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Article

Macrophages from Mice Administered *Rhus Verniciflua* Stokes Extract Show Selective Anti-Inflammatory Activity

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Abstract: The bark of *Rhus verniciflua* Stokes (RVS) is used as a food additive and herbal medicine for various inflammatory disorders and cancer in Eastern Asia. RVS exerted anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated RAW264.7 cells, but whether this effect occurs in macrophages after oral administration has not been determined. We used a thioglycollate-induced peritonitis model to obtain macrophages from mice given RVS. We examined the systemic inflammatory response to intraperitoneal LPS. RVS-treated mice had an increased population of peritoneal exudate cells expressing CD11b and SRA. Increased uptake of Alexa Fluor ^{488}-labeled acetylated lipoprotein was observed in monocyte-derived macrophages from RVS-treated mice. When these cells from the RVS group were stimulated with LPS, the levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the supernatant decreased, but the level of IL-12 increased. The surface expression of CD86 was reduced, but surface expression of class II MHC molecules was increased. RVS suppressed the serum levels of LPS-induced TNF-α and IL-6. RVS enhanced monocyte differentiation in thioglycollate-induced peritonitis by increasing scavenger receptor expression and activity. Macrophages isolated from mice given RVS responded differently to LPS. These findings demonstrate that RVS confers selective anti-inflammatory activity without causing the overall inhibitory effects on immune cells.

Keywords: *Rhus verniciflua* Stokes; in vivo; inflammation; macrophage; monocyte differentiation; MHC II; IL-12

1. Introduction

Monocytes and macrophages are collectively called mononuclear phagocytes, and the function of these cells is to maintain immunity and tissue integrity [1]. Mononuclear phagocytes are involved in phagocytosis, inflammatory response, and regulation of T cell activity throughout the body [1]. In the past, the consensus was that monocytes and macrophages were produced in the bone marrow, and that tissue macrophages were simply replenished by circulating monocytes. However, recent studies have shown that tissue macrophages are created prior to birth and are maintained through local proliferation except in some tissues, such as the intestines and dermis, where monocytes constantly migrate [2]. As inflammation progresses, circulating monocytes are recruited to the inflamed tissue and differentiate into inflammatory macrophages [3].

Upon encountering microorganisms, macrophages detect and remove them using various phagocytic receptors including scavenger receptors [4]. At the same time, macrophages produce inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-12. TNF-α

and IL-6 activate endothelial cells, attract circulating leukocytes to the site, and induce the acute phase response in the liver [5]. IL-12 activates natural killer (NK) cells and enhances the development of type 1 T helper (Th1) cells [6]. Further, such activated macrophages upregulate class II major histocompatibility complex (MHC) molecules and costimulatory molecules to activate antigen-specific Th1 cells, which in turn enhance the macrophage's function by producing interferon (IFN)-γ, a cytokine once termed "macrophage activation factor" [6]. For these reasons, any strategy for controlling inflammation must include regulation of macrophages, which are the ultimate target of many effective anti-inflammatory agents.

Thioglycollate is one of the agents that increase peritoneal macrophage yield by inducing sterile peritonitis in mice [7]. These peritoneal macrophages are highly phagocytic but do not produce inflammatory mediators unless they are stimulated with lipopolysaccharide (LPS) or other inflammatory stimuli [8,9]. By three to four days after intraperitoneal injection of thioglycollate, the number of peritoneal exudate cells reaches a peak, and the major population in the peritoneal cavity is monocyte-derived macrophages [8,10]. The process of monocyte-to-macrophage differentiation *in vitro* is characterized by upregulation of CD11b, SRA, and CD36 [11-13]. CD11b is a macrophage-specific integrin that mediates the adherence of tissue macrophages to the extracellular matrix [13]. CD36 and SRA function as scavenger receptors that recognize polyanionic macromolecules [14]. Peritoneal exudate cells from thioglycollate-injected mice show gradual upregulation of CD11b and CD36 with time [15]. In this regard, thioglycollate-induced peritonitis can be an *in vivo* model for evaluating monocyte-to-macrophage differentiation [15].

The bark of *Rhus verniciflua* Stokes (RVS), or *Toxicodendron vernicifluum*, which belongs to the Anacardiaceae family, is used as food additive and herbal medicine for inflammatory disorders and cancer in Eastern Asia. RVS demonstrates various biological activities, including anti-oxidant, anti-inflammatory, neuroprotective, and anti-cancer effects, and some of which have been verified *in vitro* [16-23]. At the cellular level, RVS extract and its phenol-rich fraction inhibit lipopolysaccharide (LPS)-induced TNF-α and IL-6 expression in RAW264.7 cells, a mouse macrophage cell line [17-19,23]. Fisetin, butein, and sulfuretin are the known anti-inflammatory polyphenols present in RVS [24-26]. Animals orally administered RVS extract exhibit reduced edema in chemically-induced dermatitis models [18,23], but whether this anti-inflammatory effect occurs in macrophages following oral administration of RVS extract has not been determined. A pharmacokinetic profile study showed that the majority of fisetin, sulfuretin, and other polyphenols in RVS extract were present in blood in their conjugated forms [27]. This indicates that inferences drawn from the *in vitro* anti-inflammatory activity of RVS extract may not be relevant to *in vivo* conditions.

