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Ginsenoside Rg3 promotes Fc gamma receptor-mediated phagocytosis of bacteria by macrophages via an extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase-dependent mechanism

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ABSTRACT

Ginsenoside Rg3 is a steroidal saponin abundant in Korean red ginseng that has high anti-inflammatory activity. Rg3 exerts an immunomodulatory effect in acute inflammatory conditions such as bacterial infections. In this study, we determined the effect of Rg3 on bacterial uptake by macrophages and the related intracellular signaling pathways. Rg3 increased macrophage phagocytosis of IgG-opsonized *Escherichia coli* and IgG-opsonized beads (IgGbeads), but not of non-opsonized beads. Rg3 also enhanced the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and p38 mitogen-activated protein kinase (p38 MAPK), but not that of Akt. The inclusion of IgGbeads in macrophage cultures also increased the phosphorylation of ERK1/2 and p38, but co-culture of macrophages with non-opsonized beads did not affect the phosphorylation of ERK1/2 and p38. The Rg3-induced promotion of phagocytosis was inhibited by PD98059, an ERK1/2 inhibitor, and SB203580, a p38 inhibitor. PD98059 inhibited Rg3-induced p38 MAPK phosphorylation, but SB203580 did not suppress ERK1/2 phosphorylation. Culture of macrophages with Rg3 increased actin polymerization, and this effect was inhibited by SB203580 and PD98059. The Rg3-induced increase in phagocytosis was also inhibited by NSC23766, a Rac1 inhibitor and CASIN, a Cdc42 inhibitor. Intraperitoneal injection of Rg3 increased the phosphorylation of ERK1/2 and p38 as well as the phagocytosis of bacteria by lung cells. These results demonstrate that ginsenoside Rg3 enhances macrophage phagocytosis of bacteria by activating the ERK1/2 and p38 MAPK pathways.

1. Introduction

Phagocytes, such as macrophages, play an essential role in host protection against invading pathogens and in the innate immune response, and present antigens to induce adaptive immunity [1]. A large number of highly selective receptors are involved in the recognition and uptake of foreign particles [2]. Among them, opsonic receptors Fcγ are the key phagocytic receptor for elimination of invading pathogens. The binding of microbes to Fcγ receptors (FcgRs) on the surface of macrophages induces several intracellular events; *e.g.*, actin filament polymerization, during phagocytosis by transducing cellular signals. Specifically, FcgR-mediated phagocytosis activates the Rho family GTPases Cdc42 and Rac, which regulate actin cytoskeletal organization in the phagocytic cup [3]. Disruption of actin polymerization during phagocytosis reduces macrophage phagocytosis [4,5].

During phagocytosis, diverse signaling molecules, such as mitogen-activated protein kinases (MAPKs), function as downstream effectors of Rho family GTPases. Activation of extracellular signal-regulated kinase (ERK) 1/2 plays an important role in phagocytosis by neutrophils, and expression of dominant negative Rac/Cdc42 by neutrophils significantly diminishes ERK1/2 activation and phagocytosis [6]. Also, p21 (RAC1)-activated kinase 1 (PAK1), a downstream target of Rac1/Cdc42, regulates the activity of ERK1/2 and is involved in the actin cytoskeleton rearrangement related to lamellipodium formation by macrophages [7], which is essential for phagocytosis [8]. Indeed, inhibition of ERK1/2 activity diminished the macrophage phagocytosis

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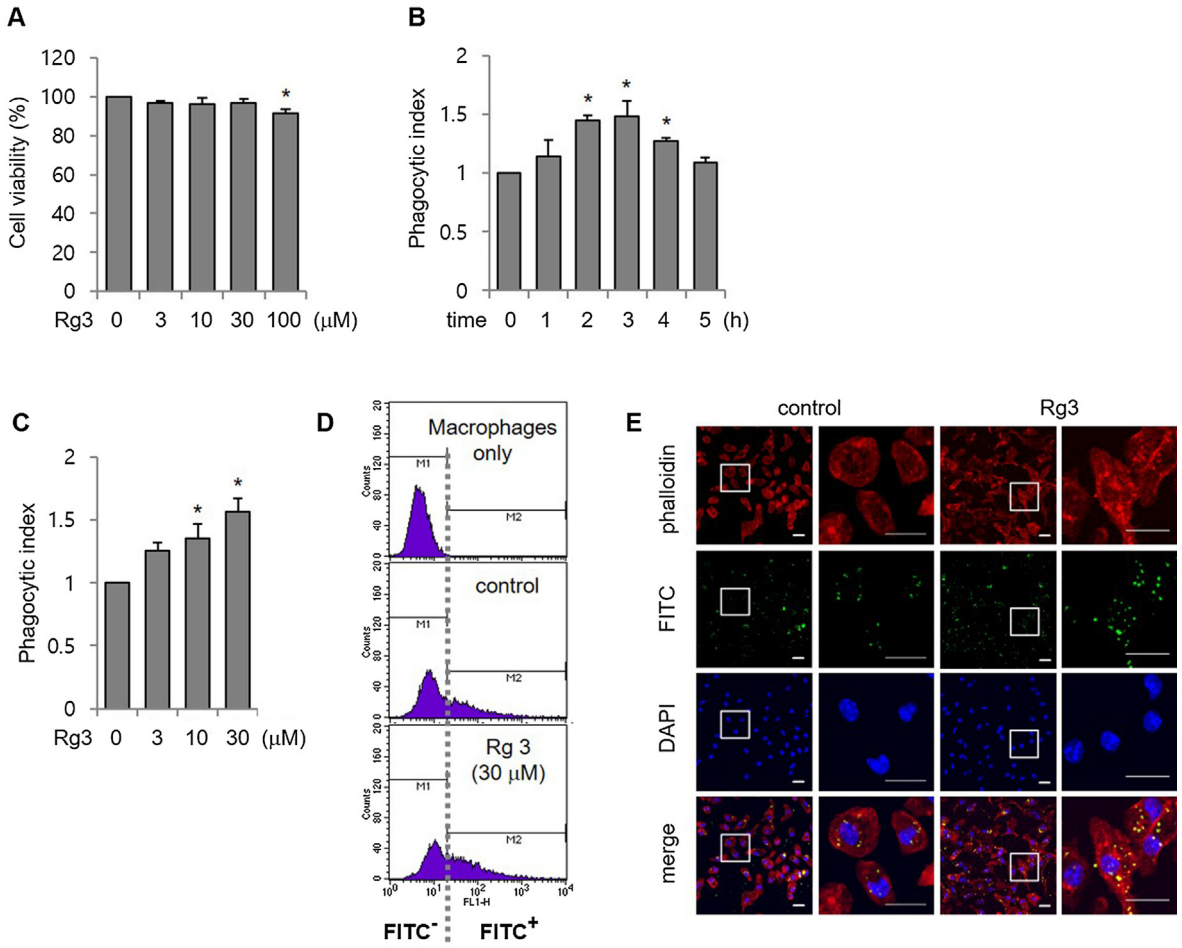

