Sulforaphane reduces lipopolysaccharide-induced inflammation and enhances myogenic differentiation of mouse embryonic myoblasts via the toll-like receptor 4 and NLRP3 pathways

*Manfeng Wang[1,A,B,D,F], *Meiling Liu[1,E,F], Wanzhen Xu[2,C,F], Zongyan Teng[1,A,F], Xiaowei Wu[1,C,F], Lu Gan[1,B,F], Yina Zhang[1,A,F]

[1] Department of Geriatrics, Second Affiliated Hospital of Harbin Medical University, China
[2] Department of Neurosurgery, The First Hospital of Jilin University, Changchun, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2023;32(4):457–467

Address for correspondence
Yina Zhang
E-mail: zhangyina12345@hrbmu.edu.cn

Funding sources
None declared

Conflict of interest
None declared

* Manfeng Wang and Meiling Liu contributed equally to this work.

Received on March 20, 2022
Reviewed on June 3, 2022
Accepted on September 12, 2022

Published online on November 18, 2022

Abstract

Background. Muscle loss and muscle weakness are manifestations of infection-induced sepsis, a condition that can lead to organ failure and death. Toll-like receptor 4 (TLR4) signaling and the NLRP3 inflammasome are involved in the inflammatory storm and the development of sarcopenia during sepsis. They are also potential targets for sepsis treatment.

Objectives. To explore the effects and molecular mechanisms of sulforaphane (SFN) on sepsis-associated inflammation and sarcopenia.

Materials and methods. Mouse C2C12 embryonic myoblasts were treated with lipopolysaccharide (LPS) to simulate sepsis-induced sarcopenia. Molecular mechanisms were investigated using quantitative real-time polymerase chain reaction (qRT-PCR), western blot, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA).

Results. Sulforaphane significantly reduced the secretion of the inflammatory cytokine interleukin-1β (IL-1β) by C2C12 cells after LPS treatment, and inhibited the production of intracellular reactive oxygen species (ROS). It also increased the expression of E-myosin heavy chain, myosin ID heavy chain, and myogenin, and induced myogenic differentiation of LPS-treated C2C12 cells. Mechanistically, SFN reduced messenger ribonucleic acid and protein levels of TLR4, NLRP3, apoptosis-associated speck-like protein, and Caspase-1 in C2C12 cells, thereby inhibiting the inflammatory response and promoting myogenic differentiation. In addition, the TLR4 inhibitor TAK-242 induced myogenic differentiation in LPS-pretreated C2C12 cells in a similar manner.

Conclusions. Sulforaphane can reduce sepsis-induced inflammatory responses and enhance myogenic differentiation by regulating the TLR4 and NLRP3 inflammasome pathways.

Key words: sepsis, LPS, sulforaphane, C2C12, TLR4-NLRP3 pathway

Cite as
Wang M, Liu M, Xu W, et al. Sulforaphane reduces lipopolysaccharide-induced inflammation and enhances myogenic differentiation of mouse embryonic myoblasts via the toll-like receptor 4 and NLRP3 pathways. *Adv Clin Exp Med*. 2023;32(4):457–467. doi:10.17219/acem/155342

DOI
10.17219/acem/155342

Background

Sepsis is a life-threatening organ dysfunction caused by an unbalanced response of the body to infection following severe trauma or surgery. It can develop into multiple organ failure and even lead to death.[1,2] Muscle tissue is the main target of damage during sepsis, and this leads to the development of sepsis-related sarcopenia and myasthenia.[3,4] Due to a lack of clear diagnostic markers and early clinical indications, sarcopenia often leads to severe respiratory muscle weakness and dysfunction, prolonged mechanical ventilation time, and other severe complications such as lung infection and lower extremity thrombosis. This leads to higher care costs and increases the risk of patient death.[5,6] Therefore, an in-depth study of the pathogenesis and prevention of septic sarcopenia and myasthenia has significance in clinical practice.

The pathogenesis of septic sarcopenia and myasthenia is complicated, and the exact molecular mechanisms are not clear. It is generally believed that the mechanisms of myogenic dysfunction caused by sepsis include muscle damage by inflammatory factors, mitochondrial dysfunction, hyperactivity of the NLRP3 inflammasome, release of inducible nitric oxide, and muscle cell apoptosis.[7–10] In recent years, new research has found that the reduced muscle production capacity caused by sepsis is also involved in the occurrence of sarcopenia.[11] Skeletal muscle cells express multiple toll-like receptors (TLRs) that recognize bacterial cell wall components, such as lipopolysaccharide (LPS). In sepsis, LPS expressed by Gram-negative bacteria binds specifically to TLR4 on the surface of skeletal muscle cells to initiate intracellular signaling, thereby mediating an inflammatory response and reducing protein synthesis.[12] The TLR4 activation upregulates autophagosome formation and expression of ubiquitin ligases atrogin-1 and muscle-specific RING finger protein-1 (MuRF1), which induces muscle protein hydrolysis and mediates muscle atrophy.[13] The development of sepsis is also accompanied by the activation of the NLRP3 inflammasome.[14] The NLRP3 is highly expressed in muscle and its activity has been found to significantly increase in myopathy.[15] Huang et al. determined from muscle morphology, organ weight, gene expression, and protein content of atrogin-1 and MuRF1, that NLRP3 knockout mice with sepsis had less muscle atrophy than wild-type sepsis mice.[16] Therefore, targeting TLR4 and NLRP3 may be a viable approach to treating sepsis-related sarcopenia and myasthenia.

Sulforaphane (SFN) is the hydrolyzed active product of glucosinolate that is extracted from cruciferous vegetables, such as broccoli.[17] Sulforaphane is known to have anti-inflammatory, anticancer and antioxidative stress properties.[18–20] Previous studies have shown that SFN treatment modulated the release of LPS-induced high mobility group protein B1, and thus reduced mortality in a mouse model of sepsis.[21] Moreover, SFN inhibited muscle atrophy and promoted the differentiation of C2C12 cells by increasing myosin ID heavy chain (myoD) and messenger ribonucleic acid (mRNA) levels, as well as through activating Akt/FoxO signaling.[22] Therefore, we hypothesized that SFN may alleviate the inflammation and sarcopenia caused by sepsis.

In this study, the mouse embryonic myoblast cell line C2C12 was treated with LPS to simulate sepsis-induced sarcopenia. Secretion of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS) was measured by quantitative real-time polymerase chain reaction (qRT-PCR). The expression of IL-1β and the activation of nuclear factor kappa-B (NF-κB) were measured by western blot assay, whilst the effects of LPS on reactive oxygen species (ROS) were measured using a ROS kit. In addition, the effects of different concentrations of LPS on myoblast differentiation of C2C12 cells were also observed through the assessment of morphology. The expression of *myoD* and *myogenin* was measured using qRT-PCR and western blot assay. Meanwhile, the expression of E-myosin heavy chain (E-MHC) and myogenin was determined by immunofluorescence assay.

Objectives

To investigate the effect of SFN on sepsis-related sarcopenia and uncover its molecular mechanisms.

Materials and methods

Cell culture and reagents

Mouse embryonic myoblast C2C12 cell line was obtained from Shanghai Cell Resource Center of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a 37°C, 5% CO_2 incubator. Horse serum (Beyotime Biotechnology, Shanghai, China), LPS (Solarbio, Beijing, China), SFN (HY-13755; MedChemExpress, South Brunswick, USA), and TAK-242 (T125887; Aladdin-E, Shanghai, China) were also used. For cell differentiation experiments, the conditioned medium was replaced daily with fresh DMEM medium containing 2% horse serum after the cells adhered.

