

# Inhibitory effects of spermidine on lipopolysaccharide-induced inflammation in RAW 264.7 cells

HAO YUAN<sup>1\*</sup>, FANG PENG<sup>1\*</sup>, YI-FENG ZHOU<sup>2</sup>, SI-XIAN WU<sup>1</sup>,  
ZHEN-YI LONG<sup>1</sup>, YA-MENG PENG<sup>1</sup> and YI-ZHAO ZHOU<sup>3</sup>

<sup>1</sup>Clinical Laboratory, Hunan Provincial People's Hospital, The First-Affiliated Hospital of Hunan Normal University, Changsha, Hunan 410005, P.R. China; <sup>2</sup>Operating Room, Hunan Provincial People's Hospital, The First-Affiliated Hospital of Hunan Normal University, Changsha, Hunan 410005, P.R. China; <sup>3</sup>Department of Orthopedics, Hunan Provincial People's Hospital, The First-Affiliated Hospital of Hunan Normal University, Changsha, Hunan 410005, P.R. China

Received September 1, 2025; Accepted January 5, 2026

DOI: 10.3892/etm.2026.13119

**Abstract.** Spermidine (SPD) is a naturally occurring polyamine with anti-inflammatory and antioxidant properties. Given the pivotal role of macrophages in inflammatory pathogenesis (such as rheumatoid arthritis and autoimmune encephalomyelitis), the present study hypothesized that SPD exerts its anti-inflammatory effects by suppressing macrophage polarization. Briefly, RAW 264.7 cells were activated using lipopolysaccharide (LPS) and were treated with a vehicle control (complete DMEM) or varying SPD concentrations. The ratio of M1 and M2 macrophages in each group was determined using flow cytometry. Inflammatory gene expression and cytokine levels were determined using reverse transcription quantitative PCR and ELISA. The levels of NF- $\kappa$ B pathway-associated proteins were measured through western blotting. The findings revealed that the proportion of M1 cells gradually decreased with increasing SPD concentrations. In LPS-stimulated RAW 264.7 cells, SPD markedly decreased the expression of M1 marker genes and notably increased that of M2 marker genes. Furthermore, SPD decreased IL-6 levels and increased IL-10 levels. In addition, the levels of phosphorylated (p)-p65 and p-I $\kappa$ B $\alpha$ , which are NF- $\kappa$ B pathway-associated proteins, were

markedly decreased after 24 h of SPD treatment. Overall, SPD treatment inhibited LPS-induced M1 polarization, reduced pro-inflammatory gene/cytokine expression and enhanced anti-inflammatory markers, potentially through NF- $\kappa$ B pathway modulation.

## Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disorder with an incidence rate of up to 1% globally among population, which results in irreversible cartilage and bone damage if left untreated (1). The efficacy of conventional anti-RA medications (such as non-steroidal anti-inflammatory drugs) is restricted and some specific drugs (such as methotrexate and sulfasalazine) have been associated with adverse effects, such as nausea, alopecia, stomatitis and hepatotoxicity (2). Although numerous studies have explored the pathogenesis of RA, research focusing on the specific role of macrophages in this process remains limited. Notably, macrophages have been implicated in the autoimmune inflammatory response of RA (3-5). Furthermore, an important pathological characteristic of RA is the accumulation and activation of monocytes/macrophages within the synovial tissue (6,7).

Activated macrophages are classified into two types, namely classically activated M1 macrophages and alternatively activated M2 macrophages (8,9). M1 macrophages are capable of directly phagocytosing cancer cells and are induced by TNF and IFN- $\gamma$  (10). By contrast, M2 macrophages are induced by steroids, IL-4 and IL-10 released by type 2 helper T cells (11), and secrete anti-inflammatory factors, diminish pro-inflammatory factors and augment scavenger receptor levels (12).

Spermidine (SPD) is a polyamine synthesized from putrescine, which is indispensable for DNA synthesis and cell proliferation (13,14). In addition to its anti-inflammatory and antioxidant capabilities SPD has a key role in cell autophagy and transcription (15-17), as well as in suppressing the secretion of pro-inflammatory cytokines and related chemokines (such as CCL3 and CXCL8) (18,19). SPD has also been shown to impede the NF- $\kappa$ B pathway in macrophages within an autoimmune encephalomyelitis mouse model (19).