We wanted to determine whether macrophages isolated from mice orally given RVS extract showed anti-inflammatory activity similar to that seen in macrophages directly treated with RVS extract or its polyphenol fractions. For this purpose, we used the thioglycollate-induced peritonitis model to obtain a high yield of macrophages and to evaluate whether RVS extract affected monocyte differentiation. In addition, we investigated the effects of RVS extract on macrophage function relating to Th cell activity. Finally, we examined the systemic response to LPS in mice administered RVS extract.

2. Materials and Methods

2.1. Sample preparation

Ten-year-old RVS bark was collected in Jecheon, Chungbuk Province, South Korea, and was dried and chopped. A voucher specimen (# 2013-RVS) was deposited at the laboratory of herbal immunology, Kyung Hee University. Because RVS contains urushiol, an allergen that can cause contact dermatitis, the removal of urushiol was performed as described by Choi et al [22]. In brief, the RVS bark pieces were roasted in an iron pot at 240 ºC for 50 min. They were then extracted in water using a heating mantle and reflux apparatus for 2 h at 95ºC and filtered through filter paper. The extract was concentrated using a rotary evaporator and freeze-dried under vacuum. The yield was 2.25%.

2.2. Qualitative and quantitative analyses of RVS extract

High performance liquid chromatography combined with mass spectrometry was used to analyze the amount and identity of the compounds in the RVS extract. The monoisotopic masses of protonated fisetin [M + H]+ (m/z = 287.1) and sulfuretin [M+H]+ (m/z = 271.1) were detected with sufficient resolution. The integrals of mass using SIM (selected ion monitoring) showed the amount of the compounds in the RVS extract. The quantitative analysis was performed by comparison with commercially available fisetin and sulfuretin after calibration curves of those compounds were obtained with a reliable coefficient of determination ($R^2 > 0.996$) (Supplementary figure S1). The liquid chromatography was performed using a Waters Acquity UPLC BEH C18 column (2.1 100 mm; Milford, MA, USA). Mobile phase A was 0.1% formic acid in water, and phase B was 0.1% formic acid in acetonitrile. The mass spectrometry was performed using a Micromass Quattro Micro API mass spectrometer (Milford). The amounts of fisetin and sulfuretin in the RVS extract are presented in Table 1.

Table 1 Quantitative analysis of *Rhus verniciflua* Stokes (RVS) extract

	MS [M+H]+		Amount in 1 mg RVS (μg)	Content (%)
Fisetin	m/z=287.1	559272	5.6	0.56
Sulfuretin	m/z=271.1	23312	0.14	0.01

2.3. Animals

Seven-week-old male Balb/c mice were obtained from Samtako (Osan, South Korea) and housed in a temperature- and humidity-controlled pathogen-free animal facility with a 12-h light-dark cycle. All animals underwent 1 week of adjustment prior to experiments and were randomly allocated to the experimental groups. RVS extract was given via oral gavage once daily for 10 days. There were no differences in body weight among the groups during the experimental period. The animal protocol was approved by the Institutional Animal Care and Use Committee at Kyung Hee University (KHUASP(SE)-15-012), and the mice were cared for according to the US National Research Council for the Care and Use of Laboratory Animals (1996) specifications.

2.4. Macrophage preparation

For macrophage isolation, mice were injected intraperitoneally with 2 ml of 3.5% sterile thioglycollate (BD, Sparks, MD, USA) 4 days before sacrifice. At the end of the experiment, the mice were sacrificed via cervical dislocation and the peritoneal exudate cells were aseptically isolated via peritoneal lavage with cold DMEM (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone) and 1% penicillin-streptomycin. After centrifugation, the cells were resuspended and counted using a TC20 Cell Counter (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Cell culture

Peritoneal exudate cells were plated in 6-well plates overnight at 37 ºC, and the non-adherent cells were removed. The cells were then stimulated with 100 ng/ml LPS for 24 h. The supernatant and cells were collected for subsequent assays. For the measurement of scavenger receptor activity, adherent cells were cultured for 4 h with Alexa Fluor ^{488}-labeled acetylated lipoprotein.

2.6. Flow cytometry

The cells were washed twice in phosphate buffered saline (PBS) and resuspended at 1×10^6 cells/ml in FACS buffer (PBS/0.1% NaN_3/1% FBS). The cells were blocked with rat anti-mouse CD16/CD32 antibody (BD Biosciences, San Diego, CA, USA) at 4ºC for 5 min and then stained with fluorescein-conjugated anti-mouse SR-AI (R&D Systems, Minneapolis, MN, USA), PE-conjugated anti-mouse CD36, FITC-conjugated CD11b, PE-conjugated anti-CD11b, PE-conjugated anti-mouse

CD86, or FITC-conjugated anti-mouse MHC II (IA/IE) (BD Biosciences) for 30 min on ice in the dark. Matched isotype antibodies were used to show non-specific binding. The cells were washed and resuspended in FACS buffer. For the measurement of intracellular uptake of Alexa Fluor^{488}-labeled acetylated lipoprotein, harvested cells were washing twice in PBS. A total of 10,000 events were acquired using a Navios flow cytometer (Beckman Coulter, La Brea, CA, USA), and the data were processed using Kaluza software (Beckman Coulter).