Quantitative real-time polymerase chain reaction

The RNA of treated C2C12 cells was extracted using TRIpure (BioTeke, Beijing, China), and then reverse-transcribed using Super M-MLV Reverse Transcriptase Kit (BioTeke) to obtain the corresponding complimentary deoxyribonucleic acid. The expression levels of mRNA

Table 1. Primers used in qPCR

Primer	Forward sequence	Reverse sequence
IL-6	ATGGCAATTCTGATTGTATG	GACTCTGGCTTTGTCTTTCT
TNF-α	CAGGCGGTGCCTATGTCTCA	GCTCCTCCACTTGGTGGTTT
iNOS	CACCACCCTCCTCGTTC	CAATCCACAACTCGCTCC
myoD	TCTATGATGACCCGTGTTTCG	TGCACCGCAGTAGGGAAGT
Myogenin	GAATGCAACTCCCACAGCG	AGGCAACAGACATATCCTCCA
TLR4	AGCAGGTGGAATTGTATCGC	TCAGGTCCAAGTTGCCGTTT
NLRP3	GAGTTCTTCGCTGCTATGT	ACCTTCACGTCTCGGTTC
ASC	TCTGGAGTCGTATGGCTTGG	TGCTTGCCTGTGCTGGTC
Caspase-1	CAGAACAAAGAAGATGGCACA	CCAACCCTCGGAGAAAGA
β-actin	CTGTGCCCATCTACGAGGGCTAT	TTTGATGTCACGCACGATTTCC

qPCR – quantitative polymerase chain reaction; IL – interleukin; TNF-α – tumor necrosis factor alpha; iNOS – inducible nitric oxide synthase; myoD – myosin ID heavy chain; TLR4 – toll-like receptor 4; ASC – apoptosis-associated speck-like protein.

in C2C12 cells were determined by use of the Exicycler™ 96 fluorescent quantitative PCR system (Bioneer, Daejeon, South Korea). Primer sequences of genes are listed in Table 1.

Western blotting

Total protein of the treated C2C12 cells was extracted using a protein extraction kit (Solarbio) and quantified using a bicinchoninic acid kit (Solarbio). A total of 40 μg of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyl difluoride (PVDF) membrane (Beyotime Biotechnology). The PVDF membrane was blocked with 5% skimmed milk for 1 h, and incubated with the corresponding primary antibodies overnight at 4°C. Primary antibodies used included: p-NF-κB p65 (WL02169; Wanleibio, Shenyang, China), NF-κB p65 (WL01980; Wanleibio), IL-1β (12507S; Cell Signaling Technology, Danvers, USA), myoD (ab16148; Abcam, Cambridge, UK), myogenin (ab1835; Abcam), TLR4 (WL00196; Wanleibio), NLRP3 (AG-20B-0014; Adipogen, San Diego, USA), apoptosis-associated speck-like protein (ASC) (67824T; Cell Signaling Technology), Caspase-1 (22915-1-AP; Proteintech, Wuhan, China), and β-actin (WL01845; Wanleibio). The PVDF membrane and horseradish peroxidase (HRP)-coupled secondary antibodies were incubated for 1 h at room temperature. Finally, the protein signal on the PVDF membrane was detected with an electrochemiluminescence developer (Haigene Bio, Harbin, China), and the optical density value of the target band was measured using a gel image processing system (Gel-Pro-Analyzer software; Media Cybernetics Inc., Silver Spring, USA).

Cell proliferation

The C2C12 cells in the logarithmic growth phase were cultured at a density of 3×10^4 cells/L in each well. After incubating overnight at 37°C in 5% CO_2, the cells were exposed to 0 μM, 1 μM, 5 μM, 10 μM, 20 μM, or 30 μM SFN for 6 h, 24 h, 72 h, or 120 h. Dimethyl sulfoxide (DMSO) was used as a solvent control. Each treatment group was repeated 5 times. To evaluate cell proliferation, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was used. Each well of the microplate had 20 μL of MTT solution added (Beyotime Biotechnology), and plates were incubated at 37°C for 4 h. Then, the medium was removed and formazan dissolving solution was added to all wells. The MTT colorimetric value was obtained by measuring the absorbance at 570 nm with a microplate reader (TECAN, Männedorf, Switzerland).

Enzyme-linked immunosorbent assay

The IL-6 protein content in cell culture supernatant was evaluated using an IL-6 enzyme-linked immunosorbent assay (ELISA) detection kit (Wanleibio). First, a 96-well plate was coated overnight at 4°C with the IL-6 antibody (10 μg/mL). Samples and standards were then diluted and 100 μL was added to the wells and incubated at 37°C for 2 h. Then, the liquid was discarded from wells and 100 μL of capture antibody was added. Plates were washed 3 times with 300 μL phosphate buffered saline with Tween 20 (PBST; pH 7.3, 0.5% Tween 20) washing solution, and were soaked for 2 min each wash. The HRP-Streptavidin (100 μL), 3,3',5,5'-tetramethylbenzidine (TMB) chromogenic solution (100 μL) and TMB stop solution D (50 μL) were added to the wells sequentially. The absorbance was read at 450 nm in the microplate reader, and the linear regression curve of the standard product was drawn to calculate the concentration.

Cell morphology and immunofluorescence

For the assessment of cell morphology, the medium was replaced with DMEM containing 2% horse serum in order to induce the differentiation of the C2C12 cells

when they reached 70%–80% confluence. At the same time, different dosages of LPS or SFN were added to the cells. Morphology was observed and photographed at 0 h, 24 h, 72 h, and 120 h after the differentiation had commenced. For immunofluorescence, treated cells were fixed to a microscope slide with 4% paraformaldehyde for 15 min and then washed with PBS. Next, the cells were permeabilized with 0.1% Triton X-100 at room temperature for 30 min. Goat serum was added dropwise to the cells to block nonspecific binding of the antibodies, and slides were incubated at room temperature for 15 min. Sufficient amount of E-MHC antibody (1:50) and myogenin antibody (1:500) were added to the slides, and incubated overnight at 4°C. Fluorescent secondary antibody was added and incubated for 1 h at room temperature. Cell nuclei were stained by incubating the cells with 4',6-diamidino-2-phenylindole (DAPI) for 5 min, and slides were then sealed with an anti-fluorescence quencher. Images were collected under a fluorescence microscope (Olympus IX53; Olympus Corp., Tokyo, Japan).

Reactive oxygen species detection

A ROS assay kit (Beyotime Biotechnology) was used to quantify ROS levels in C2C12 cells. Diluted dichlorodihydroflourescein diacetate (DCFH-DA) was added to wells and incubated at 37°C for 20 min. Cells were washed 3 times with serum-free cell culture medium to remove the free DCFH-DA. Then, the cells were trypsinized and collected as a single cell suspension. The fluorescence intensity of DCFH-DA in the cell suspension was measured at an excitation wavelength of 500 nm and an emission wavelength of 525 nm.

Statistical analyses

The statistical analysis was carried out using GraphPad Prism v. 7.0 software (GraphPad Software, San Diego, USA). For the MTT assay, data were collected from 5 biological replications. The comparisons between the groups at different time points were performed using two-way analysis of variance (ANOVA) with the Tukey's post hoc analysis. Interactions between variables were also analyzed using Type III sums of squares, after testing the distribution and homogeneity of the data. Data in Fig. 1A are presented as median (interquartile range (IQR)) with each individual datum shown. For other experiments, data were collected from 3 biological replications and comparisons between the groups were performed using ordinary one-way ANOVA with the Holm–Sidak method used for post hoc analysis, after testing the distribution and homogeneity of data. Data in other figures are presented as individual datum. The value of $p < 0.05$ was considered statistically significant.

Results

Sulforaphane reduced the inflammatory response induced by lipopolysaccharide in mouse embryonic myoblasts

Initially, the effect of SFN on the viability of mouse embryonic myoblast cell line C2C12 was evaluated. The C2C12 cells were treated with SFN at different concentrations (1 μM, 5 μM, 10 μM, 20 μM, and 30 μM), and there was little difference in cell viability between the various concentrations after 6 h of treatment. After treatment for 24 h with increased SFN concentration, C2C12 cell viability clearly decreased. Furthermore, 120-hour treatment with 10 μM SFN resulted in lower viability compared to the control group. Nonetheless, the cell survival rate was still greater than 90% (Fig. 1A). Therefore, SFN concentrations of 1 μM, 5 μM and 10 μM were selected for subsequent experimental studies. Expression levels of IL-6 gene and protein in supernatant of C2C12 cells were significantly increased 6 h after the LPS treatment. However, 6 h of SFN treatment, especially at 10 μM, inhibited the increased expression levels of the *IL-6* gene (Fig. 1B) and IL-6 protein (Fig. 1C) induced by LPS. Therefore, 10 μM SFN was used for subsequent experiments.