---

*Correspondence to:* Dr Hao Yuan, Clinical Laboratory, Hunan Provincial People's Hospital, The First-Affiliated Hospital of Hunan Normal University, 61 Jiefang West Road, Furong, Changsha, Hunan 410005, P.R. China  
E-mail: yuanhao666@hunnu.edu.cn

\*Contributed equally

*Abbreviations:* SPD, spermidine; LPS, lipopolysaccharide; RA, rheumatoid arthritis; CCK-8, Cell Counting Kit-8; FSC, forward scatter; RT-qPCR, reverse transcription-quantitative PCR; p-I $\kappa$ B $\alpha$ , phosphorylated-I $\kappa$ B $\alpha$

*Key words:* SPD, LPS, macrophage polarization, NF- $\kappa$ B pathway, inflammation

Paoletti *et al* (6) indicated that the number of M1 macrophages is elevated in the synovium of patients with RA. In addition, macrophages tend to polarize towards the M1 phenotype, augment the release of pro-inflammatory factors and promote synovitis in mice with collagen-induced arthritis (20). These findings suggest that M1 macrophages are predominant in the synovium of patients with RA. Therefore, based on the documented anti-inflammatory and antioxidant properties of SPD, and its reported role in suppressing macrophage pro-inflammatory cytokine secretion and regulating the NF- $\kappa$ B pathway in an autoimmune encephalomyelitis mouse model, SPD may alleviate RA symptoms by modulating macrophage polarization and cytokine release; however, the underlying mechanisms of this remain to be elucidated. Accordingly, the present study hypothesized that SPD may function by regulating activation of the NF- $\kappa$ B pathway. Suitably, RAW 264.7 cells were stimulated with lipopolysaccharide (LPS) to establish a relevant inflammatory cell model, and were subsequently treated with varying SPD concentrations. The cytokine levels (TNF- $\alpha$ , IL-1 $\beta$  and IL-6), mRNA expression levels (*iNOS* for M1 phenotype and *Arg-1* for M2 phenotype) and phosphorylation levels of NF- $\kappa$ B-associated proteins in RAW 264.7 macrophages from different groups were then determined to evaluate the impact of SPD on LPS-induced inflammation and the associated underlying mechanisms. The findings of the present study may offer an experimental basis for exploring SPD as a potential natural anti-inflammatory agent against RA; however, further *in vivo* and clinical studies are still required.

## Materials and methods

**Cell culture.** RAW 264.7 cells were obtained from the Institute for Advanced Study of Central South University (Changsha, China). DMEM (Shanghai BasalMedia Technologies Co., Ltd.) supplemented with 10% FBS (Shanghai BasalMedia Technologies Co., Ltd.), 100 U/ml penicillin and 25  $\mu$ g/ml amphotericin B (Hyclone<sup>TM</sup>; Cytiva) was used as the cell culture medium, with incubation conditions set at 37°C and 5% CO<sub>2</sub> (21).

**Cell cytotoxicity assay.** Cytotoxicity assays were performed using the Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions (NCM Biotech Co., Ltd.). Briefly, RAW 264.7 cells were seeded into 96-well plates at a density of 1.0x10<sup>4</sup> cells/well for 24 h, followed by treatment with varying concentrations (0, 100, 200, 400, 800 or 1,600  $\mu$ M) of SPD ( $\geq$ 99% gas chromatography grade; MilliporeSigma) for additional durations of 6, 12, 24 and 48 h at 37°C in a 5% CO<sub>2</sub> incubator. Subsequently, for the cytotoxicity assay, 10  $\mu$ l CCK-8 solution was added to each well and incubated for 1 h. Finally, the absorbance was measured at a wavelength of 450 nm and cell viability was calculated based on the measured optical density values.

