

Article

# Immune-Enhancing Effects of Red *Platycodon grandiflorus* Root Extract via p38 MAPK-Mediated NF- $\kappa$ B Activation

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**Abstract:** *Platycodon grandiflorus* (PG) root extract has been widely used as an oriental herbal medicine. Red PG root extract (RPGE), which is made by steaming and drying PG several times, contains more saponin than raw (white) PG. Although RPGE has been known to have anti-inflammatory activity, the effects of RPGE on the immune-enhancing response remain unclear. In this study, we aimed to investigate the immune-enhancing effects of RPGE and its mechanism in macrophage cells and splenocytes. Our results revealed that cell proliferation of both macrophages and splenocytes correlate positively with the concentration of RPGE. Moreover, RPGE treatment increased the phagocytic activity of macrophage cells, as well as nitric oxide and cytokines production. Furthermore, RPGE induced phosphorylation of the p38 mitogen-activated protein kinase, which contributed to nuclear factor-kappa B activation. Thus, our findings suggest that RPGE may be a potential functional food for improving immune function.

**Keywords:** red *platycodon grandiflorus* root extract; immune-enhancing effect; p38 MAPK; NF- $\kappa$ B

## 1. Introduction

Herbal immunostimulants are generally categorized as natural compounds that can enhance immune responses by activating immune cells, such as macrophages [1,2]. By promoting the phagocytosis of macrophages, immunostimulants induce the secretion of cytokines, such as tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-1 beta (IL-1 $\beta$ ), and interleukin-6 (IL-6), thereby improving immune function [3].

Nuclear factor-kappa B (NF- $\kappa$ B)-mediated cellular changes are known to be closely associated with both the innate and adaptive immune responses [4,5]. In innate immune cells, such as macrophages, NF- $\kappa$ B activation is initiated by the degradation of inhibitory kappa B alpha (I $\kappa$ B) proteins, following which the free NF- $\kappa$ B enters the nucleus to perform various downstream functions, including immune regulation and proliferation [4,6]. Generally, immunostimulants activate the immune cell function through specific binding with receptors [7]. Moreover, immunostimulants trigger several downstream signals, such as the NF- $\kappa$ B pathway, p38 mitogen-activated protein kinase (MAPK), and the c-Jun N-terminal kinase (JNK) pathway, increasing the secretion of nitric oxide (NO), as well as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 [1,7].

The spleen plays a role in the immune system by protecting against blood-derived antigens and removing aged red blood cells and damaged cells [8]. The populations of cells in the spleen are mainly composed of various immune lymphocytes, including T cell, B cell, and macrophages [9], which are widely used for immunomodulatory research.

*Platycodon grandiflorus* (PG) has a long history of being widely used as a traditional medicine [10]. It has been reported, inter alia, to reduce inflammation and improve liver function [8,10]. Saponins, which are part of single compounds of PG, have also been studied actively, mainly focusing on their anti-cancer properties [10,11]. Among them, platycodin D showed immunoadjuvant activity in ovalbumin-immunized mice [12] and increased serum cytokine levels in h22 hepatocellular carcinoma-bearing mice [11], providing the evidence of immune enhancing effect. Red PG, which is made by steaming and drying PG several times, increases the saponin content during processing when compared to raw (white) PG [8]. Red PG with a high content of platycodin D might have potential as a herbal immunostimulant, however no study has been conducted on the pharmacologic effects and molecular mechanisms of red PG in the immune-enhancing response. In this study, we aimed to evaluate the immune-enhancing effects of red PG extract (RPGE) in RAW 264.7 macrophage cells and mouse splenocytes. We also investigated its effects on the NF- $\kappa$ B and MAPK signaling pathways to determine the underlying mechanisms of the immune-enhancing effects of RPGE in immune cells. Thus, the results of our study provide novel insights regarding the immunomodulatory effects and the mechanism action of RPGE.

## 2. Materials and Methods

### 2.1. Red *Platycodon grandiflorus* Root Extract (RPGE) Preparation

RPGEs used in the experiments were supplied by SK Bioland (Ansan, Korea) in two types of formulation, concentrate and powder, which are representative formulations of functional foods. Both types of RPGEs were prepared by standard production processes. Briefly, Korean PG roots were washed twice and steamed for 2 h. Following that, the roots were subjected to 4 cycles of drying for 24 h and steaming for 90 min and then dried for 72 h to prepare red PG. The red PG was mixed with 50% ethanol (Korea ethanol supplies company, Seoul, Korea) at a ratio of 1:15 (*w/v*) and extracted at 80 °C for 8 h. The primary extract was recovered, and the remaining residues were subjected to a secondary extraction at 80 °C for 8 h with 50% ethanol (ratio 1:15, *w/v*). All extracts were then mixed and filtered using a filter press. The filtered extract was concentrated at reduced pressure until the solid content reached 60% or more and subsequently sterilized to obtain RPGE-concentrate (RPGE-C). To prepare RPGE-powder (RPGE-P), RPGE-C was mixed with dextrin at a 1:1 ratio and then spray-dried.