Lipopolysaccharide increased the gene expression levels of TNF-α and iNOS in C2C12 cells. At the same time, 10 μM SFN was observed to reduce inflammation by significantly downregulating the expression of LPS-induced TNF-α (Fig. 1D) and iNOS (Fig. 1E). The activity of phosphorylated NF-κB protein in the LPS-treated cells was significantly higher than in the control cells, and was inhibited by SFN treatment (Fig. 1F). Lipopolysaccharide significantly increased IL-1β protein levels in the supernatant of C2C12 cells. However, after SFN treatment, IL-1β protein levels were significantly reduced (Fig. 1G). In addition, the ROS fluorescence-labeling assay showed that SFN significantly reduced the production of LPS-induced ROS (Fig. 1H,I). The above results suggest that LPS induces the production of multiple inflammatory mediators in mouse embryonic myoblasts, which is inhibited by SFN treatment.

Lipopolysaccharide inhibited the myogenic differentiation of mouse embryonic myoblasts

The differentiation of C2C12 cells was achieved by the addition of medium containing 2% horse serum. Compared with the control group, LPS treatment at multiple concentrations inhibited the formation of multinucleated myotubes that are normally observed during the myogenic differentiation. This manifested as a significant decrease in the mean myotube width. Notably,

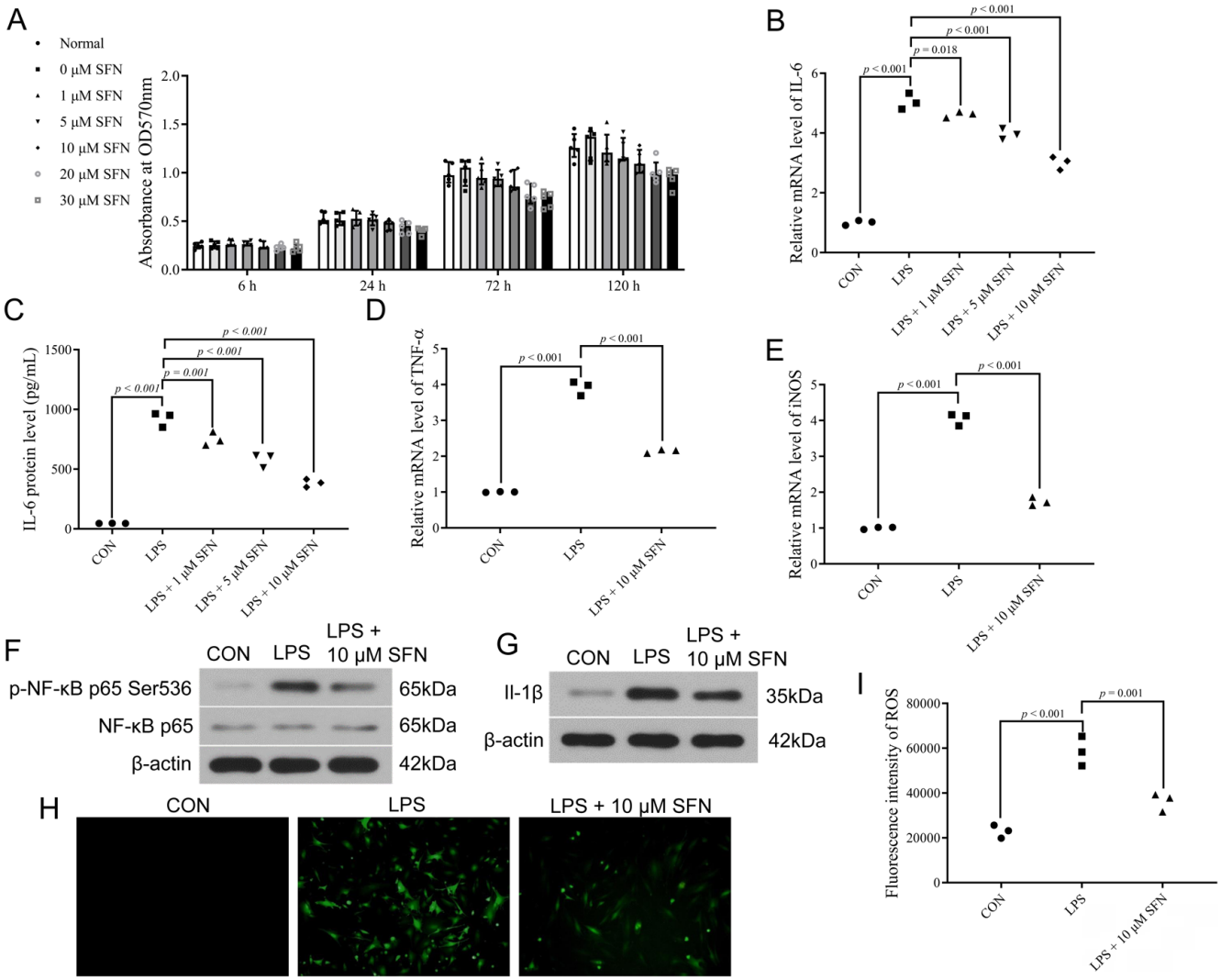

Fig. 1. Sulforaphane (SFN) reduced the lipopolysaccharide (LPS)-induced inflammatory response in C2C12 cells. A. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction (MTT) assay was performed after SFN treatment of C2C12 cells (0 μM, 1 μM, 5 μM, 10 μM, 20 μM, or 30 μM) for 6 h, 24 h, 72 h, or 120 h; B. C2C12 cells were treated with the following drugs: control (CON, equal volume of dimethylsulfoxide (DMSO)), 25 ng/mL LPS, 25 ng/mL LPS + 1 μM SFN, 25 ng/mL LPS + 5 μM SFN, and 25 ng/mL LPS + 10 μM SFN. Messenger ribonucleic acid (mRNA) levels of interleukin-6 (IL-6) were detected with quantitative real-time polymerase chain reaction (qRT-PCR); C. According to the grouping in B, expression levels of IL-6 protein in culture supernatants were detected using an enzyme-linked immunosorbent assay (ELISA) kit; D. C2C12 cells were treated with the following drugs: control (CON, equal volume of DMSO), 25 ng/mL LPS, and 25 ng/mL LPS + 10 μM SFN. The mRNA levels of tumor necrosis factor alpha (TNF-α) were detected using qRT-PCR; E. The mRNA levels of inducible nitric oxide synthase (iNOS) were detected using qRT-PCR; F. According to the grouping in D, expression levels of nuclear factor kappa-B (NF-κB) p65 and p-NF-κB p65 (Ser536) were detected with western blotting; G. Western blotting was used to detect the expression levels of IL-1β protein in the culture supernatants; H. Fluorescence images of intracellular reactive oxygen species (ROS) in each group; I. Fluorescence signal intensity of ROS

treatment with 1000 ng/mL LPS for 120 h had the most significant inhibitory effect on myogenic differentiation of C2C12 cells (Fig. 2A). Compared with the control group, LPS significantly reduced the expression of *myoD* (Fig. 2B) and *myogenin* (Fig. 2C), genes that are specific for myogenic differentiation in C2C12 cells. Compared with lower concentrations, LPS at a concentration of 1 μg/mL had the most significant impact on the downregulation of expression of these genes. Therefore, these results suggest that LPS inhibits the myogenic differentiation of mouse embryonic myoblast cell line C2C12. Subsequent experiments used 1 μg/mL LPS to inhibit myogenic differentiation.

