

Immunostimulatory activity of *Syneilesis palmata* leaves through macrophage activation via TLR2/4-dependent p38 activation and macrophage autophagy via TLR2/4 stimulation

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Abstract

Syneilesis palmata (Thunb.) Maxim (*S. palmata*) has been used as a traditional medicinal plant and vegetable. It has been found that *S. palmata* has anti-inflammatory activity, anti-cancer activity, and anti-HIV-1 activity. However, there are no studies on the immunostimulatory activity of *S. palmata*. Thus, in this study, we report that *S. palmata* leaves (SPL) induce the activation of macrophages. SPL increased the production of immunostimulatory factors and phagocytosis in RAW264.7 cells. TLR2/4 inhibition blocked SPL-mediated production of immunostimulatory factors and phagocytosis. In addition, the p38 inhibition reduced the SPL-mediated production of immunostimulatory factors, and the SPL-mediated p38 activation was blocked by the TLR2/4 inhibition. SPL increased the level of LC3-II and p62/SQSTM1. TLR2/4 inhibition blocked SPL-mediated increase in the level of LC3-II and p62/SQSTM1. These findings indicate that SPL may activate macrophage activation through TLR2/4-mediated activation of p38, and activate macrophage autophagy through TLR2/4 stimulation.

Introduction

Due to the recent COVID-19 pandemic, interest in strengthening the immune system continues to grow. It has recently been reported that improving the aging immune system can reduce death and long-term disability caused by COVID-19 [1]. In particular, numerous studies have shown that strengthening innate immunity is considered an important defender against COVID-19 because innate immunity can increase resistance to COVID-19 and prevent severe diseases caused by COVID-19 [2].

The intake of vegetables is known to protect the human body from foreign pathogens by exhibiting a long-lasting immune-boosting effect [3]. *Syneilesis palmata* (Thunb.) Maxim (*S. palmata*) is an herbaceous perennial plant belonging to the Asteraceae family and has been used as a traditional medicinal plant and vegetable for a long time in Korea. In terms of medicinal properties, the young leaves, and flowers of *S. palmata* have been used to treat pain, arthritis, gout, back pain, and bruises. In addition, *S. palmata* was used for the purpose of improving blood circulation [4, 5]. So far, through various pharmacological activity studies of *S. palmata*, it has been found that *S. palmata* has anti-inflammatory activity [4], anti-cancer activity [5, 6], and anti-HIV-1 activity [7]. However, there are no studies on the immunostimulatory activity of *S. palmata*. Thus, in this study, we report that *S. palmata* induces the activation of macrophages, which are innate immune cells.

Materials And Methods

Chemical reagents

MTT for the evaluation of the cytotoxicity, neutral red for the evaluation of macrophage phagocytosis, and Griess for the evaluation of NO production were purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemical inhibitors such as TAK-242 (TLR4 inhibitor), C29 (TLR2 inhibitor), PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), and SP600125 (JNK inhibitor) were purchased from Sigma-Aldrich.

The primary antibody such as p-p38, LC3, p62/SQSTM1, and β -actin and the secondary antibody such as anti-rabbit IgG, HRP-linked antibody were purchased from Cell Signaling (Beverly, MA, USA).

SPL preparation

S. palmata leaves were provided by Forest Medicinal Resources Research Center, Korea. After grinding *S. palmata* leaves, 10 g of the powder was immersed in 200 ml of distilled water and left at 40°C for 24 h. After extraction for 24 h, the extracts were centrifuged at 15,000 rpm at 4 °C for 10 min, and then the clear supernatant was freeze-dried. The freeze-dried extracts from aerial parts of *S. palmata* leaves (SPL) were dissolved in distilled water for treatment to the cells and stored at -80 °C.