**Flow cytometry.** RAW 264.7 cells were inoculated into a 6-well plate at a density of 4x10<sup>5</sup> cells/well and cultured for 24 h until they adhered to the well walls. Pretreatment with varying concentrations (100, 200, 400 or 800) of SPD or vehicle control (complete DMEM) for 1 h was followed

by stimulation with 1  $\mu$ g/ml LPS 24 h at 37°C in a 5% CO<sub>2</sub> incubator, and this cell treatment protocol was applied uniformly prior to all subsequent experiments in this study. Prior to antibody staining, cells were stained with Zombie NIR<sup>TM</sup> Fixable Viability Kit (1:1,000; cat. no. 423105; BioLegend) for 15 min at room temperature in the dark to distinguish live/dead cells. Subsequently, the cells were gently harvested, washed twice with PBS and stained with CD86-phycoerythrin (1:100; clone GL-1; cat. no. 553692; BD Biosciences) and CD206-BUV395 (1:100; clone Y17-505; cat. no. 568817; BD Biosciences) for 30 min at 4°C in the dark. Fluorescence intensity was assessed using the BD FACSCanto<sup>TM</sup> II flow cytometer (BD Biosciences), and the collected data were analyzed using BD FACSDiva<sup>TM</sup> software (Version 8.0; BD Biosciences).

Gating hierarchy was as follows: i) Forward scatter (FSC)/side scatter gating to exclude debris; ii) singlet gating (FSC-area vs. FSC-height); iii) live cell gating (Zombie NIR<sup>TM</sup>-negative); and iv) CD86/CD206 double staining gating. Compensation was performed using single-stained controls and fluorescence minus one control and isotype-matched antibodies (PE Rat IgG2a; 1:100; cat. no. 553930; and BUV395 Rat IgG2a; 1:100; cat. no. 563556; both BD Biosciences) were used to set the gating thresholds.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was isolated from the cells using the Cell Total RNA Extraction Kit (cat. no. G3694; Wuhan Servicebio Technology Co., Ltd.) according to the manufacturer's instructions. The concentration of total RNA was quantified using a NanoDrop spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc.), whereas cDNA was synthesized using the PrimeScript<sup>TM</sup> RT kit (Takara Bio, Inc.). Gene-specific primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. and qPCR was carried out using the TB Green<sup>®</sup> Premix Ex Taq<sup>TM</sup> II kit as per the manufacturer's instructions (Wuhan Servicebio Technology Co., Ltd.). The primer sequences are listed in Table I. RNA amplification reactions were performed using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Inc.) with an initial denaturation step at 95°C for 30 sec, followed by 40 cycles of amplification (denaturation at 95°C for 5 sec and annealing at 60°C for 34 sec), and finally, a dissociation curve was generated. After amplification, the data were analyzed using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (22) with the mouse *Gapdh* gene as the reference (23).

**ELISA.** The cells were resuspended in complete DMEM, seeded into 6-well plates and incubated for 24 h. After a 1 h pretreatment with SPD, LPS was added to the co-culture system. Cytokine levels in the cell supernatants were quantified using a ELISA kits (Mouse IL-6 ELISA kit, cat. no. EMC004.96; Mouse IL-10 ELISA kit, cat. no. EMC005.96; both Neobioscience Technology Co., Ltd.) according to the manufacturer's instructions.

**Western blotting.** The cells were seeded into 6-well plates and incubated for 24 h. Subsequently, the cells were pretreated with SPD for 1 h and then stimulated with LPS for another 24 h. Immediately after stimulation, total proteins were extracted on ice using RIPA Lysis Buffer (Wuhan Servicebio Technology

Table I. Primer sequences.

Primer name	Sequence (5'-3')
<i>Gapdh</i>	Forward: TCAACGGCACAGTCAAGG Reverse: ACTCCACGACATACTCAGC
<i>Tnf</i>	Forward: TACTGAAC TTCGGGTGATCGGTC Reverse: CAGCCTTGTCCTTGAAGAGAACC
<i>Il6</i>	Forward: CTCCAACAGACCTGTCTATAC Reverse: CCATTGCACA ACTCTTTTCTCA
<i>Il1b</i>	Forward: CACTACAGGCTCCGAGATGAAC AAC Reverse: TGTCGTTGCTTGGTTCTCCTTGAC
<i>Nos2</i>	Forward: CACCTTGGAGTTCACCCAGT Reverse: ACCACTCGTACTTGGGATGC
<i>Arg1</i>	Forward: CATATCTGCCAAGACATCGTG Reverse: GACATCAAAGCTCAGGTGAATC
<i>Il10</i>	Forward: CACAAAGCAGCCTTGCAGAA Reverse: AGAGCAGGCAGCATAGCAGTG

*Nos2*, nitric oxide synthase; *Arg1*, arginase 1.

