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**Running Title:**  $\alpha$ -LA attenuates LPS-induced NLRP3 inflammasome

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**ABSTRACT**

Microglial cells are known as the main immune cells in the central nervous system, both regulating its immune response and maintaining its homeostasis. Furthermore, the antioxidant  $\alpha$ -lipoic acid (LA) is a recognized therapeutic drug for diabetes because it can easily invade the blood–brain barrier. This study investigated the effect of  $\alpha$ -LA on the inflammatory response in lipopolysaccharide (LPS)-treated BV-2 microglial cells. Our results revealed that  $\alpha$ -LA significantly attenuated several inflammatory responses in BV-2 microglial cells, including pro-inflammatory cytokines, such as tumor necrosis factor- $\alpha$  and interleukin (IL)-6, and other cytotoxic molecules, such as nitric oxide and reactive oxygen species. In addition,  $\alpha$ -LA inhibited the LPS-induced phosphorylation of ERK and p38 and its pharmacological properties were facilitated via the inhibition of the nuclear factor kappa B signaling pathway. Moreover,  $\alpha$ -LA suppressed the activation of NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasomes, multiprotein complexes consisting of NLRP3 and caspase-1, which are involved in the innate immune response. Finally,  $\alpha$ -LA decreased the genes accountable for the M1 phenotype, IL-1 $\beta$  and ICAM1, whereas it increased the genes responsible for the M2 phenotype, MRC1 and ARG1. These findings suggest that  $\alpha$ -LA alleviates the neuroinflammatory response by regulating microglial polarization.

## INTRODUCTION

In pathological status, activation of microglial cells is essential in neuroinflammatory response as the resident phagocytes in the central nervous system (CNS) [1]. Specifically, microglial cells are classified as either classically activated (M1 phenotype) or alternatively activated (M2 phenotype). Most previous studies indicated that microglial cells in the M1 phenotype release pro-inflammatory mediators, including cytokines, such as tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6, and other cytotoxic molecules, such as nitric oxide (NO) and reactive oxygen species (ROS) [2]. In contrast, microglial cells in the M2 phenotype were shown to downregulate the release of pro-inflammatory cytokines and protect against inflammation [3]. Therefore, the differentiation of microglial cells polarization suggests whether they are capable of inducing anti-inflammatory responses.

Furthermore, recent studies found both nuclear factor- $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinase (MAPK) signaling to contribute to the activation of the nucleotide binding and oligomerization domain-like receptor containing a pyrin domain (NLRP3) inflammasome [4]. NLRP3 inflammasomes are immune complexes consisting of NLRP3, the apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC), and pro-caspase-1. Although they regulate the immune response in microglial cells by activating both pro-caspase-1 and interleukin (IL)-1 $\beta$  [5], the process by which NF- $\kappa$ B and MAPK signaling activate NLRP3 inflammasome is unknown.

The antioxidant  $\alpha$ -lipoic acid ( $\alpha$ -LA) is considered an attractive drug candidate for anti-neuroinflammatory therapy. In fact, several studies reported the beneficial effects of  $\alpha$ -LA in various disorders, including hypertension [6], diabetes mellitus [7], while  $\alpha$ -LA was used as a safe supplement for humans in various countries. However, most studies on such  $\alpha$ -LA effects did not discuss microglial activation, specifically through NLRP3 inflammasome mediation.

In this study, we aimed to investigate the anti-inflammatory effects of  $\alpha$ -LA in relation to its regulatory role on several inflammatory responses and NLRP3 inflammasome activation through increased M2 phenotype.

## RESULTS

### $\alpha$ -LA decreased pro-inflammatory cytokines in LPS-induced BV-2 microglial cells

Cytotoxicity of  $\alpha$ -LA was evaluated prior to the analysis of pro-inflammatory cytokines in BV-2 cells. Cells were incubated with  $\alpha$ -LA (100, 200, 500, and 1000  $\mu$ M), with or without LPS (1  $\mu$ g/ml), for 24 h.  $\alpha$ -LA did not present cytotoxicity at any of the concentrations employed (Fig. 1A). These results suggest that  $\alpha$ -LA did not affect the viability of BV-2 cells *in vitro*. To further analyze the effects of  $\alpha$ -LA on pro-inflammatory cytokines production, BV-2 cells were incubated with both the indicated concentration of  $\alpha$ -LA and LPS. Although both TNF- $\alpha$  and IL-6 levels were found to be increased in LPS-induced BV-2 cells, their expression was considerably decreased in  $\alpha$ -LA treated BV-2 cells (Fig. 1B and C). These results suggest that  $\alpha$ -LA inhibits the production of pro-inflammatory cytokines without affecting cell viability.

