

# *Ex Vivo Rumex Crispus and Cordyceps Sinensis Mixture Regulates Immune Cells Responses to Pro-inflammatory Cytokines in C57BL/6 mice Splenocytes*

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## ABSTRACT

We investigated the efficacy of a *Rumex crispus* and *Cordyceps sinensis* mixture made using the Beopje (Korea traditional processing method to remove anti-nutrients and enhance phytochemicals) method to regulate immune cell responses toward nitric oxide (NO) production, pro-inflammatory cytokines, and inflammation related genes in mice splenocytes. The six experimental groups were as follows: control (control), Rc-Cs (*Rumex crispus* (Rc) and *Cordyceps sinensis* (Cs) mixture, 6:4), TMC (Taemyeongcheong, commercial healthy drink containing Rc-Cs), LPS (lipopolysaccharide), LPS+Rc-Cs, and LPS+TMC. The Rc-Cs mixture reduced nitric oxide (NO) production in LPS-induced splenocytes. Moreover, Rc-Cs enhanced production of the pro-inflammatory cytokines TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , and IL-6 compared to the control (no treatment). However, Rc-Cs inhibited production of pro-inflammatory cytokines in LPS-induced splenocytes. In addition, LPS+Rc-Cs also significantly suppressed mRNA expression of IL-1 $\beta$  and IL-6 compared to LPS treatment. Interestingly, Rc-Cs did not increase mRNA levels of iNOS and COX-2, which are inflammation related genes compared to the control, while LPS+Rc-Cs reduced mRNA levels of iNOS and COX-2 compared LPS alone ( $p < 0.05$ ). TMC showed a similar pattern compared to Rc-Cs. Therefore, Rc-Cs treatment in splenocytes enhanced NO production and pro-inflammatory cytokines compared to the control, whereas Rc-Cs treatment in LPS-induced splenocytes reduced NO production, pro-inflammatory cytokines, and inflammation related genes. Thus, Rc-Cs regulated immune cells responses by increasing pro-inflammatory cytokines in splenocytes and reducing toxin (LPS)-induced inflammation. These results indicate that a *Rumex crispus* and *Cordyceps sinensis* mixture (Rc-Cs) and TMC containing Rc-Cs promote immune cells responses and anti-inflammatory activities.

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## 1. Introduction

*Rumex crispus* (Rc) is a perennial plant belonging to the Polygonaceae family. It has been reported that the roots and leaves of Rc have functional activities [1]. Rc is commonly consumed as a dried or processed herb or used as a medicinal product [2], and it is known to inhibit arachidonic acid induced inflammation in mice [3]. Rc also protects against liver injury [3] and exhibits anti-oxidant [4], anti-cancer [5], and anti-obesity [6] effects. *Cordyceps sinensis* (Cs) has been used since ancient times as an herbal ingredient [7], and has been reported to have anti-inflammatory and anti-cancer effects [8,9]. Both Rc and Cs have been shown to have anti-inflammatory effects when mixed at various ratios. Specifically, a 6:4 ratio was shown to have the most effective and synergistic anti-inflammation effects in a human mast cell line (HMC-1) [10]. Jeong et al. [10] reported that Rc-Cs reduced production of interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in phorbol 12-myristate 13-acetate and calcium ionophore A23187 (PMACI)-treated HMC-1 cells.

Chronic inflammatory reactions induce metabolic disease in various organs, resulting in obesity, diabetes, cardiovascular disease (CVD), cancer, neurodegeneration, and asthma [11]. Among inflammatory factors, nitric oxide (NO) has been reported to increase production of iNOS and reactive oxygen species (ROS), resulting in activation of inflammation [12]. Lipopolysaccharide (LPS) stimulates mammalian cells to interact with multiple protein molecules such as LPS binding protein (LBP) and toll-like receptor-4 (TLR4) [13,14]. TLR4 activated by LPS activates nuclear factor  $\kappa$ B (NF- $\kappa$ B), and activated NF- $\kappa$ B promotes transcription of pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , type 1 interferons (IFNs) such as IFN- $\beta$  and IFN- $\gamma$ , and inflammation-related enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the nucleus [15-17]. Upon initiation of inflammation, an immune response occurs, and a variety of immune cells are activated. Among them, T helper cells (Th) are important in the immune response and are known to produce various cytokines [18]. Th can be classified based on the type of cytokine produced. Type 1 T helper cells (Th1) produce TNF- $\alpha$ , IFN- $\gamma$ , and IL-12, whereas type 2 T helper cells (Th2) produce IL-4, IL-5, IL-6, and IL-10 [18]. IL-1 $\beta$  stimulates Th2 and promotes cytokine production by Th2 [19]. Naïve T cells are transformed into four different Th cells (Th1, 2, 17, and Treg) by various cytokines [20]. Each Th cell plays a different role depending on its type.