2.7. Cytokine analysis

The levels of TNF- α, IL-6, IL-12p70, and IFN- γ in the supernatants or sera were determined using BD OptEIA mouse ELISA sets (BD Biosciences) according to the manufacturer's protocol.

2.8. Intraperitoneal injection of LPS

Mice were intraperitoneally injection with 1.3 mg/kg of LPS (serotype 055:B5, Sigma) at the end of the experiment. After 1 h, the mice were anesthetized with ether, and blood was collected via cardiac puncture. Serum was obtained and stored at -20 ºC for further analysis.

2.9. Statistical analysis

The data were presented as mean±standard error of the mean (SEM). Two-sided Student's t-test or two-way analysis of variance was applied to compare the differences between groups. If the statistical analysis showed that the differences between multiple groups were significant, Tukey's *post-hoc* test was used for further comparison. All statistical analysis was performed using IBM SPSS software, version 22.0 (Chicago, IL, USA). *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Assessment of the degree of monocyte differentiation in peritoneal exudate cells following thioglycollate injection

We obtained peritoneal exudate cells four days after thioglycollate injection and examined the cells expressing both CD11b and CD36 using flow cytometry. Cells isolated from the RVS extract-treated group showed an increase in this cell population with 77.49±1.79% (*P*=0.247) and 79.94±1.52% (*P*=0.080) of cells expressing both CD11b and CD36 in the 200 and 1000 mg/kg RVS extract groups, respectively, compared to 72.86±3.31% in the control group (Figure 1A,B). However, the difference did not reach statistical significance. The cell population expressing both CD11b and SRA was also assessed. The proportion of SRA(+)CD11b(+) cells in the control group was 78.87±2.03%, which increased to 84.37±1.18% (*P*=0.047) and 82.31±1.43% (*P*=0.350) in the 200 mg/kg and 1000 mg/kg RVS groups, respectively (Figure 1C,D). These data show that RVS extract treatment tends to enhance thioglycollate-induced monocyte differentiation in the peritoneum.

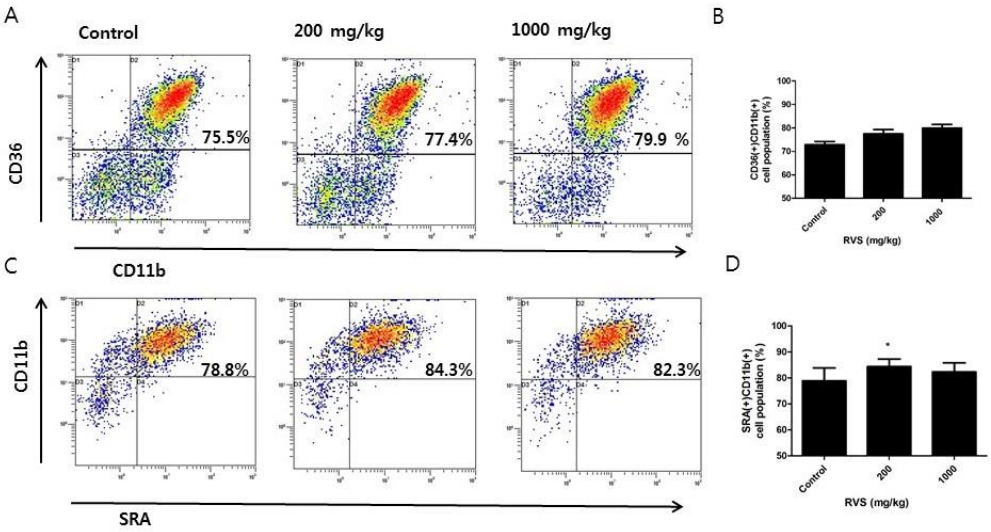

Figure 1. Effects of *Rhus verniciflua* Stokes (RVS) extract on scavenger receptor expression in peritoneal exudate cells. Mice were orally given RVS extract (200 or 1000 mg/kg) for 10 days, and intraperitoneal injection of thioglycollate was performed 4 days before the isolation of peritoneal exudate cells. A, B: Peritoneal exudate cells were double-stained with FITC-labeled anti-CD11b and PE-labeled anti-CD36 antibodies (A and B) or FITC labeled anti-SRA and PE labeled anti-CD11b antibodies (C and D) and analyzed via flow cytometry. Representative dot plots are shown. B,D: The bars represent the mean±SEM (N=6). * P <0.05 vs. control

3.2. RVS extract enhanced the activity of scavenger receptors expressed by peritoneal macrophages

Because we found that scavenger receptor expression was increased in CD11b(+) cells from the RVS group, we examined their scavenger receptor activity. In our laboratory, we routinely find over 90% of adherent peritoneal exudate cells to be CD11b(+) (data not shown). We cultured these adherent peritoneal macrophages with Alexa Fluor ^{488}-labeled acetylated lipoprotein, a ligand for scavenger receptors, and measured the intracellular uptake of the labeled acetylated lipoprotein. A dose-dependent increase in mean fluorescence intensity from the Alexa Flour ^{488}-labeled acetylated lipoprotein was observed in the RVS extract group, with 37.43±1.26 (P=0.018) and 43.71±2.73 (P=0.003) in the 200 mg/kg and 1000 mg/kg groups, respectively, compared to 32.91±0.97 in the control group (Figure 2). The increased uptake of Alexa Fluor ^{488}-labeled acetylated lipoprotein indicates that RVS extract enhanced the activity of scavenger receptors expressed by monocyte-derived peritoneal macrophages.

