Original Article

Iranian Journal of Immunology

March 2026; 23(2):

Polysaccharide from *Momordica charantia* Induces Immunostimulatory Responses and Reveals Mechanisms in Macrophages

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ABSTRACT

Background: Immunity protects organisms from pathogens, diseases, and external noxious agents. Naturally derived polysaccharides are well-documented to exhibit potent immunomodulatory effects. However, the exact immunostimulatory properties and underlying mechanisms of polysaccharides derived from *Momordica charantia* (MCPS) remain to be clearly elucidated.

Objective: To investigate the immunomodulatory effects of MCPS on murine macrophages and to elucidate the underlying molecular mechanisms.

Methods: MCPS was isolated from *Momordica charantia*. RAW 264.7 murine macrophages were treated with varying concentrations of MCPS, and its effects on cell viability, nitric oxide (NO) production, cytokine secretion, surface marker expression, and key intracellular signaling pathways were evaluated using standardized analytical assays. Potential endotoxin contamination was systematically excluded using a polymyxin B neutralization assay.

Results: MCPS exhibited no cytotoxicity across all tested concentrations. Notably, MCPS significantly stimulated NO production and enhanced the secretion of pro-inflammatory cytokines, indicative of robust macrophage activation. This was accompanied by a marked upregulation of macrophage surface activation markers. Furthermore, MCPS activated the MAPK and NF-κB signaling pathways. This cytokine production was significantly attenuated by pathway-specific inhibitors, confirming the functional involvement of these cascades. Importantly, polymyxin B suppressed LPS-induced responses but did not alter MCPS- stimulated activation, indicating that the immunomodulatory effects were independent of endotoxin contamination.

Conclusion: MCPS effectively promotes murine macrophage activation without cytotoxicity by stimulating NO production, cytokine secretion, and activation marker expression through the MAPK and NF-κB signaling pathways. These findings demonstrate that MCPS holds strong potential as a natural immunostimulatory agent capable of enhancing host immune responses.

Keywords: Cytokines; MAP kinases; *Momordica charantia*; NF-kappa B; RAW 264.7

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Cite this article as:
Cho HJ, Byun EH. Polysaccharide from *Momordica charantia* Induces Immunostimulatory Responses and Reveals Mechanisms in Macrophages. *Iran J Immunol.* 2026; 23(2): , doi: 10.22034/iji.2026.109873.3154.

Received: 2025-11-26
Revised: 2026-01-21
Accepted: 2026-01-26

INTRODUCTION

Immunity refers to the biological mechanisms through which organisms protect themselves from pathogens, diseases, and external noxious agents (1). The host defense system comprises two complementary, interconnected subsystems: innate immunity and adaptive immunity (2). Innate immunity, present in virtually all multicellular organisms, serves as the first line of defense by providing a rapid and non-specific response to cellular insults. In contrast, adaptive immunity, exclusive to jawed vertebrates, is orchestrated and activated by innate immune signals and is characterized by high antigen specificity and immunological memory (3).

Momordica charantia (bitter gourd), a tendril-bearing climbing vine of the Cucurbitaceae family, is widely distributed throughout tropical and subtropical regions. Its fruit, characterized by a distinct bitterness, is consumed as a functional food and has historically been utilized in traditional medicine (4). While contemporary pharmacological research has primarily focused on the bioactive proteins and saponins of *M. charantia* (5), scientific interest in its polysaccharide components has increased considerably (6). Polysaccharides, high-molecular-weight carbohydrate macromolecules composed of monosaccharide units linked by glycosidic bonds (7), are ubiquitous biomolecules essential across plants, animals, and microorganisms.

Numerous natural polysaccharides exhibit notable antiviral, antioxidant, antitumor, and immunomodulatory properties (8). For example, polysaccharides isolated from *Chrysanthemum zawadskii* Herbich var. latilobum leaves have been shown to activate dendritic cells and elicit antigen-specific T cell responses, thereby enhancing immune activity (9). Similarly, a polysaccharide derived from *Cistanche deserticola* activates dendritic cells via the TLR4 signaling pathway to augment antibody production and T cell mediated responses, demonstrating potent immunostimulatory effects (10). Owing to their characteristically low cytotoxicity, polysaccharides are considered highly attractive candidates for therapeutic development, and subsequent chemical modifications can further optimize or confer novel bioactivities (11).

Although polysaccharides isolated from *M. charantia* have been extensively evaluated for their antioxidant (12), nuclear factor kappa B (NF-κB) signaling pathway-inhibitory (13), and antidiabetic (14) activities, their immunostimulatory potential remains relatively underexplored. *M. charantia* polysaccharides hold promise as valuable functional biomaterials; however, definitive evidence confirming their specific immunomodulatory mechanisms remains limited.

Therefore, this study aims to elucidate the immunostimulatory effects of *M. charantia*-derived polysaccharides (MCPS) on murine macrophages and to uncover the underlying cellular and molecular mechanisms. Additionally, this investigation evaluates the potential of MCPS as functional biomaterials or nutraceutical ingredients designed to promote host immune health.