Sulforaphane rescued inhibition of myogenic differentiation by lipopolysaccharide

It was found that SFN decreased the inhibitory effect of LPS on the formation of multinucleated myotubes. In C2C12 cells treated with LPS, 10 μM SFN treatment for 72 h or 120 h significantly restored myotube formation compared to cells treated with LPS alone (Fig. 3A). Compared with the control group, LPS significantly inhibited the expression of *myoD* and *myogenin*, both of which are genes that are specific to myogenic differentiation in C2C12 cells. However, SFN treatment increased

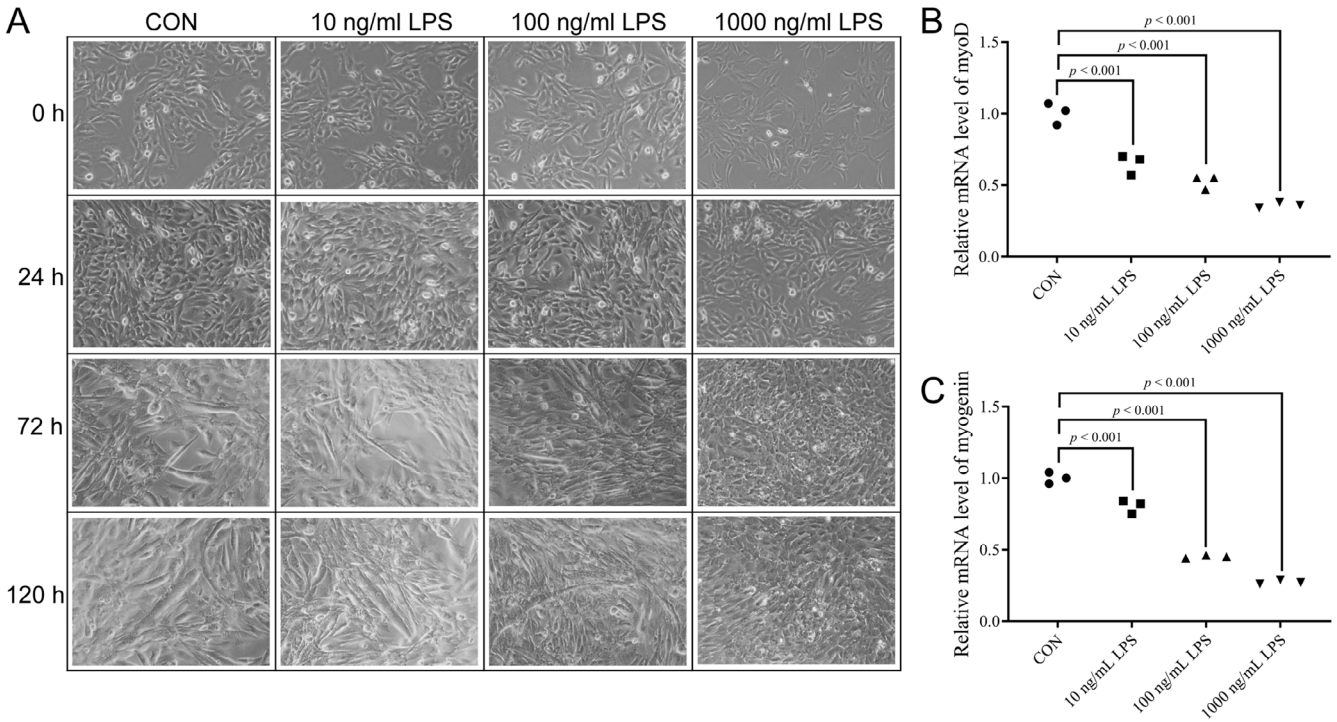

Fig. 2. Lipopolysaccharide (LPS) inhibited the myogenic differentiation of C2C12 cells. A. C2C12 cells were induced to differentiate in a conditioned medium containing 2% horse serum, and were treated with 0 ng/mL, 10 ng/mL, 100 ng/mL, or 1000 ng/mL of LPS. Images of cell morphology were taken at 0 h, 24 h, 72 h, and 120 h after the induction of differentiation; B. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the messenger ribonucleic acid (mRNA) levels of myosin ID heavy chain (myoD) after 72-hour differentiation of C2C12 cells; C. The mRNA level of myogenin was detected with qRT-PCR

CON – control.

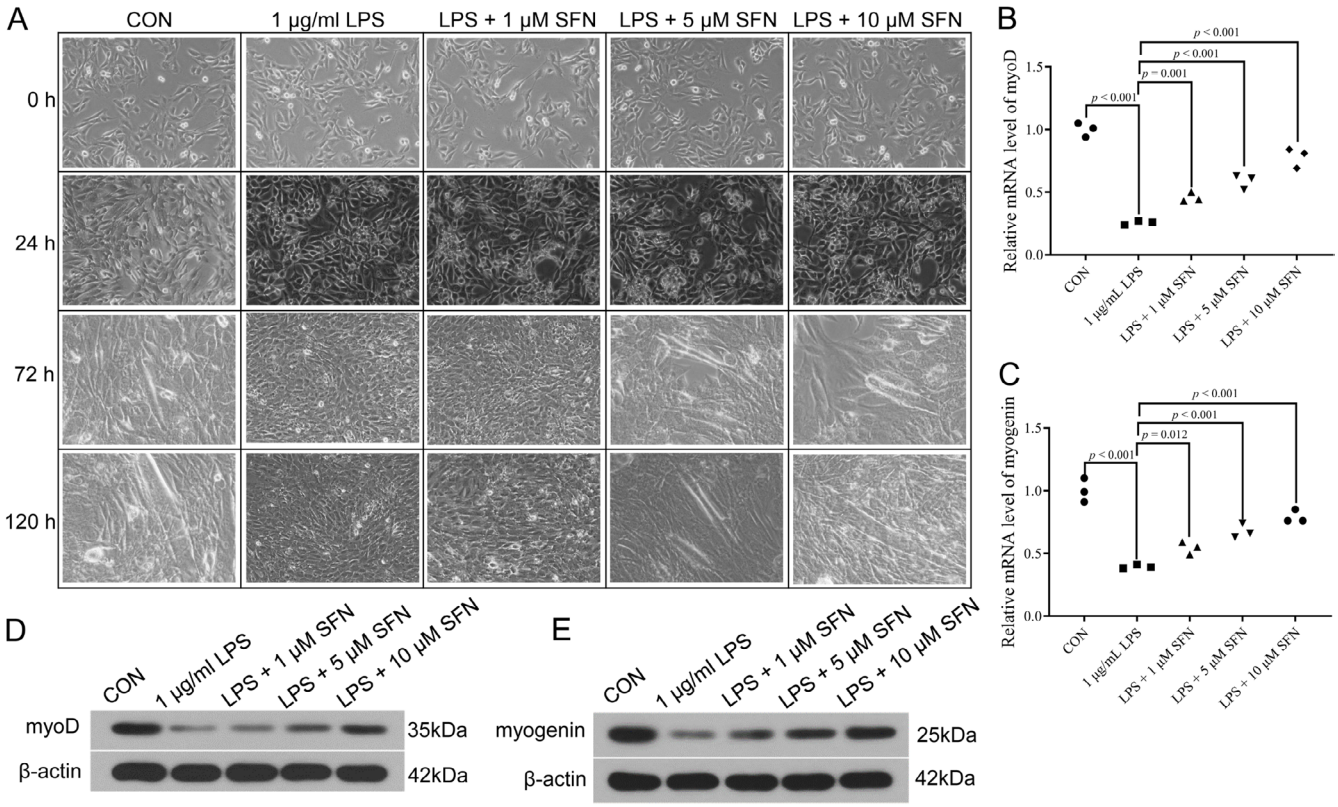

Fig. 3. Sulforaphane (SFN) rescued myogenic differentiation of C2C12 cells inhibited by lipopolysaccharide (LPS). A. C2C12 cells were treated with the following drugs during the differentiation process using 2% horse serum: control group (CON), 1 μg/mL LPS, 1 μg/mL LPS + 1 μM SFN, 1 μg/mL LPS + 5 μM SFN, and 1 μg/mL LPS + 10 μM SFN. Images of cell morphology were taken at 0 h, 24 h, 72 h, and 120 h after the induction of differentiation; B. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the messenger ribonucleic acid (mRNA) level of myosin ID heavy chain (myoD) after 72-hour differentiation of C2C12 cells; C. The mRNA level of myogenin was detected with qRT-PCR; D,E. Protein level of myoD (D) and myogenin (E) was detected with western blotting