### Effects of $\alpha$ -LA on both production of ROS and NO, and expression of NOS in LPS-stimulated BV-2 microglial cells

The protective effect of  $\alpha$ -LA on LPS-induced ROS production was further investigated with H<sub>2</sub>DCF-DA. As shown in Fig. 2A, a ~2-fold increase in ROS production was found in LPS treated cells compared to its levels in untreated cells. However, the LPS-induced ROS production in BV-2 cells was significantly reduced by  $\alpha$ -LA (Fig. 2A). To test whether  $\alpha$ -LA has an anti-inflammatory effect on microglia, the level of NO production in BV-2 cell culture supernatant was measured. LPS was seen to highly increase the production of NO, whereas

$\alpha$ -LA treatment significantly blocked its formation (Fig. 2B). Successively, the effects of  $\alpha$ -LA on NOS expression were analyzed, considering its role as predominant pro-inflammatory enzyme and its correction in LPS-stimulated BV-2 cells. Furthermore, western blotting data indicated that  $\alpha$ -LA decreased the expression levels of NOS in LPS-treated BV-2 cells (Fig. 2C). Altogether, our results imply that  $\alpha$ -LA reduced the enhanced LPS-induced ROS generation and that it may inhibit NO formation, downregulating its expression.

### **$\alpha$ -LA inhibits both phosphorylation of ERK and p38, and the activation of NLRP3 inflammasome and NF- $\kappa$ B in LPS-induced BV-2 cells**

We examined whether the repressive effect of  $\alpha$ -LA is a result of the MAPK signaling pathways. Following the identification of an inhibition of the LPS-induced phosphorylation of both ERK and p38 by  $\alpha$ -LA (Fig. 3A), whether  $\alpha$ -LA could prevent the activation of the NF- $\kappa$ B pathway in LPS-treated BV-2 cells was explored. As shown in Fig. 3B, LPS stimulation provoked the translocation of NF- $\kappa$ B into the nucleus, whereas the  $\alpha$ -LA treatment blocked such a process. In addition, the LPS-induced phosphorylation of I $\kappa$ B $\alpha$  was suppressed in  $\alpha$ -LA-treated BV-2 microglial cells (Fig. 3C).

Previous studies have reported that the activation of NF- $\kappa$ B mediates the activation of NLRP3 inflammasomes in microglia [4], which induces pro-caspase-1 cleavage. While western blot analysis demonstrated that NLRP3, pro-caspase-1, and active caspase-1 were markedly increased in response to LPS stimulation, treatment with  $\alpha$ -LA significantly reduced their LPS-induced protein expression when compared to those in control cells (Fig. 3D). This indicated that  $\alpha$ -LA, not only reduces the immune response associated with NF- $\kappa$ B signaling, but also decreased NLRP3 inflammasome activation.

### **Effect of $\alpha$ -LA on both the M1 and M2 phenotypes in LPS-induced BV-2 cells**

Microglia phenotype is distinguished by the expression of either the surface marker or specific molecules. Specifically, both ICAM-1 (CD54) and IL-1 $\beta$  are expressed in M1-positive microglial cells, whereas the MRC1 (CD206) surface marker and Th2-related molecules, such as Arginase 1, are expressed in M2 microglial cells [2]. RT-qPCR and immunofluorescence analysis demonstrated that the transcription levels of both the ICAM-1 and IL-1 $\beta$  genes were upregulated in LPS-treated BV-2 microglial cells. However,  $\alpha$ -LA treatment effectively inhibited their expression levels (Fig. 4A and B, respectively). In contrast,  $\alpha$ -LA treatment slightly increased MRC1-positive cells in LPS-induced BV-2 cells (Fig. 4C). Similarly, the expression of [ARG1](#) mRNA was increased in all  $\alpha$ -LA treated cells when compared to LPS-induced BV-2 cells (Fig. 4D). These results indicate that  $\alpha$ -LA switched microglia cells phenotype from M1 to M2.

## DISCUSSION

Several studies have indicated that  $\alpha$ -LA exerts a neuroprotective effect in several neurologic diseases, Alzheimer's disease [8]. Moreover, LPS-induced microglial cells damage neurons by releasing ROS, NO, and other cytokines such as TNF- $\alpha$  and IL-6 [9]. ROS, a major factor in the pathological features of neurodegenerative diseases, may be linked to activation of NLRP3 inflammasome and IL-1 $\beta$  expression[10,11]. Also, elevated NO levels contributes to nitritative stress and NOS is the key enzyme responsible for NO production after exposure to LPS [12]. Our results confirmed the effect of  $\alpha$ -LA on microglial cell activation following LPS treatment, considering that the  $\alpha$ -LA treated group reported significantly decreased ROS, NO, and NOS expression and inhibition of TNF- $\alpha$  and IL-6.