Cell line and cell culture

RAW264.7 cells, which are mouse macrophages, were purchased from the American Type Culture Collection (Manassas, VA, USA). RAW264.7 cells were cultured in a CO₂ incubator (5% CO₂, 37 °C, Humidified atmosphere) with DMEM/F-12 medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Measurement of the cytotoxicity

To evaluate the cytotoxicity of SPL, an MTT assay was performed. Briefly, RAW264.7 cells (5×10^5 cells/ml) cultured for 24 h in a 96-well plate were treated with SPL, and then further cultured in an incubator (5% CO₂, 37 °C, Humidified atmosphere) for 24 h. After 24 h, 50 µl of MTT solution (1 mg/ml) was added to each well and then incubated in an incubator (5% CO₂, 37 °C, Humidified atmosphere) for 4 h. After 4 h, the culture medium was removed, and then 100 µl of dimethyl sulfoxide was added to each well to dissolve the purple formazan. The absorbance of dissolved purple formazan was measured at 570 nm using a UV/Visible spectrophotometer (Human Cop., Xma-3000PC, Seoul, Korea).

Measurement of NO production

To evaluate the effect of SPL on NO production, a Griess assay was performed. Briefly, RAW264.7 cells (5×10^5 cells/ml) cultured for 24 h in a 96-well plate were treated with SPL, and then further cultured in an incubator (5% CO₂, 37 °C, Humidified atmosphere) for 24 h. After 24 h, the cell culture medium and Griess solution were mixed in a 1:1 ratio and left at room temperature for 15 min. After 15 min, the absorbance of the mixture was measured at 540 nm using a UV/Visible spectrophotometer (Human Cop., Xma-3000PC).

Measurement of phagocytosis

To evaluate the effect of SPL on phagocytosis, a neutral red assay was performed. Briefly, RAW264.7 cells (5×10^5 cells/ml) cultured for 24 h in a 96-well plate were treated with SPL, and then further cultured in an incubator (5% CO₂, 37 °C, Humidified atmosphere) for 24 h. After 24 h, the cells were cultured with

0.01% of neutral red solution in an incubator (5% CO₂, 37 °C, Humidified atmosphere) for 2 h. After 2 h, the cells were washed with 1 X PBS three times and then treated with lysis buffer (50% ethanol acid: 1% acetic acid = 1:1) at room temperature for 2 h to elute the neutral red adsorbed on the cells. The absorbance of the eluted neutral red was measured at 540 nm using a UV/Visible spectrophotometer (Human Cop., Xma-3000PC).

SDS-PAGE and Western blot analysis

The total protein extracted from RAW264.7 cells was quantified using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA USA). The protein was separated on 12% SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Thermo Fisher Scientific). After blocking at room temperature for 1 h, the membranes were incubated with the primary antibodies (1:1,000) at 4 °C overnight. After that, the membranes were incubated with anti-rabbit IgG, HRP-linked antibody at room temperature for 1 h. After treating ECL Western blotting substrate on the membrane, the protein band was visualized using LI-COR C-DiGit Blot Scanner. Quantitative analysis of visualized protein bands was performed using the software UN-SCAN-IT gel version 5.1 (Silk Scientific Inc. Orem, UT, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total mRNA from RAW264.7 cells was extracted using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Extracted mRNA was quantified using a GeneQuantTM 1300 (Biochrom, Cambridge, England). Total mRNA (1 µg) was reverse transcribed into cDNA using a Verso cDNA Kit (Thermo Scientific). The PCR amplification of the cDNA was performed using a PCR Master Mix Kit (Promega, Madison, WI, USA). The sequences of primers used in the PCR amplification of the cDNA were as follows: iNOS: forward, iNOS, IL-1β, TNF-α, and GAPDH were as follows : iNOS: forward 5'-ttgtgcatcgacctaggctggaa-3' and reverse 5'-gacctttcgattagcatggaagc-3', IL-1β: forward 5'-ggcaggcagtatcactcatt-3' and reverse 5'-cccaaggccacaggtattt-3', TNF-α: forward 5'-tggaactggcagaagaggca-3' and reverse 5'-tgctcctccacttggtggtt-3', GAPDH: forward 5'-ggactgtggtcatgagcccttcca-3' and reverse 5'-actcacggcaaattcaacggcac-3'. The PCR bands were visualized using agarose gel electrophoresis. The density of the PCR bands was calculated using the software UN-SCAN-IT gel version 5.1 (Silk Scientific Inc. Orem).