Co., Ltd.). The protein concentration was determined using a BCA assay (BCA Protein Assay Kit; cat. no. P0012; Beyotime Biotechnology). Equal amounts of protein (20 µg per lane) were separated by 12% SDS-PAGE (cat. no. BL522A; Labgic Technology Co., Ltd.) and transferred onto PVDF membranes (cat. no. IPVH10100, Merckmillipore Co., Ltd.). The membranes were blocked with 5% skimmed milk in Tris-Buffered Saline with Tween-20 (TBST; cat. no. G0004-1L; Wuhan Servicebio Technology Co., Ltd.) for 1 h at room temperature. The membranes were then incubated with the following primary antibodies overnight at 4°C: p65 (1:1,000; cat. no. 8242; Cell Signaling Technology, Inc.), phosphorylated (p)-p65 (1:1,000; cat. no. 3033; Cell Signaling Technology, Inc.), IκBα (1:1,000; cat. no. 4814; Cell Signaling Technology, Inc.), p-IκBα (1:1,000; cat. no. 2859; Cell Signaling Technology, Inc.) and β-tubulin (1:1,000; cat. no. 10094-1-AP; Proteintech Group, Inc.). After washing with TBST, the membranes were incubated with the corresponding HRP-linked secondary antibodies for 1 h at room temperature, namely anti-rabbit IgG (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.) and anti-mouse IgG (1:5,000; cat. no. 7076; Cell Signaling Technology, Inc.). The protein bands were visualized using a super-sensitive ECL chemiluminescent substrate (cat. no. BL5965A; Labgic Technology Co., Ltd.) and detected with a chemiluminescence imaging system (ChemiDoc Imaging System, Bio-Rad Laboratories, Inc.). The gray values of the target bands were semi-quantified using ImageJ software version 1.53 (National Institutes of Health) to determine the relative protein expression levels and β-tubulin was used as an internal control.

**Statistical analysis.** Statistical analysis and data presentation were performed using SPSS 26.0 (IBM Corp.) and GraphPad Prism 8.0 (Dotmatics) software. Differences among multiple groups were evaluated using one-way ANOVA, followed by

Tukey's multiple comparison test for pairwise comparisons. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Cytotoxic effects of SPD on RAW 264.7 cells.** To evaluate whether SPD treatment induced cytotoxicity, cells were incubated with different concentrations of SPD for 6, 12, 24 and 48 h. As shown in Fig. 1, treatment with 100 and 200 µM SPD for 6 h significantly enhanced RAW 264.7 cell viability (P<0.05). After 12 h of treatment, only 100 µM SPD significantly increased cell viability (P<0.001). After 24 h of 0-800 µM SPD treatment, RAW 264.7 macrophage viability increased significantly (P<0.001), however the difference in cell viability between different concentration subgroups (200, 400, 600 and 800 µM) was not significant (P>0.05). Treatment with 1,600 µM SPD induced cytotoxicity compared with that of the control group (DMEM) (P<0.001). After 48 h of treatment, none of the SPD concentrations improved cell viability. Therefore, SPD concentrations of 0-800 µM and a 24 h treatment duration were chosen for subsequent experimentation.

**Effect of SPD on LPS-induced polarization of RAW 264.7 cells.** M1-type macrophages express high levels of CD86, whereas M2-type macrophages express CD206 (24). The proportions of M1 and M2 cells after LPS-induced polarization of RAW 264.7 cells were ascertained using flow cytometry. CD86<sup>-</sup>CD206<sup>-</sup> represents M0 macrophages, CD86<sup>+</sup>CD206<sup>-</sup> represents M1 macrophages and CD86<sup>-</sup>CD206<sup>+</sup> represents M2 macrophages. The findings revealed that SPD treatment diminished macrophage CD86 molecule levels and shifted macrophage polarization from a pro-inflammatory M1 phenotype towards an anti-inflammatory M2 phenotype after 24 h; this effect was most notable in response to 800 µM SPD (Fig. 2A-E). The results revealed that LPS stimulation significantly increased the percentage of M1 macrophages and decreased the percentage of M0 or M2 macrophages compared with the control. Co-treatment with SPD dose-dependently reversed this effect. Specifically, increasing SPD concentrations (200, 400 and 800 µM) gradually decreased the M1 percentage while concurrently increasing the M2 percentage. The percentage of M0 macrophages remained largely unchanged across SPD groups. These data indicate that SPD can redirect LPS-induced M1 polarization toward an M2 phenotype in a dose-dependent manner (P<0.05; Fig. 2F-H).