Further, NLRP3 inflammasome are highly organized intracellular sensors for neuronal alarming signals, such as DAMP, LPS, and  $\beta$ -amyloid. They consist of NLRP3, ASC, and pro-caspase-1, which activate both caspase-1 and IL-1 $\beta$  when stimulated [13]. Additionally,

our group showed in previous studies that NLRP3 inflammasome expressed in LPS-stimulated BV-2 microglial cells can be reduced by drugs [14]. Surprisingly, the present study confirmed the effect of  $\alpha$ -LA on NLRP3 inflammasome activation. Specifically, our data indicated that  $\alpha$ -LA considerably reduced NLRP3, pro-caspase-1 and cleaved caspase-1. Considering that various studies suggested that NLRP3 inflammasome activate NF- $\kappa$ B, MAPK signaling, and ROS, renowned inflammatory responses [5, 16], the effect of  $\alpha$ -LA on the regulation of the NF- $\kappa$ B and MAPK signaling pathway was investigated. Other studies showed that LPS stimulates the phosphorylation of p38 and JNK not ERK, or only p38 [17,18]. In this study, LPS activated p38 and ERK MAPKs in BV-2 microglial cells as observed previously [18]. Simultaneously, our findings reported the inhibition of both NF- $\kappa$ B translocation to the nucleus and MAPK signaling molecules, such as p-ERK and p-p38, in  $\alpha$ -LA-treated BV-2 microglial cells.

Activated microglial cells present the classical activation (M1 phenotype), which is associated with pro-inflammatory mediators, including IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . In contrast, those with the alternative activation (M2 phenotype) are associated with the induction of specific proteins, such as Arg1 and the MRC1 [19]. Considering that microglial activation is important in several chronic neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease [20], monitoring microglial activation may represent an innovative proposal for the treatment of neurodegenerative disease. Moreover, the NLRP3 inflammasome-related components links with the changes in the polarization state of macrophage [21]. In fact, our results suggest  $\alpha$ -LA-treated LPS-stimulated BV-2 microglial cells have reduced markers of the M1 phenotype, namely the IL-1 $\beta$  and ICAM1, whereas the M2 phenotype markers, i.e., MRC1 and ARG1, were higher in the group treated with  $\alpha$ -LA than in that stimulated with LPS.  $\alpha$ -LA is involved in controlling M1 polarization through IL-1 $\beta$ , a key proinflammatory cytokine produced by inflammasome activation in BV-2



microglial cells. Furthermore, increased expression level of ARG1 mRNA and MRC1 protein shows that  $\alpha$ -LA inhibits NLRP3 inflammasome activation and promote M1 microglia induced by LPS toward M2 phenotype.

In summary, the current study suggests that  $\alpha$ -LA both reduces NLRP3 inflammasome activation, NF- $\kappa$ B, MAPK signaling, and pro-inflammatory cytokine release in LPS-induced BV-2 microglial cells and modulates microglial cells M1/M2 polarization. Although the mechanism underlying the regulation of NLRP3 inflammasome activation by microglial cells polarization is yet to be fully elucidated, our study indicates that  $\alpha$ -LA may represent a candidate drug for the treatment of both inflammatory and chronic neurodegenerative diseases.

## MATERIALS AND METHODS

### Materials

Both  $\alpha$ -lipoic acid and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). In contrast, Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Hyclone (Logan, UT, USA). Penicillin (100 U/ml)/streptomycin (100  $\mu$ g/ml) was purchased from Gibco (Life Technologies Inc., Gaithersburg, MD, USA), and heat-inactivated fetal bovine serum (FBS) was obtained from Pan Biotech (Aidenbach, Germany). Both TNF- $\alpha$  and IL-6 were quantitatively measured through an enzyme-linked immunosorbent assay (ELISA) using the mouse TNF- $\alpha$  and IL-6 DuoSet ELISA kit (R&D systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Additionally, intracellular ROS production was estimated with H<sub>2</sub>DCF-DA from molecular probes (Carlsbad, OR, USA). The following antibodies were used for western blotting: anti-NOS, anti-ERK, anti-pERK, anti-p38, anti-p-p38, anti-NF- $\kappa$ B, anti-I $\kappa$ B $\alpha$ , anti-p-I $\kappa$ B $\alpha$ , anti-NLRP3 (Cell Signaling Technology, Beverly, MA, USA), anti-caspase-1 (p20),

anti-ASC (AdipoGen life science, [USA](#)) anti-JNK, anti-p-JNK, anti-caspase-1, anti- $\beta$ -actin and anti-Lamin B1 (Santacruz Biotechnology, Santa Cruz, CA, USA). In contrast, the antibodies employed for immunofluorescence labeling analysis are the anti-Mannose receptor, goat anti-mouse IgG H&L Alexa Fluor® 488, and goat anti-rabbit IgG H&L Alexa Fluor® 488 (Abcam, Milton, Cambridge, UK). All cells were counterstained with DAPI (Thermo Fisher Scientific, Pittsburgh, PA, USA).