Statistical analysis

All the data are shown as mean ± SD (standard deviation). Statistical significance was determined by Student's t-test. Differences with *P <0.05 and #P <0.05 were considered statistically significant.

Results And Discussion

Effect of SPL on macrophage activation in RAW264.7 cells

The immune system that protects the human body from foreign pathogens consists of an innate immune response and an adaptive immune response [8]. Innate immune cells such as neutrophils, macrophages, and dendritic cells recognize and eliminate foreign pathogens in the early stages of infection [8]. T cells and B cells that make up the adaptive immune system remove foreign pathogens by cytotoxic reaction and production of antigen-specific antibodies [8]. Among innate immune cells, macrophages are activated when foreign pathogens invade and remove foreign pathogens through phagocytosis and secretion of immunostimulatory factors such as nitric oxide (NO), inducible nitric oxide synthase (iNOS), interleukin-1 β (IL-1 β), and tumor necrosis factor- α (TNF- α), which contributes to the maintenance of tissue homeostasis [9]. In addition, it is known that activated macrophages have the antigen-presenting ability, and immunostimulatory factors secreted by activated macrophages play a positive role in the activation of T cells and B cells [10]. Therefore, the activation of macrophages is considered a major target that can strengthen the innate and adaptive immune systems. We analyzed the production of immunostimulatory factors such as NO, iNOS, IL-1 β , and TNF- α in SPL-treated RAW264.7 cells to verify the effect of SPL on macrophage activation. As shown in Fig. 1A and 1B, it was observed that the production of NO, iNOS, IL-1 β and TNF- α increased in SPL-treated cells. Since the increased secretion of these immunostimulatory factors means the activation of macrophages, we analyzed the effect of SPL on phagocytosis, an indicator of macrophage activation. As shown in Fig. 1C, SPL increased the phagocytosis of RAW264.7 cells in a concentration-dependent manner. However, SPL was found to have no cytotoxicity against RAW264.7 cells (Fig. 1D). From these results, SPL induces the activation of macrophages, so it can be used as a potential food agent for enhancing the immune response of the human body.

Effect of TLR2/4 on the production of immunostimulatory factors by SPL in RAW264.7 cells

The body's immune response upon invasion of foreign pathogens begins when pattern recognition receptors (PRRs) of innate immune cells recognize pathogen-associated molecular patterns of foreign pathogens [11]. Among the various PRRs of macrophages, Toll-like receptors (TLRs) are known to be essential for the activation of innate and adaptive immune responses [12, 13]. Among TLRs, TLR2/4 involved in the initiation of innate immune responses and the development of adaptive immunity are known to be the most specialized PRRs for the recognition of foreign pathogens [14]. In addition, stimulation of TLR2 and TLR4 in macrophages increases the secretion of immunostimulatory factors through macrophage activation [15]. Therefore, we investigated whether TLR2 and TLR4 are involved in the secretion of immunostimulatory factors by SPL. As shown in Fig. 2A, SPL increased the production of NO, iNOS, IL-1 β , and TNF- α , but inhibition of TLR2 by C29 slightly decreased the production of NO and IL-1 β by SPL. However, inhibition of TLR4 by TAK-242 markedly blocked the SPL-induced production of NO, iNOS, and IL-1 β . Interestingly, the production of TNF- α by SPL did not change with the inhibition of TLR2 and TLR4. And we investigated the effect on TLR2 and TLR4 on phagocytosis by SPL. As shown in Fig. 2B, SPL-induced macrophage phagocytosis was inhibited slightly in the inhibition of TLR2 by C29 and notably in the inhibition of TLR4 by TAK-242. From these results, TLR4 may be the major PPR involved in SPL-induced macrophage activation although both TLR2 and TLR4 are involved in SPL-induced macrophage activation.