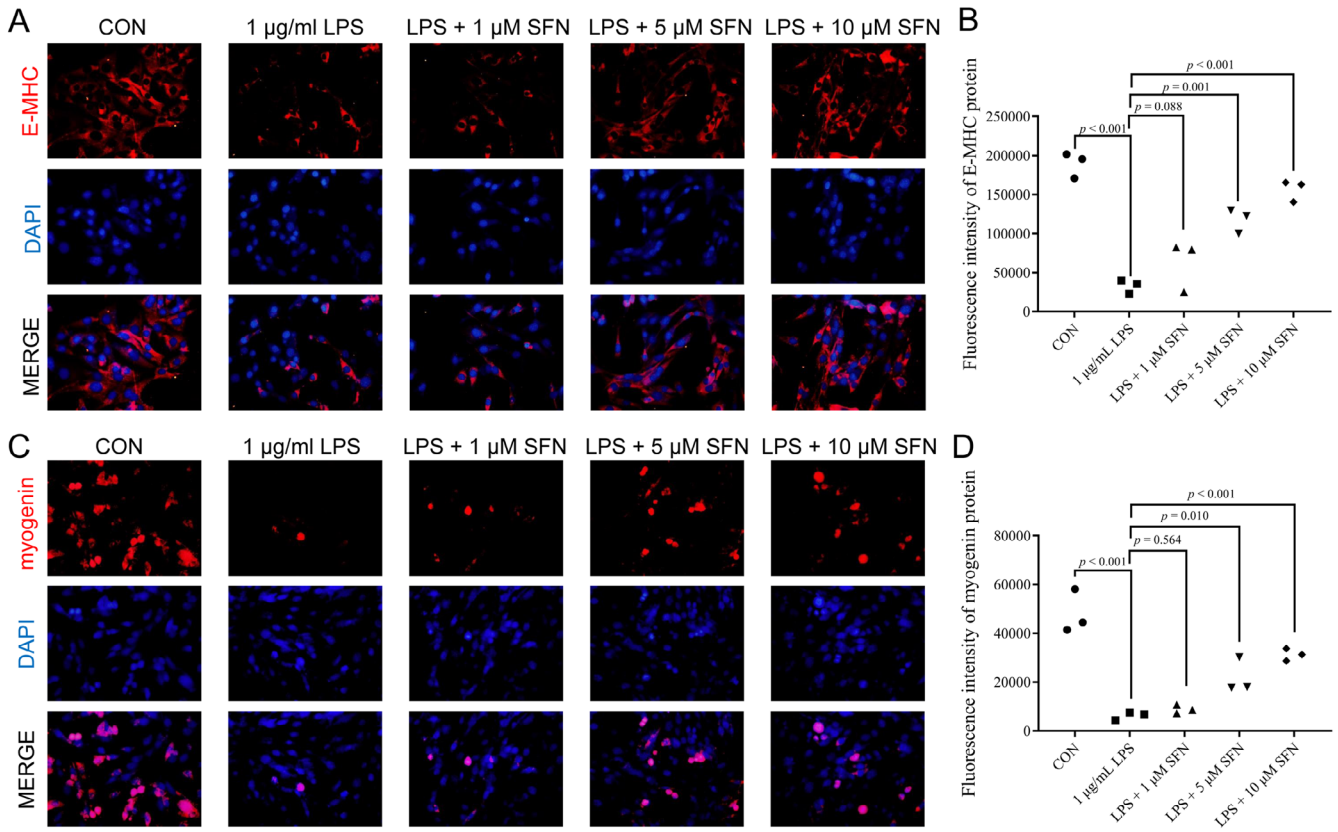

Fig. 4. Sulforaphane (SFN) increased the expression of E myosin heavy chain (E-MHC) and myogenin in differentiated C2C12 cells. A. C2C12 cells in each group (control group (CON), 1 μg/mL lipopolysaccharide (LPS), 1 μg/mL LPS + 1 μM SFN, 1 μg/mL LPS + 5 μM SFN, and 1 μg/mL LPS + 10 μM SFN) were induced to differentiate for 120 h, and immunofluorescence staining was used to detect the expression level of E-MHC. Nuclear staining was achieved with the use of 4′,6-diamidino-2-phenylindole (DAPI); B. The fluorescence intensity of E-MHC protein was quantified and analyzed; C. Immunofluorescence staining was used to detect the expression level of myogenin; D. The fluorescence intensity of myogenin protein was quantified and analyzed

the expression of *myoD* and *myogenin* compared with the LPS treatment group (Fig. 3B,C). Similarly, SFN increased the protein expression of myoD and myogenin in C2C12 cells treated with LPS (Fig. 3D,E). This indicates that SFN may ameliorate the inhibitory effects of LPS on myogenic differentiation.

The E-MHC is a landmark indicator that reflects the state of cell differentiation and the rate of cell fusion. Its expression increases with the extension of cell differentiation time. Immunofluorescence staining showed that LPS significantly reduced the expression of E-MHC during the differentiation process of C2C12 cells. However, after the addition of SFN (especially after the treatment with 10 μM SFN), the expression levels of E-MHC increased (Fig. 4A,B). Myogenin promotes the terminal differentiation of myoblasts into myotube muscle fibers. Immunofluorescence showed that LPS significantly reduced the expression of myogenin during the differentiation process of C2C12 cells. However, the treatment with SFN (especially 10 μM SFN) increased myogenin expression compared to the treatment with LPS alone (Fig. 4C,D). These results confirm that LPS inhibits myogenic differentiation, which is reversed by SFN treatment.

Sulforaphane regulated lipopolysaccharide-induced myogenic differentiation through the toll-like receptor 4 and NLRP3 signaling pathways

The C2C12 cells were treated with the TLR4 inhibitor TAK-242 to evaluate the role of TLR4 signaling in SFN therapy. Based on cellular morphology, it was shown that TAK-242 or SFN treatment of C2C12 cells significantly restored myotube formation, compared with LPS treatment (Fig. 5A). The treatment of C2C12 cells with LPS increased the gene and protein expression levels of TLR4 (Fig. 5B,C), NLRP3 (Fig. 5B,D), inflammation-related factor ASC (Fig. 5B,E), and apoptosis-related factor Caspase-1 (Fig. 5B,F). Compared with the LPS treatment group, TAK-242 and SFN reduced the gene and protein expression of TLR4, NLRP3, ASC, and Caspase-1. This indicates that LPS activates the TLR4 signaling pathway and causes the NLRP3 inflammasome cascade. These results also suggest that SFN may attenuate the activation of this inflammatory cascade that occurs through the TLR4 and NLRP3 signaling pathways.

It was also observed that LPS reduced the expression of myoD and myogenin to inhibit myogenic differentiation.