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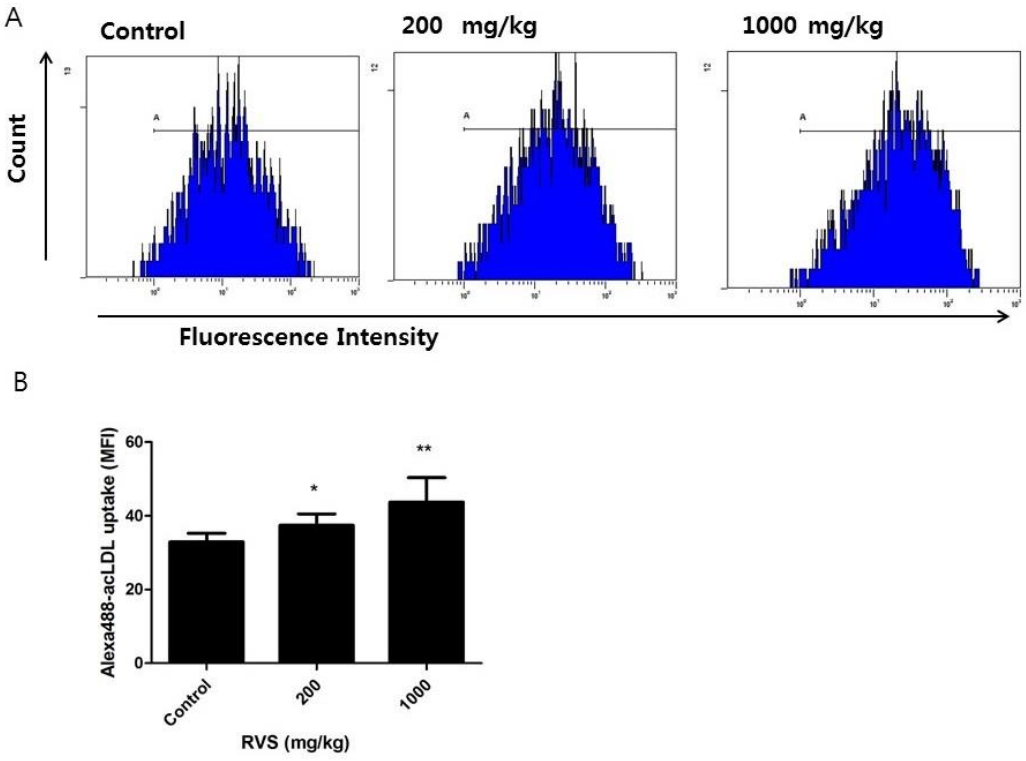

Figure 2. Effects of oral administration of RVS extract on scavenger receptor activity in monocyte-derived peritoneal macrophages. Peritoneal exudate cells isolated from thioglycollate-injected mice were cultured overnight. After removal of non-adherent cells, the remaining adherent cells were incubated with Alexa Fluor ^{488}-labeled acetylated lipoprotein for 4 h and analyzed via flow cytometry. A: Representative histograms are shown. B: The bars represent the mean±SEM (N=6). * P <0.05, ** P <0.01 vs. control

3.3. Macrophages from the RVS extract groups had reduced LPS-induced tumor necrosis factor (TNF)-α and interleukin (IL)-6 production

Peritoneal macrophages from the control and RVS extract groups were stimulated with LPS for 24 h, and the levels of secreted TNF-α and IL-6 were measured. A significant reduction in TNF-α production was noted in cells from the 200 mg/kg group (35.10±1.61 ng/ml, P=0.001) compared to 43.82±5.20 ng/ml in the control group and 42.10±0.89 ng/ml in the 1000 mg/kg group (Figure 3A). The levels of IL-6 also decreased in a dose-dependent manner with 116.62±7.11 ng/ml in the control group, 99.21±11.10 ng/ml in the 200 mg/kg group (P=0.001), and 86.90±3.95 ng/ml in the 1000 mg/kg group (P=0.000) (Figure 3B). Peritoneal macrophages from the RVS group show anti-inflammatory activity similar to that seen in RAW264.7 cells treated with the RVS extract [19,23].