Effect of MAPKs on the production of immunostimulatory factors by SPL in RAW264.7 cells

Mitogen-activated protein kinases (MAPKs) are known to play an important role in the human immune system by participating in TLR signaling. Especially, MAPKs contribute to the activation of macrophages and the production of immunostimulatory factors [16, 17]. Among the three types of MAPKs such as extracellular signal-regulated protein kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal Kinase (JNK), p38 and ERK have been reported to play an important role in the production of immunostimulatory factors [18, 19]. Thus, we investigated whether MAPKs are involved in the production of immunostimulatory factors by SPL. As shown in Fig. 3A, inhibition of ERK1/2 by PD98059 had no effect on the production of NO, iNOS, IL-1 β , and TNF- α by SPL. Inhibition of p38 by SB203580 significantly reduced the production of NO, iNOS, and IL-1 β by SPL, but not the production of TNF- α . Among NO, iNOS, IL-1 β , and TNF- α induced by SPL, inhibition of JNK by SP600125 only inhibited NO production. From these results, p38 is considered to be a major signaling pathway related to the production of immunostimulatory factors by SPL. Thus, we investigated whether SPL activates p38 and whether TLR2 and TLR4 are involved in SPL-induced p38 activation. As shown in Fig. 3B, phosphorylation of p38, the active form of p38, increased from 5 min after SPL treatment, reached a maximum at 1 h, and continued to decrease thereafter, which indicates that SPL activates p38. And both inhibition of TLR2 by C29 and the inhibition of TLR4 by TAK-242 decreased the phosphorylation of p38 induced by SPL (Fig. 3C). From these results, SPL may increase the production of immunostimulatory factors through TLR2/4-mediated p38 activation.

Effect of SPL on macrophage autophagy in RAW264.7 cells

Autophagy is known to be closely related to the enhancement of innate immunity because it is involved in the activation of macrophage functions such as antigen recognition, antigen presentation, and phagocytosis [20]. In autophagy, microtubule-associated protein 1A/1B-light chain 3 (LC3) is widely used to monitor autophagosome and autophagic activity and plays a role in recruiting cargo to autophagosomes [21]. It is known that the amount of LC3-II reflects the number of autophagosomes and autophagy-related structures because LC3-II formed by conjugation of LC3-I with PE is localized to autophagosomes [22]. p62/sequestosome 1 (SQSTM1) is a selective autophagy receptor and plays a role in the delivery of a wide range of ubiquitinated cargo to the autophagy pathway [23]. Thus, the increase in the conversion of LC3-I to LC3-II and the expression of p62/SQSTM1 are known to be major indicators of autophagy. Therefore, to investigate whether SPL induces macrophage autophagy, we measured changes in LC3-II and p62/SQSTM1 in SPL-treated RAW264.7 cells. As shown in Fig. 4A, the level of LC3-II increased from 15 min of SPL treatment, reached a maximum at 3 h of SPL treatment, and then decreased thereafter. However, p62/SQSTM1 increased from 3 h of SPL treatment, reached a maximum at 10 h of SPL treatment, and decreased slightly thereafter. In addition, SPL dose-dependently increased the levels of LC3-II and p62/SQSTM1 (Fig. 4B). The autophagy process of macrophages is activated during the invasion of foreign pathogens, and it is reported that TLRs play an essential role in the formation of autophagosomes [17]. Especially, TLR4-mediated autophagy of macrophages is known to enhance the innate and adaptive immune responses of the human body by enhancing the antigen processing and antigen presentation functions of macrophages [24]. Thus, we investigated whether

TLR2/4 is involved in SPL-mediated increases in LC3-II and p62/SQSTM1. As shown in Fig. 4C, the SPL-mediated increase in LC3-II was reduced by inhibition of TLR2 by C29 and TLR4 by TAK-242. On the other hand, the increase of p62/SQSTM1 by SPL was reduced only by TLR4 by TAK-242. From these results, it is thought that SPL may induce autophagy by stimulating TLR2/4.