**Effect of SPD on LPS-activated mRNA expression of inflammatory factors in RAW 264.7 cells.** RT-qPCR was performed to assess the ability of LPS to suppress the expression of inflammatory cytokines. LPS significantly elevated the mRNA expression levels of *Tnfa*, *Il6*, *Il1b* and nitric oxide synthase (*Nos2*) compared with those in the control group (Fig. 3A-D). The mRNA levels of *Tnfa*, *Il6*, *Il1b* and *Nos2* then gradually diminished with increasing SPD concentrations, thereby suggesting a significant dose-dependent effect (P<0.01). Relative to those in the control group, LPS significantly elevated the mRNA expression levels of arginase 1 (*Arg1*) and *Il10*. Compared with those in the LPS group, the mRNA expression levels of arginase 1 (*Arg1*) and *Il10* were

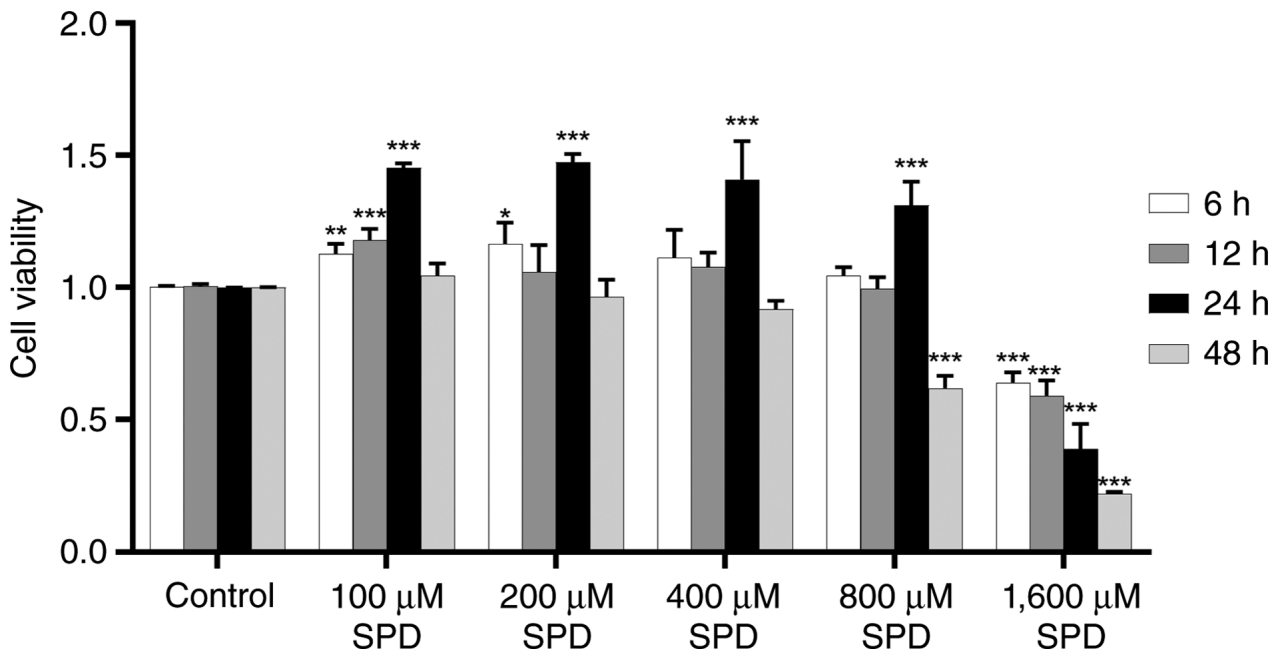


Figure 1. Impact of different concentrations and treatment durations of SPD on the viability of RAW 264.7 cells (n=3; mean  $\pm$  SD; one-way ANOVA with Tukey's post-hoc test). \*\*\*P<0.001, \*\*P<0.01 and \*P<0.05 vs. control group. SPD, spermidine.

significantly increased in the 200 and 400  $\mu$ M SPD groups (P<0.01), and peaked after treatment with 800  $\mu$ M SPD (Fig. 3E and F). Furthermore, SPD treatment reduced the mRNA expression levels of *Tnfa*, *Il6*, *Il1b* and *Nos2* at all tested SPD concentrations. These results implied that SPD inhibited autoinflammation.