Fig. 1. Ginsenoside Rg3 increases macrophage phagocytosis. (A) Peritoneal macrophages were treated with the indicated concentrations of Rg3 for 24 h and their viability was evaluated by MTT assay. (B and C) Peritoneal macrophages were cultured (B) with Rg3 (30 μM) for the indicated times or (C) with Rg3 (0, 3, 10, or 30 μM) for 3 h, and subsequently with IgG-opsonized fluorescein isothiocyanate (FITC)-conjugated *E. coli* for 20 min, followed by flow cytometry. (D) Representative flow cytometry histograms are shown. (E) Representative microscopic images show that Rg3 (30 μM) increased internalization of IgG-opsonized FITC-conjugated E.coli by macrophages. Original magnification, 40×. Scale bar = 20 μm. The right panels in each group are magnified from the white box of the left panels. Red indicates phalloidin; FITC, *E. coli*; DAPI, nucleus. Bars are means ± SD (n = 5). $^*P < 0.05$ compared with the control.

enhanced by stimulation with a Toll-like receptor (TLR) agonist [9]. P38 MAPK is also reportedly involved in actin polymerization and lamellipodium formation in various cell types [10–13]. For example, the activity of p38 and heat shock protein (HSP) 27, a downstream target of p38, are reported to be involved in platelet-derived growth factor-induced actin polymerization and lamellipodia formation in smooth muscle cells [10]. Also, engagement of FcgR activates the MAPK3/6-p38 pathway, which enhances actin polymerization and macrophage phagocytosis [5].

Panax ginseng is used as an herbal medicine in Asian countries. Ginsenosides are a class of glycosylated triterpenoid saponins and are the major active compounds of *Panax ginseng.* Among the various ginsenoside saponins, ginsenoside Rg3 has a wide range of pharmacological activities such as anticancer, anti-inflammatory, antioxidant, and antidiabetic effects [14,15]. For example, Rg3 has been shown to decrease the intracellular level of reactive oxygen species (ROS), increase the antioxidant glutathione pool, and suppress mitochondrial dysfunction in hepatocytes injured by lipopolysaccharide (LPS) [16]. Rg3 exerts an immunoregulatory effect on acute lung injury induced by LPS. Rg3 diminished LPS-induced histopathologic lung damage and decreased the production of inflammatory cytokines (*e.g.*, tumor necrosis factor (TNF)-α, interleukin [IL]-1β, and IL-6), but increased that of anti-inflammatory cytokines (*e.g.*, IL-10 and transforming growth factor-β) [17]. These results suggest that Rg3 exerts a protective effect in inflammatory conditions associated with bacterial infection. In the present study, we investigated the effect of Rg3 on FcgR-mediated phagocytosis and evaluated the underlying intracellular mechanisms.

2. Materials and methods

2.1. Mice

Male BALB/c mice of 20–25 g weight and 8–10 weeks of age were obtained from Samtako Science (Daejeon, South Korea). The mice were maintained under a 12/12 h light/dark cycle and were provided food and water *ad libitum*. The study was approved by The Animal Care and Ethics Committee of Chonnam National University Medical School (CNUIACUC-H-2018-61).

2.2. Reagents and antibodies

Ginsenoside Rg3 from *P. ginseng*, LY294002, STO609, CASIN, NSC23766, and PD98059 were obtained from Sigma-Aldrich (St Louis, MO, USA) and SB203580 from Calbiochem (La Jolla, CA, USA). Bovine serum albumin (BSA) and Roswell Park Memorial Institute (RPMI) 1640 medium containing L-glutamine, penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA). Antibodies specific for phosphor- or total-p38, Akt, ERK1/2, and HSP27