Conclusions

Whenever an infectious disease such as COVID-19 occurs, interest in strengthening the immune system of the human body is increasing. Although the optimal strategy to fight these infectious diseases is considered to be vaccine development, the natural enhancement of the immune system through diet has also been regarded as an important defense strategy. Vegetables are widely used food ingredients to boost the immune system of the human body. *S. palmata* leaves (SPL) is a vegetable widely used for food in Korea. In this study, SPL increased macrophage-released immunostimulatory factors such as NO, iNOS, IL-1 β , and TNF- α through TLR2/4-mediated activation of p38 and activated macrophage autophagy through TLR2/4 stimulation in RAW264.7 cells. These findings indicate that SPL has immunostimulatory activity. Thus, it is believed that SPL can be used as a functional ingredient for the development of health functional foods that enhance the immune system. However, animal-based preclinical studies related to SPL's immunostimulatory activity are required.

Declarations

Author Contributions S.J.P. performed the experiments and wrote the manuscript, J.H.Y. and M.Y.C. performed the experiments, J.W.L. performed the experiments and revised the manuscript, J.B.J. conceived the article, wrote, and revised the manuscript.

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Data Availability Not applicable.

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication All authors have given their consent for publication.

Conflict of Interest Authors have no potential conflicts of interest to disclose.

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Figures

Figure 1

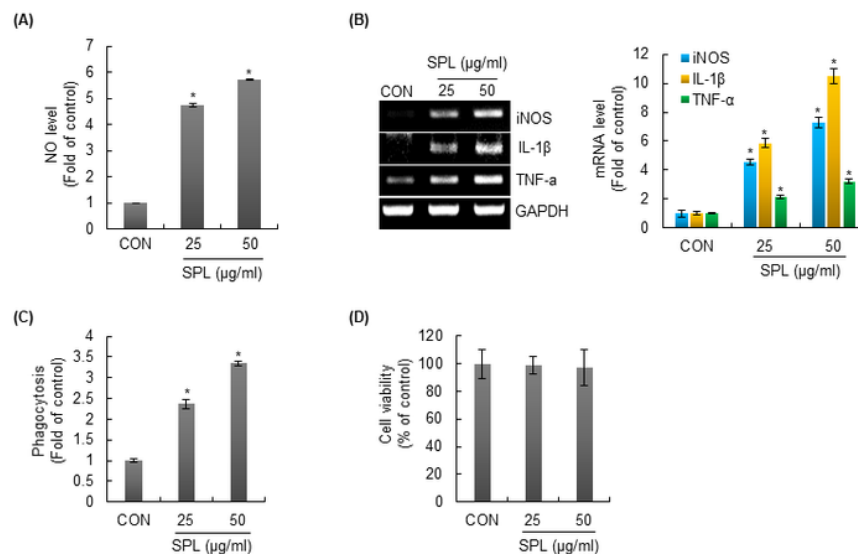


Figure 1

Effect of SPL on macrophage activation in RAW264.7 cells. RAW264.7 cells were treated with SPL for 24 h. NO level (A), mRNA level (B), phagocytotic activity (C), and cell viability (D) were measured by Griess assay, RT-PCR, neutral red assay, and MTT assay, respectively.