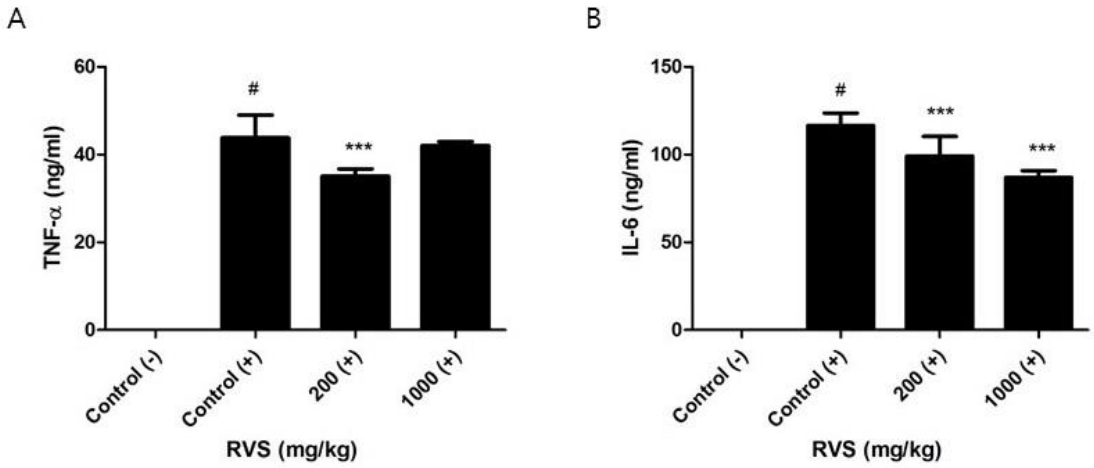

Figure 3. Effects of oral administration of RVS extract on tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels in the supernatants of lipopolysaccharide (LPS)-stimulated peritoneal macrophages. Adherent peritoneal macrophages were stimulated with LPS for 24 h, and the concentrations of TNF-α (A) and IL-6 (B) were measured using ELISA (N=6). (-): without LPS, (+): with LPS. # P <0.005 vs. control (-). *** P <0.005 vs control (+)

3.4. Differential effects on LPS-induced upregulation of CD86 and class II MHC were observed in macrophages from RVS extract-treated mice

We investigated whether RVS extract affected the surface expression of CD86 and class II MHC molecules, which link macrophages to Th cells. The mean fluorescence intensity of CD86 in the control group was 10.87±0.71 compared to 10.87±0.86 and 9.44±0.79 (P=0.005) in the 200 mg/kg and 1000 mg/kg groups, respectively (Figure 4A, C). The mean fluorescence intensity of class II MHC molecules in the control group was 8.79±1.04 compared to 8.91±0.61 and 10.92±0.91 (P=0.010) in the 200 mg/kg and 1000 mg/kg groups, respectively (Figure 4B,D). These data show that the higher dose of RVS extract induces a differential response in surface molecules relating to Th cells.

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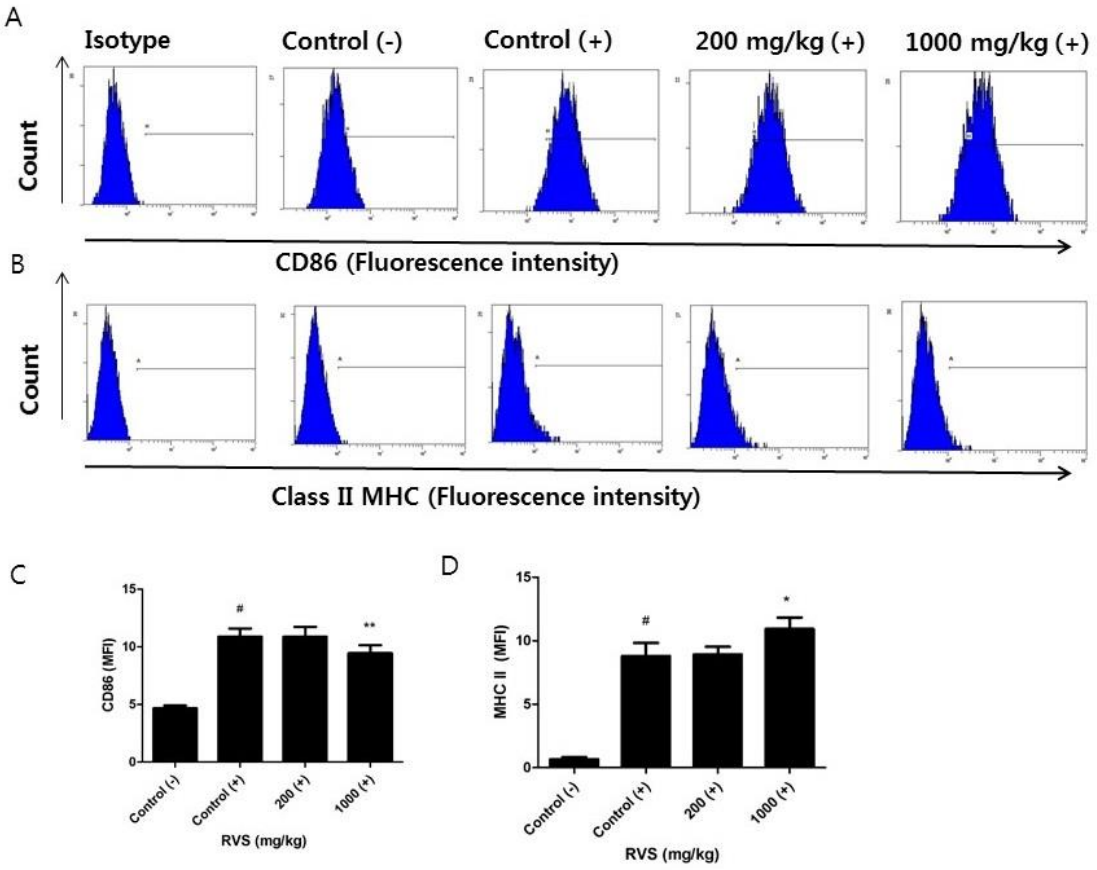