### Cell Culture

The BV-2 microglial cell line was obtained from the Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine (Seoul, South Korea) and was maintained in DMEM with penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and 10% FBS at 37 °C. BV-2 cells were incubated in a humidified atmosphere containing 5% CO<sub>2</sub>.

### MTT Assay

BV-2 microglial cells were seeded in a 96-well plate ( $1 \times 10^4$  cells/well) and cultured overnight. Successively, they were incubated with LPS (1  $\mu$ g/ml) for 30 min, prior to the addition of the  $\alpha$ -lipoic acid (100, 200, 500, and 1000  $\mu$ M). After incubation for 24 h, cells were assayed for cell viability by adding the Ez-Cytox reagent (20  $\mu$ l/well) and further incubated for 1 h at 37 °C. Thereafter, the 96-well plate was measured with an enzyme-linked immunosorbent assay microplate reader (Molecular Device, Sunnyvale, CA, USA) at 450 nm.

### Enzyme-Linked Immunosorbent Assay

BV-2 microglial cells were seeded in a 6-well plate ( $5 \times 10^5$  cells/well) and incubated with LPS (1  $\mu$ g/ml) for 30 min, followed by treatment with  $\alpha$ -LA (500  $\mu$ M). After incubation for 24 h, cell-free supernatants were collected and both TNF- $\alpha$  and IL-6 levels were measured in

the extracellular medium with the mouse TNF- $\alpha$  and IL-6 DuoSet ELISA kit, according to the manufacturer's instructions. Absorbance was measured at 450 nm using the enzyme-linked immunosorbent assay microplate reader. All assays were performed in three independent experiments. The concentration of both TNF- $\alpha$  and IL-6 was calculated employing the standard value obtained from a linear regression equation.

### **Measurement of ROS through Flow Cytometry**

The measurement of DCF was performed to estimate the generation of intracellular ROS in BV-2 microglial cells. Briefly, BV-2 microglial cells were seeded in a 6-well plate ( $5 \times 10^5$  cells/well) and cultured overnight. Thereafter, they were incubated with LPS (1  $\mu$ g/ml) for 30 min prior to the treatment of  $\alpha$ -LA (500  $\mu$ M). After incubation for 24 h, H<sub>2</sub>DCF-DA was treated during the last hour of incubation. Successively, cells were harvested and washed twice with PBS. Finally, ROS levels were analyzed through flow cytometry (NOVOCYTE flow cytometer, ACEA Biosciences Inc). Data were analyzed using the Novoexpress software (ACEA Biosciences Inc).

### **Measurement of Nitric Oxide through a Biochemical Assay**

The NO concentration in the conditioned culture medium was indirectly assessed by measuring the amount of generated nitrite. BV-2 microglial cells were seeded in a 6-well plate ( $5 \times 10^5$  cells/well) and incubated with LPS (1  $\mu$ g/ml) for 30 min, followed by treatment with  $\alpha$ -LA (500  $\mu$ M) for 24 h. Cell-free conditioned culture medium was collected and measured using the nitric oxide detection kit, based on the Griess method.

### **Western Blot Analysis**

Following the treatment with both LPS (1  $\mu$ g/ml) and  $\alpha$ -LA (500  $\mu$ M) at their specific times,

BV-2 microglial cells pellets were lysed using either the cytosolic extraction or RIPA buffer. Thereafter, protein samples (30 µg/lane) were separated by 10-12% SDS-polyacrylamide gel. After transfection at the nitrocellulose membrane, they were incubated overnight at 4 °C with the primary antibody, which was followed by incubation for 1 h at room temperature with either the anti-rabbit IgG HRP or the anti-mouse IgG HRP secondary antibodies. The membrane was developed using the enhanced chemiluminescence detection system (Vilber Lourmat, Marne la Vallee, France).