Figure 2

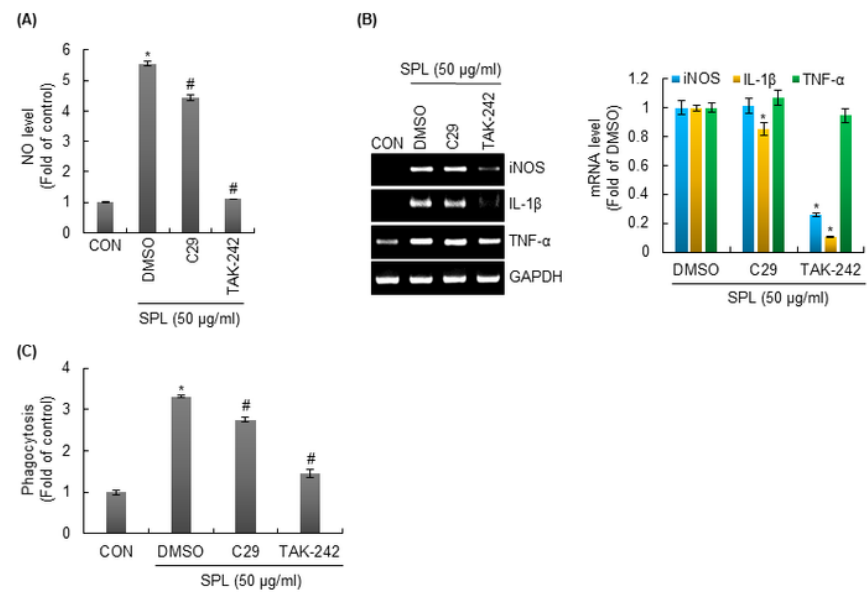


Figure 2

Effect of TLR2/4 on SPL-mediated macrophage activation in RAW264.7 cells. RAW264.7 cells were pretreated with C29 (TLR2 inhibitor, 100 μ M) or TAK-242 (TLR4 inhibitor, 5 μ M) for 2 h and co-treated with SPL for 24 h. NO level (A) and mRNA level (B), and phagocytotic activity (C) were measured by Griess assay, RT-PCR, and neutral red assay, respectively.