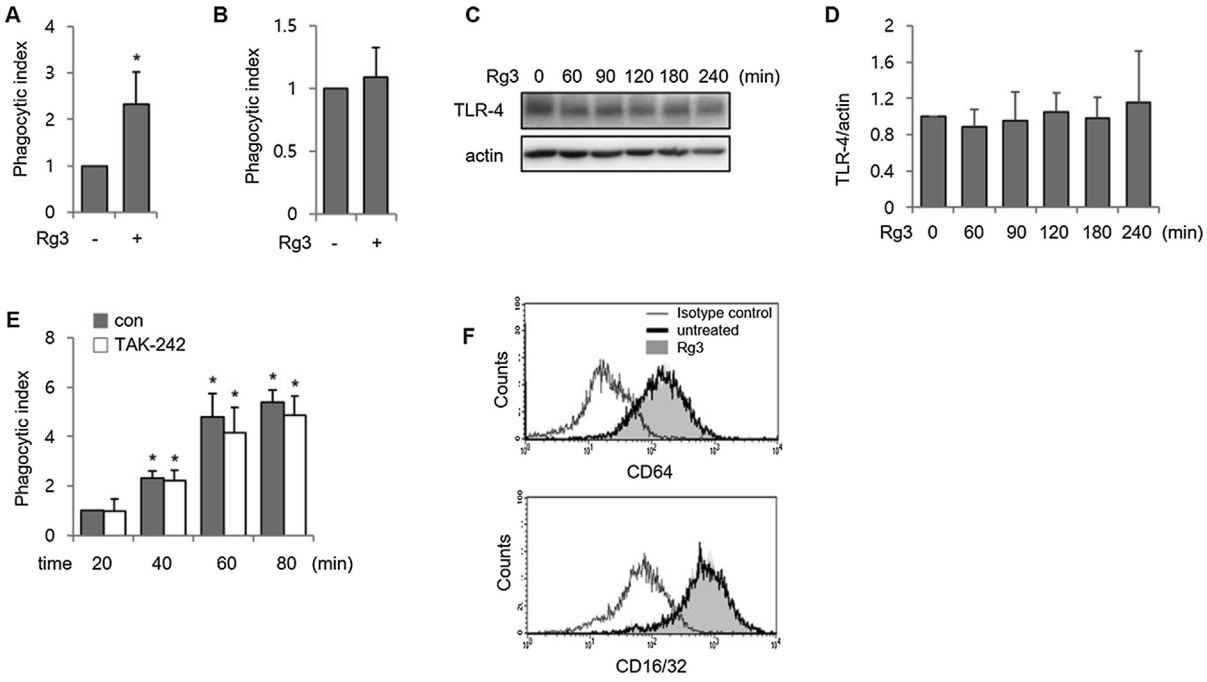

Fig. 2. Rg3 promotes FcgR-mediated phagocytosis. (A and B) Peritoneal macrophages were cultured with Rg3 (0 or 30 μM) for 3 h and then with (A) IgG-opsonized FITC-conjugated fluorescent beads or (B) unopsonized FITC-conjugated fluorescent beads for 30 min, followed by flow cytometry. Bars are means ± SD (n = 4). *$P < 0.05$ compared with the control. (C and D) Peritoneal macrophages were cultured with Rg3 for the indicated times and whole-cell lysates were subjected to Western blotting using antibodies for Toll-like receptor-4 (TLR-4) and actin. (C) Representative Western blots show TLR-4 and actin levels. (D) The ratios of the TLR-4 to actin band densities were calculated from four independent experiments. (E) Macrophages were cultured with TAK242 (0 or 1 μM) for 2 h before incubation with IgG-opsonized FITC-conjugated *E. coli* for the indicated times, followed by flow cytometry. Bars are means ± SD (n = 5). *$P < 0.05$ compared with the control. (F) Peritoneal macrophages were cultured with Rg3 (0 or 30 μM) for 3 h and incubated with the FcgR1 marker CD64 conjugated to phycoerythrin (PE), or the FcgR2 and FcgR3 marker CD16/32 conjugated to PE. Representative flow cytometry histograms are shown.

were from Cell Signaling Technology (Beverly, MA, USA). Fluorescein isothiocyanate (FITC)-conjugated *Escherichia coli* K-12, 1 μm yellow-green fluorescent (excitation 505 nm, emission 515 nm) sulfate FluoSpheres® (F8852), and opsonizing reagent (E2870; rabbit polyclonal IgG antibodies specific for *E. coli*) were obtained from Invitrogen (Eugene, OR, USA). PE anti-mouse CD16/CD32 (clone 2.4G2) and PE anti-mouse CD64 were purchased from BD Biosciences (San Jose, CA, USA) and BioLegend (San Diego, CA, USA), respectively.

2.3. Isolation and culture of cells

Brewer thioglycollate (1 mL, 4% w/v) was injected intraperitoneally in the mice. Four days later, macrophages were isolated by peritoneal lavage and cultured in 12- or 24-well plates (1×10^6 or 5×10^5 cells/well, respectively) in RPMI 1640 medium supplemented with 5% FBS, penicillin (100 U/mL), and streptomycin sulfate (100 μg/mL) in a 5% CO_2 incubator at 37 °C for 1 h. The cells were washed three times with culture medium to remove non-adherent cells, and cultured in serum-free RPMI 1640 medium for 1 h before Rg3 administration.

2.4. Cell viability

The viability of macrophages was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Macrophages were cultured with Rg3 (0–100 μM) for 24 h in 96-well plates (1×10^4 cells/well). MTT (10 μL) was added for 4 h, the medium was removed, and DMSO (100 μL) was added. Absorbance at 570 nm was measured using an enzyme calibrator at 570 nm and cell viability was calculated as the ratio of the absorbance of the test and control groups.

2.5. Western blot analysis

Cells were lysed by incubation in PRO-PREP™ Protein Extraction Solution (Intron Biotechnology, Seoul, Korea) for 20 min on ice. A BCA protein assay kit (Thermo Fisher Scientific, Pierce, WI, USA) was used to quantify the protein concentration in whole-cell lysates according to the manufacturer's instructions. Equal amounts of proteins (50 μg/sample) were resolved by electrophoresis in polyacrylamide gels containing 10–12% sodium dodecyl sulfate and transferred to polyvinylidene difluoride membranes. The membranes were incubated with blocking buffer (5% nonfat dry milk in 20 mM TBS with 0.1% Tween) for 1 h at room temperature, then with specific primary antibodies as described in the figure legends, a dilution of 1/1000 in 5% BSA in TBST overnight at 4 °C, and finally with horseradish peroxidase-conjugated secondary antibodies at a dilution of 1/1000 in 5% nonfat dry milk in TBST. Bands were detected using enhanced chemiluminescence Western Blotting Detection Reagents (Millipore, Billerica, MA, USA), and imaged using a LAS-3000 (Life Science Systems, Fujifilm Global). Densitometry was performed using a Multi-Gauge v. 3.0 chemiluminescence system and associated software (Life Science Systems, Fujifilm Global) to quantify the phosphorylated to total protein ratio.

2.6. In vitro phagocytic assay

The phagocytic index was calculated as described previously [18]. The FITC-conjugated *E. coli* or fluorescent beads were opsonized using an IgG-opsonizing reagent for 1 h at 37 °C, according to the manufacturer's instructions, before adding them to the macrophage cultures. Peritoneal macrophages were cultured with FITC-conjugated opsonized *E. coli* for 20 min and fluorescent beads or opsonized fluorescent beads (1:2) for 30 min at 37 °C. The cells were washed vigorously three times with cold phosphate-buffered saline (PBS), and the fluorescent signals