MATERIALS AND METHODS

Isolation and Extraction of Polysaccharides from Momordica charantia

Dried *Momordica charantia* was pulverized into a fine powder using a laboratory grinder (NSG-1002SS, Hanil, Seoul, Korea). A 100-g aliquot of the powder was subjected to hot-water extraction with 900 mL of distilled water (DW) at 100°C for 2 h. The crude extract was filtered through Whatman No. 4 filter paper (Whatman, Kent, UK), To precipitate the polysaccharides, the resulting filtrate was mixed with pre-diluted 70% ethanol and incubated at 4°C for 12 h. The precipitate was collected via centrifugation at 1700 × g for 20 min at 4°C

using a Combi 514R centrifuge (Hanil, Seoul, Korea). The isolated polysaccharide pellet was subsequently lyophilized and stored at –70°C prior to experimental use. This purified polysaccharide fraction was designated as *Momordica charantia* polysaccharide (MCPS).

Cell Culture and Maintenance

The RAW 264.7 murine macrophage cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Dongdaemun-gu, Seoul, Korea) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin. Cultures were incubated at 37°C in a humidified incubator with 5% CO_2 and subcultured when they reached 80% confluence.

RAW 264.7 macrophage viability assay

Cell viability in response to MCPS treatment was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 macrophages were seeded into 96-well plates at a density of 1×10^4 cells per well and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 12 h to ensure complete cell adherence. Subsequently, the cells were treated with lipopolysaccharide (LPS; positive control) or various concentrations of MCPS (62.5, 125, 250, 500, and 1000 µg/mL) for 24 h to compare their respective effects. Following incubation, the culture medium was replaced with100 µL of fresh medium containing MTT solution (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) at a 1:10 dilution, followed by incubation for 30 min at 37°C. The supernatant was then carefully aspirated, and 100 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to each well to completely solubilize the purple formazan crystals. The absorbance was measured at 570 nm using a microplate spectrophotometer (BioTek, Winooski, VT, USA).

Determination of Nitric Oxide (NO) production

Nitric oxide (NO) production was quantified using the Griess assay. RAW 264.7 cells were seeded in 48-well plates at a density of 5×10^4 cells per well and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 12 hours. Subsequently, the cells were stimulated with lipopolysaccharide (LPS) or varying concentrations of MCPS (62.5 to 1000 µg/mL) for an additional 12 h. Following incubation, the culture supernatants were harvested, and equal volumes (100 µL) of each cell-free supernatant and Griess reagent (Sigma-Aldrich) were mixed. After incubating the reaction mixture in the dark at room temperature for 10 min, the absorbance was measured at 540 nm using a microplate reader. Extracellular nitrite concentration—reflecting absolute NO production—was determined by interpolation from a standard curve generated using sodium nitrite ($NaNO_2$; Sigma-Aldrich).

Quantification of cytokine Secretion

Cytokine levels in culture supernatants were measured using enzyme-linked immunosorbent assays (ELISA). RAW 264.7 cells were seeded into 48-well plates at a density of 5×10^4 cells per well and incubated at 37°C with 5% CO_2 for 12 h. The cells were then treated with lipopolysaccharide (LPS) or increasing concentrations of MCPS (62.5–1000 µg/mL) for an additional 12 h. Following treatment, the culture supernatants were collected, and the concentrations of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) were determined using commercial ELISA kits (eBioscience, San Diego, CA, USA) in accordance with the manufacturer's instructions. The optical density was recorded at 450 nm, and absolute cytokine concentrations were calculated via regression analysis against standard curves generated using corresponding recombinant cytokine standards.

Confirmation of endotoxin decontamination

To rule out the possibility that the immunostimulatory effects of MCPS on macrophages were artifacts caused by exogenous endotoxin contamination, RAW 264.7 cells were seeded into 48-well plates at a density of 5×10^4 cells per well and incubated for 12 h at 37°C in a humidified atmosphere containing 5% CO_2. The cells were then pretreated with polymyxin B (PMB; 50 μg/mL, Sigma-Aldrich) at room temperature for 1 h prior to stimulation with either LPS (100 ng/mL) or MCPS (1000 μg/mL). After 24 h of incubation, the culture supernatants were harvested, and the extracellular concentrations of TNF-α and IL-6 were quantified using ELISA.

Analysis of Cell Surface Marker Expression via Flow Cytometry

To evaluate the effects of MCPS on the expression of surface activation markers, RAW 264.7 cells were seeded into 12-well plates at a density of 1×10^6 cells and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 12 h to ensure complete adherence. The cells were then treated with MCPS (500 and 1000 μg/mL), using LPS (100 ng/mL) as a positive control. After 24 hours, cells were harvested and incubated with an FcγRI/III blocking reagent (1 μg/mL; BD Biosciences, San Diego, CA, USA) at 4°C for 20 min to minimize non-specific antibody binding via Fc receptors. Following a washing with phosphate-buffered saline (PBS), the cells were resuspended in fluorescence-activated cell sorting (FACS) buffer—consisting of PBS supplemented with 2% FBS and 0.1% sodium azide—and subsequently blocked with 0.5% bovine serum albumin (BSA) in PBS for 30 min at 4°C. After an additional wash with PBS, the cells were stained with fluorochrome-conjugated monoclonal antibodies specific for cluster of differentiation 80 (CD80)-PE, CD86-PE, and anti-major histocompatibility complex (MHC) class I and II-PE (all from BD Biosciences), each diluted 1:1000, and incubated at 4°C for 30 min. The stained cells were subsequently analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Immunoblotting Analysis