### 2.2. Cell Culture and Reagents

RAW 264.7 cells purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic in 5% CO<sub>2</sub> at 37 °C. All cell culture reagents were obtained from GIBCO (Gaithersburg, MD, USA). SB203580 and SP600125 were purchased from Calbiochem (San Diego, CA, USA). Concanavalin A (Con A) and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### 2.3. Splenocyte Isolation

The five-week-old imprinting control region (ICR) male mice were purchased from Orient Bio Co. (Seongnam, Korea). The spleens were isolated and rinsed with RPMI 1640 medium. After that, the spleens were physically crushed and filtered through a 200  $\mu$ m cell strainer (BD Biosciences, San Jose, CA, USA) and centrifuged at 3000 rpm for 10 min at 4 °C. The cell pellets were resuspended and incubated for 5 min with lysis buffer to remove erythrocytes. The cells were then washed with RPMI 1640 medium and centrifuged (3000 rpm, 10 min, 4 °C) to obtain splenocytes. Isolated

splenocytes were cultured for each experiment in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotic-antimycotic in an atmosphere containing 5% CO<sub>2</sub> at 37 °C. The animal protocols used in this work were evaluated and approved by the Institutional Animal Care and Use Committee (IACUC) of the Gachon University (reference number: GIACUC-R2019019). They are in accordance with Korean Animal Protection Act (Act No. 16075).

#### 2.4. Cell Viability Assay

RAW 264.7 cells or splenocytes isolated from mice were seeded at a concentration of  $2 \times 10^4$  cells/well and  $5 \times 10^5$  cells/well into 96-well plates, respectively. After 24 h (RAW 264.7 cells) or immediately (splenocytes), the cells were treated with various concentrations of RPGE-C or RPGE-P. For the splenocytes proliferation assay, Con A and LPS were treated as positive mitogens. After incubating for 24 h, the Cell Counting Kit-8 (CKK-8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan) solution was added (10 µL/well) and cells were further incubated at 37 °C for 2 h. The absorbance of each well was measured at 450 nm on an Epoch Microplate Spectrophotometer (Biotek Inc., Winooski, VT, USA).

#### 2.5. Phagocytosis Assay

The phagocytic ability was determined using the CytoSelect™ 96-well phagocytosis assay kit (Cell Biolabs, Inc., San Diego, CA, USA), according to the manufacturer's instructions. Briefly, RAW 264.7 cells were seeded at a concentration of  $2 \times 10^4$  cells/well into 96-well plates. The cells were preincubated with the phagocytosis inhibitor (PI, 2 µM of Cytochalasin D) or various concentrations of RPGE-C or RPGE-P for 24 h before addition of the *E. coli* suspension. After incubating for 4 h, we performed a removal and blockage of external particles step. The internalized particles were detected at 450 nm using an Epoch Microplate Spectrophotometer (Biotek Inc., Winooski, VT, USA).

#### 2.6. Measurement of Nitric Oxide (NO) Production

RAW 264.7 cells were seeded in 6-well plates at  $4.0 \times 10^5$  cells/well. The cells were treated with various concentrations of RPGE-C or RPEG-P for 24 h. NO levels in the media were assayed using the Griess Reagent System (Promega, Madison, WI, USA). Cell culture supernatant aliquots with a volume of 50 µL were incubated with the substrate solution for 10 min, followed by incubation avoiding light with the coloring solution for another 10 min. The absorbance was measured at 520 nm using an Epoch Microplate Spectrophotometer (Biotek Inc., Winooski, VT, USA). NO production was determined by comparison to the dilution of a sodium nitrite standard.

#### 2.7. RNA Isolation and Real-Time Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using the easy-spin™ total RNA extraction kit (iNtRON Biotechnology, Gyeonggi-do, Korea), and cDNA was synthesized using the GoScript™ Reverse Transcriptase (Promega, Madison, WI, USA). Real-time PCR was performed on the synthesized cDNA using the TB Green™ Premix Ex Taq™ II (TaKaRa Bio, Otsu, Japan) on the ABI QuantStudio 3 (Applied Biosystems, Foster City, CA, USA). The primer sequences (5'-3') used in the experiments were shown in Table 1. All gene expression values were normalized to *Actb* (β-actin).

**Table 1.** The primer sequences (5'–3') used in the experiments.

Genes	Forward Sequence	Reverse Sequence	Ref.
<i>Nos2</i>	GCGAAAGGTCATGGCTTCAC	CTGGTCCATGCAGACAACCT	This study
<i>Ptgs2</i>	CATCCCCTCCTGCGAAGTT	GGCCCTGGTGTAGTAGGAGA	This study
<i>Tnf-α</i>	TGTCCCTTCACTCACTGGC	CATCTTTTGGGGGAGTGCCT	[13]
<i>IL-1b</i>	AACTGTTCCCTGAACCTCACTGT	GAGATTGAAGCTGGATGCTCT	[14]
<i>IL-6</i>	GGGACTGATGCTGGTGACAA	TCCACGATTCCCAGAGAACA	[13]
<i>Actb</i>	GACGTTGACATCCGTAAAG	CAGTAACAGTCCGCCT	[2]

### 2.8. Quantification of Cytokine Levels

RAW 264.7 cells or mouse splenocytes were seeded in 6-well plates at  $4.0 \times 10^5$  cells/well. The cells were treated with various concentrations of RPGE-C or RPEG-P for 24 h. To determine the cytokine levels, media was collected, and IL-6 and IL-10 levels were measured using enzyme-linked immunosorbent assays kits (ELISA; R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions.