**Effect of SPD on inflammatory factor levels in RAW 264.7 cells.** A major function of M1-polarized macrophages is the production of pro-inflammatory cytokines. Initially, IL-6 secreted by RAW 264.7 cells into the conditioned medium was examined using ELISA. LPS notably stimulated IL-6 secretion by macrophages compared with that in the control group (Fig. 4A). This suggested that RAW 264.7 cells were activated by LPS stimulation, thereby inducing an inflammatory response. In addition, SPD markedly reduced IL-6 production in a dose-dependent manner compared with that in the LPS group. LPS stimulation led to an elevation in IL-10 levels compared with those in the control group. IL-10 was not significantly induced at the lower SPD concentration (200  $\mu$ M) but was significantly enhanced compared with that in the LPS group at higher concentrations (400 and 800  $\mu$ M) (Fig. 4B). These results indicated that SPD may promote anti-inflammatory effects by both inhibiting IL-6 and inducing IL-10 secretion.

**Influence of SPD on LPS-induced phosphorylation of p65 and I $\kappa$ B $\alpha$  in macrophages.** NF- $\kappa$ B is a key target pathway activated by inflammation (25). Therefore, NF- $\kappa$ B activation was investigated through western blotting to determine whether SPD inhibited its activation (Fig. 5A). The levels of the NF- $\kappa$ B pathway-associated proteins p65, p-p65, I $\kappa$ B $\alpha$  and p-I $\kappa$ B $\alpha$  were analyzed after 24 h of treatment with LPS and SPD. The expression levels of p-p65 and p-I $\kappa$ B $\alpha$  were significantly increased in macrophages after 24 h of LPS stimulation

compared with that in the control group (Fig. 5B and C). Notably, in cultured RAW 264.7 cells, three SPD concentrations (200, 400 and 800  $\mu$ M) inhibited the LPS-induced increase in p-p65 and p-I $\kappa$ B $\alpha$  protein expression after 24 h of incubation in a dose-dependent manner. Treatment with all tested SPD concentrations for 24 h markedly inhibited the activation of both p-p65 and p-I $\kappa$ B $\alpha$  proteins. These results suggested that the mechanism underlying the anti-inflammatory effect of SPD may involve inhibition of NF- $\kappa$ B activation, which in turn inhibits the expression of inflammatory genes.

## Discussion

Within the present study, SPD treatment was demonstrated to impede the shift of RAW 264.7 cells towards the M1 phenotype upon LPS stimulation. This was evidenced by the downregulation of the M1 phenotype marker CD86 and upregulation of the M2 phenotype marker CD206, along with the modulation of relevant cytokine production. Compared with previous studies on the anti-inflammatory effects of SPD (18-20), the present study systematically provided dose-response data regarding the effects of SPD (0-800  $\mu$ M) on macrophage polarization, combining transcriptional (RT-qPCR) and secretory (ELISA) analyses to demonstrate the regulatory effect of SPD on pro-inflammatory and anti-inflammatory factors. The findings of the present study further provide a mechanistic understanding of the anti-inflammatory properties of SPD. Alongside the reduction in the M1 phenotype-specific surface marker, SPD reduced M1 phenotype cytokine secretion, as determined by measuring the downregulation of *Tnfa*, *Il6* and *Il1b* gene expression. All of these genes are implicated in RA (26).