Immunoblotting was conducted to evaluate alterations in the expression of intracellular signaling proteins induced by MCPS treatment. RAW 264.7 cells were seeded into 6-well plates at a density of 1×10^6 cells per well and incubated under standard culture conditions (37°C, 5% CO_2) for 12 hours. The cells were subsequently exposed to MCPS (500 and 1000 μg/mL) or LPS (100 ng/mL) for 24 h. Following incubation, cells were lysed using NP-40 lysis buffer (Biosource, Seoul, Korea) supplemented with protease and phosphatase inhibitor cocktails, and the crude lysates were clarified via centrifugation at 16000 × g for 20 minutes at 4°C. Total protein concentrations were quantified using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein (20 μg) were resolved via 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Merck KGaA, Darmstadt, Germany). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween-80 (TBS-T) for 1 h at room temperature, rinsed, and incubated overnight at 4°C with primary antibodies (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA) targeting phospho-extracellular signal-regulated kinase (p-ERK)1/2, phospho-c-Jun N-terminal kinase (p-JNK), phospho-p38, inhibitor of kappa B alpha (IκB-α), and nuclear factor kappa B (NF-κB). After washing thrice with TBS-T, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:3000; Calbiochem, La Jolla, CA, USA) for 1 hat room temperature. The protein bands were visualized using an enhanced chemiluminescence system (EZ-Western Lumi Femto, DoGen, Seoul, Korea)

and captured using a digital imaging system.

Preparation of Nuclear and cytoplasmic extracts preparation

Nuclear extracts were isolated from MCPS-treated cells using a specialized differential lysis approach. Following treatment with MCPS (500 and 1000 μg/mL), the cells were washed three times with ice-cold PBS and harvested via centrifugation. The resulting cell pellets were resuspended in 100 μL of ice-cold hypotonic buffer containing of 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was incubated on ice for 10 min to allow cellular membrane lysis while keeping nuclear envelopes intact. The lysates were then centrifuged at 5,000 × g for 5 minutes at 4°C to pellet the intact nuclei; the resulting supernatant was collected as the cytoplasmic fraction. The crude nuclear pellet was washed once in hypotonic buffer, resuspended in 100 μL of high-salt extraction buffer (20 mM HEPES, pH 7.9; 400 mM NaCl; 1 mM EDTA; 1 mM DTT; and 1 mM PMSF), and incubated at 4°C for 30 min with continuous gentle agitation to solubilize nuclear proteins. Finally, nuclear debris was pelleted by centrifugation at 13,000 × g for 30 min at 4°C and the clarified nuclear extract supernatant was collected and stored at –80°C until further analysis.

Pharmacological Signaling Pathway inhibitor assay

To delineate the intracellular signaling pathways involved in MCPS-mediated cytokine secretion, RAW 264.7 macrophages were pretreated with pathway-specific pharmacological inhibitors prior to MCPS exposure. All chemical inhibitors utilized in this study were obtained from Calbiochem (San Diego, CA, USA). Cells were incubated with each respective inhibitor in DMEM medium supplemented with FBS for 1 h at 37°C. The final concentrations for the selective inhibitors were established as follows: U0126 (selective ERK1/2 inhibitor), 10 μM; SP600125 (selective JNK inhibitor), 10 μM; SB203580 (selective p38 inhibitor), 20 μM; and Bay 11-7082 (selective NF-κB inhibitor), 20 μM. Following the 1-h pretreatment interval, the cells were stimulated with MCPS(1000 μg/mL) for an additional 12 h. Post-stimulation, the culture supernatants were harvested, and the extracellular secretion levels of TNF-α and IL-6 were measured using ELISA.

Statistical analysis

All experimental data are reported as the mean ± standard deviation (SD) from at least three independent biological replicates. Statistical comparisons among multiple experimental groups were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc multiple comparison test. All statistical analyses were executed using GraphPad Prism software (version 8.0; GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

MCPS sustains macrophage viability and activates immunomodulatory phenotypes via secretion of nitric oxide (NO) and pro-inflammatory cytokines

To evaluate the effect of MCPS on macrophage viability, RAW 264.7 cells were treated with various concentrations of MCPS (62.5, 125, 250, 500, and 1000 μg/mL) for 24 h. LPS, a well-characterized Toll-like receptor 4 (TLR4) agonist that elicits robust inflammatory responses in immune cells, served as a positive control. Cell viability was assessed using the MTT assay to rule out potential cytotoxicity as a confounding factor for subsequent analyses. The results demonstrated that MCPS exposure did not compromise cell viability at any tested concentration,