Th1 regulates cellular immunity and intracellular pathogen clearance, Th2 humoral immunity, extracellular pathogen clearance and allergies, Th17 tissue information and autoimmunity, and Treg immune suppression [20].

Taemyeongcheong (TMC) is a health functional beverage made using traditional methods in Korea, and it is commonly found in various foods and herbal ingredients. TMC inhibits the allergic effects induced by 48/80 [21] and acetaminophen-induced hepatic damage *in vivo* [22]. We already reported that TMC inhibited Th2 cytokines such as IL-1 $\beta$ , IL-6, and IL-10 as well as iNOS and COX-2 in LPS-induced RAW 264.7 cells [23].

The spleen participates in immune responses, inflammation, and degenerative diseases throughout the whole body [24]. Especially, the spleen contains T and B cells, macrophages, and dendritic cells and controls immune responses [24]. Splenocytes, which are the cell type of the spleen, generally participate in immune cells functions. In this study, we investigated the immune response regulatory effects of nitric oxide (NO) concentration, protein levels of TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , and IL-6 in medium, mRNA expression levels of IL-1 $\beta$ , IL-6, iNOS, and COX-2 in Rc-Cs, and TMC treatment in LPS-induced *ex vivo* mice splenocytes.

## 2. Materials and Methods

### 2.1. Sample preparation

*Rumex crispus* (Rc), *Cordyceps sinensis* (Cs), and Taemyeongcheong (TMC, healthy drink) were provided by Gawha Wellfood Co. (Jincheon, Chungcheongbuk-do, Korea), and the samples were freeze-dried. The Rc-Cs (6:4) samples were mixed at a ratio of 6:4. TMC was made from the following ingredients: *Rumex crispus* (13.62%), *Camellia Sinensis* (11.14%), *Saururus chinensis* (11.14%), *Viscum album* (11.14%), *Houttuynia cordata* (11.14%), *Atractylodes ovata* (9.90%), *Capsella bursa-pastoris* (8.67%), *Cornus officinalis* (8.67%), *Phyllostachys bambusoides* leaf (7.43%), and *Cordyceps sinensis* (7.15%). Rc-Cs and TMC were processed using a traditional Korean processing method called Beopje [25]. The Beopje process consists of washing, steaming, dehydration, parching, and then dehydration [25].

### 2.2. Ex vivo splenocytes collection and culture

Collection and culturing of mouse splenocytes were performed with slight modification [26,27]. Ten 5-week-old C57BL/6 mice were purchased from Orient Bio (Seongnam, Gyunggi-do, Korea) and used in the experiment. Mouse spleens were aseptically removed and immersed in Dulbecco Modified Eagle's Medium (DMEM). To make a single cell suspension, spleens were chopped into small pieces using sterilized scissors. Cells were centrifuged at 1500 rpm for 10 min to obtain a cell pellet, after which resuspension was performed to obtain free

cells. To quantify splenocytes proliferation, cells were counted using a hemocytometer and dispersed at a concentration of  $2 \times 10^6$  cells/mL in 6-well plates. Cells were cultured in DMEM containing 10% Fetal Bovine Serum (FBS) and 100 units/mL of penicillin-streptomycin. Cells were cultured at 37°C with 5% CO<sub>2</sub> [22]. This experiment was approved by the Cha University Animal Ethics Committee (IACUC-170149).

### 2.3. NO production

NO production was measured by nitrite concentration in media using griess reagent (Sigma-Aldrich, St. Louis, MO, USA). Splenocytes plated in 6-well plates were incubated for 24 h. The medium was removed and exchanged for medium without FBS and penicillin-streptomycin for 24 h to induce starvation. After the medium was removed, 0.05 mg/mL of Rc-Cs and 0.1 mg/mL of TMC were added to each well. To induce inflammation, LPS (2 µg/mL) was treated to each well. After incubation for 24, 48, and 72 h, the medium was collected. The collected media were reacted with griess reagent, and absorbance was measured at 550 nm using a Wallac Victor3 1420 Multilabel Counter (Perkin-Elmer, Wellesley, Mass.) [23].