### **Immunofluorescence Analysis**

BV-2 microglial cells were grown on coverslips and treated with LPS (1 µg/ml) for 30 min followed by treatment with  $\alpha$ -LA at the indicated times. Thereafter, cells were fixed with 4% paraformaldehyde at room temperature for 10 min and permeabilized with 0.25% Triton X-100 at room temperature for additional 10 min. Successively, they were blocked with 1% BSA, 22.52 mg/ml glycine in 0.1% PBST solution for 30 min at room temperature. Subsequently, the coverslips containing cells were incubated with the anti-MRC1 (Abcam, Milton, Cambridge, UK) antibody at 4 °C overnight and the corresponding secondary antibody at room temperature for an additional hour. Finally, nuclei were counterstained with DAPI and cells were observed through a fluorescence microscope (Eclipse Ts2R, Nikon, Tokyo, Japan).

### **Real-Time Quantitative PCR analysis**

BV-2 microglial cells were harvested and lysed in the TRIzol (Life Technologies) reagent, according to the manufacturer's instructions, and quantified using the NanoDrop™ One. The synthesis of the cDNA template was performed with the DiaStar™ 2X RT Pre-Mix (SolGent). Quantitative RT-PCR (qRT-PCR) was accomplished using GAPDH as an internal control to

normalize the expression level. Real-time PCR was conducted in a CFX96™ real-time system employing the SsoAdvanced™ Universal SYBR® Green Supermix for cDNA quantification. Furthermore, the following primers were used: IL-1 $\beta$  (Forward- GCCCATCCTCTGTGACTCAT, Reverse- AGGCCACAGGTATTTTGTCG), ICAM-1 (Forward- AGCACCTCCCCACCTACTTT, Reverse- AGCTTGCACGACCCTTCT AA), ARG1 (Forward- GTGAAGAACCCACGGTCTGT, Reverse- CTGGTTGTCA GGGGAGTGTT) and GAPDH (Forward- TGTCCGTCGTGGATCTGAC, Reverse- CCTGCTTCACCACCTTCTTG)

### Statistical Analyses

Data were presented as means  $\pm$  standard error and were representative of three independent experiments. SPSS statistical software package (Version 18.0, Chicago, IL, USA) was used for analysis of variance (ANOVA), as appropriate. Additionally, individual differences among each group were compared through one-way ANOVAs, followed by the Scheffe method and Dunnett T3 method. A threshold of  $p < 0.05$  was considered statistically significant.

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### CONFLICTS OF INTEREST

The authors declare that there are no conflict of interest.

## FIGURE LEGENDS

### **Figure. 1. $\alpha$ -LA inhibits the expression of pro-inflammatory cytokines in LPS-treated BV-2 microglial cells**

(A) Effects of  $\alpha$ -LA on cell viability. BV-2 microglial cells were incubated with LPS (1  $\mu$ g/ml) for 30 min followed by treatment with the indicated concentrations of  $\alpha$ -LA for 24 h. Thereafter, cell viability was assessed through the MTT assay. (B and C) BV-2 microglial cells were treated with LPS (1  $\mu$ g/ml) for 30 min followed by treatment with the indicated concentrations of  $\alpha$ -LA at the suggested times. The cell-free conditioned culture medium was collected and analyzed with ELISA for TNF- $\alpha$ , IL-6. Data from three independent experiments are presented as means  $\pm$  S.D. \* $\leq$ 0.05, \*\* <0.01, \*\*\* <0.001 and are related to both LPS-induced cells and  $\alpha$ -LA treated cells.

### **Figure. 2. Effects of $\alpha$ -LA on both the production of ROS and NO, and the expression of NOS in LPS-stimulated BV-2 microglial cells**

(A) LPS-induced ROS levels were measured through flow cytometry. H<sub>2</sub>DCF-DA was treated during the last hour of incubation with both LPS (1  $\mu$ g/ml) and  $\alpha$ -LA. (B) Cells were pretreated with LPS (1  $\mu$ g/ml) followed by  $\alpha$ -LA for 24 h, while the Griess reagent was used to measure NO. (C) NOS expression levels were detected by western blotting, while  $\beta$ -actin was used as the internal control. Densitometric analysis showed the suppression of NOS protein expression compared with LPS-induced BV-2 cells. Data from three independent experiments are presented as means  $\pm$  S.D. \* $p$ <0.05, \*\* $p$ <0.01 and \*\*\* $p$ <0.001 and are related to both LPS-induced cells and  $\alpha$ -LA treated cells.

**Figure. 3. Effects of  $\alpha$ -LA on ERK, p38, NLRP3 inflammasome expression and NF- $\kappa$ B translocation in LPS-induced BV-2 microglial cells.**

BV-2 microglial cells were stimulated with LPS (1  $\mu$ g/ml) for 30 min followed by treatment with the indicated concentrations of  $\alpha$ -LA for 2 hours. (A) BV-2 microglial cells were lysed to whole lysates. Activation of ERK and p38 was detected by western blotting. (B) The translocation of NF- $\kappa$ B was also analyzed through western blotting. BV-2 microglial cells were lysed to cytosolic extracts and nucleic extracts, while both  $\beta$ -actin and Lamin-B1 were used as internal controls. (C) LPS and  $\alpha$ -LA treatment was performed as described in the above legends. Finally, I $\kappa$ -B $\alpha$  phosphorylation was detected by western blotting. (D) The western blot was performed to investigate the presence of NLRP3, pro-caspase-1 and cleaved caspase-1.