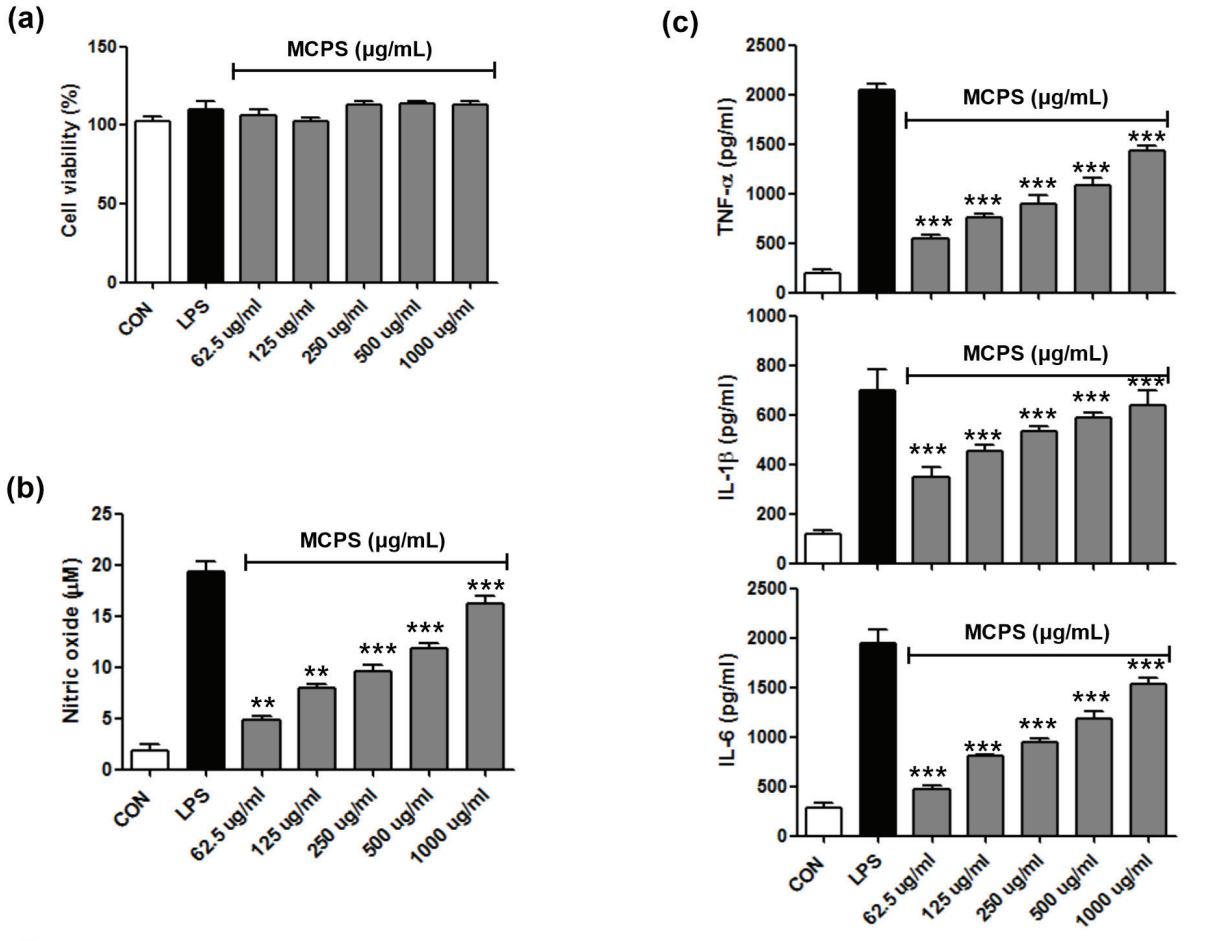

Fig. 1. Effects of *Momordica charantia* polysaccharide (MCPS) on cell viability, nitric oxide production, and pro-inflammatory cytokine secretion in RAW 264.7 macrophages. (A) RAW 264.7 macrophages were treated with various concentrations of MCPS (62.5, 125, 250, 500, and 1000 μg/mL) for 24 h, with lipopolysaccharide (LPS, 100 ng/mL) serving as the positive control. Cell viability was subsequently evaluated using the MTT assay.(B) Nitric oxide (NO) production was measured in the culture supernatants after 24 h of treatment with the indicated concentrations of MCPS using the Griess reagent assay. (C) The secretion of pro-inflammatory cytokines into the culture supernatants was quantified by enzyme-linked immunosorbent assay (ELISA). Data are presented as the mean ± standard deviation of three independent experiments (n = 3). Statistical significance compared with the control group is indicated as **p < 0.01 and ***p < 0.001.

confirming its biocompatibility with RAW 264.7 macrophages (Fig. 1A). Following the confirmation of non-cytotoxicity, macrophage activation was evaluated by measuring nitric oxide (NO) production via the Griess assay. MCPS treatment induced a concentration-dependent increase in extracellular nitrite generation, establishing an activation profile that paralleled robust LPS-mediated stimulation (Fig. 1B). To further characterize the immunomodulatory potential of MCPS, ELISAs were performed to quantify the secretion of key pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β). MCPS significantly elevated the release of these cytokines in a dose-dependent manner, closely mirroring the cytokine profile induced by LPS (Fig. 1C).