### 2.9. Luciferase Assay

RAW 264.7 cells were cotransfected with the 3 $\times$ κB-Luc [15] and pNL1.1.TK vector using FuGENE<sup>®</sup> hD (Promega, Madison, WI, USA) for 24 h. The transfected cells were then incubated with RPGEs (0, 20, 100, or 500 μg/mL). After incubation for 24 h, the cells were assayed using a Nano-Glo<sup>®</sup> Dual-Luciferase<sup>®</sup> Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The luciferase activity was detected using a GloMax<sup>®</sup> Discover Multimode Microplate Reader (Promega, Madison, WI, USA).

### 2.10. Western Blot Analysis

The crude extracts from RAW 264.7 cells were prepared using the lysis solution (PRO-PREP<sup>™</sup> Protein Extraction Solution plus the halt<sup>™</sup> phosphatase inhibitor cocktail, iNtRON Biotechnology and Thermo Scientific, Waltham, MA, USA, respectively). The crude extracts were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with the indicated antibodies. Antibodies against p-NF-κB p65 (Ser536), NF-κB p65, phospho-IκBα (Ser32), phospho-p38 (Thr180/tyr182), p38, phospho-JNK (Thr183/Try185), and JNK were purchased from Cell Signaling Technology (Danvers, MA, USA). The β-actin antibody was purchased from Abcam (Cambridge, MA, USA). Secondary antibodies were obtained from Promega. ImageQuant LAS 500 (GE healthcare Life Sciences, Little Chalfont, UK) were used for visualization.

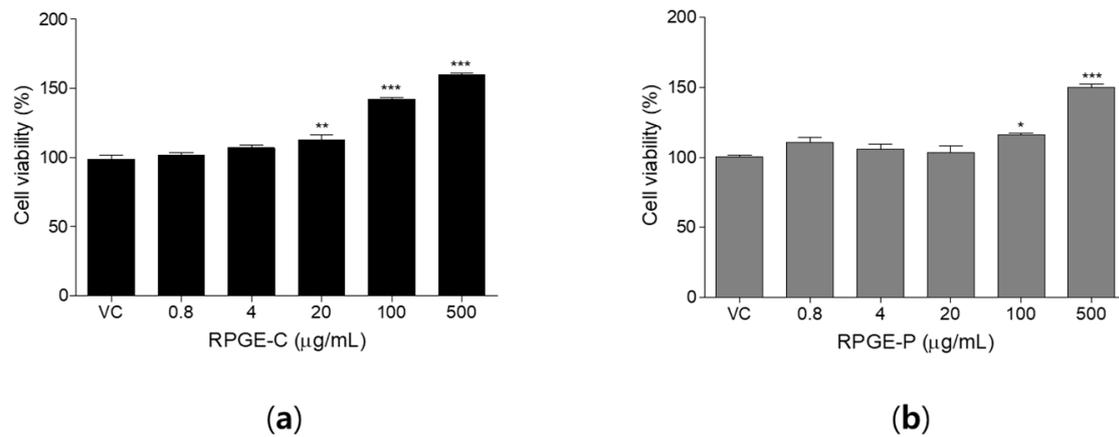
### 2.11. Statistical Analysis

All data are expressed as mean  $\pm$  SEM. Multiple comparisons of means among experimental groups were carried out with one-way ANOVA, followed by a post-hoc test using the GraphPad Prism 5 software (Graph Pad Software Inc., San Diego, CA USA). A *p*-value of <0.05 was considered statistically significant.