Figure 4. Effects of oral administration of RVS extract on surface expression of CD86 and class II MHC in LPS-stimulated peritoneal macrophages. Adherent peritoneal macrophages were stimulated with LPS for 24 h and surface expression of CD86 and class II MHC molecules was determined via flow cytometry. A, B: Representative histograms are shown. C,D: The bar represent the mean±SEM (N=6). (-): without LPS, (+): with LPS. # P <0.005 vs. control (-). *P <0.05, **P <0.01 vs control (+)

3.5. Increased production of LPS-induced IL-12 was found in supernatants of macrophages from RVS extract-treated mice

IL-12 (IL-12p70) is produced by macrophages and other phagocytes stimulated by microorganisms and their byproducts and is a potent inducer of IFN-γ production in natural killer (NK) cells and Th1 cells [28]. Because IL-12 is not produced by macrophage cell lines, previous studies on the anti-inflammatory activity of RVS extract using RAW264.7 cells did not detect an IL-12 response [19,23,29]. In contrast to TNF-α and IL-6, increases in LPS-induced IL-12 production were observed in macrophages from the RVS extract-treated groups with levels of 424.15±83.12 pg/ml (P=0.027) and 453.36±126.11 59.29 pg/ml (P=0.000) in the 200 mg/kg and 1000 mg/kg groups, respectively, compared to 353.47±45.94 pg/ml in the control group (Figure 5A). Macrophages are not the dominant source of IFN-γ, but IL-12 has been reported to induce the secretion of IFN-γ in peritoneal macrophages [30]. Therefore, we measured the level of IFN-γ in the supernatants of the activated macrophage cultures. Similar to but less marked than IL-12, increases in IFN-γ were found in the supernatants of the RVS extract-treated groups, but they did not reach statistical significance with 241.75±85.69 pg/ml (P=0.892) and 359.00±126.11 pg/ml (P=0.106) in the 200 mg/kg and 1000 mg/kg groups, respectively, compared to 236.66±90.41 pg/ml in the control group (Figure 5B). RVS extract differentially regulated the production of macrophage-derived cytokines in response to LPS.

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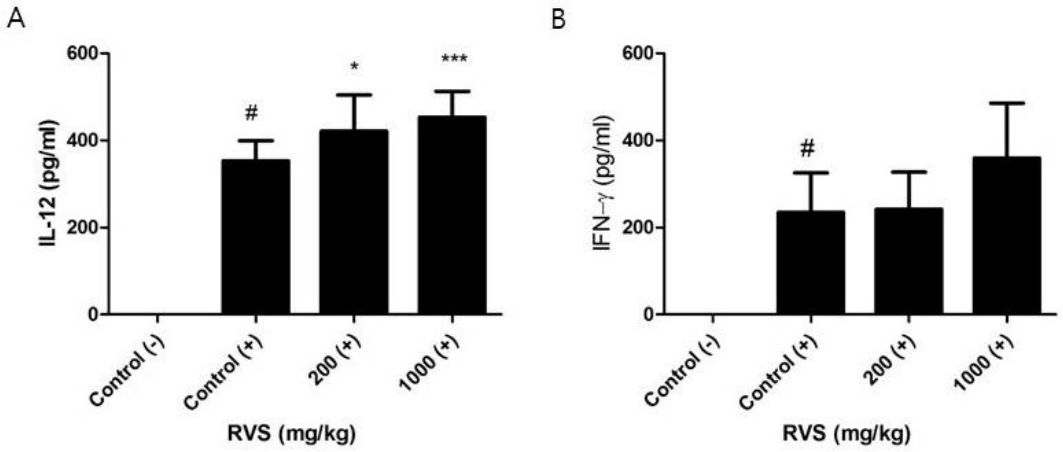

Figure 5. Effects of oral administration of RVS extract on IL-12 and interferon (IFN)-γ levels in the supernatants of LPS-stimulated peritoneal macrophages. Adherent peritoneal macrophages were stimulated with LPS for 24 h, and the concentrations of IL-12 (A) and IFN-γ (B) were measured via ELISA (N=6). (-): without LPS, (+): with LPS. # *P* <0.005 vs. control (-). **P* <0.5, ****P* <0.005 vs control (+)

3.6. RVS extract suppressed the systemic response to LPS

We tested whether the systemic cytokine response after LPS challenge is altered in RVS extract-treated mice. Intraperitoneal injection of a sublethal dose of LPS was performed on mice that had been administered 1000 mg/kg of RVS extract. Since the peak serum TNF-α level occurs approximately 1 h after LPS challenge, we obtained serum at this time point [31]. The levels of serum TNF-α in the control group was 6.43±1.60 ng/ml, which was decreased to 2.56±1.08 ng/ml in the RVS extract group (*P*=0.000) (Figure 6A). The level of serum IL-6 in the RVS extract group was 5.71± 1.21 ng/ml (*P*=0.000) compared to 10.26±2.68 ng/ml in the control group (Figure 6B). Because of the delayed production of serum IL-12 and IFN-γ in comparison to TNF-α, we were not able to detect those cytokines.