**Figure. 4.  $\alpha$ -LA inhibits the expression of pro-inflammatory cytokines in M1 polarized BV-2 microglial cells following LPS treatment.**

The effects of  $\alpha$ -LA on microglial polarization was analyzed through both immunofluorescence and RT-qPCR. BV-2 microglial cells were treated with LPS (1  $\mu$ g/ml) for 30 min and by indicated concentrations of  $\alpha$ -LA at the suggested times. (A, B and D) The cytokine IL-1 $\beta$ , the cell surface marker ICAM1 and ARG1 mRNA expression in the total RNA were assessed through real-time quantitative PCR. (C) BV-2 microglial cells were visualized using an anti-MRC1 antibody and an Alexa Fluor® 488-linked secondary antibody. Successively, coverslips were detected via fluorescence microscopy. Scale bar = 100  $\mu$ m.

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[Fig. 1]

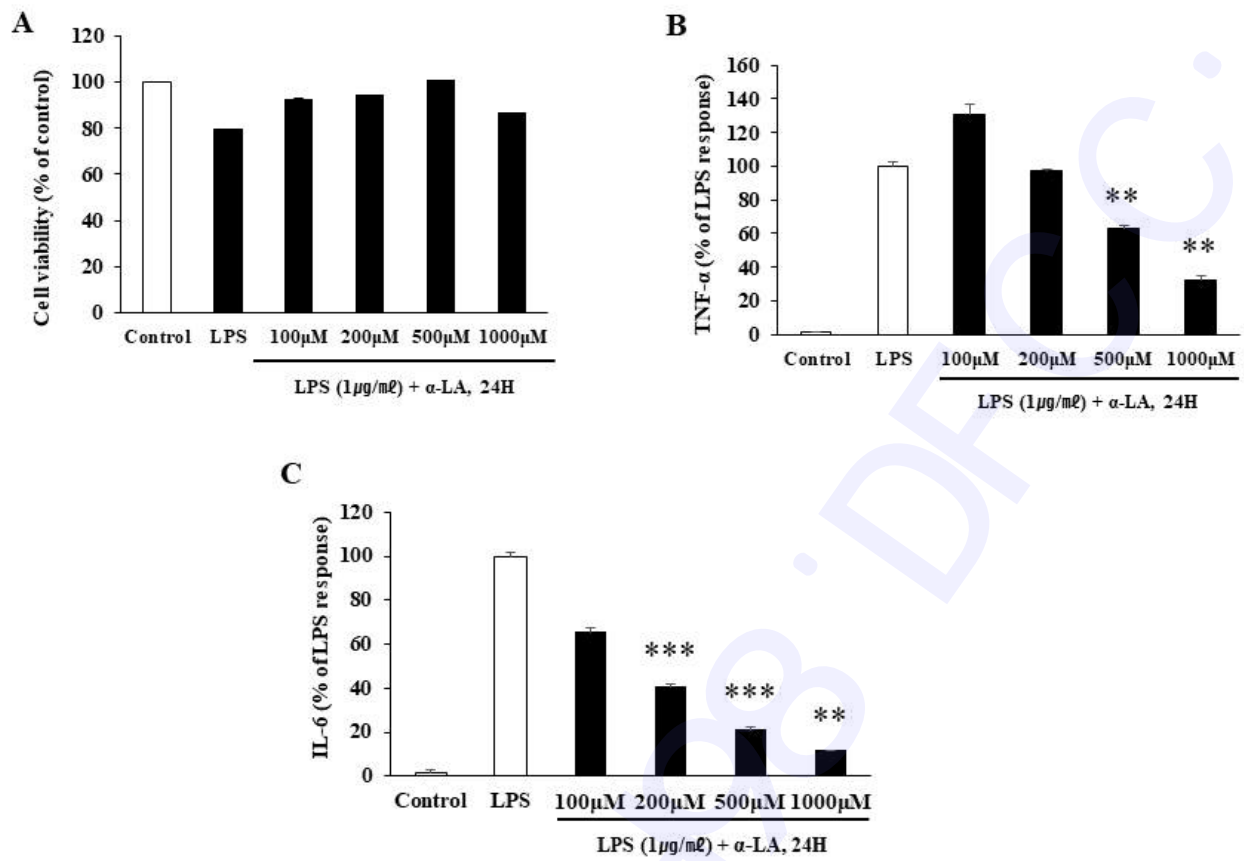


Fig. 1. Figure. 1.