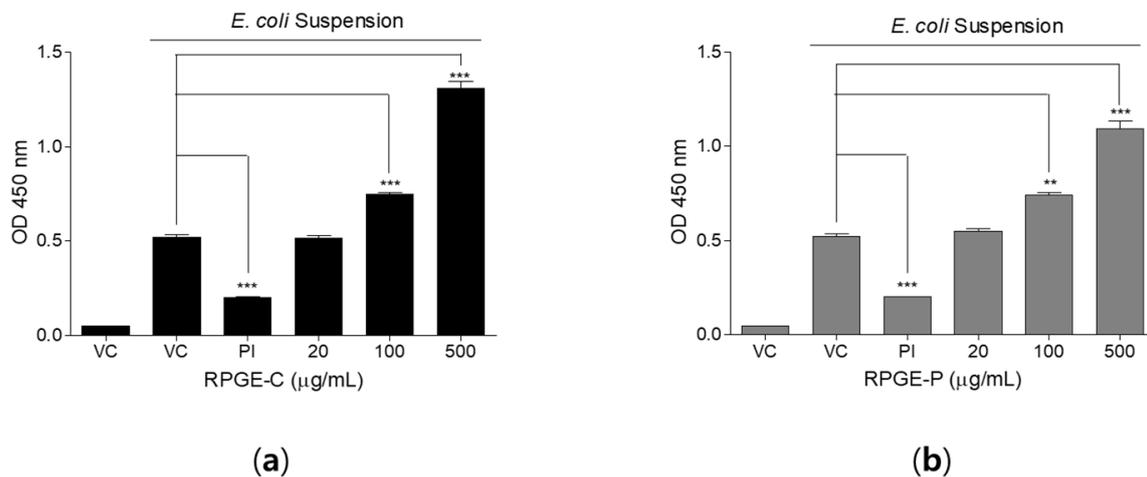
## 3. Results

### 3.1. RPGE Increases Phagocytic Activity in RAW 264.7 Cells

To assess whether the RPGE affects cell viability, we performed the CCK-8 assay. Both RPGE-C and RPGE-P were shown to have any cytotoxic effect on RAW 264.7 cells. Interestingly, the viable cell population was increased by RPGEs in a concentration-dependent manner (Figure 1a,b). To ascertain whether RPGE-induced cell proliferation in RAW 264.7 cells is related to macrophage activation, we first analyzed the phagocytic ability of RPGE treated cells. The phagocytic activity was monitored by measuring the amount of internalized *E. coli* in macrophages. RAW 264.7 cells were pretreated with PI or RPGEs (20, 100, or 500 μg/mL) for 24 h before addition of the *E. coli* suspension. After treatment with RPGE-C or RPGE-P at concentrations above 100 μg/mL, the phagocytic activity of cells was found to be significantly increased than in cells treated with the vehicle control (VC) (Figure 2a,b).



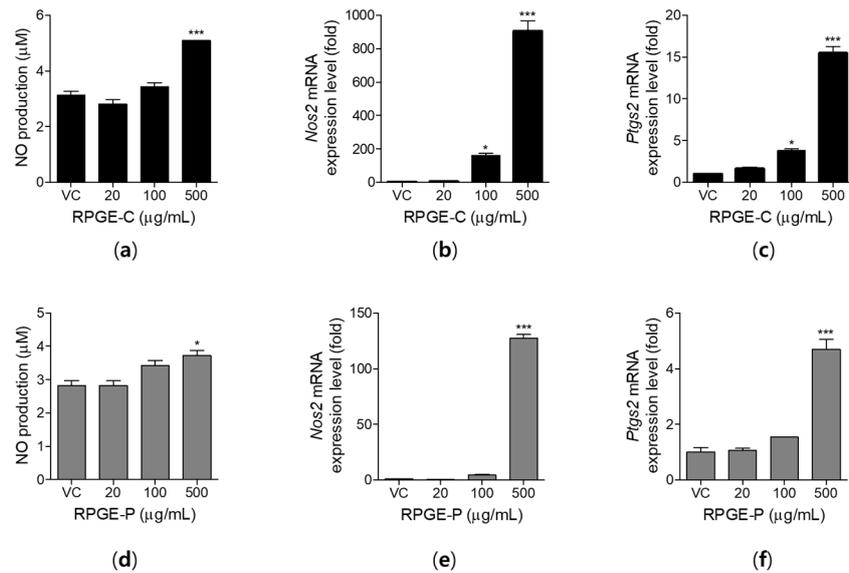
**Figure 1.** Effect of Red *Platycodon grandiflorus* Root Extract (RPGE) on cell viability in RAW 264.7 cells. (a) RPGE-concentrate (RPGE-C); (b) RPGE-powder (RPGE-P). The results are expressed as relative values to that of the vehicle-treated cells (vehicle control, VC), which is set to 100%. The data are represented as the mean  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  when compared with the VC.



**Figure 2.** Effect of RPGE on the phagocytic activity of RAW 264.7 cells. (a) RPGE-C; (b) RPGE-P. PI, phagocytosis inhibitor. Values are represented as the mean  $\pm$  SEM. \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  when compared with the VC treated with an *E. coli* suspension.