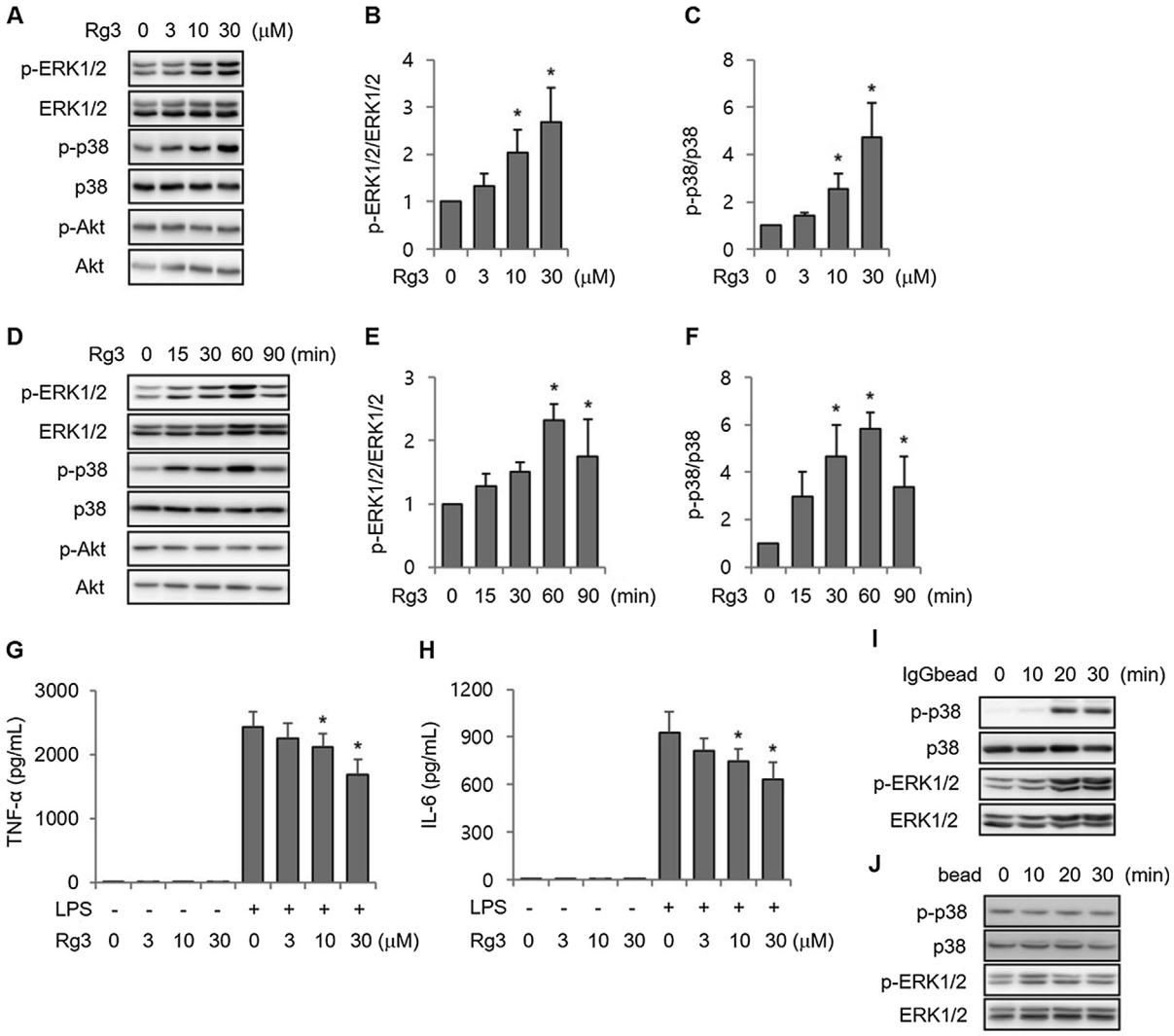

Fig. 3. Rg3 increases the activation of ERK1/2 and p38 in macrophages. (A–F) Peritoneal macrophages were cultured with Rg3 (0, 3, 10, or 30 μM) for 1 h or with Rg3 (30 μM) for the indicated times. (A and D) Representative Western blots of phosphorylated ERK1/2, p38, and Akt and total ERK1/2, p38, and Akt are shown. (B, C, E, and F) Density ratios of phosphorylated to total protein from five independent experiments. *$P < 0.05$ compared with the control. (G and H) Peritoneal macrophages were cultured with the indicated concentration of Rg3 for 1 h before adding LPS (0 or 100 ng/mL) for 4 h. TNF-α and IL-6 protein levels were measured in the culture medium by ELISA. *$P < 0.05$ compared with LPS only. Bars are means ± SD (n = 5). (I and J) Peritoneal macrophages were incubated with IgGbeads or unopsonized beads for the indicated times, and whole-cell lysates were subjected to electrophoresis in polyacrylamide gels.

from cell-attached *E. coli* and beads were quenched using 0.2% trypan blue solution. The cells were isolated using 0.25% trypsin/EDTA in PBS, and the cell suspension was centrifuged and the cell pellet resuspended in PBS for flow cytometry analysis. The percentage of macrophages positive for fluorescent *E. coli* from 10,000 gated events was used as a measure of the phagocytic activity. The phagocytic index was expressed as the ratio relative to the phagocytic activity of the control cells.

2.7. In vivo assay of phagocytic activity

Phagocytosis was assayed *in vivo* as described previously [18]. Briefly, Rg3 was injected intraperitoneally into the mice 3 h before administration of FITC-labeled opsonized *E. coli* in PBS (50 μL, 1×10^7 cells/mouse) into the lungs of the mice via tracheotomy under sevoflurane anesthesia. After 2 h intratracheal administration of *E. coli,* lung cells were collected by bronchoalveolar lavage (BAL). Ice-cold PBS (1 mL) containing 5 mM EDTA was slowly infused and withdrawn three times through the trachea. The cells were washed with ice-cold PBS and treated with 0.2% trypan blue solution, and then washed twice with cold PBS. Finally, flow cytometry was performed to quantify cells that reacted positively to *E. coli.*

2.8. Actin polymerization

Actin polymerization was evaluated as described previously [5]. Briefly, peritoneal macrophages were fixed in PBS containing 4% paraformaldehyde for 20 min, permeabilized in 0.1% Triton X-100 for 4 min, and washed with PBS. The washed cells were blocked with 3% BSA and Alexa Fluor 594-conjugated phalloidin for 20 min at room temperature, washed, and stained with 4′,6-diamidino-2-phenylindole (DAPI) in emulsion oil solution. Fluorescence was measured using a fluorescence plate reader (Infinite M200 PRO; Tecan Life Science, Männedorf, Switzerland). Actin polymerization was assessed as the fluorescence units of phalloidin (excitation, 580 nm; emission, 610 nm) relative to DAPI (excitation, 355 nm; emission, 460 nm).

2.9. Confocal microscopy

Confocal microscopic images were obtained as described previously [19]. Macrophages were incubated with 4% paraformaldehyde in PBS

