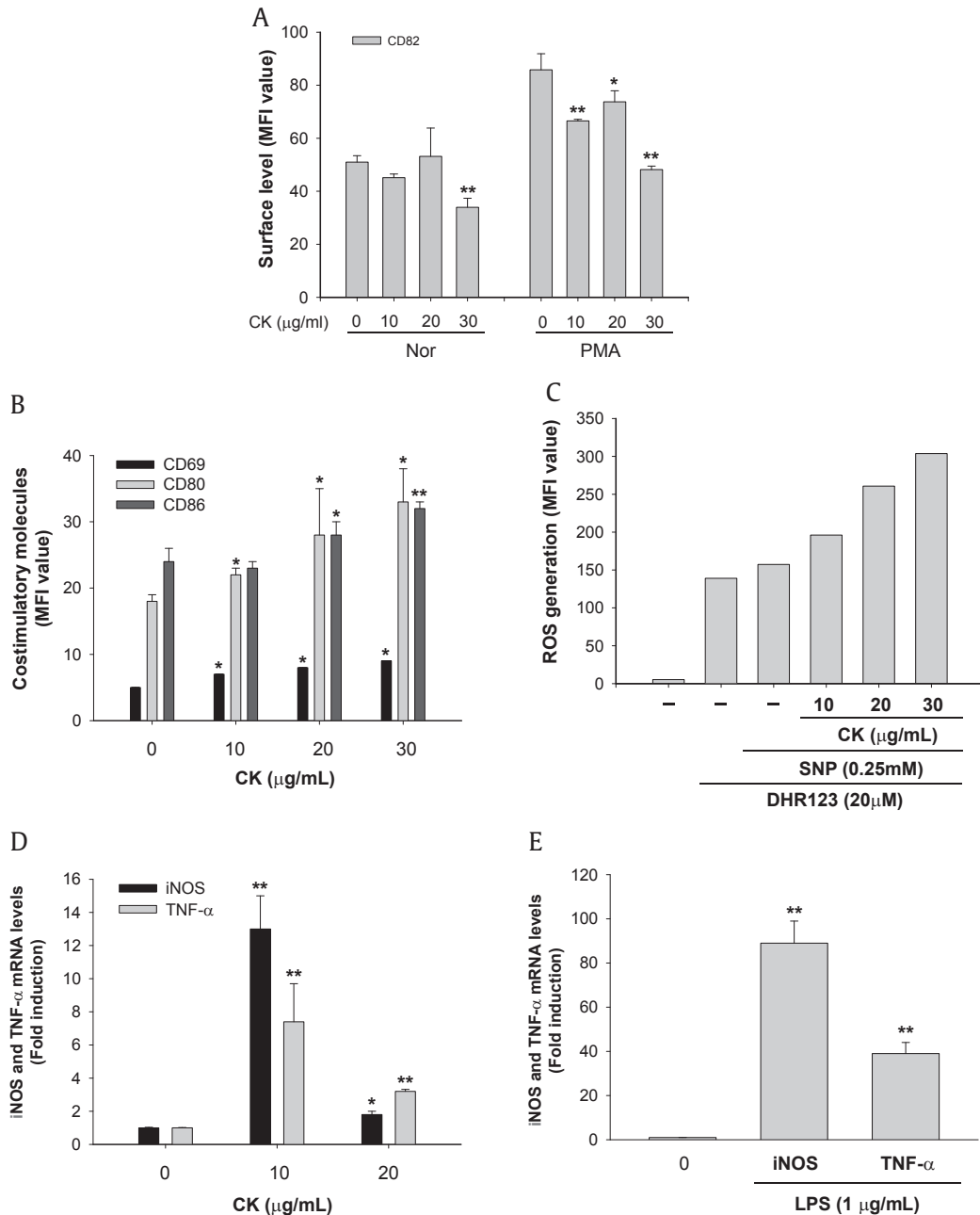

Figure 3. Effect of compound K (CK) on Raw264.7 cell migration, phagocytic activity, and cell cluster formation. (A and B) RAW264.7 cells were grown as a confluent monolayer, scratched, and incubated in the absence or presence of the indicated concentration of CK for 48 h. The migrated cells were photographed by an inverted phase microscope (A) and were quantified by a homotypic cell-cell adhesion assay using a hemocytometer (B). (C) RAW264.7 cells pretreated with the indicated concentration of CK were incubated with FITC-dextran (1 mg/mL) for 2 h. The uptake levels of FITC-dextran were determined by flow cytometry analysis. (D) RAW264.7 cells were treated with the indicated concentration of CK for 72 h, and the cluster formation of the cells was analyzed by imaging the cells using an inverted light microscope equipped with a high-performance charge-coupled device video camera. FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity. * $p < 0.01$, compared with the control.

significantly suppressed the migration of RAW264.7 cells at a concentration of 30 μg/mL. We also examined whether CK was able to regulate the phagocytic activities of macrophages. Phagocytic activity was determined by measuring the levels of fluorescence of FITC-labeled dextran in RAW264.7 cells by flow cytometry analysis. As shown in Fig. 3C, CK induced the phagocytic activity of RAW264.7 cells at 20 and 30 μg/mL concentrations. Moreover, whether CK is capable of controlling cell cluster formation by inducing cell-cell adhesion was further examined in RAW264.7 cells. In accordance with U937 results (Figs. 2A—2D), CK induced

the cluster formation of RAW264.7 cells through facilitating cell-cell adhesion (Fig. 3D). These results strongly indicate that CK modulates morphological change-mediated cellular responses such as cell migration, phagocytic uptake, and cell cluster formation through cell-cell adhesion in macrophages.