### 3.2. RPGE Enhances NO Production in RAW 264.7 Cells

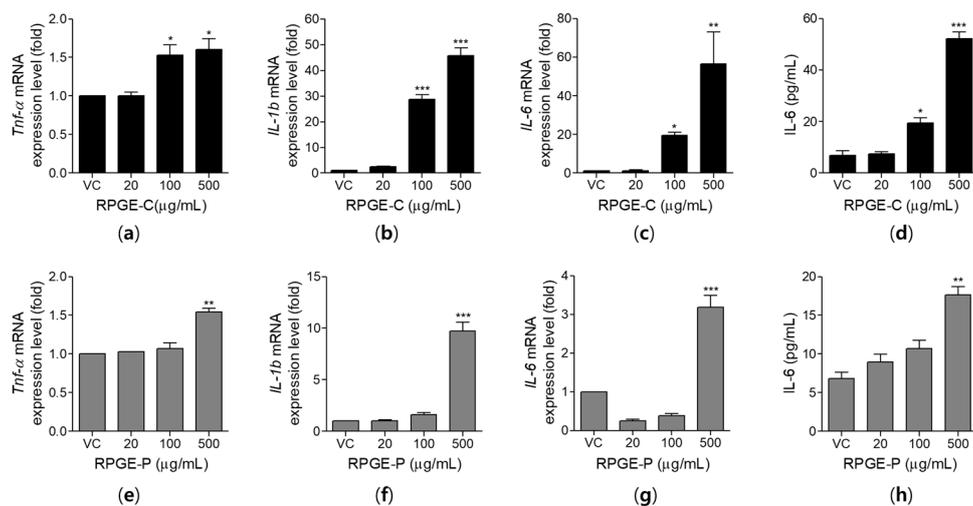
The immune-enhancing effects of RPGEs were confirmed by measuring the nitrite level in cell culture medium using the Griess Reagent System. As shown in Figure 3, we observed a slight but significant enhancement in NO production after treatment with the highest concentration (500 µg/mL) of RPGE-C (Figure 3a) or RPGE-P (Figure 3d). Inducible nitric oxide synthase (iNOS, gene name *Nos2*) is a NO synthesis enzyme, and cyclooxygenase (COX-2, gene name *Ptgs2*) is the enzyme that converts arachidonic acid to prostaglandin E2 [16]. The *Nos2* and *Ptgs2* mRNA expression levels in RAW 264.7 cells were measured after RPGEs treatment. As shown in Figure 3b,c,e,f, treatment with RPGEs for 24 h markedly increased *Nos2* and *Ptgs2* mRNA expression levels compared to the vehicle control.



**Figure 3.** Effect of RPGE on NO production and related mRNA expression in RAW 264.7 cells. (a–c) RPGE-C; (d–f) RPGE-P. (a,d) NO production; (b,e) *Nos2* mRNA expression level; (c,f) *Ptgs2* mRNA expression level. NO, Nitric oxide. All gene expression values were normalized to *Actb*, and the results were expressed as relative values to that of the VC, which is set to 1. The data are represented as the mean ± SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  when compared with the VC.

### 3.3. RPGE Increases Cytokine Levels in RAW 264.7 Cells

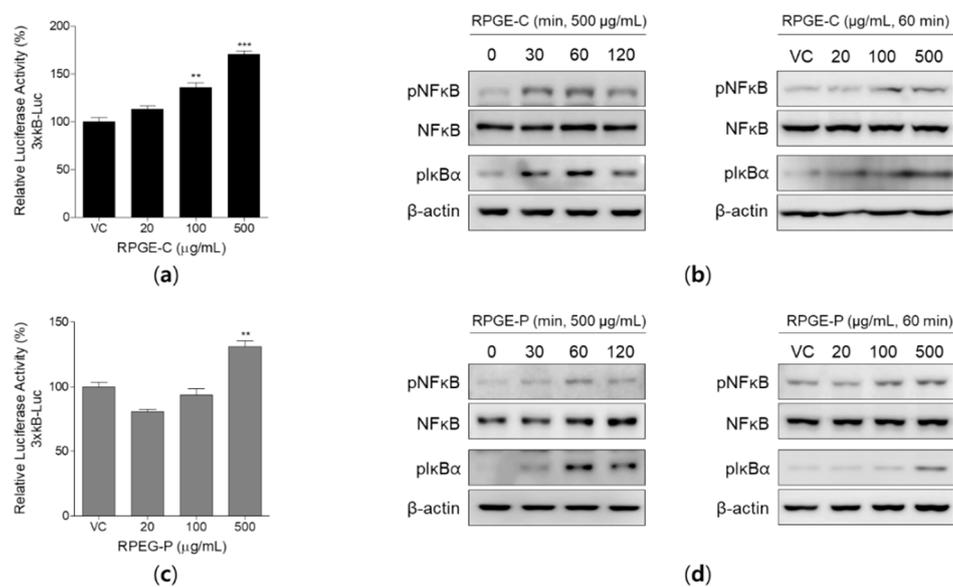
To assess the modulation of cytokines related to innate immunity due to RPGE-induced macrophage activation, we measured TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 mRNA expression levels by real-time RT-PCR and IL-6 protein levels by ELISA. Compared to the VC, RPGE-C treatment increased the mRNA expression of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, as well as IL-6 production, in a concentration-dependent manner (Figure 4a–d). Compared to the RPGE-C treatment, the effects of RPGE-P on the mRNA expression levels of cytokines and production level of IL-6 were less pronounced but statistically significant compared with the VC at the highest concentration (500 µg/mL, Figure 4e–h). These data indicated that both RPGE-C and RPGE-P play a role in cytokine regulation.