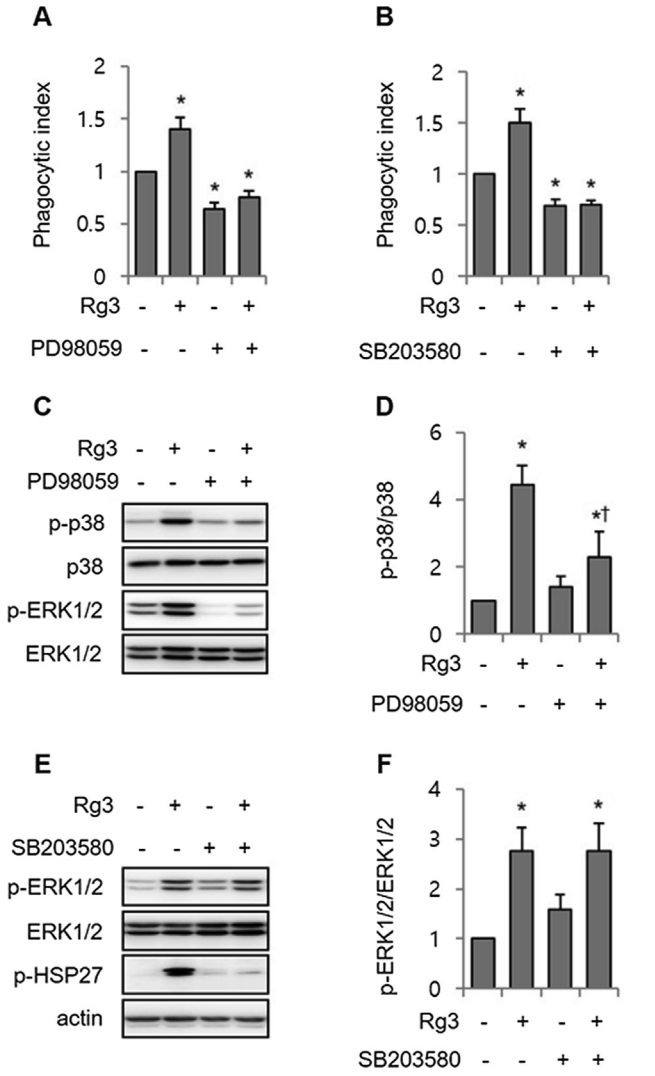

Fig. 4. Rg3 increases macrophage phagocytosis by activating ERK1/2 and p38. (A and B) Peritoneal macrophages were cultured with PD98059 (0 or 50 μM) or SB203580 (0 or 10 μM) for 30 min before adding Rg3 (0 or 30 μM) for 3 h, and with IgG-opsonized FITC-conjugated *E. coli* for 20 min. The phagocytic index was determined by flow cytometry. (C–F) Peritoneal macrophages were cultured with PD98059 (0 or 50 μM) or SB203580 (0 or 10 μM) for 30 min before adding Rg3 (0 or 30 μM) for 1 h, and whole-cell lysates were subjected to Western blotting. (C and E) Representative Western blots show the levels of phosphorylated ERK1/2, p38, and heat shock protein (HSP) 27, a downstream target of p38, and total ERK1/2, p38, and actin. (E and F) Density ratios of phosphorylated to total protein from four independent experiments. Bars are means ± SD (n = 5). $^{*}P < 0.05$ compared with the control. †$P < 0.05$ compared to cells treated with Rg3 only.

for 20 min, washed with PBS and permeabilized using 0.1% TritonX-100/PBS for 4 min. The cells were then washed and incubated with 3% BSA in PBS for 45 min followed by Alexa Fluor 594-conjugated phalloidin (25 μL/mL, Invitrogen) for 20 min. Cells were mounted using an emulsion oil solution containing DAPI after washing with PBS. Confocal microscopy was performed using a LSM510 inverted epifluorescence/Nomarski microscope (LSM510, ZEISS, Oberkochen, Germany) outfitted with Leica TCS NT laser confocal optics.

2.10. Statistical analysis

Data are expressed as means ± standard deviation (SD). The Kolmogorov–Smirnov test was used to determine whether data were normally distributed. Statistical significance was evaluated by Student's *t*-test between two groups and one-way analysis of variance (ANOVA) with Tukey's *post hoc* test among more than two groups if values were normally distributed. The Kruskal–Wallis *H*-test was used, and individual differences were detected by Bonferroni-corrected Mann–Whitney *U* tests if values were not normally distributed. A value of $P < 0.05$ was considered indicative of significance. All analyses were performed using SPSS v. 21.0.

3. Results

3.1. Rg3 promotes FcgR-mediated phagocytosis by macrophages

We first evaluated the effect of Rg3 on macrophage viability by MTT assay. As shown in Fig. 1A, treatment with Rg3 at 100 μM, but not 0–30 μM, reduced macrophage viability. Because FcgR plays a pivotal role in the elimination of invading pathogens, we examined its effect on FcgR-mediated phagocytosis. As shown in Fig. 1B, the increase in phagocytic ability of macrophages to IgG-opsonized *E. coli* was first observed at 1 h after Rg3 treatment, peaking at 3 h. At 0 to 30 μM, Rg3 dose-dependently increased macrophage phagocytosis of IgG-opsonized *E. coli* after 3 h (Fig. 1C and D). Confocal microscopy demonstrated a significant increase in internalized *E. coli* in macrophages treated with Rg3 compared with the vehicle-treated cells (Fig. 1E). To exclude any confounding effect of bacterial toxins or LPS, peritoneal macrophages were cultured with IgG-opsonized FITC-conjugated fluorescent beads (IgGbeads) or unopsonized FITC-conjugated fluorescent beads. As shown in Fig. 2A and B, Rg3 enhanced macrophage phagocytosis of the IgGbeads, but not the unopsonized beads. In addition, because LPS-TLR4 signaling modulates the phagocytosis of bacteria [20,21], TLR4 expression was evaluated. Rg3 did not affect the expression of TLR4 (Fig. 2C and D), and the addition of TAK242, an inhibitor of TLR4 signaling, to the macrophage cultures did not affect the macrophage phagocytic activity toward IgG-opsonized *E. coli* (Fig. 2E). To determine whether the effect of Rg3 on FcgR-mediated phagocytosis by macrophages is due to increased surface expression of FcgR, Rg3-treated macrophages were subjected to flow cytometry. As shown in Fig. 2F, the results of flow cytometry suggested that little change in the expression of phagocytic receptors, such as FcgR1, FcgR2, and FcgR3, was observed on the surface of macrophages.

3.2. Rg3 increases macrophage phagocytosis by activating ERK1/2 and p38

The phagocytosis of bacteria by professional phagocytes, such as macrophages and neutrophils, involves several protein kinases [6,18,22–24]. Therefore, we explored the effect of Rg3 on ERK1/2, p38, and Akt activity in macrophages. Rg3 increased the phosphorylation of ERK1/2 and p38, but not of Akt, in a time- and dose-dependent manner (Fig. 3A–F). Previous studies have shown that drugs with anti-inflammatory effects can affect phagocytic ability of macrophages [25,26], and Rg3 has been reported to have anti-inflammatory effects in several experimental models [16,17,27,28]. Therefore, we investigated the effect of Rg3 on the LPS-induced production of inflammatory cytokines in macrophages. As shown in Fig. 3G and H, Rg3 reduced the levels of TNF-α and IL-6 induced by LPS, consistent with previous findings [17], although Rg3 increased the phosphorylation of p38 and ERK1/2 in macrophages. However, Rg3 alone did not affect the production of TNF-α and IL-6, within the range of concentrations evaluated in this experiment. These results suggest that the effect of Rg3 on macrophage phagocytosis is not due to its anti-inflammatory effect, although the possibility that Rg3 could increase the expression of anti-inflammatory cytokines such as IL-10 cannot be ruled out. Next, to determine whether ERK1/2 and p38 are involved in FcgR-mediated phagocytosis, macrophages were cultured with IgGbeads. As shown in Fig. 3I and J, the addition of IgGbeads, but not unopsonized beads, to macrophage cultures increased the phosphorylation of ERK1/2 and p38. The addition of PD98059, an ERK1/2 inhibitor, or SB203580, a