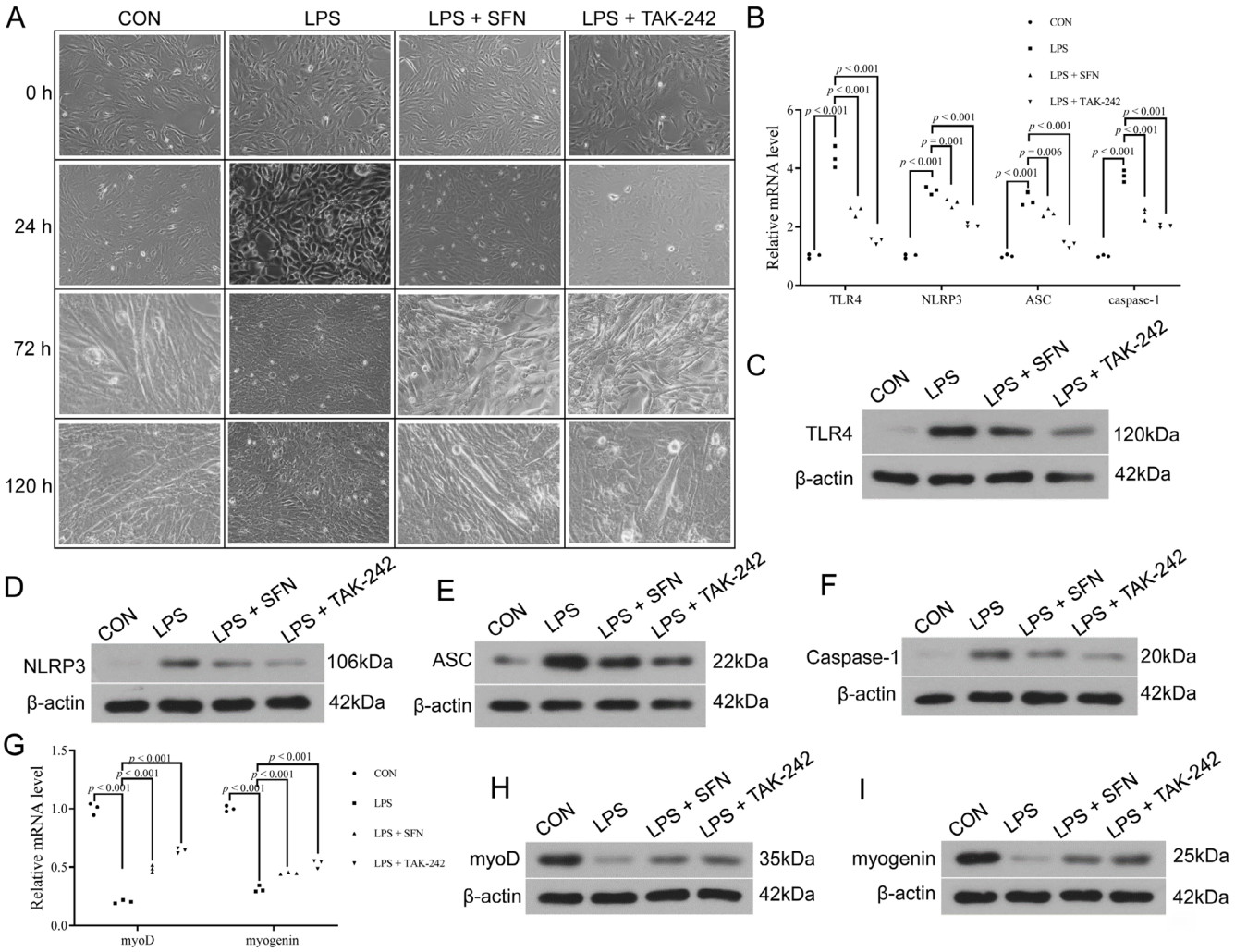

Fig. 5. Sulforaphane (SFN) regulated lipopolysaccharide (LPS)-induced differentiation through the toll-like receptor 4 (TLR4) and NLRP3 pathways in C2C12 cells. A. C2C12 cells were induced to differentiate in a conditioned medium containing 2% horse serum and treated with different concentrations of LPS (control (CON), 1 μg/mL LPS, 1 μg/mL LPS + 10 μM SFN, and 1 μg/mL LPS + 1 μM TAK-242). Images of cell morphology were taken at 0 h, 24 h, 72 h, and 120 h after the induction of differentiation; B. C2C12 cells treated with the aforementioned drugs were induced to differentiate for 72 h, and the messenger ribonucleic acid (mRNA) levels of TLR4, NLRP3, apoptosis-associated speck-like protein (ASC), and Caspase-1 were detected using quantitative real-time polymerase chain reaction (qRT-PCR); C–F. The protein levels of TLR4 (C), NLRP3 (D), ASC (E), and caspase-1 (F) were detected using western blotting; G. The mRNA levels of myosin ID heavy chain (myoD) and myogenin were detected using qRT-PCR; H,I. The protein level of myoD (H) and myogenin (I) were detected using western blotting

However, compared with the LPS treatment group, TAK-242 or SFN treatment significantly increased gene and protein expression of myoD and myogenin (Fig. 5G–I). This indicates that SFN alleviates the inhibition of myogenic differentiation induced by LPS, and is dependent upon silencing of the TLR4 signaling axis.

The immunofluorescence analysis of myogenic differentiation markers E-MHC and myogenin showed that LPS treatment significantly reduced their expression in C2C12 cells. However, compared with the LPS treatment, TAK-242 and SFN significantly increased the expression of E-MHC (Fig. 6A,B) and myogenin (Fig. 6C,D). These data indicate that the promotion of myogenic differentiation by SFN may be achieved by repressing the activation of the TLR4 pathway.

Discussion

Muscle tissue is the main target for sepsis injury, and muscular atrophy is a serious long-term complication frequently seen in patients with sepsis in the intensive care unit. Furthermore, muscular atrophy is an important cause of sepsis-related sarcopenia and myasthenia,[23,24] and occurs as a result of many factors. The increased expression of systemic inflammatory mediators is a prerequisite for the occurrence of skeletal muscle depletion,[25] while decreased musculogenic capacity and increased muscle proteolysis[26,27] are important determinants of skeletal muscle atrophy. Studies have shown that patients with sepsis exhibit high levels of pro-inflammatory cytokines that cause changes in the muscle microenvironment, abnormal levels of muscle fiber autophagy and oxidative stress damage, thereby accelerating muscle protein

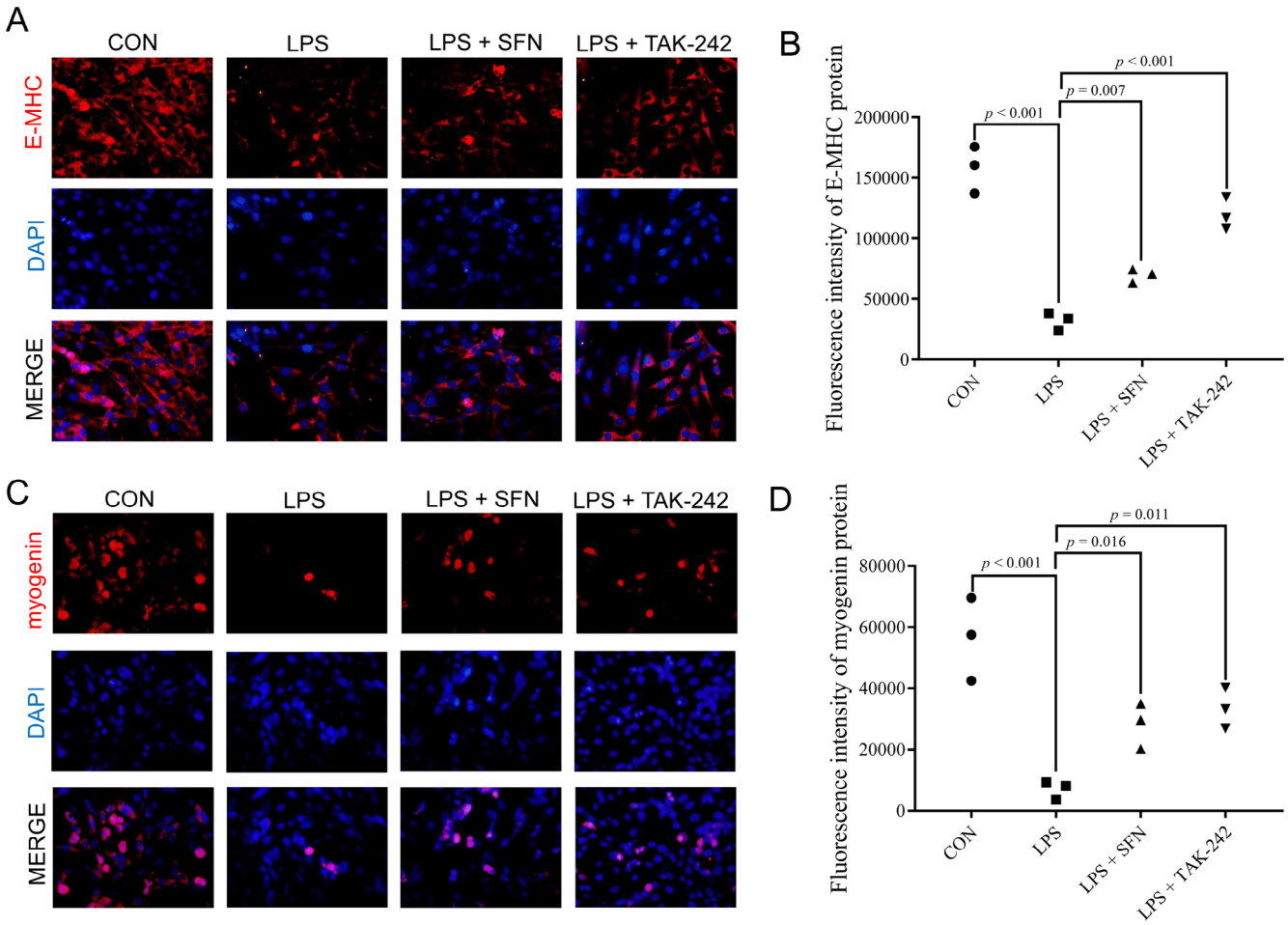

Fig. 6. Toll-like receptor 4 (TLR4) inhibitor increased the expression levels of E myosin heavy chain (E-MHC) and myogenin in differentiated C2C12 cells. A. C2C12 cells in each group (control (CON), 1 μg/mL lipopolysaccharide (LPS), 1 μg/mL LPS + 10 μM sulforaphane (SFN), and 1 μg/mL LPS + 1 μM TAK-242) were induced to differentiate for 120 h, and immunofluorescence staining was used to detect the expression levels of E-MHC. Nuclear staining was carried out using 4′,6-diamidino-2-phenylindole (DAPI); B. The fluorescence intensity of E-MHC protein was quantified and analyzed; C. Immunofluorescence staining was used to detect the expression levels of myogenin; D. The fluorescence intensity of myogenin protein was quantified and analyzed

degradation.[28–30] Under the conditions of sepsis, regeneration potential of activated myosatellite cells is impaired, and their proliferation and differentiation are inhibited, meaning that they cannot compensate for muscle loss. At present, the clinical treatment for sepsis-related sarcopenia and myasthenia is mainly aimed at appropriate nutrition and physical rehabilitation therapy. However, their efficacy is limited, and both have shown limitations in the repair of muscle loss before atrophy or depletion occurs. Therefore, to control the adverse effects of sepsis, the discovery of effective drugs and combinational therapies against multiple targets is urgently needed. Through a series of cytological experiments, we confirmed the effect of SFN in alleviating LPS-induced myoblast damage, which suggests that SFN may be a potential therapy for sepsis-related sarcopenia.