[Fig. 2]

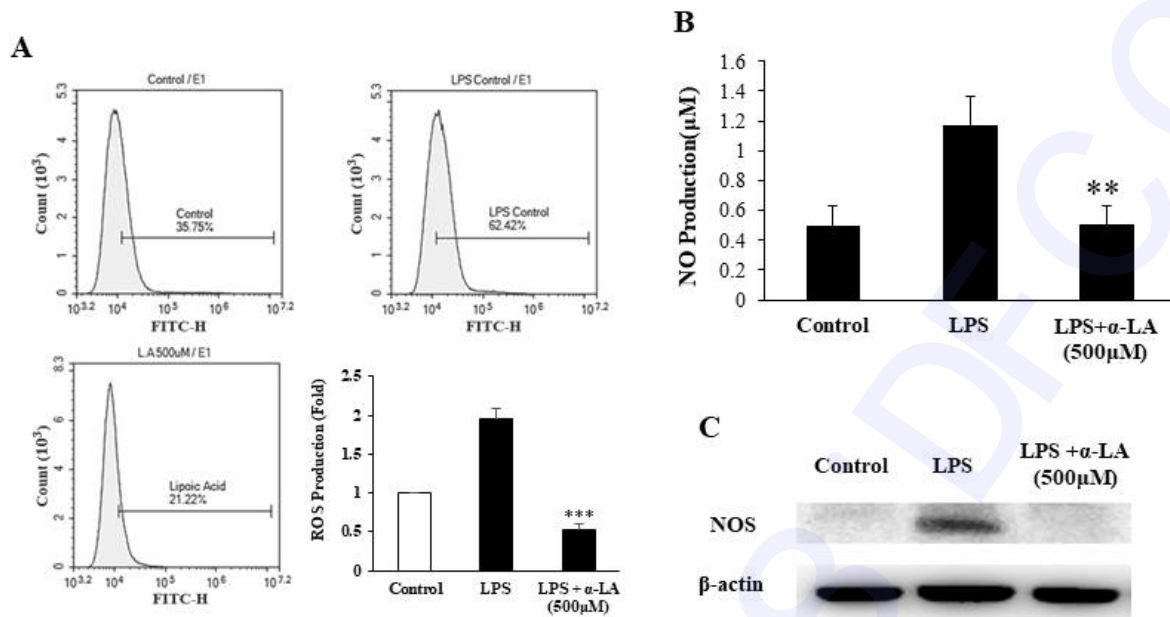


Fig. 2. Figure. 2. Effects of

[Fig. 3]