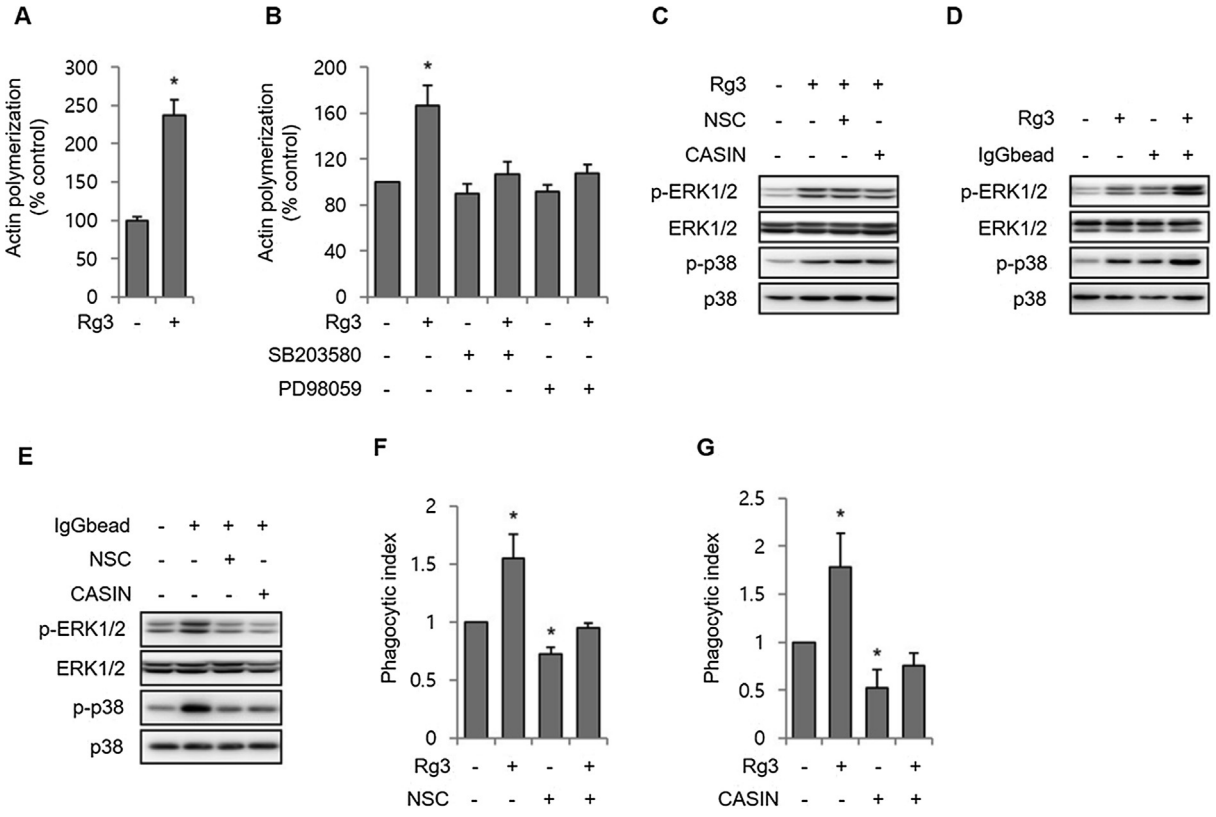

Fig. 5. Rac1 and Cdc42 are required for macrophage phagocytosis. (A) Peritoneal macrophages were cultured with Rg3 (0 or 30 μM) for 3 h. The cells were stained with DAPI for nuclei and Alexa Fluor 594-conjugated phalloidin for filamentous actin. Fluorometric analysis of actin polymerization (fluorescence units of phalloidin relative to DAPI) was performed using a fluorescence plate reader. (B) Peritoneal macrophages were cultured with PD98059 (PD; 0 or 50 μM) or SB203580 (SB; 0 or 10 μM) for 30 min before exposure to Rg3 (0 or 30 μM) for 3 h, followed by fluorometric analysis of actin polymerization. Bars are means ± SD (n = 4). *$P < 0.05$ compared with the control. (C) Peritoneal macrophages were cultured with NSC23766 (NSC; 0 or 100 μM) or CASIN (0 or 5 μM) for 30 min before adding Rg3 (0 or 30 μM) for 1 h. (D) Macrophages were cultured with Rg3 (0 or 30 μM) for 3 h and subsequently with IgG beads for 20 min. (E) Peritoneal macrophages were cultured with NSC23766 (NSC; 0 or 100 μM), a Rac1 inhibitor, or CASIN (0 or 5 μM), a Cdc42 inhibitor, for 30 min before adding IgGbeads for 20 min. (F and G) Macrophages were cultured with NSC (0 or 100 μM) or CASIN (0 or 5 μM) for 30 min before adding Rg3 (0 or 30 μM) for 3 h, followed by culture with IgG-opsonized FITC-conjugated *E. coli* for 20 min. Bars are means ± SD (n = 4). *$P < 0.05$ compared with the control.

p38 inhibitor, before the addition of Rg3 to macrophage cultures, diminished macrophage phagocytosis (Fig. 4A and B). Furthermore, addition of PD98059 to macrophage cultures inhibited Rg3-induced phosphorylation of p38, but SB203580 did not block Rg3-induced phosphorylation of ERK1/2 although it suppressed phosphorylation of heat shock protein 27 (HSP27), a downstream target of p38 (Fig. 4C–F). These results suggest that Rg3-induced p38 activation is mediated by ERK1/2 in macrophages.

3.3. Rac1 and Cdc42 are required for the Rg3-induced increase in macrophage phagocytosis

Rearrangement of the actin cytoskeleton is essential for FcgR-mediated phagocytosis [29]. Therefore, we examined whether Rg3 increased actin polymerization, resulting in formation of lamellipodia or filopodia. As shown in Fig. 5A, Rg3 increased actin polymerization in macrophages. However, when the activity of ERK1/2 and p38 was blocked, Rg3-induced actin polymerization was inhibited (Fig. 5B). These results suggest that ERK1/2 and p38 are involved in actin polymerization during Rg3-induced macrophage phagocytosis. Next, we examined whether Rg3 enhances the activity of ERK1/2 and p38 by activating Rac1 and Cdc42. Addition of NSC23766, a Rac1 inhibitor, or CASIN, a Cdc42 inhibitor, to macrophage cultures did not inhibit Rg3-induced phosphorylation of ERK1/2 and p38 (Fig. 6A). Also, co-culture of macrophages with Rg3 and IgGbeads further increased the phosphorylation of ERK1/2 and p38 compared with Rg3 or IgGbeads alone (Fig. 5E). These results suggest that Rg3 promotes FcgR-mediated phagocytosis by increasing the activity of ERK1/2 and p38 and that Rg3 increases the activity of ERK1/2 and p38 independently of Rac1 and Cdc42. Because Rho family GTPases, such as Rac1 and Cdc42, are essential for actin polymerization during internalization of IgG-opsonized particles [30], we examined whether engagement of FcgR with IgGbeads enhances the activity of ERK1/2 and p38 by activating Rac1 and Cdc42. As shown in Fig. 5C, addition of NSC23766, a Rac1 inhibitor, or CASIN, a Cdc42 inhibitor, to macrophage cultures suppressed IgGbead-induced phosphorylation of ERK1/2 and p38. These results suggest that Rac1 and Cdc42 are essential for FcgR-mediated phagocytosis but not required for Rg3-mediated activation of ERK1/2 and p38. Indeed, the Rg3-induced increase in macrophage phagocytosis was inhibited by NSC23766 or CASIN (Fig. 5F and G).