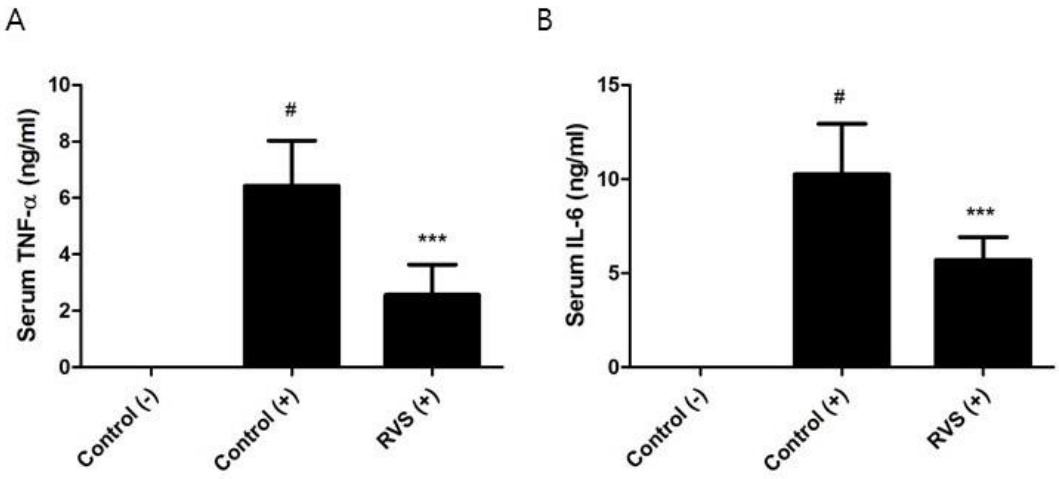

Figure 6. Effects of oral administration of RVS extract on the systemic response to LPS. Following oral administration of RVS extract, mice were intraperitoneally injected with a sublethal dose of LPS (1.3 mg/kg), and serum was obtained one hour later. The cytokine levels were measured via ELISA (N=10). (-): without LPS, (+): with LPS. # *P* <0.005 vs. control (-). **** P* <0.005 vs control (+)

4. Discussion

Previous studies have shown that *in vitro* treatment of macrophages with RVS extract or its active components suppresses LPS-induced inflammatory mediators. Here, we demonstrated that macrophages isolated from mice orally given RVS extract showed a similar activity to that seen in vitro. Further, we discovered that RVS extract stimulated the differentiation of monocytes in the peritoneum and the immune response of macrophages to affect Th cells.

In the normal mouse peritoneum, the major cell types are resident macrophages and B-1 cells, with the remaining minor groups consisting of T cells, NK cells, NKT cells, and other immune cells [8,10]. Following intraperitoneal injection of thioglycollate, neutrophils are initially recruited, and then monocyte-derived macrophages become the predominant cell type in the peritoneum [8,10,15]. In thioglycollate-induced peritonitis, the number of eosinophils is also increased [8]. Because eosinophils express a low level of CD11b, we defined macrophages as cells that express both CD11b and scavenger receptors [10]. Scavenger receptors were originally discovered based on their ability to detect and remove acetylated lipoprotein [14]. Their major roles are to clear pathogens and endogenous waste products, aiding in phagocytosis, antigen presentation, and clearance of apoptotic cells by macrophages [14]. We found over 70% of peritoneal exudate cells obtained four days after thioglycollate injection to be either CD11b(+)CD36(+) cells or CD11b(+)SRA(+) cells. Mice orally given RVS extract showed an increased population of CD11b(+)SRA(+) cells in response to thioglycollate and a concurrent increase in uptake of Alexa Fluor 488-labeled acetylated lipoprotein by peritoneal macrophages. These results suggest that RVS extract may promote the process of monocyte-to-macrophage differentiation and the clearance of unwanted products upon inflammatory insults.