Circulating monocytes are recruited to the inflamed areas of pathogen-infected tissues and differentiate into functional macrophages in response to various signals. During these biological processes, functional macrophages induce surface expression of the monocyte differentiation marker CD82 and costimulatory

Figure 4. The effect of compound K (CK) on macrophage activation. (A) RAW264.7 cells pretreated with the indicated concentration of CK were incubated with PMA for 24 h, and the surface expression levels of CD82 were determined by flow cytometry analysis. (B) RAW264.7 cells were treated with the indicated concentration of CK for 12 h, and the surface expression levels of CD69, CD80, and CD86 were determined by flow cytometry analysis. (C) RAW264.7 cells pretreated with the indicated concentration of CK were treated with DHR 123 (20 μm) in the absence or presence of SNP (0.25 mM) for 2 h. Intracellular levels of ROS were determined by flow cytometry analysis. (D) RAW264.7 cells were treated with the indicated concentration of CK for 6 h or (E) treated with LPS (1 μg/mL) for 6 h. The messenger RNA (mRNA) levels of iNOS and TNF-α expressed in these cells were determined by quantitative real-time PCR analysis. CD, cluster of differentiation; DHR, dihydrorhodamine; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; Nor, normal; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SNP, sodium nitroprusside; TNF-α, tumor necrosis factor-alpha. * $p < 0.05$ and **$p < 0.01$, compared to the control.
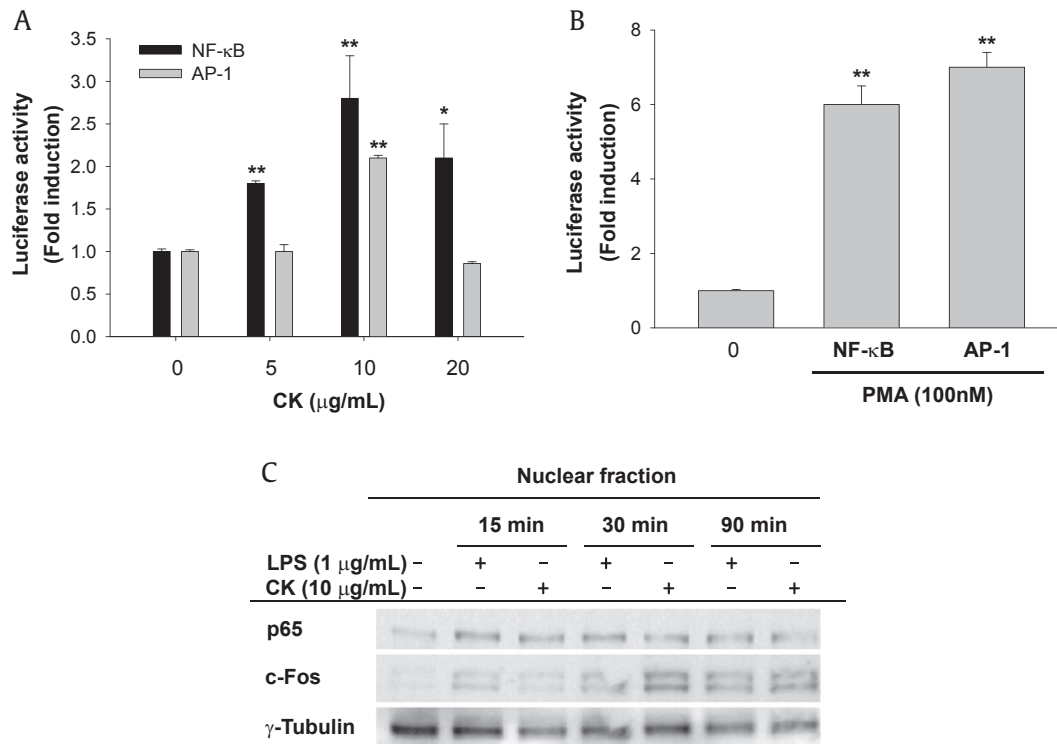
Figure 5. The effect of compound K (CK) on the activation of nuclear factor kappa-B (NF-κB) and activator protein 1 (AP-1) pathways. (A) HEK293 cells transfected with β-galactosidase constructs (0.1 μg/mL, as the transfection control) and either NF-κB-luciferase (NF-κB-Luc) (1 μg/mL) or AP-1-luciferase (AP-1-Luc) (1 μg/mL) constructs for 24 h were treated with the indicated concentration of CK for another 24 h or (B) by PMA (100 nM) for 24 h. Luciferase activities were measured using a luminometer. (C) RAW264.7 cells were treated with CK (10 μg/mL) or LPS (1 μg/mL) for the indicated time, and nuclear lysates were prepared from the cells, as described in the Materials and methods section. Protein levels of p65, c-Fos, and γ-tubulin in the nuclear lysates were determined by western blot analysis using the antibodies specific for the target proteins. LPS, lipopolysaccharide; MFI, mean fluorescence intensity; PMA, phorbol 12-myristate 13-acetate. * $p < 0.05$, **$p < 0.01$, compared with the control.