### 2.4. Quantitation of pro-inflammatory cytokines by enzyme-linked immunosorbent assay (ELISA)

Splenocytes plated in 6-well plates were incubated for 24 h. After the medium was removed, 0.05 mg/mL of Rc-Cs and 0.1 mg/mL of TMC were added to each well. To induce inflammation, LPS (2 µg/mL) was treated to each well. After incubation for 24, 48, and 72 h, the medium was collected. Concentrations of IL-6, IL-1β, IFN-γ, and TNF-α were measured in collected medium using an ELISA kit (BioLegend, San Diego, CA). This experiment was performed according to the method provided by the manufacturer [28].

### 2.5. mRNA quantitation of pro-inflammatory cytokines and inflammation related genes in splenocytes by real time-quantitative polymerase chain reaction (RT-qPCR)

Splenocytes plated in 6-well plates were incubated for 24 h. After the medium was removed, 0.05 mg/mL of Rc-Cs and 0.1 mg/mL of TMC were added to each well. To induce inflammation, LPS (2 µg/mL) was treated to each well. After incubation for 48 h, RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The isolated RNA was quantified using NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE). Quantified RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and synthesized as cDNA. The synthesized cDNA was amplified using a thermal cycler BioRad CFX-96 real time system (BioRad, USA), and the expressed gene was identified. The primers used were 18s rRNA, IL-

1 $\beta$ , IL-6, iNOS, and COX-2, and the primer sequence was as follows: 18s rRNA forward 5' - TCGAGGCCCTGTAATTGGAA-3' and reverse 5'-CCCTCCAATGGATCCTCGTT-3', IL-1 $\beta$  forward 5'-AAGGGCTGCTTCCAAAC-3' and reverse 5'-CTCCACAGCCACAATGA-3', IL-6 forward 5'-ATGAAGTTCCCTCTCTGCAA-3' and reverse 5'-AGTGGTATCCTCTGTGAAG-3', iNOS forward 5'-ATGGCTTGCCCCTGGAA-3' and reverse 5'-TATTGTTGGGCTGAGAA-3', COX-2 forward 5'-GGCAGCAAATCCTTGC-3' and reverse 5'-TATTGTTGGGCTGAGAA-3' [22].

### 2.5. Statistical analysis

All data are presented as the mean  $\pm$  standard deviation (SD). Differences between the mean values for individual groups were assessed by one-way analysis of variance (ANOVA) of Duncan's multiple range tests. Differences were considered significant when  $p < 0.05$ . The SPSS v18 statistical software package (SPSS Inc. Westlands, Hong Kong) was used to perform these analyses [23].

## 3. Results

### 3.1. NO production in mice splenocytes

Treatment with Rc-Cs at a concentration of 0.005 ~ 0.05 mg/mL did not significantly affect proliferation of RAW 264.7 cells after 24, 48, and 72 h of incubation (data not shown). In a previous study, TMC showed no cytotoxicity in RAW 264.7 cells at a concentration of 0.01 ~ 0.1 mg/mL [23].

Inflammation induced by LPS in animal tissue results in the activation of nitric oxide synthase (NOS) and increased production of NO [29]. As shown in Table 1, LPS treated splenocytes showed significantly elevation of NO depending on treatment time and compared to the control, Rc-Cs and TMC treatments. Rc-Cs ( $2.32 \pm 0.09$   $\mu$ M) increased NO levels compared to the control ( $1.38 \pm 0.20$   $\mu$ M) at 72 h ( $p < 0.05$ ), and splenocytes treated with LPS+Rc-Cs ( $2.66 \pm 0.27$   $\mu$ M) and LPS+TMC ( $1.54 \pm 0.23$   $\mu$ M) showed significantly lower NO levels than LPS treated splenocytes ( $3.15 \pm 0.20$   $\mu$ M) at 72 h ( $p < 0.05$ ).