Figure 3

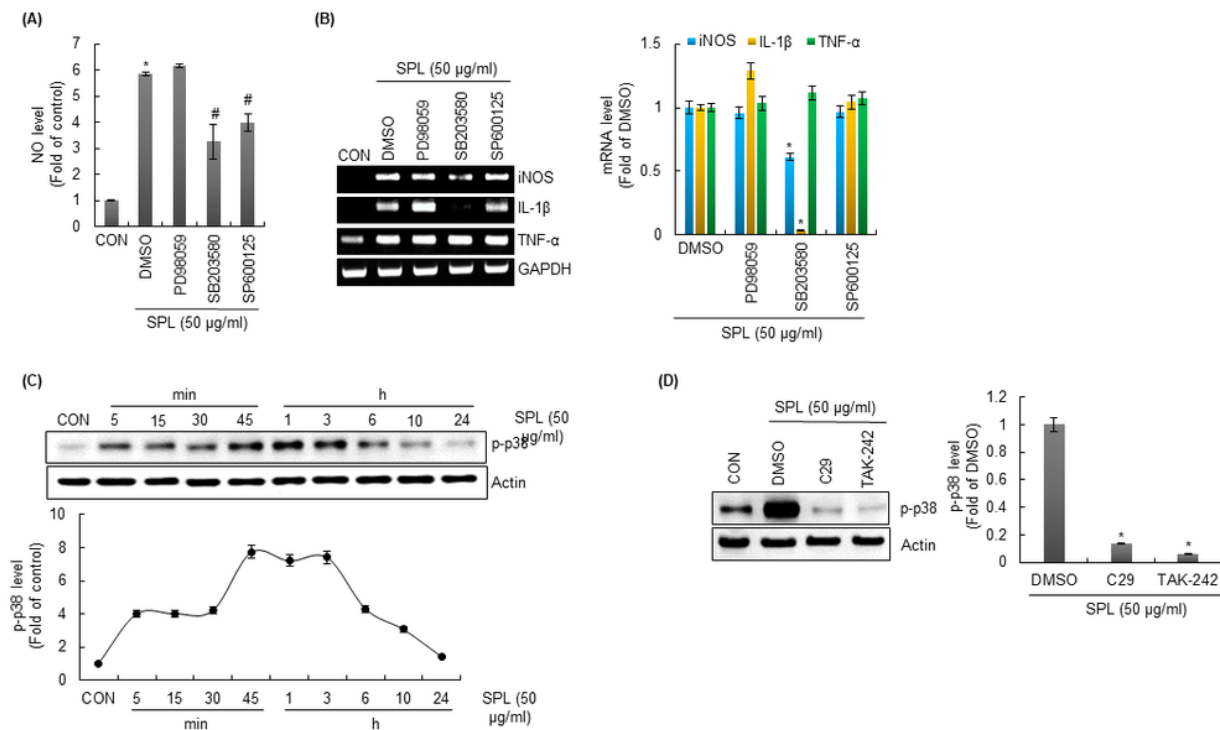


Figure 3

Effect of MAPK signaling pathway on SPL-mediated production of immunostimulatory factors in RAW264.7 cells. RAW264.7 cells were pretreated with PD98059 (ERK1/2 inhibitor, 40 μ M), SB203580 (p38 inhibitor, 40 μ M), or SP600125 (JNK inhibitor, 40 μ M) for 2 h and then co-treated with SPL for 24 h. NO level (A) and mRNA level (B) were measured by Griess assay and RT-PCR, respectively. (C) RAW264.7 cells were treated with SPL for the indicated times. The protein levels were determined by Western blot analysis. Actin was used as a loading control. (D) RAW264.7 cells were pretreated with C29 (TLR2 inhibitor, 100 μ M) or TAK-242 (TLR4 inhibitor, 5 μ M) for 2 h and co-treated with SPL for 1 h. Protein levels were measured by Western blot analysis. Actin was used as a loading control.

Figure 4

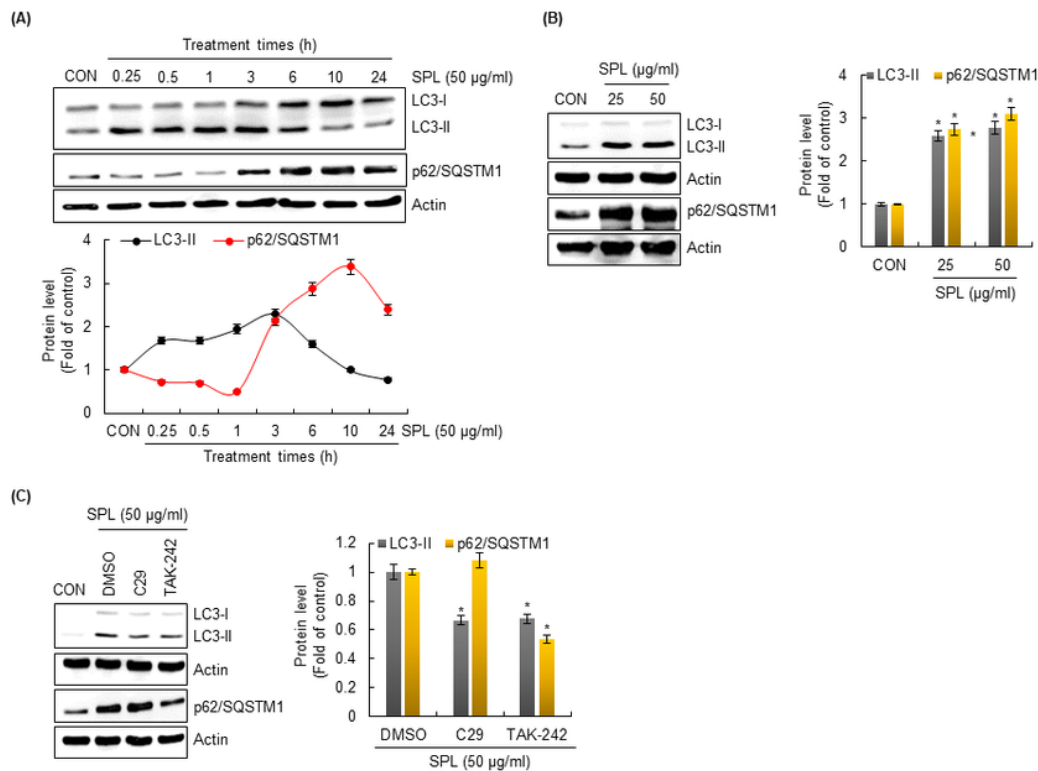


Figure 4

Effect of SPL on macrophage autophagy. (A) RAW264.7 cells were treated with SPL for the indicated times. (B) RAW264.7 cells were treated with SPL for 1 h or 6 h. (C) RAW264.7 cells were pretreated with C29 (TLR2 inhibitor, 100 µM) or TAK-242 (TLR4 inhibitor, 5 µM) for 2 h and then co-treated with SPL for 1 h or 6 h. The protein levels were determined by Western blot analysis. Actin was used as a loading control. The protein levels were determined by Western blot analysis. Actin was used as a loading control.

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