molecules such as CD69, CD80, and CD86, and the release of toxic radical molecules such as ROS [4,9,10]. Therefore, we examined whether CK is able to modulate these functional changes in macrophages. The effect of CK on the surface expression of CD82 in PMA-stimulated macrophages was examined by flow cytometry analysis. As Fig. 4A indicates, CK inhibited the surface expression of the monocyte differentiation marker CD82, which was induced by PMA in RAW264.7 cells. This finding suggests that CK suppresses the differentiation process of monocytes into macrophages.

The effect of CK on the surface expression of costimulatory molecules CD69, CD80, and CD86 was examined in RAW264.7 cells by flow cytometry analysis. CK dose-dependently increased the surface expression of all costimulatory molecules (e.g., CD69, CD80, and CD86) in RAW264.7 cells (Fig. 4B), which indicated that macrophages are functionally activated by CK.

Whether CK could modulate the generation of ROS in macrophages was also investigated. We employed fluorescence probe, DHR 123, and measured its fluorescence levels in RAW264.7 cells by flow cytometry analysis. RAW264.7 cells were treated with SNP in the absence or presence of CK, and ROS generation in RAW264.7 cells was determined. As shown in Fig. 4C, CK enhanced the generation of ROS in SNP-stimulated RAW264.7 cells in a dose-dependent manner, which indicated that CK could activate macrophages, and thereby generate toxic radicals. We further examined whether CK is capable of regulating the expression of proinflammatory genes in RAW264.7 cells. The mRNA levels of inflammatory genes such as iNOS and TNF-α were determined by quantitative real-time PCR analysis in CK-treated RAW264.7 cells. As expected, the mRNA expression levels of

iNOS and TNF-α were significantly upregulated by LPS in RAW264.7 cells (Fig. 4E). CK also induced mRNA expression of iNOS and TNF-α, although mRNA expression levels of iNOS and TNF-α were significantly lower in CK-treated RAW264.7 cells than in LPS-stimulated RAW264.7 cells (Fig. 4D). This finding indicated that, similar to ROS generation, CK functionally activates macrophages and induces mRNA expression of proinflammatory genes, although it's macrophage-activating effect is not as strong as that of LPS in macrophages.

We investigated the molecular mechanism by which CK modulates the cellular responses of macrophages. It is well established that ROS generation and mRNA expression of proinflammatory genes are predominantly induced through activation of NF-κB and AP-1 pathways [4,5,42—44]. To examine whether CK has a role in modulating the transcriptional activities of NF-κB and AP-1, we employed a luciferase reporter gene assay using NF-κB-Luc and AP-1-Luc constructs in HEK293 cells. In accordance with the results of ROS generation and mRNA expression of proinflammatory genes (Figs. 4C—4E), CK significantly increased both NF-κB and AP-1 luciferase activities (Fig. 5A). However, the increased levels of NF-κB and AP-1 luciferase activities due to CK were lower than those induced by PMA (Fig. 5B). Since CK modulated the transcriptional activities of NF-κB and AP-1, we further examined whether CK regulates nuclear translocation of NF-κB and AP-1 transcription factors p65 and c-Fos in RAW264.7 cells. As expected, CK induced nuclear translocation of both p65 and c-Fos from 15 min and 30 min, respectively, while LPS induced nuclear translocation of both transcription factors from 15 min (Fig. 5C). These results further support previous results indicating that CK induces ROS generation and mRNA expression of proinflammatory genes

through activation of both NF-κB and AP-1 pathways in macrophages.

In conclusion, we have demonstrated that CK was able to modulate various cellular events in monocytes and macrophages such as cell cluster formation through facilitating cell-cell adhesion rather than through cell-tissue interaction, cell migration, phagocytic uptake, and expression of a monocyte differentiation marker and costimulatory molecules. Moreover, CK induced ROS generation and mRNA expression of proinflammatory genes through activation of both NF-κB and AP-1 pathways in macrophages. These cellular responses are critical in innate immune responses mediated by monocytes and macrophages, as well as by certain ginseng-derived components [45,46]; therefore, our results strongly suggest that CK may be a potential immunomodulatory target for the functional activation of monocytes and macrophages to enhance innate immunity. Further studies regarding the detailed molecular mechanism of CK-mediated immunomodulatory activity and *in vivo* effects of CK on innate immune conditions are required.

Conflicts of Interest

All authors have no conflicts of interest to declare.

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