**Figure 4.** Effect of RPGE on cytokine levels in RAW 264.7 cells. (a–d) RPGE-C; (e–h) RPGE-P. (a,e) *Tnf-α* mRNA expression level; (b,f) *IL-1β* mRNA expression level; (c,g) *IL-6* mRNA expression level. The gene expression values were normalized to *Actb*, and the results were expressed as relative values to that of the VC, which is set to 1. (d,h) IL-6 protein levels in the culture supernatant. All data are represented as the mean ± SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  when compared with the VC.

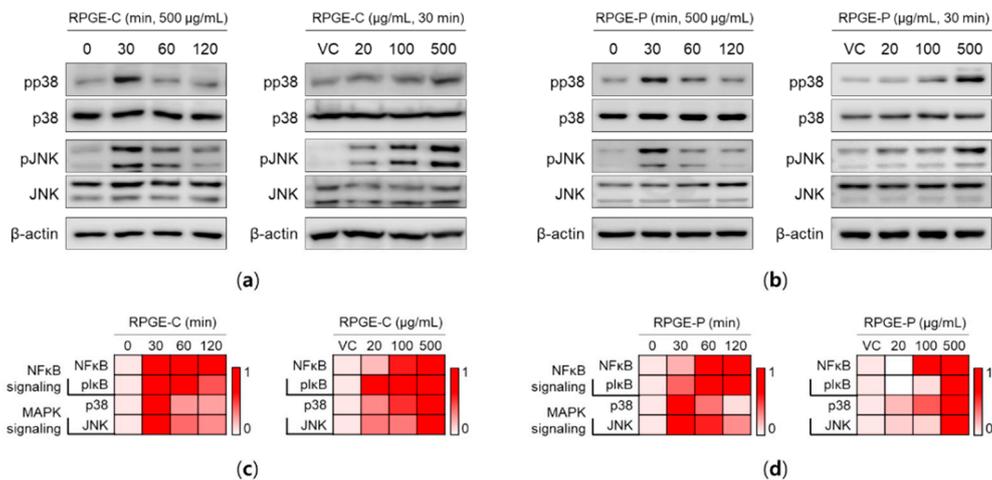
### 3.4. RPGE Activates NF- $\kappa$ B and MAPK Signaling in RAW 264.7 Cells

To identify the effect of RPGE on NF- $\kappa$ B signaling, we performed a transcriptional activity assay using reporter gene constructs of 3 $\times$ NF- $\kappa$ B-binding elements in RAW 264.7 cells. Treatment with RPGE-C at concentrations of 100 and 500  $\mu$ g/mL (Figure 5a) or RPGE-P at a concentration 500  $\mu$ g/mL (Figure 5c) led to the induction of luciferase activity, indicating that RPGE activates NF- $\kappa$ B signaling. To understand the molecular mechanisms of the effect of RPGE on NF- $\kappa$ B signaling, we examined the phosphorylation status of NF- $\kappa$ B and I $\kappa$ B by Western blot. Our results showed that RPGE-C increased the levels of phospho-NF- $\kappa$ B and phospho-I $\kappa$ B $\alpha$  in a dose-dependent manner (Figure 5b). In the case of RPGE-P, phosphorylation levels of NF- $\kappa$ B and I $\kappa$ B $\alpha$  were also increased at the highest concentration (500  $\mu$ g/mL, Figure 5d). Moreover, the presence of an NF- $\kappa$ B inhibitor (pyrrolidine dithiocarbamate, PDTC) with RPGE inhibited NO production, cytokine levels, and phosphorylation of NF- $\kappa$ B (Figure S1). Therefore, our results indicated that RPGE can induce macrophage activation via NF- $\kappa$ B signaling.



**Figure 5.** Effect of RPGE on NF- $\kappa$ B activation in RAW 264.7 cells. **(a)** RPGE-C; **(c)** RPGE-P. Luciferase activity was expressed as a relative value to that of the VC, which is set to 100%. The figures show the mean  $\pm$  SEM. \*  $p < 0.05$  and \*\*\*  $p < 0.001$ . **(b)** The cells were treated with 500  $\mu$ g/mL of RPGE-C for the indicated times or indicated concentrations for 60 min prior to Western blot analysis; **(d)** The cells were treated with 500  $\mu$ g/mL of RPGE-P for the indicated times or indicated concentrations prior to Western blot analysis.

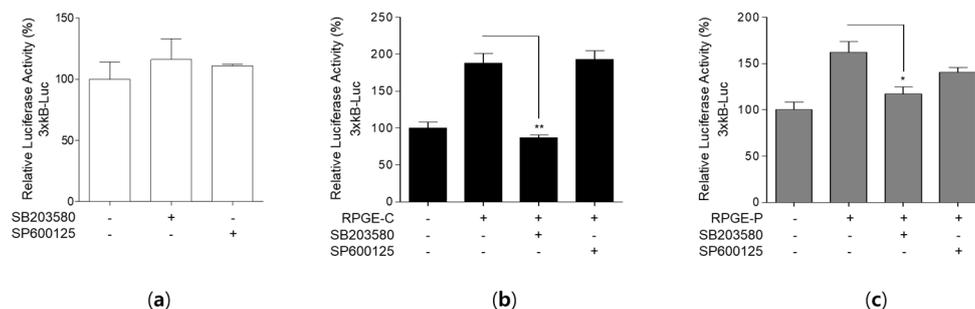
Since NF- $\kappa$ B activation is mediated via MAPK (p38 MAPK and JNK) signaling [17], we assessed whether RPGE induces MAPK activation in RAW 264.7 cells. Figure 6a,b (left) showed that the activation of p38 MAPK and JNK signaling were observed following treatment with 500  $\mu$ g/mL of RPGE-C or RPGE-P. Maximum levels of p38 MAPK and JNK phosphorylation appeared at 30 min post-treatment and decreased thereafter. Not only RPGE-C but also RPGE-P treatment was shown to increase the phosphorylation level of MAPK in a concentration-dependent manner (Figure 6a,b, right).