### 3.2. Pro-inflammatory cytokine levels of TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , and IL-6 in mice splenocytes

NF- $\kappa$ B activated by LPS regulates pro-inflammatory cytokines such as TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , and IL-6 [15]. In the current study, LPS treated groups showed increased production of pro-inflammatory cytokines compared to the control group (Fig. 1).

As shown in Fig. 1A, B, LPS treatment significantly increased TNF- $\alpha$  and IFN- $\gamma$  levels compared to the control ( $p < 0.05$ ). Rc-Cs ( $173.8 \pm 72.4$  pg/mL) and TMC ( $267.0 \pm 81.6$  pg/mL) treatments also significantly increased TNF- $\alpha$  protein levels compared to the control ( $2.0 \pm 2.5$  pg/mL) at 72 h ( $p < 0.05$ ) (Fig. 1A). In addition, LPS+Rc-Cs ( $342.8 \pm 102.2$  pg/mL) and LPS+TMC ( $379.3 \pm 5.7$  pg/mL) treatments significantly reduced TNF- $\alpha$  protein levels compared to LPS ( $544.4 \pm 54.0$  pg/mL) at 72 h ( $p < 0.05$ ) (Fig. 1A). Rc-Cs ( $60.9 \pm 5.0$  pg/mL) and TMC ( $51.4 \pm 9.7$  pg/mL) treatments significantly increased IFN- $\gamma$  protein levels compared to the control ( $44.4 \pm 1.1$ ) at 72 h ( $p < 0.05$ ) (Fig. 1B). In addition, LPS+Rc-Cs ( $63.4 \pm 13.3$  pg/mL) and LPS+TMC ( $62.2 \pm 2.2$  pg/mL) treatments significantly reduced IFN- $\gamma$  protein levels compared to the LPS ( $108.3 \pm 4.8$  pg/mL) at 72 h ( $P < 0.05$ ) (Fig. 1B). However, LPS+TMC treatment showed similar a TNF- $\alpha$  level compared to LPS treatment at 48 h. As shown in Fig. 1C, D, LPS significantly increased IL-1 $\beta$  and IL-6 levels compared to the control ( $p < 0.05$ ). LPS+Rc-Cs ( $47.9 \pm 1.6$  pg/mL) and LPS+TMC ( $45.2 \pm 6.7$  pg/mL) treatments significantly reduced IL-1 $\beta$  protein levels compared to LPS ( $81.2 \pm 18.7$  pg/mL) at 72 h ( $p < 0.05$ ) (Fig. 2A). Rc-Cs ( $446.2 \pm 209.4$  pg/mL) and TMC ( $328.4 \pm 83.6$  pg/mL) treatments significantly increased IL-6 protein levels compared to the control ( $54.2 \pm 21.3$  pg/mL) at 72 h ( $p < 0.05$ ) (Fig. 2B). In addition, LPS+Rc-Cs ( $570.7 \pm 264.4$  pg/mL) and LPS+TMC ( $326.0 \pm 159.8$  pg/mL) treatments significantly reduced IL-6 protein levels compared to LPS ( $1050.2 \pm 128.7$  pg/mL) at 72 h ( $p < 0.05$ ) (Fig. 2B). Thus, Rc-Cs and TMC reduced production of pro-inflammatory cytokines in LPS induced splenocytes but increased pro-inflammatory cytokine production in naïve splenocytes.

### 3.3. mRNA expression of pro-inflammatory cytokines of IL-1 $\beta$ and IL-6 in mice splenocytes

Previously, we reported that TMC inhibited Th2 cytokines in RAW 264.7 cells [23]. Therefore, in this study, we investigated whether or not Rc-Cs and TMC interfere with mRNA levels of Th2 cytokines in splenocytes. The mRNA expression levels of IL-1 $\beta$  and IL-6 are shown in Fig. 2. Rc-Cs and TMC significantly increased IL-1 $\beta$  and IL-6 levels compared to the control, which is similar to the results in Fig. 2. LPS increased mRNA levels of these cytokines in splenocytes compared to the control ( $p < 0.05$ ). In addition, LPS+Rc-Cs and LPS+TMC significantly reduced IL-1 $\beta$  (0.27 and 0.08) and IL-6 (0.12 and 0.13) levels compared to LPS (1.00) ( $P < 0.05$ ).