Sulforaphane is an isothiocyanate found in cruciferous vegetables such as broccoli and cabbage.[31] Sulforaphane has an anti-inflammatory activity and is therefore used in the treatment of various diseases.[32] Indeed, SFN has been shown to inhibit a variety of tumors by intervening in oxidative stress, inflammation, cell cycle, proliferation, apoptosis, and metastasis.[33,34] It has also been demonstrated to have a neuroprotective effect, and is used for preventing and treating various acute and chronic neurodegenerative diseases.[20] Furthermore, SFN can inhibit the expression of myostatin in porcine skeletal muscle satellite cells and stem cells in a dose-dependent manner.[35] Additionally, SFN increased the number and function of skeletal muscle stem cells, preventing age-related cardiac and muscle dysfunction.[36] Other studies have reported that SFN can prevent dexamethasone-induced muscle atrophy by regulating the Akt/FoxO1 axis in C2C12 myotubes.[22] However, the therapeutic efficacy of SFN has not been previously studied in the process of sepsis-related sarcopenia. Our study has demonstrated that SFN promotes LPS-induced myogenic differentiation of mouse myoblasts and further clarified that myogenic differentiation was achieved through the TLR4–NLRP3 signaling pathway. In the search for a therapeutic drug that could reverse the decline in skeletal muscle strength and quality that occurs in sepsis, SFN has demonstrated the potential

to reduce disabling muscular dystrophy and the incidence of sepsis mortality.

Toll-like receptor 4 was identified as the first human homologue of the *Drosophila Toll* gene and is well known as a receptor for LPS. In addition to being highly expressed in immune cells, TLR4 is also abundant in adipose tissue, liver and skeletal muscles.[37] The TLR4-mediated signal transduction activates NF-κB and promotes the production of pro-inflammatory cytokines, such as TNF-α and IL-6. These factors are well-known modulators of protein renewal in muscle and contribute to the development of muscle atrophy under various conditions. The NLRP3 inflammasome is a collection of cytosolic receptor proteins, including NLRP3, ASC and Caspase-1. Furthermore, the NLRP3 inflammasome is an important part of innate immunity, and is activated by pathogens, secreted toxins, crystals, and endogenous danger signals.[38] Studies have found that the regulation of the NLRP3 inflammasome alleviated the disease process and even blocked disease pathologies. For example, in NLRP3 knockout mice, the suppression of the inflammatory response contributed to the development of sarcopenia in aging mice and reduced muscle glycolysis.[39] Therefore, inhibiting the activation of the NLRP3 inflammasome could be an important strategy for the prevention and treatment of sarcopenia.[40] In sepsis patients, circulating LPS binds to TLR4 to activate the TLR4 signaling pathway. This leads to the release of a large amount of inflammatory mediators and the activation of the NLRP3 inflammasome.[8] Our data show that SFN inhibited LPS-induced cytokine release and expression of TLR4 and NLRP3 in C2C12 cells. We revealed that SFN may play a key role in improving septic sarcopenia and myasthenia by regulating the TLR4 signaling pathway and NLRP3 inflammasome activation.

Additionally, the concentration of circulating LPS usually increases in sepsis. The activation of the TLR4 signaling pathway by LPS led to the release of a large number of inflammatory factors and inhibited the proliferation of C2C12 cells. Lipopolysaccharide also downregulated the expression of *myoD* and *myogenin* in a dose-dependent manner, and inhibited the myogenic differentiation of C2C12 cells. Lipopolysaccharide binds to the TLR4 receptor on myoblast cells to activate the TLR4 signaling pathway and cause local inflammation. At the same time, LPS also causes the release of inflammatory mediators and activates the NLRP3 inflammasome, which is closely associated with the occurrence of sarcopenia. As an isothiocyanate, SFN exists in natural cruciferous vegetables and has potential anti-inflammatory effects. Indeed, SFN suppressed the release of inflammatory factors caused by LPS in C2C12 myoblasts, and restored cell proliferation and myogenic differentiation that was inhibited by LPS. Investigation of the underlying mechanisms revealed that SFN attenuated the expression of TLR4, NLRP3 and their related regulatory proteins in C2C12 myoblasts, which demonstrates the activation of the TLR4 and NLRP3 signaling pathways. This was similar to the effects brought about by the administration of the TLR4 inhibitor TAK-242. Therefore, dietary supplementation with vegetables containing SFN, such as broccoli and cabbage, may reduce sarcopenia and myasthenia in patients with sepsis through inhibiting the TLR4 and NLRP3 inflammasome pathways.

Limitations

Due to the instability of the animal model of sepsis, the study was not conducted in vivo. Further experiments are needed in future research.

Conclusions

Sulforaphane can improve the expression of myogenic factors myoD and myogenin by regulating TLR4 and NLRP3 signaling pathways, so as to ameliorate the inhibition of myogenic differentiation induced by LPS.

Supplementary data

The supplementary statistical data are available as Supplementary Tables (https://doi.org/10.5281/zenodo.7234725). The package consists of the following files:

Supplementary Table 1. Post hoc comparisons for MTT assay.

Supplementary Table 2. All statistical data for all figures.

ORCID iDs

Manfeng Wang https://orcid.org/0000-0002-7556-8739
Meiling Liu https://orcid.org/0000-0002-3418-7104
Wanzhen Xu https://orcid.org/0000-0001-6602-963X
Zongyan Teng https://orcid.org/0000-0002-4247-9609
Xiaowei Wu https://orcid.org/0000-0002-5725-9123
Lu Gan https://orcid.org/0000-0002-0731-004X
Yina Zhang https://orcid.org/0000-0001-5981-2626

References

1. Ma XY, Tian LX, Liang HP. Early prevention of trauma-related infection/sepsis. *Military Med Res*. 2016;3(1):33. doi:10.1186/s40779-016-0104-3
2. Rello J, Valenzuela-Sánchez F, Ruiz-Rodriguez M, Moyano S. Sepsis: A review of advances in management. *Adv Ther*. 2017;34(11):2393–2411. doi:10.1007/s12325-017-0622-8
3. Koga Y, Fujita M, Yagi T, et al. Early enteral nutrition is associated with reduced in-hospital mortality from sepsis in patients with sarcopenia. *J Crit Care*. 2018;47:153–158. doi:10.1016/j.jcrc.2018.06.026
4. Onyekwulu FA, Onwuekwe IO. Critical care of myasthenia gravis in a resource poor setting: A study of southeast Nigeria. *Neurologist*. 2010;16(6):368–370. doi:10.1097/NRL.0b013e3181c29f25
5. Goossens C, Marques MB, Derde S, et al. Premorbid obesity, but not nutrition, prevents critical illness-induced muscle wasting and weakness: Obesity attenuates CI-induced muscle wasting. *J Cachexia Sarcopenia Muscle*. 2017;8(1):89–101. doi:10.1002/jcsm.12131
6. Xu J, Zheng B, Zhang S, et al. Effects of preoperative sarcopenia on postoperative complications of minimally invasive oesophagectomy for oesophageal squamous cell carcinoma. *J Thorac Dis*. 2019;11(6):2535–2545. doi:10.21037/jtd.2019.05.55
7. Ding M, Huang T, Zhu R, et al. Immunological behavior analysis of muscle cells under IFN-γ stimulation in vitro and in vivo. *Anat Rec*. 2018;301(9):1551–1563. doi:10.1002/ar.23834