3.4. Rg3 increases phagocytosis of bacteria in the mouse lung

We investigated the effect of Rg3 on ERK1/2 and p38 activity and phagocytosis of bacteria in the mouse lung. As shown in Fig. 6A–C, Rg3 increased the phosphorylation of ERK1/2 and p38 in cells obtained by BAL and enhanced phagocytosis of bacteria in the mouse lung (Fig. 6D and E).

4. Discussion

We report that ginsenoside Rg3 enhanced macrophage phagocytosis of IgG-opsonized bacteria *in vitro* and *in vivo*, possibly by activating ERK1/2 and p38. Rg3 did not affect the surface expression of FcgRs.

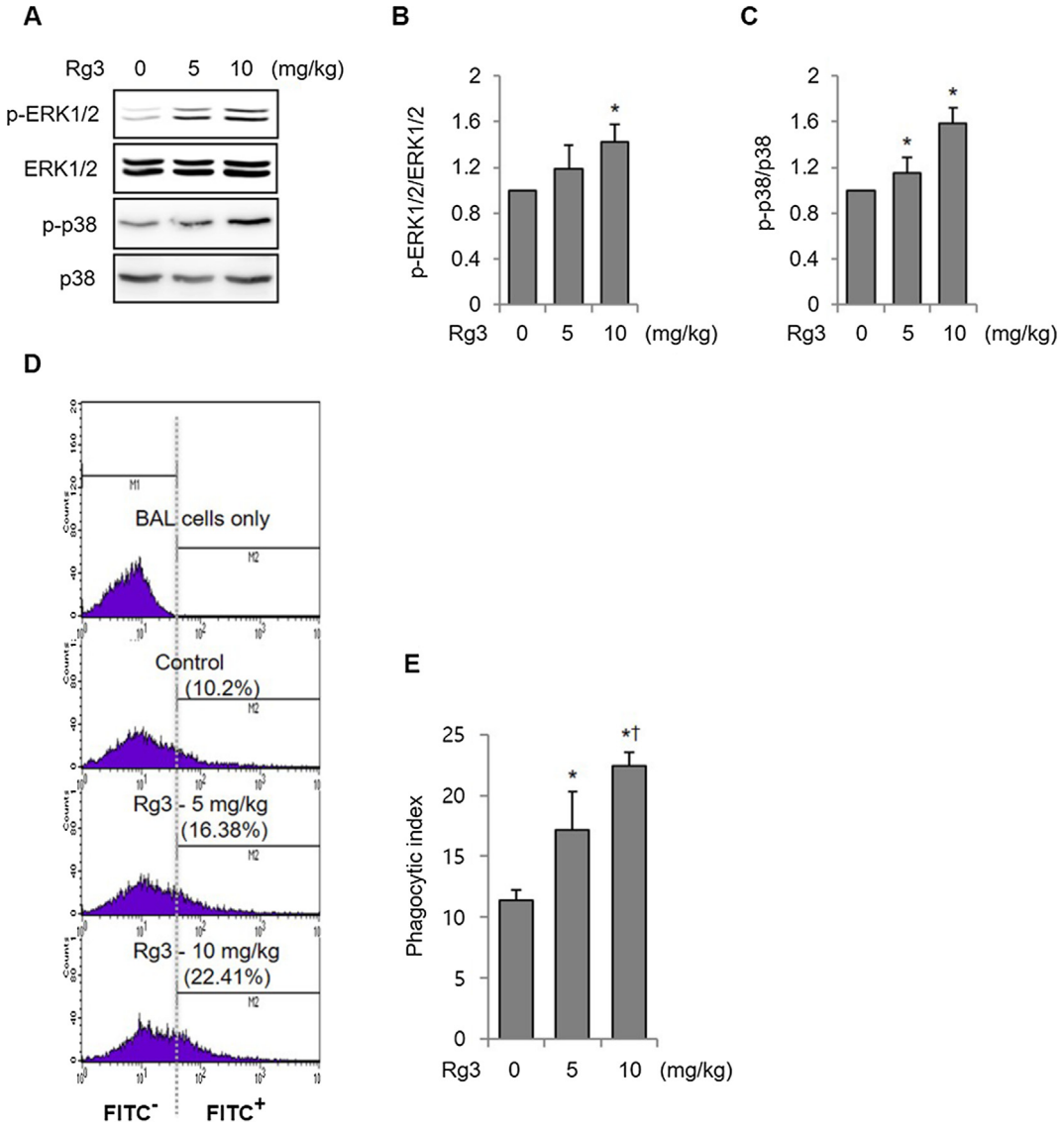

Fig. 6. Rg3 increases bacterial uptake in the mouse lung. (A–C) Rg3 (0, 5, or 10 mg/kg) was injected into the intraperitoneal cavity and bronchoalveolar lavage (BAL) cells were isolated from the lung 3 h later. Lysates of BAL cells were subjected to Western blotting. (A) Representative Western blots, and (B and C) quantitative density ratios, of phospho- and total-ERK1/2 and p38 of five mice per group. $^{*}P < 0.05$ compared with the control. (D and E) After intraperitoneal injection of Rg3 into mice for 3 h and FITC-conjugated *E. coli* was added to the lungs for 2 h. Lung cells were isolated by BAL and assayed by flow cytometry. (D) Representative flow cytometry histograms are shown. (E) Bars are means ± SD (n = 5). $^{*}P < 0.05$ compared with the control. †$P < 0.05$ compared to 5 mg/kg.

Rg3-induced macrophage phagocytosis and actin polymerization were diminished by PD98059, an ERK1/2 inhibitor, and SB203580, a p38 inhibitor. These results suggest that the effect of Rg3 on phagocytosis is mediated by its effect on downstream signaling of FcgR rather than on FcgR itself.