### 3.4. mRNA expression of inflammation-related genes in mice splenocytes

mRNA expression levels of iNOS and COX-2 are shown in Fig. 3. Rc-Cs and TMC did not significantly affect iNOS and COX-2 expression compared to the control (no significant difference in Duncan's multiple range tests (ANOVA)). LPS-induced mice splenocytes showed significantly increased mRNA levels compared to the control

( $p < 0.05$ ). LPS+Rc-Cs and LPS+TMC significantly reduced iNOS (0.39 and 0.08) and COX-2 (0.52 and 0.14) levels compared to LPS (1.00) ( $p < 0.05$ ). Thus, Rc-Cs and TMC decreased inflammation related genes in LPS-induced splenocytes but not in naïve splenocytes.

#### 4. Discussion

*Rumex crispus* and *Cordyceps sinensis* are currently studied for their efficacies in a variety of diseases. *Rumex crispus* was shown to inhibit histamine activity by 82% in RAW 264.7 cells [30] and suppress inflammation in arachidonic acid (AA) and carrageenan (CA) induced mice [3]. *Cordyceps sinensis* inhibited IL-1 $\beta$  and TNF- $\alpha$  in focal cerebral ischemic injury rats [31] and TNF- $\alpha$  in LPS-induced RAW 264.7 cells [32]. These findings appear to be similar to the *Rumex crispus* and *Cordyceps sinensis* mixture in this study. However, the mechanisms involved in *Rumex crispus* have not been elucidated yet, and *Cordyceps sinensis* has been shown to inhibit pro-inflammatory cytokines. Therefore, it can be indirectly confirmed that *Rumex crispus* inhibits inflammation similar to *Cordyceps sinensis*, and Rc-Cs has good anti-inflammatory effects in LPS-induced splenocytes.

According to current studies, naïve T cells differentiate into four different Th cells (Th1, 2, 17, and Treg) upon induction by various cytokines [20]. Among them, Th1 and Th2 are well-known factors, and the balance between Th1 and Th2 is important [33]. In this study, Rc-Cs inhibited Th2 (especially IL-6) more strongly than Th1, whereas TMC did not significantly decrease Th1 (especially TNF- $\alpha$ ). It was previously reported that TMC reduces production of Th2 cytokines [23]. Th1 regulates cellular immunity and intracellular pathogen clearance, Th2 humoral immunity, extracellular pathogen clearance, and allergies [20]. Thus, a Rc-Cs containing health functional beverage may inhibit atopy through suppression of Th2 cytokines.

*Rumex crispus* inhibited inflammation in AA and CA induced mice [3]. AA produces prostaglandins (PGs), which are metabolites produced through COX and lipoxygenase (LOX) pathways [34]. CA, which is a sulfated polysaccharide, is involved in the induction and progression of inflammatory factors such as histamine, serotonin, PGs, and kinin as a chemical mediator [35]. *Cordyceps sinensis* inhibited iNOS and COX-2 in rats [31] as well as iNOS and COX-2 in LPS-induced RAW 264.7 cells [32]. In this study, Rc-Cs suppressed iNOS and COX-2 production in LPS-induced splenocytes. These results indicate that Rc-Cs reduced production of Th1 and Th2 cytokines and the inflammation related genes iNOS and COX-2 in LPS-induced mice splenocytes. *Rumex crispus* and *Cordyceps sinensis* in TMC are major ingredients with anti-inflammatory effects.

Rc-Cs may gain enhanced anti-inflammatory effects through the Beopje method. Beopje is a traditional Korean method of removing anti-nutrients and enhancing phytochemicals [28, 36]. *Rumex crispus* made using the Beopje

method had higher anti-inflammatory effects than *Rumex crispus* without Beopje in LPS-induced RAW 264.7 cells (data not shown) and mouse splenocytes [37]. In addition, *Rumex crispus* with Beopje (120 µg/g) showed increased chrysophanol production compared to *Rumex crispus* without Beopje (70.8 µg/g) (data not published). In addition, *Cordyceps sinensis* containing cordycepin suppressed phytohemagglutinin-induced inflammation in peripheral blood mononuclear cells [38]. Joeng et al. [10] reported that a 6:4 ratio for Rc-Cs and chrysophanol had anti-inflammation effects in PMACI-treated HMC-1 cells. Thus, a 6:4 ratio had synergistic anti-inflammatory effects since chrysophanol and cordycepin regulate inflammation.