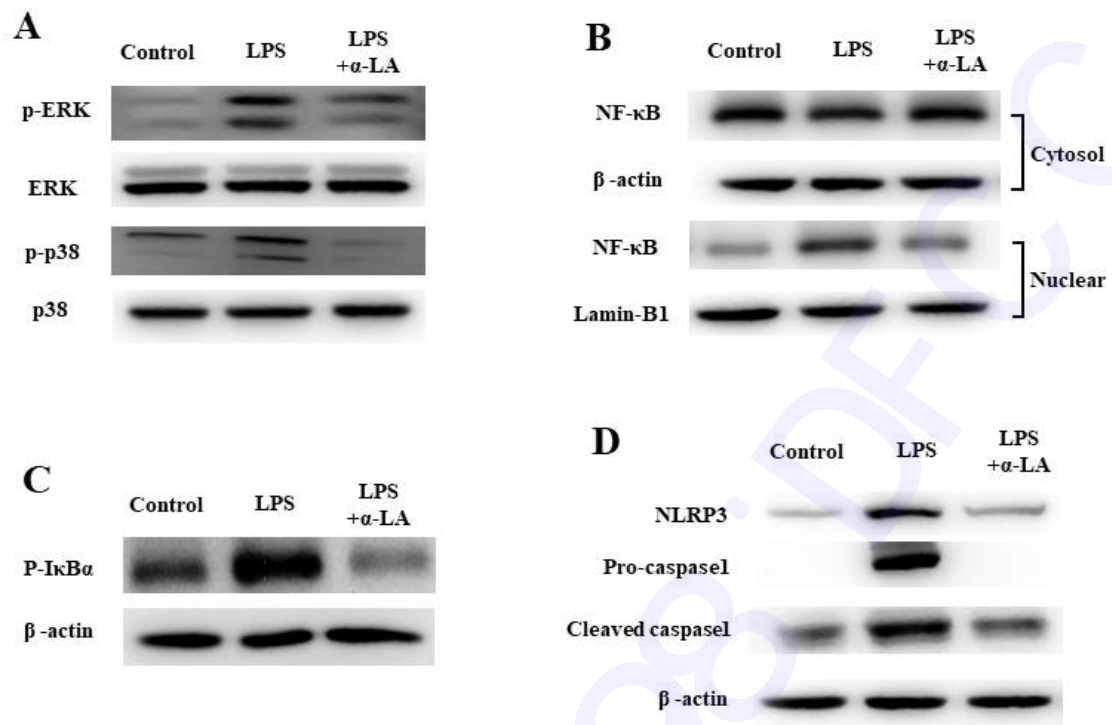


Fig. 3. Figure. 3. Effects of

[Fig. 4]

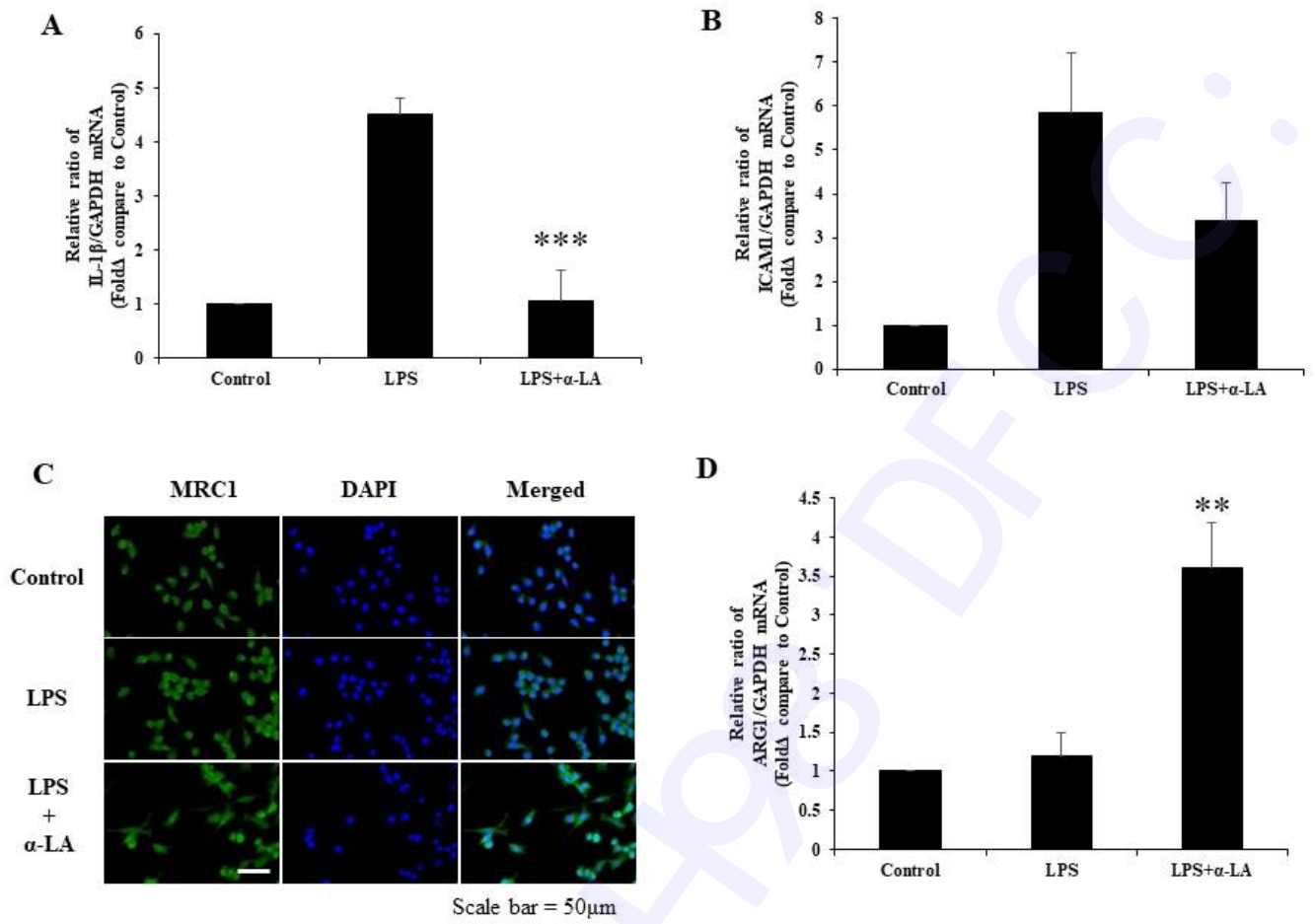


Fig. 4. Figure. 4.