Actin polymerization during FcgR-mediated phagocytosis is controlled by activation of Rho family GTPases, such as Rac1 and Cdc42 [31]. Rac1 and Cdc42 are reportedly responsible for ERK1/2 activation [32]. For example, the activation of ERK1/2 required Rac1 and Cdc42 activation, which is responsible for phagocytosis by neutrophils [6]. In addition, deletion of PAK1, a downstream effector of Rac1 and Cdc42, diminished ERR1/2 activation [7] and inhibition of ERK1/2 diminished TLR-induced FcR-mediated phagocytosis by macrophages [9]. Also, p38 activation in macrophages may be associated with enhancement of phagocytosis [5,18,19]. Inhibition of p38 activity has been reported to reduce the expression of intercellular adhesion molecule-1 and impair phagocytosis [23]. p38 also plays an important role in actin cytoskeleton reorganization in various cell populations [10–13]. In the present study, the Rg3-induced increase in phagocytosis and actin polymerization in macrophages was suppressed in the presence of PD98059 or SB203580. Interestingly, the inhibition of Rg3-induced ERK1/2 activation by PD98059 suppressed p38 activation, but p38 inhibition did not block the Rg3-induced activation of ERK1/2. These results suggest that Rg3-induced actin polymerization and phagocytosis are mediated by activation of the ERK1/2/p38 pathway.

Rg3 increased the phosphorylation of ERK1/2 and p38. However, flow cytometry results (Fig. 2F) suggested that Rg3 had little effect on FcgR expression. FcgR engagement with IgGbeads, but not unopsonized beads, in macrophage culture also increased the activation of ERK1/2 and p38. Furthermore, co-culture of macrophages with Rg3 and IgGbeads further increased the phosphorylation of ERK1/2 and p38. These results suggest that p38 and ERK1/2 are involved in FcgR-mediated phagocytosis, and that Rg3 affects downstream targets of FcgR rather than acting directly on FcgR during FcgR-mediated macrophage phagocytosis. The inhibition of Rac1 and Cdc42 activity suppressed the ERK1/2 and p38 activation induced by FcgR-mediated phagocytosis, but not that induced by Rg3. However, Rg3-induced macrophage phagocytosis was inhibited by the inhibition of Rac1 and

Cdc42 activity. These results suggest that the activity of Rac1 and Cdc42 is essential for Rg3-induced FcgR-mediated phagocytosis, although Rg3 increases the activity of ERK1/2 and p38 independently of Rac1 and Cdc42 activity and promotes FcgR-mediated phagocytosis by further increasing the activity of ERK1/2 and p38.

In this experiment, Rg3 increased macrophage phagocytosis by further increasing the activities of p38 and ERK1/2, which are involved in FcgR-mediated phagocytosis. However, in our preliminary laboratory data, Rg3 increased macrophage phagocytosis of unopsonized *E. coli* (data not shown). Also, a previous study observed that the activity of p38 was also increased during macrophage phagocytosis of unopsonized *E. coli* [33]. These results suggest that Rg3-induced p38 activity may be involved in macrophage phagocytosis of unopsonized *E. coli*. In this experiment, however, we studied the mechanisms involved in the association of Rg3 with FcgR rather than the effect of Rg3 on all receptors involved in phagocytosis, since FcgR is known to play a very important role in the elimination of invading pathogens. Several types of receptors are involved in the ingestion of particles and are co-expressed within a single macrophage. Opsonic receptors such as Fc and complement are involved in the phagocytosis of particles coated with opsonins, such as IgG or complement proteins. However, pattern-recognition receptors such as dectin-1, CD14, mannose receptor, MARCO, and scavenger receptor A are involved in the internalization of unopsonized particles [2]. Therefore, although Rg3 showed increased macrophage phagocytosis via FcgRs in this experiment, the possibility that another receptor involved in particle internalization, including non-opsonic receptors, increases phagocytosis cannot be ruled out.

Rg3 reportedly exerts beneficial effects by inhibiting macrophage phagocytosis of the intracellular bacterium *Brucella abortus* [34]. In that study, Rg3 suppressed ERK1/2 and p38 activation and actin polymerization in RAW264.7 cells, a murine macrophage cell line, which is not in agreement with our findings. At this stage, we cannot explain these contrasting results. However, in the previous study, Rg3 was used at 0.1 mg/mL (~130 μM). But, in this study, 30 μM Rg3 or less were used to determine the effect of Rg3 on macrophage phagocytic ability. Interestingly, Rg3 at ≥ 100 μM suppressed phagocytosis by peritoneal macrophages (data not shown). Also, the previous study [34] used RAW264.7 macrophages, which were derived from tumors induced in male BALB/c mice by the Abelson murine leukemia virus, whereas in this experiment we used thioglycollate-activated peritoneal macrophages to measure phagocytic ability. Two distinct subsets of macrophages exist in the mouse peritoneal cavity [35,36]. Resident peritoneal macrophages, so called large peritoneal macrophages (LPMs), originate from an embryonic precursor and are maintained via self-renewal, and LPMs account for most of the peritoneal macrophages under unstimulated conditions. By contrast, the predominant macrophages in the peritoneal cavity following intraperitoneal thioglycollate injection, so-called small peritoneal macrophages (SPMs), are formed from differentiating blood monocytes entering the peritoneal cavity. At 4 days after thioglycollate injection, as in this experiment, SPM occupies most of the peritoneal macrophages. LPMs and SPMs differ functionally, including in their phagocytic ability and level of nitric oxide production, and exhibit distinct surface marker expression. Therefore, the differences in the characteristics in the cell populations and Rg3 concentrations between our and the previous study may be responsible for the discrepant effects of Rg3 observed on macrophage phagocytosis.

In the present study, administration of Rg3 increased phagocytosis of bacteria by macrophages from the mouse lung. Rg3 reportedly reduces apoptosis and reactive oxygen species (ROS) production, preserves the antioxidant glutathione pool, and reverses mitochondrial dysfunction in injured hepatocytes in a model of LPS-induced sepsis [16]. Rg3 has also been reported to suppress LPS-induced ROS and nitric oxide production by macrophages [37]. Moreover, Rg3 decreased and increased the levels of proinflammatory and anti-inflammatory cytokines, respectively, and diminished LPS-induced acute lung injury via the Mer tyrosine kinase-dependent signaling pathway [17]. Therefore, Rg3 can help alleviate acute inflammatory conditions and eliminate invading pathogens.

Combined, our findings suggest that ginsenoside Rg3 increases the phagocytic activity of macrophages by activating ERK1/2 and p38. Therefore, Rg3 may exert a protective effect against bacterial infection and may be a candidate adjuvant therapy for such infections and related inflammatory conditions.

Declaration of Competing Interest

No conflict of interest declared.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105945.

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