MCPS upregulates macrophage surface co-stimulatory and antigen-presenting molecules associated with immune activation

To assess the phenotypic maturation of macrophages induced by MCPS, flow cytometry was employed to evaluate the surface expression of key co-stimulatory and antigen-presenting molecules (Fig. 2).

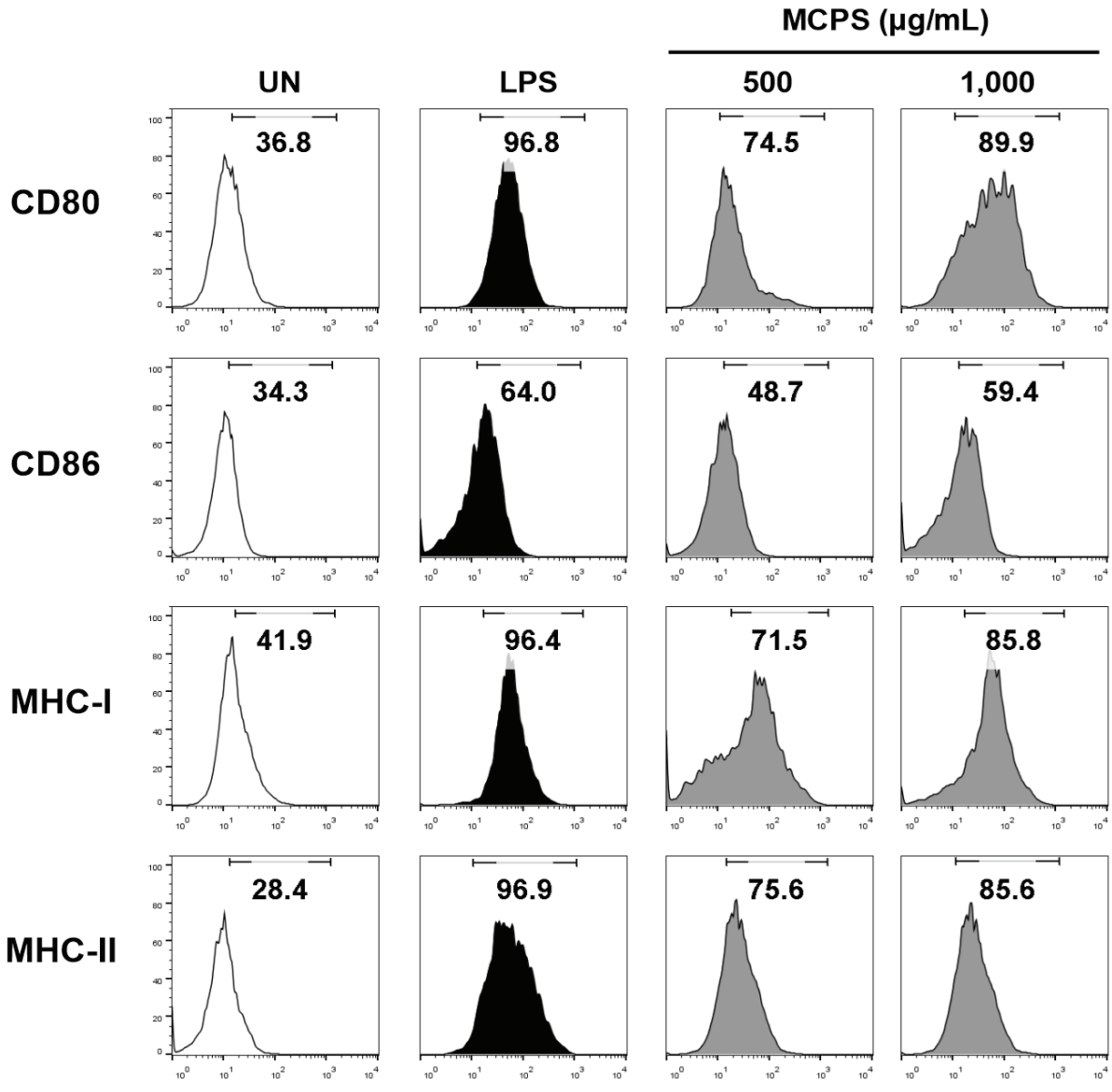

Fig. 2. Effects of *Momordica charantia* polysaccharide (MCPS) on the expression of surface markers in RAW 264.7 macrophages. RAW 264.7 macrophages were treated with MCPS or lipopolysaccharide (LPS) for 24 h. The cells were then stained with monoclonal antibodies against CD80, CD86, MHC class I, and MHC class II and incubated for 30 minutes. Surface marker expression was subsequently quantified using flow cytometry analysis.