In this study, Rc-Cs treatment increased NO production and pro-inflammatory cytokines in control splenocytes but decreased NO production and pro-inflammatory cytokines in LPS-induced splenocytes. Interestingly, Rc-Cs did not increase iNOS and COX-2 levels in control splenocytes. Linehan et al. [39] reported that low levels of TNF- $\alpha$  inhibited iNOS activation in an epithelial cell line (HT-29) induced by a pro-inflammatory cytokine mixture of IL-1 $\alpha$ /TNF- $\alpha$ /IFN- $\gamma$  [39]. Therefore, Rc-Cs stimulates pro-inflammatory cytokine production in Th1 and Th2 cells. These results indicate that Rc-Cs may promote immune cells activities and regulate a toxic agent(LPS) induced inflammation in mice splenocytes.

However, herbal ingredients are composed of numerous functional ingredients, and it is difficult to determine which processing methods and materials have health beneficial effects. Therefore, further research on Rc-Cs is needed to identify what ingredients have health beneficial effects.

## 5. Conclusion

Rc-Cs regulated increased immune cells responses and LPS-induced inflammation in mice splenocytes. These result indicates that Rc-Cs affect immune cell responses and have anti-inflammatory effects at a proper ratio (6:4) as well as enhanced health beneficial compounds (chrysophanol) through the Beopje method. In addition, *Rumex crispus* and *Cordyceps sinensis* in TMC are major ingredients in immune cells responses and have anti-inflammatory effects.

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### **Conflicts of Interest**

The authors declare no conflict of interest

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Table 1. Nitric oxide (NO) production of mice splenocytes during different incubation times.

	24H	48H	72H
Control	0.26 ± 0.05 <sup>c</sup>	0.93 ± 0.08 <sup>c</sup>	1.38 ± 0.20 <sup>d</sup>
Rc-Cs	0.27 ± 0.06 <sup>c</sup>	1.06 ± 0.07 <sup>c</sup>	2.32 ± 0.09 <sup>c</sup>
TMC	0.43 ± 0.15 <sup>c</sup>	0.79 ± 0.34 <sup>c</sup>	1.48 ± 0.20 <sup>d</sup>
LPS	0.59 ± 0.07 <sup>b</sup>	2.39 ± 0.51 <sup>a</sup>	3.15 ± 0.20 <sup>a</sup>
LPS+Rc-Cs	0.77 ± 0.06 <sup>a</sup>	1.59 ± 0.31 <sup>b</sup>	2.66 ± 0.27 <sup>b</sup>
LPS+TMC	0.76 ± 0.18 <sup>a</sup>	1.04 ± 0.33 <sup>c</sup>	1.54 ± 0.23 <sup>d</sup>

Control : no treatment, LPS : 2 µg / mL of LPS added mice splenocytes, Rc-Cs : 0.05 mg/mL of *Rumex crispus* and *Cordyceps* mixture (Rc-Cs) added mice splenocytes, LPS+Rc-Cs : 2 µg / mL LPS + 0.05 mg/mL of *Rumex crispus* and *Cordyceps* mixture (Rc-Cs) added mice splenocytes, TMC : 0.1 mg/mL of Taemyeongcheong added mice splenocytes, LPS+TMC : 2 µg / mL of LPS + 0.1 mg/mL Taemyeongcheong added mice splenocytes.

<sup>a-d</sup>Means with different letters on right side of mark are significantly different ( $p < 0.05$ ) by Duncan's multiple range tests.