**Figure 6.** Effect of RPGE on MAPK signaling in RAW 264.7 cells. (a) The cells were treated with 500 µg/mL of RPGE-C for the indicated times or indicated concentrations for 60 min prior to Western blot analysis; (b) The cells were treated with 500 µg/mL of RPGE-P for the indicated times or indicated concentrations for 60 min prior to Western blot analysis; (c) The time–response and concentration–response relationships of RPGE-C were represented as heat maps. (d) The time–response and concentration–response relationships of RPGE-P were represented as heat maps.

### 3.5. RPGE-Induced NF-κB Activation Is Associated with p38 MAPK in RAW 264.7 Cells

To identify the association between the MAPK and NF-κB signaling by RPGE, we performed a luciferase assay using SB203580 (a p38 MAPK inhibitor) and SP600125 (a JNK inhibitor). Firstly, we confirmed that these inhibitors did not influence luciferase activity (Figure 7a). Treatment with 10 µM SB203580 (Figure 7b) markedly reduced RPGE-C or RPGE-P induced NF-κB signaling. However, cotreatment with 10 µM SP600125 (Figure 7c) had no effect on NF-κB signaling. These results suggested that the p38 MAPK is associated with RPGE-induced NF-κB activation.

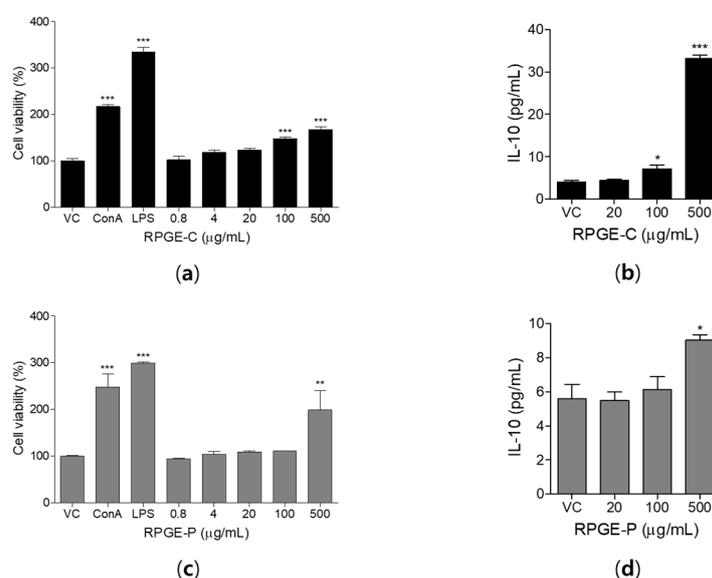


**Figure 7.** Effect of MAPK signaling on NF-κB activation in RAW 264.7 cells. (a) without RPGEs; (b) RPGE-C; (c) RPGE-P. Luciferase activity was expressed as a relative value to that of the VC (-), which is set to 100%. The figures show the mean ± SEM. \*  $p < 0.05$  and \*\*  $p < 0.01$  when compared with cells treated with RPGEs alone. SB203580, p38 MAPK inhibitor and SP600125, JNK inhibitor.

### 3.6. RPGE Induces Cell Proliferation and Increases IL-10 Expression Levels in Mouse Splenocytes

To assess the immune-enhancing effect of RPGE in splenocytes, we isolated whole spleen cells from mice. The cell viability of splenocytes treated with reference mitogens (T-lymphocyte mitogen, Con A or B-lymphocyte mitogen, LPS) was increased by approximately 230% and 300% of the VC (100%), respectively. Viable cell populations were gradually increased by treatment with RPGE-C in a concentration-dependent manner (Figure 8a) and by treatment with the highest concentration (500 µg/mL) of RPGE-P (Figure 8c). To determine whether RPGE could affect IL-10 production, we investigated the secretion of IL-10 in isolated mouse splenocytes. Both RPGE-C and RPGE-P

treatment induced the secretion of IL-10 (Figure 8b,d), which suggested that RPGE could enhance immunity via modulation of immune lymphocytes, such as B cells.