RAW 264.7 macrophages were treated with MCPS at 500 and 1000 μg/mL for 24 h, and the surface levels of CD80, CD86, MHC class I, and MHC class II molecules were quantified. LPS was included as the positive control. As shown in Fig. 2, MCPS treatment induced a significant dose-dependent upregulation of these activation markers. Specifically, exposure to 500 μg/mL of MCPS elevated the mean fluorescence intensity (MFI) of CD80, CD86, MHC I, and MHC II by 74.5%, 48.7%, 71.5%, and 75.6%, respectively, compared to the untreated control. These stimulatory effects were further enhanced at the maximum concentration of 1000 μg/mL, with MFIs increasing by 89.9% for CD80, 59.4% for CD86, 85.8% for MHC I, and 85.6% for MHC II.

MCPS activates macrophage immune responses via the mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways

Having established that MCPS does

not exert cytotoxic effects but significantly enhances nitric oxide production and pro-inflammatory cytokine secretion, we next investigated the underlying molecular mechanisms driving this macrophage activation. Immunoblotting was performed to assess the activation status of the MAPK and NF-κB cascades, which are critical intracellular hubs for immune gene transcription. RAW 264.7 macrophages treated with MCPS at 500 and 1000 µg/mL exhibited a marked increase in the phosphorylation of ERK1/2, JNK1/2, and p38 MAPKs, alongside the concurrent nuclear translocation of the NF-κB p65 subunit (Fig. 3). These findings confirm robust activation of both the MAPK and NF-κB signaling pathways (Fig. 3A, 3B). To definitively verify whether the activation of these pathways is causally linked to MCPS-induced cytokine secretion,, pharmacological blockade experiments were executed using specific small-molecule inhibitors: U0126 (ERK1/2), SP600125 (JNK), SB203580 (p38), and Bay11-7082 (NF-κB) were used (Fig. 3C). Macrophages were pretreated with each respective inhibitor for 1 h prior to MCPS