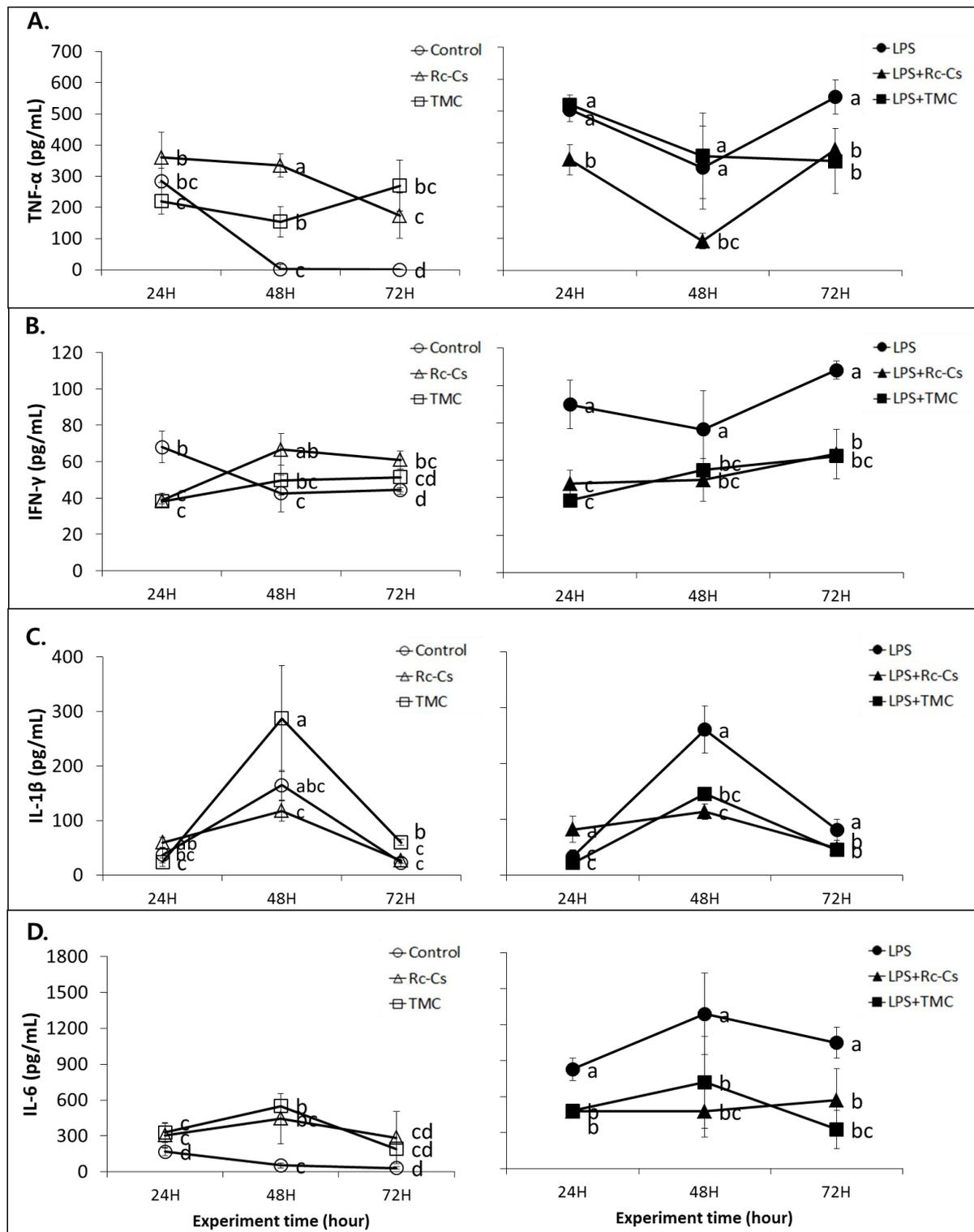


Figure 1. Pro-inflammatory cytokines TNF- $\alpha$ (A), IFN- $\gamma$ (B), IL-1 $\beta$ (A), and IL-6(B) in mice splenocytes during different incubation times.

Control : no treatment, LPS : 2  $\mu$ g / mL of LPS added mice splenocytes, Rc-Cs : 0.05 mg/mL of *Rumex crispus* and *Cordyceps* mixture (Rc-Cs) added mice splenocytes, LPS+Rc-Cs : 2  $\mu$ g / mL of LPS + 0.05 mg/mL *Rumex*

*crispus* and *Cordyceps* mixture (Rc-Cs) added mice splenocytes, TMC : 0.1 mg/mL of Taemyeongcheong added mice splenocytes, LPS+TMC : 2  $\mu$ g / mL of LPS + 0.1 mg/mL Taemyeongcheong added mice splenocytes.

<sup>a-d</sup>Means with different letters are significantly different ( $p < 0.05$ ) by Duncan's multiple range tests.