8. Ono Y, Sakamoto K. Lipopolysaccharide inhibits myogenic differentiation of C2C12 myoblasts through the Toll-like receptor 4-nuclear factor-κB signaling pathway and myoblast-derived tumor necrosis factor-α. *PLoS One*. 2017;12(7):e0182040. doi:10.1371/journal.pone.0182040
9. Zhang P, Li W, Wang L, et al. Salidroside inhibits myogenesis by modulating p-Smad3-induced Myf5 transcription. *Front Pharmacol*. 2018;9:209. doi:10.3389/fphar.2018.00209
10. Baradaran Rahim V, Khammar MT, Rakhshandeh H, Samzadeh-Kermani A, Hosseini A, Askari VR. Crocin protects cardiomyocytes against LPS-induced inflammation. *Pharmacol Rep*. 2019;71(6):1228–1234. doi:10.1016/j.pharep.2019.07.007
11. Rocheteau P, Chatre L, Briand D, et al. Sepsis induces long-term metabolic and mitochondrial muscle stem cell dysfunction amenable by mesenchymal stem cell therapy. *Nat Commun*. 2015;6(1):10145. doi:10.1038/ncomms10145
12. Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*. 1997;388(6640):394–397. doi:10.1038/41131
13. Doyle A, Zhang G, Fattah EAA, Eissa NT, Li Y. Toll-like receptor 4 mediates lipopolysaccharide-induced muscle catabolism via coordinate activation of ubiquitin-proteasome and autophagy-lysosome pathways. *FASEB J*. 2011;25(1):99–110. doi:10.1096/fj.10-164152
14. Cornelius DC, Baik CH, Travis OK, et al. NLRP 3 inflammasome activation in platelets in response to sepsis. *Physiol Rep*. 2019;7(9):e14073. doi:10.14814/phy2.14073
15. Rawat R, Cohen TV, Ampong B, et al. Inflammasome upregulation and activation in dysferlin-deficient skeletal muscle. *Am J Pathol*. 2010;176(6):2891–2900. doi:10.2353/ajpath.2010.090058
16. Huang N, Kny M, Riediger F, et al. Deletion of Nlrp3 protects from inflammation-induced skeletal muscle atrophy. *Intensive Care Med Exp*. 2017;5(1):3. doi:10.1186/s40635-016-0115-0
17. Wilcox A, Murphy M, Tucker D, et al. Sulforaphane alters the acidification of the yeast vacuole. *Microb Cell*. 2020;7(5):129–138. doi:10.15698/mic2020.05.716
18. Myzak MC, Tong P, Dashwood WM, Dashwood RH, Ho E. Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. *Exp Biol Med (Maywood)*. 2007;232(2):227–234. PMID:17259330.
19. Vanduchova A, Anzenbacher P, Anzenbacherova E. Isothiocyanate from broccoli, sulforaphane, and its properties. *J Med Food*. 2019;22(2):121–126. doi:10.1089/jmf.2018.0024
20. Huang C, Wu J, Chen D, Jin J, Wu Y, Chen Z. Effects of sulforaphane in the central nervous system. *Eur J Pharmacol*. 2019;853:153–168. doi:10.1016/j.ejphar.2019.03.010
21. Lee IC, Kim DY, Bae JS. Sulforaphane reduces HMGB1-mediated septic responses and improves survival rate in septic mice. *Am J Chin Med*. 2017;45(6):1253–1271. doi:10.1142/S0192415X17500690
22. Son YH, Jang EJ, Kim YW, Lee JH. Sulforaphane prevents dexamethasone-induced muscle atrophy via regulation of the Akt/Foxo1 axis in C2C12 myotubes. *Biomed Pharmacother*. 2017;95:1486–1492. doi:10.1016/j.biopha.2017.09.002
23. Wollersheim T, Grunow JJ, Carbon NM, et al. Muscle wasting and function after muscle activation and early protocol-based physiotherapy: An explorative trial. *J Cachexia Sarcopenia Muscle*. 2019;10(4):734–747. doi:10.1002/jcsm.12428
24. Schefold JC, Bierbrauer J, Weber-Carstens S. Intensive care unit – acquired weakness (ICUAW) and muscle wasting in critically ill patients with severe sepsis and septic shock. *J Cachexia Sarcopenia Muscle*. 2010;1(2):147–157. doi:10.1007/s13539-010-0010-6
25. Tuttle CSL, Thang LAN, Maier AB. Markers of inflammation and their association with muscle strength and mass: A systematic review and meta-analysis. *Ageing Res Rev*. 2020;64:101185. doi:10.1016/j.arr.2020.101185
26. Sandri M, Sandri C, Gilbert A, et al. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell*. 2004;117(3):399–412. doi:10.1016/S0092-8674(04)00400-3
27. Bodine SC, Latres E, Baumhueter S, et al. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science*. 2001;294(5547):1704–1708. doi:10.1126/science.1065874
28. van Hall G. Cytokines: Muscle protein and amino acid metabolism. *Curr Opin Clin Nutr Metab Care*. 2012;15(1):85–91. doi:10.1097/MCO.0b013e32834e6ea2
29. Lang CH, Krawiec BJ, Huber D, McCoy JM, Frost RA. Sepsis and inflammatory insults downregulate IGFBP-5, but not IGFBP-4, in skeletal muscle via a TNF-dependent mechanism. *Am J Physiol Regul Integr Comp Physiol*. 2006;290(4):R963–R972. doi:10.1152/ajpregu.00684.2005
30. Maes K, Stamiris A, Thomas D, et al. Effects of controlled mechanical ventilation on sepsis-induced diaphragm dysfunction in rats. *Crit Care Med*. 2014;42(12):e772–e782. doi:10.1097/CCM.0000000000000685
31. Zhang JC, Yao W, Dong C, et al. Prophylactic effects of sulforaphane on depression-like behavior and dendritic changes in mice after inflammation. *J Nutr Biochem*. 2017;39:134–144. doi:10.1016/j.jnutbio.2016.10.004
32. Yagishita Y, Fahey JW, Dinkova-Kostova AT, Kensler TW. Broccoli or sulforaphane: Is it the source or dose that matters? *Molecules*. 2019;24(19):3593. doi:10.3390/molecules24193593
33. Yang F, Wang F, Liu Y, et al. Sulforaphane induces autophagy by inhibition of HDAC6-mediated PTEN activation in triple negative breast cancer cells. *Life Sci*. 2018;213:149–157. doi:10.1016/j.lfs.2018.10.034
34. Bose C, Awasthi S, Sharma R, et al. Sulforaphane potentiates anticancer effects of doxorubicin and attenuates its cardiotoxicity in a breast cancer model. *PLoS One*. 2018;13(3):e0193918. doi:10.1371/journal.pone.0193918
35. Fan H, Zhang R, Tesfaye D, et al. Sulforaphane causes a major epigenetic repression of myostatin in porcine satellite cells. *Epigenetics*. 2012;7(12):1379–1390. doi:10.4161/epi.22609
36. Bose C, Alves I, Singh P, et al. Sulforaphane prevents age-associated cardiac and muscular dysfunction through Nrf2 signaling. *Aging Cell*. 2020;19(11):e13261. doi:10.1111/acel.13261
37. Li H, He Y, Zhang J, Sun S, Sun B. Lipopolysaccharide regulates toll-like receptor 4 expression in human aortic smooth muscle cells. *Cell Biol Int*. 2007;31(8):831–835. doi:10.1016/j.cellbi.2007.01.034
38. Danielski LG, Giustina AD, Bonfante S, Barichello T, Petronilho F. The NLRP3 inflammasome and its role in sepsis development. *Inflammation*. 2020;43(1):24–31. doi:10.1007/s10753-019-01124-9
39. McBride MJ, Foley KP, D'Souza DM, et al. The NLRP3 inflammasome contributes to sarcopenia and lower muscle glycolytic potential in old mice. *Am J Physiol Endocrinol Metab*. 2017;313(2):E222–E232. doi:10.1152/ajpendo.00060.2017
40. Sayed RKA, Fernández-Ortiz M, Diaz-Casado ME, et al. Lack of NLRP3 inflammasome activation reduces age-dependent sarcopenia and mitochondrial dysfunction, favoring the prophylactic effect of melatonin. *J Gerontol A Biol Sci Med Sci*. 2019;74(11):1699–1708. doi:10.1093/gerona/glz079