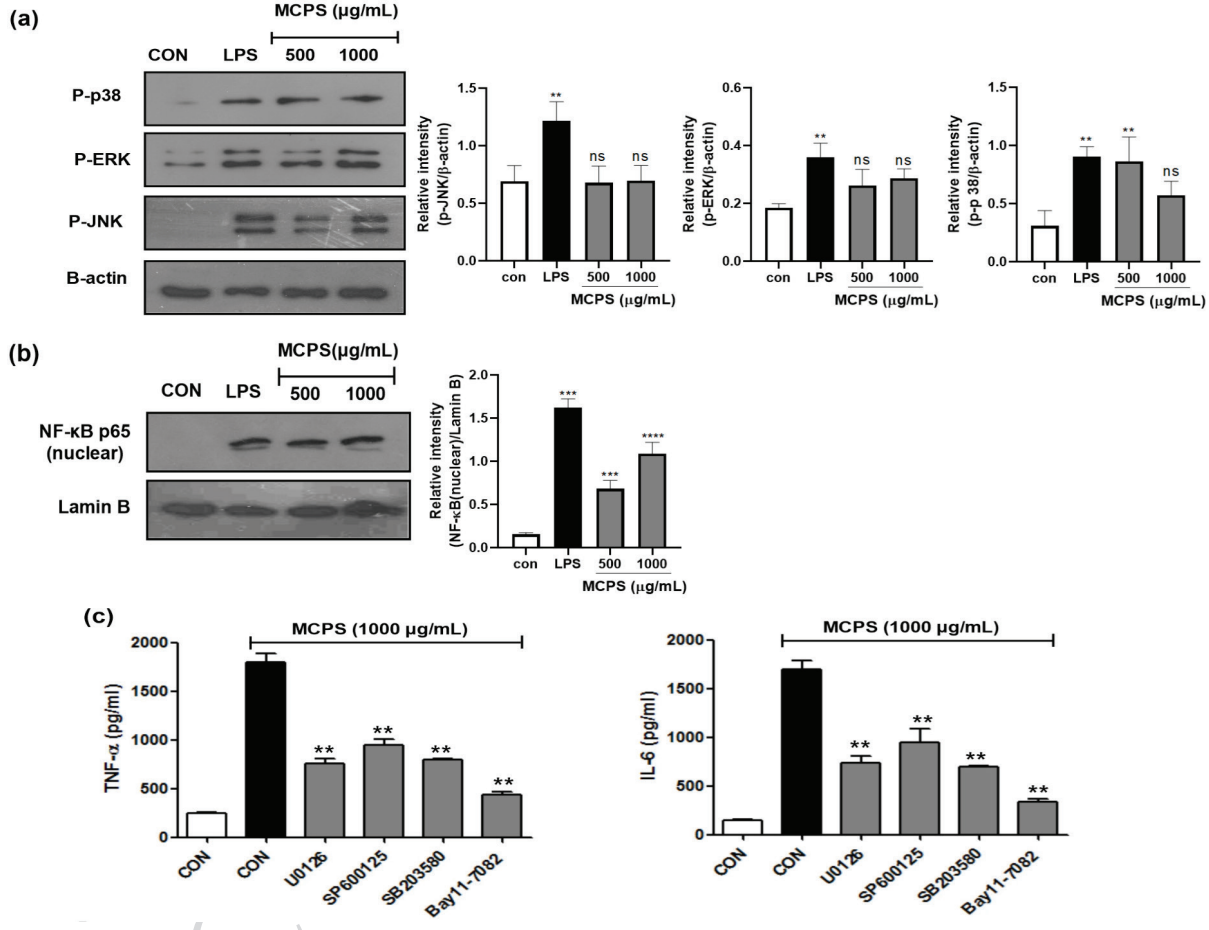

Fig. 3. Effect of *Momordica charantia* polysaccharide (MCPS) on MAPKs and NF-κB signaling pathways in RAW 264.7 Macrophages. (A). RAW 264.7 cells were treated with MCPS (500 and 1000 µg/mL) for 24 h. Whole-cell protein extracts were separated by SDS-PAGE and analyzed by immunoblotting using antibodies against phosphorylated MAPKs, including phospho-ERK1/2, phospho-JNK, and phospho-p38. (B). Nuclear protein extracts were also prepared and analyzed by immunoblotting with an antibody against NF-κB p65 to assess its nuclear translocation. (C). For inhibitor studies, cells were pretreated for 1 hour with selective inhibitors of ERK1/2 (U0126, 10 µM), JNK (SP600125, 20 µM), p38 (SB203580, 20 µM), or NF-κB (Bay 11-7082, 20 µM), with DMSO serving as the vehicle control, followed by stimulation with MCPS (1000 µg/mL) for 24 h. The concentrations of TNF-α and IL-6 in the culture supernatants were subsequently measured by enzyme-linked immunosorbent assay (ELISA). Data are expressed as the mean ± standard deviation (SD) of three independent experiments (n=3). Statistical significance was determined relative to the untreated control group (**p < 0.01).

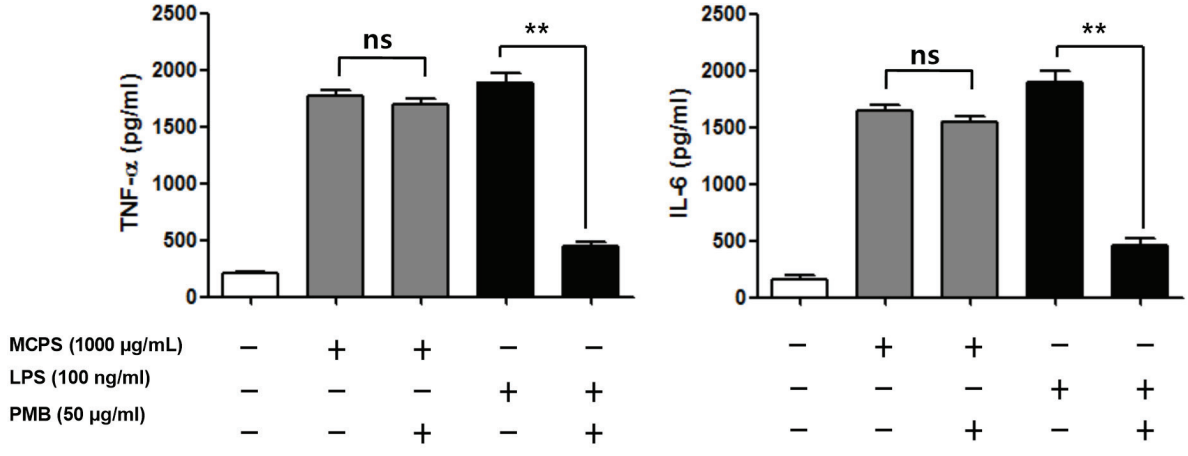

Fig. 4. Assessment of endotoxin contamination in *Momordica charantia* polysaccharide (MCPS). RAW 264.7 cells were pretreated with Polymyxin B (PMB, 50 μg/mL) for 1 hour at room temperature and subsequently stimulated with either lipopolysaccharide (LPS, 100 ng/mL) or MCPS (1000 μg/mL). After 24 h of incubation, the concentrations of (A) TNF-α and (B) IL-6 in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA). Data are presented as the mean ± standard deviation (SD) of three independent experiments (n = 3). Statistical significance (**$p < 0.01$) was determined relative to the untreated control group, whereas "ns" denotes no statistically significant difference.

stimulation. Notably, targeted inhibition of any of these individual pathways significantly reduced the MCPS-induced secretion of TNF-α and IL-6 (Fig. 3C). Taken together, these functional loss-of-function data confirm that MAPK and NF-κB signaling cascades serve as essential upstream mediators of the immunostimulatory effects of MCPS.

The immunostimulatory effects of MCPS are independent of endotoxin contamination as confirmed by polymyxin B (PMB) neutralization

To definitively exclude the possibility that MCPS-induced macrophage activation was an artifact driven by trace bacterial endotoxin contamination, an endotoxin neutralization assay was conducted using polymyxin B (PMB), a selective LPS antagonist. Preliminary screening confirmed that PMB treatment alone did not significantly compromise RAW 264.7 macrophage viability (data not shown). Individual treatments with either LPS or MCPS markedly increased the secretion of pro-inflammatory cytokines TNF-α and IL-6. Importantly, pre-incubation with PMB effectively ablated cytokine production in LPS-stimulated cells, yet it failed to alter or diminish the robust cytokine secretion induced by MCPS (Fig. 4).