Macrophages detect LPS through toll-like receptor4, which then initiates the induction of inflammatory and immune responses by using MyD88 and TRIF as adaptors to transmit signals to the nucleus [32]. The MyD88-depenent pathway activates NF-κB and MAPK to induce the expression of inflammatory proteins such as TNF-α and IL-6 [33]. The TRIF-dependent pathway is involved in inflammatory gene expression and, more importantly, is indispensable in the upregulation of costimulatory molecules, such as CD86, and class II MHC molecules that bridge macrophages to Th cells [34]. Macrophages isolated from the RVS extract-treated mice showed reduced production of TNF-α and IL-6 after stimulation with LPS, similar to that seen in macrophages directly treated with RVS water extract [19,23]. However, given that polyphenols in the blood of rats orally given RVS extract are likely to be present in their conjugated form, it remains unresolved whether the same components are responsible for the results obtained here [27]. We also found that LPS-induced serum levels of TNF-α and IL-6 were remarkably suppressed in the RVS extract group, even more so than in the supernatants of activated macrophages isolated from RVS extract-treated mice. This is likely due to the fact that macrophages are continuously exposed to LPS *in vitro*, but LPS is rapidly cleared by the liver *in vivo* [35]. A similar study in which rats were orally given RVS extract at 100 mg/kg and 500 mg/kg for 2 weeks and then challenged with intraperitoneal LPS showed a significant reduction in IL-6 protein and a marginal reduction in TNF-α protein in the liver [36]. These hepatic cytokine concentrations were measured 16 h after LPS stimulation [36]. However, the peak response of serum TNF-α is approximately 1 h after intraperitoneal challenge with LPS and rapidly declines [31]. Because of the rapid kinetic response of TNF-α, the previous study may have failed to detect a dramatic reduction in the liver.

CD86 has increased expression in macrophages stimulated with pathogenic microbes and enhances T cell activity by binding to CD28 on T cells [37]. Class II MHC molecules are required to present antigens to Th cells [6]. Interestingly, macrophages from the 1000 mg/kg RVS extract group showed increased expression of class II MHC and decreased expression of CD86. Although LPS-induced upregulation of CD86 and class II MHC molecules is defective in TRIF-deficient macrophages, their production pathways downstream of TRIF seem to be distinct. Expression of class II MHC is increased by IFN-γ [34,38]. A similar, though not statistically significant, increase in IFN-γ was found in the supernatants of activated macrophages from the 1000 mg/kg RVS extract group, which may have driven the increase in class II MHC expression. Such differential regulation of CD86 and class II MHC indicates that RVS extract may enhance the capacity of macrophages to

present antigens to Th cells but reduce their stimulatory signal to activate Th cells. This property may be beneficial for a prolonged immune response involving macrophages and Th cells together.

The principal function of IL-12 is to induce the production of IFN-γ in NK cells and Th1 cells, which play a critical role in intracellular pathogen control and anti-tumor immunity [39,40]. IL-12p70 is the active form of IL-12 consisting of p35 and p40, which are regulated by different chromosomes [41]. The production of IL-12p35, and therefore of IL-12 requires NF-κB and IRF-1, and the latter of which is activated by IFN-γ [42]. However, the cellular origin of IFN-γ in the culture of peritoneal macrophages is unclear [39]. Adherent peritoneal exudate cells contain small quantities of T cells, NK cells, and NKT cells, which produce a large amount of IFN-γ in response to LPS. Therefore, these lymphoid cells could be the main source of the IFN-γ detected in the activated macrophage culture. Whatever the source of the IFN-γ, RVS may stimulate macrophages to respond to IFN-γ and produce more IL-12. In addition, IL-12p40 is unique in comparison to other inflammatory cytokines regarding NF-κB dependence. The NF-κB family comprises Rel (p65), Rel-B, c-Rel, p50 and p52. IL-12p40 is one of the few c-Rel- dependent target genes [43]. IL-12p40 production is severely impaired in c-Rel- deficient macrophages, but TNF-α and IL-6 expression are unaltered [44]. IL-12p35 gene induction also depends more on c-Rel than on p65 [42]. Interestingly, macrophages from the RVS extract-treated mice had increased level of IL-12 and reduced levels of TNF-α and IL-6 in response to LPS. Given that IL-12 is regulated differently by the NF-κB family than are TNF-α and IL-6, RVS extract may selectively affect NF-κB signaling activity during an inflammatory response. Previous studies using RAW264.7 cells failed to detect an influence of RVS extract on IL-12 produced by LPS-stimulated macrophages [19,23]. This report sheds light on the ability of RVS extract to stimulate IL-12 in activated macrophages, although the detailed mechanism needs to be further verified.

5. Conclusions

Taken together, we demonstrated for the first time that oral administration of RVS extract enhances monocyte differentiation in the thioglycollate-induced peritonitis model, as well as increasing scavenger receptor expression and activity. When these monocyte-derived macrophages from mice given RVS extract were stimulated with LPS *ex vivo*, they showed reduced production of TNF-α and IL-6 and reduced surface expression of CD86 but increased surface expression of class II MHC and increased production of IL-12. Although detailed mechanistic studies on such differential effects are needed, these findings support the hypothesis that RVS confers anti-inflammatory activity without causing overall inhibitory effects on immune cells.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Calibration curves of fisetin and sulfuretin

Author Contributions: Bo-Geun Kim and Hee Kang wrote the paper; Mi-Gi Lee performed flow cytometric analysis; Jin-Mo Gu performed HPLC and mass spectrometry; Youngju Song performed ELISA and cell culture; SeungGwan Lee contributed reagents.

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