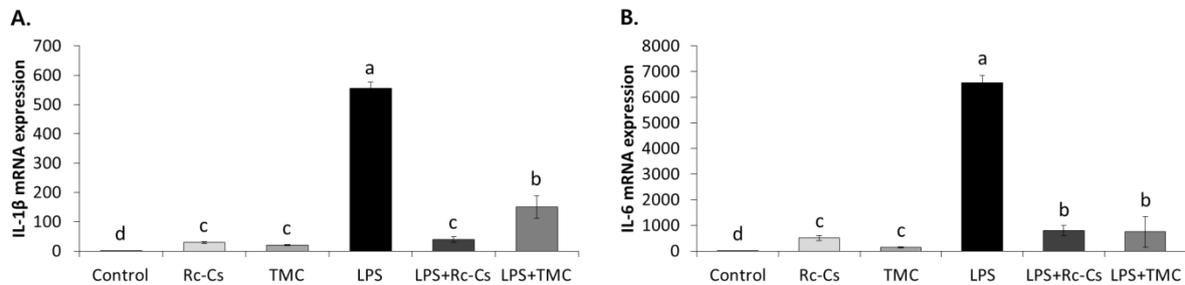


Figure 2. mRNA levels of pro-inflammatory cytokines IL-1 $\beta$  and IL-6 in mice splenocytes at 48 h.

Control : no treatment, LPS : 2  $\mu$ g / mL of LPS added mice splenocytes, Rc-Cs : 0.05 mg/mL of *Rumex crispus* and *Cordyceps* mixture (Rc-Cs) added mice splenocytes, LPS+Rc-Cs : 2  $\mu$ g / mL of LPS + 0.05 mg/mL *Rumex crispus* and *Cordyceps* mixture (Rc-Cs) added mice splenocytes, TMC : 0.1 mg/mL of Taemyeongcheong added mice splenocytes, LPS+TMC : 2  $\mu$ g / mL of LPS + 0.1 mg/mL Taemyeongcheong added mice splenocytes.

The mRNA expression levels were calculated based on 18s rRNA, which was used as a control (control fold ratio = 1).

<sup>a-d</sup>Means with different letters on the bar are significantly different ( $p < 0.05$ ) by Duncan's multiple range tests.

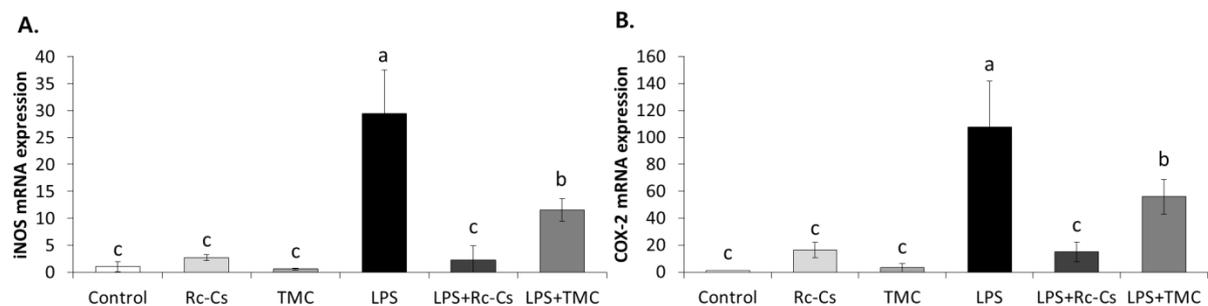


Figure 3. mRNA levels of inflammation-related genes iNOS and COX-2 in mice splenocytes at 48 h.

Control : no treatment, LPS : 2  $\mu$ g / mL of LPS added mice splenocytes, Rc-Cs : 0.05 mg/mL of *Rumex crispus* and *Cordyceps* mixture (Rc-Cs) added mice splenocytes, LPS+Rc-Cs : 2  $\mu$ g / mL of LPS + 0.05 mg/mL *Rumex crispus* and *Cordyceps* mixture (Rc-Cs) added mice splenocytes, TMC : 0.1 mg/mL of Taemyeongcheong added mice splenocytes, LPS+TMC : 2  $\mu$ g / mL of LPS + 0.1 mg/mL Taemyeongcheong added mice splenocytes.

The mRNA expression levels were calculated based on 18s rRNA, which was used as a control (control fold ratio = 1).

<sup>a-c</sup>Means with different letters on the bar are significantly different ( $p < 0.05$ ) by Duncan's multiple range tests.