DISCUSSION

The MTT assay employed in this study serves as a reliable indicator of mitochondrial NAD(P)H-dependent cellular oxidoreductase activity within metabolically active cells. It reflects overall cell viability through the enzymatic reduction of tetrazolium salt to insoluble formazan (15). Nitric oxide (NO), synthesized by inducible nitric oxide synthase (iNOS) in activated macrophages, functions as a critical pleiotropic effector molecule in antimicrobial host defense, immune regulation, and intercellular signaling cascades (16)(17). Compelling evidence from preceding studies highlights the dose-dependent role of NO in modulating immune responses and facilitating pathogen clearance by macrophages (17).

Pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 function as primary molecular coordinators that orchestrate downstream immune and inflammatory cascades. While TNF-α and IL-1β primarily initiate and amplify acute-phase inflammatory processes (18), IL-6 serves as a bridge to adaptive immunity by supporting

the differentiation of dendritic cells and promoting B cell maturation, thereby enhancing antigen-specific immunity (19)(20)(21). Collectively, these observations suggest that MCPS safely primes macrophage functionality without inducing cytotoxicity. By driving the coordinated expression of these key immunomodulatory mediators, MCPS demonstrates significant potential as a therapeutic immunostimulatory agent capable of bolstering innate host defenses.

Macrophages play a pivotal role in bridging innate and adaptive immunity through processing and presenting internalized pathogens as antigenic peptides via MHC molecules to T lymphocytes (22). The upregulation of co-stimulatory molecules CD80 and CD86 provides the mandatory secondary signal required for effective T cell activation. Concurrently, MHC class I and class II molecules facilitate the presentation of antigens to CD8+ and CD4+ T cells, respectively (23)(24)(25). Our findings demonstrate that MCPS markedly upregulates the surface density of both co-stimulatory and antigen-presenting machinery on RAW 264.7 cells. This phenotypic shift indicates that MCPS enhances the antigen-presenting capabilities of macrophages, thereby providing a potent cellular mechanism to orchestrate downstream adaptive immune responses.

Mitogen-activated protein kinases (MAPKs) serve as central regulators of cellular responses including activation, proliferation, differentiation, and apoptosis, while tightly modulating the production of TNF-α and interleukins (26)(27). Concurrently, the transcription factor NF-κB, which is activated by stimuli such as LPS, translocates to the nucleus and regulates the expression of genes involved in both innate and adaptive immune responses (28)(29). The marked phosphorylation of MAPKs and nuclear translocation of NF-κB p65 following MCPS treatment provide compelling evidence for the robust activation of these signaling pathways. Furthermore, inhibitor studies further confirm the critical roles of MAPK and NF-κB pathways in mediating the immunostimulatory effects of MCPS.

To exclude the possibility that residual endotoxin contamination contributed to the observed immune activation, polymyxin B (PMB), a potent neutralizer of LPS through high-affinity binding was employed (30)(31). The persistence of immunostimulatory activity in the presence of PMB strongly indicates that the observed effects are attributable to MCPS itself rather than to residual endotoxin contamination.

In recent years, numerous studies have explored the immunomodulatory potential of natural polysaccharides. Although the detailed molecular mechanisms underlying the immunomodulatory effects of MCPS remain to be fully elucidated, previous studies have demonstrated that MCPS and its fractions enhance immune responses by directly stimulating splenic lymphocytes in cyclophosphamide-induced immunosuppressed models (32). Consistent with these findings, a study (33) demonstrated that MCPS significantly enhances both spontaneous and mitogen-induced proliferation of splenic lymphocytes in immunocompromised mice while increasing secretion and mRNA expression of key cytokines, including IL-6, IFN-γ, and IL-12, thereby facilitating the recovery of damaged immune tissues. Together with previous studies, the findings of the present study further substantiate the potent immunomodulatory activity of natural polysaccharides as functional bioactive compounds for immune modulation.

CONCLUSION

In summary, MCPS demonstrated no cytotoxic effects on RAW 264.7 macrophages while significantly enhancing nitric oxide production, pro-inflammatory cytokine secretion, and the expression of surface markers CD80, CD86, MHC class I, and MHC class II. Immunoblotting

analysis further revealed that MCPS induces the phosphorylation of the MAPK and NF-κB signaling pathways, thereby promoting macrophage activation via distinct intracellular signaling mechanisms. Taken together, these findings endorse the potent immunomodulatory activity of MCPS and support its potential application as a functional bioactive compound for immune modulation.

ACKNOWLEDGMENTS

We sincerely appreciate the support from Editage (www.editage.co.kr) for their professional English editing services. This work was supperted by the National Reserach Foundation of Korea inder Grants NRF(RS-2025-196829693078206478000l); and by the research grant of Kongju National University in 2025.

DISCLOSURE OF INTEREST

The authors declare that they have no financial or personal relationships that could have appeared to influence this work.

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