**Figure 8.** Effect of RPGE on cell viability and secretion of IL-10 in mouse splenocytes. (a) Mouse splenocytes were incubated with Con A (5 µg/mL), LPS (1 µg/mL), or various concentrations of RPGE-C; (c) Mouse splenocytes were incubated with Con A (5 µg/mL), LPS (1 µg/mL), or various concentrations of RPGE-P. Con A, Concanavalin A; LPS, lipopolysaccharide. The results were expressed as a relative value to that of VC, which is set to 100%. The data are represented as the mean ± SEM. \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  as when compared with the VC. (b) IL-10 protein level (RPGE-C); (d) IL-10 protein level (RPGE-P). All data are represented as the mean ± SEM. \*  $p < 0.05$  and \*\*\*  $p < 0.001$  as when compared with the VC.

#### 4. Discussion

The host immune system plays a crucial role in defending or inhibiting several pathological conditions, such as infections and tumors [18,19]. However, the most important aspect of the immunity is the balance between activation and inhibition. There are some plant extracts or their components that have been reported to have immunostimulant effects under normal conditions, whereas they have an immunosuppressing effect in inflammatory environments, such as LPS treatment [7,20–22].

In the present study, we have revealed the immune-enhancing effect of RPGE. RPGE-C or RPGE-P treatment significantly increased phagocytic activity in macrophages, as well as NO production and mRNA expression levels of innate immune-related cytokines. Moreover, our previous studies have also confirmed the mitigating effects of RPGE on the LPS-induced inflammatory response in splenocytes isolated from mice [8]. Therefore, we concluded that RPGE has immunomodulatory properties in immune cells.

NF-κB and MAPK signaling play a crucial role in immune responses [1,4,23,24]. However, the relationship between the two signaling pathways remains unclear. Here, our results show that RPGE stimulates the immune responses in RAW 264.7 cells via two different mechanisms. RPGE has shown to increase phosphorylation levels of NF-κB, as well as increase p38 MAPK-induced NF-κB activation. The inhibition of NF-κB and p38 reduced RPGE-induced macrophage activation (Figure S1). Therefore, our results suggest that RPGE is a promising functional food for enhancing immunity. Moreover, further studies on the immunomodulatory effect of RPGE may further understanding of the molecular mechanisms underlying the immune response.

The functional food industry has used various processing methods to increase the active ingredients of raw materials [25–27]. Approximately 30 different active ingredients of PG are known [28], including

platycodin D, the representative saponin in PG (Figure S2A). In this study, we steamed and dried raw PG several times, thereby increasing the content of platycodin D by approximately three times when compared to white PG (Figure S2B). This process is similar to the increase in the saponin content of red ginseng compared to white ginseng [5,26,27]. Moreover, this increase in the content of active ingredients may further improve the functionality of PG.

To determine the cellular mechanism of the immune-enhancing effect of RPGE, we used the murine macrophage cell line, RAW 264.7. The viable cell population was increased by RPGE treatment (Figure 1a,b). The mean proliferation rates after treatment with the highest concentration of RPGE-C and RPGE-P (500 µg/mL) were increased by approximately 60% and 50% compared to the VC, respectively. Thus, our results demonstrate that RPGEs induce macrophage cell proliferation in RAW 264.7 cells. Interestingly, extracellular-signal-regulated kinase (ERK) activity was increased in cells treated with RPGE-C (Figure S3A) or RPGE-P (Figure S3B). ERK is a family of MAPK that plays a role in regulating cell growth signals [29,30]. When cells were cotreated with RPGEs and an ERK inhibitor (PD98059), NF-κB activity was increased (Figure S3C). This is consistent with the results obtained in the previous study, which showed that ERK inhibition activates NF-κB [31]. These results suggest that RPGE-induced ERK activation in RAW 264.7 cells might regulate cell proliferation rather than immune activity.

When comparing RPGE-C and RPGE-P, both extracts showed dose-dependent-induced phagocytosis. However, for most of pharmaceutical activities, including cytokines and NF-κB, RPGE-C was more effective at lower concentrations than RPGE-P. These results are typical, as the RPGE-P was prepared by mixing with dextrin, and experiments with higher doses of RPGE-P are not possible due to the limited solubility. Moreover, these effects were not ascribed to processing but due to variations in their content, therefore it is suggested that more powder should be consumed than concentrate for the same effect. In conclusion, we showed the immunostimulatory effects of RPGE and its mechanisms, both in vitro and ex vivo. However, further studies are necessary to confirm the effects of RPGEs in animal models and humans.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2076-3417/10/16/5457/s1>, Figure S1: Effects of PDTC or SB203580 on RPGE-induced immunomodulation in RAW 264.7 cells.; Figure S2: high-performance liquid chromatography (HPLC) analysis of RPGE.; Figure S3: Effect of RPGE on ERK activation in RAW 264.7 cells.

**Author Contributions:** E.-J.P. and h.-J.L. designed the study. E.-J.P., Y.-S.L. and S.-H.L. contributed methodology. E.-J.P., Y.-S.L., S.M.K., J.-H.Y. and A.J.J. performed the experiments. h.C.J. performed the experiment of Figure S2. E.-J.P. analyzed the data. E.-J.P. wrote the manuscript. h.-J.L. supervised the project and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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