RA is an autoimmune inflammatory disease that is primarily characterized by bilateral symmetrical joint damage and synovial inflammation (24,27). During chronic inflammation,

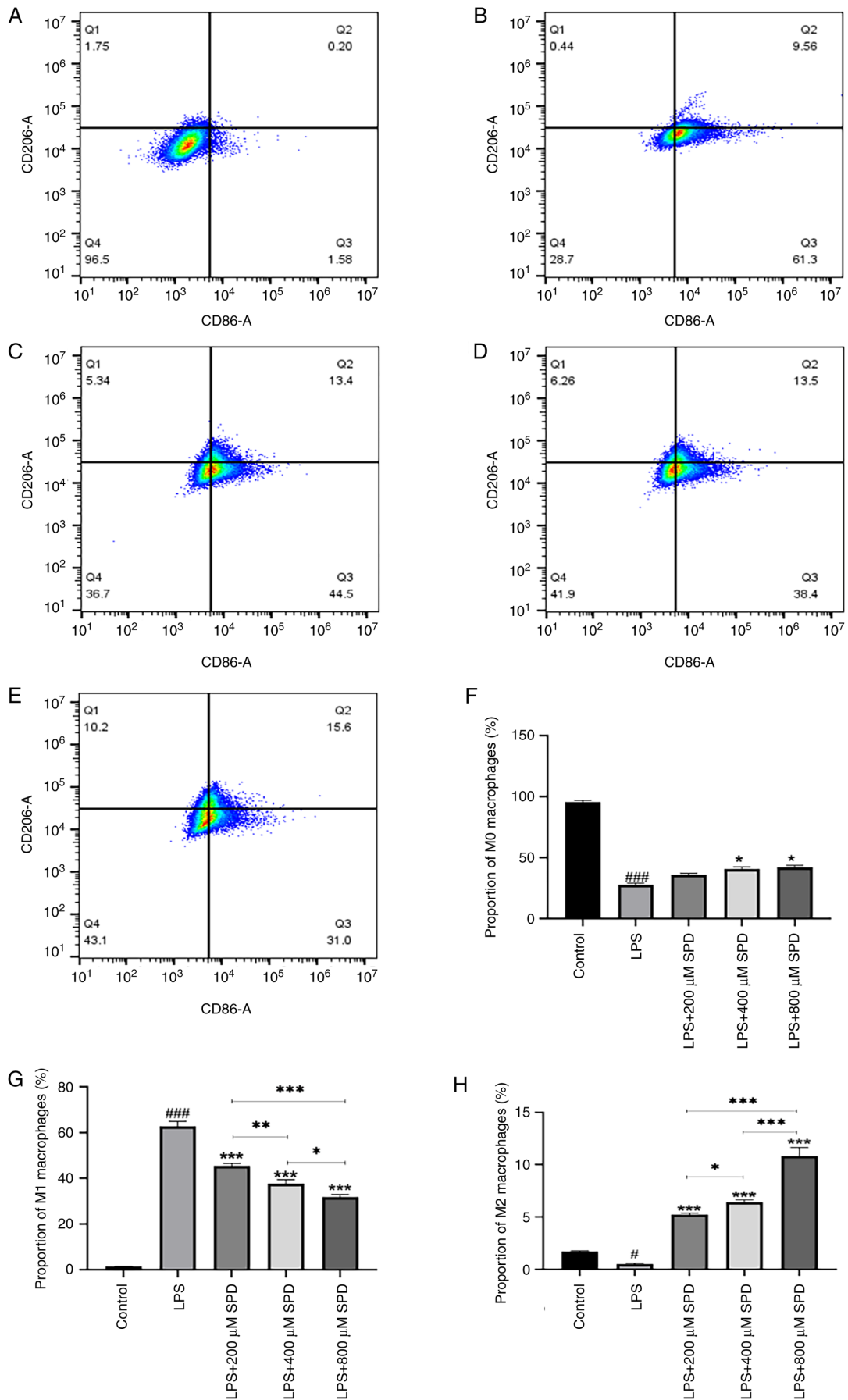


Figure 2. Influence of SPD on LPS-induced polarization of RAW 264.7 cells. Representative flow cytometry plots of the (A) control, (B) LPS, (C) LPS + 200  $\mu$ M SPD, (D) LPS + 400  $\mu$ M SPD and (E) LPS + 800  $\mu$ M SPD groups. Proportion of (F) M0, (G) M1 and (H) M2 macrophages (n=3; mean  $\pm$  SD; one-way ANOVA with Tukey's post-hoc test). ###P<0.001 and #P<0.05 vs. control group; \*\*\*P<0.001, \*\*P<0.01 and \*P<0.05 vs. LPS group or as indicated by lines between specific columns. LPS, lipopolysaccharide; SPD, spermidine.

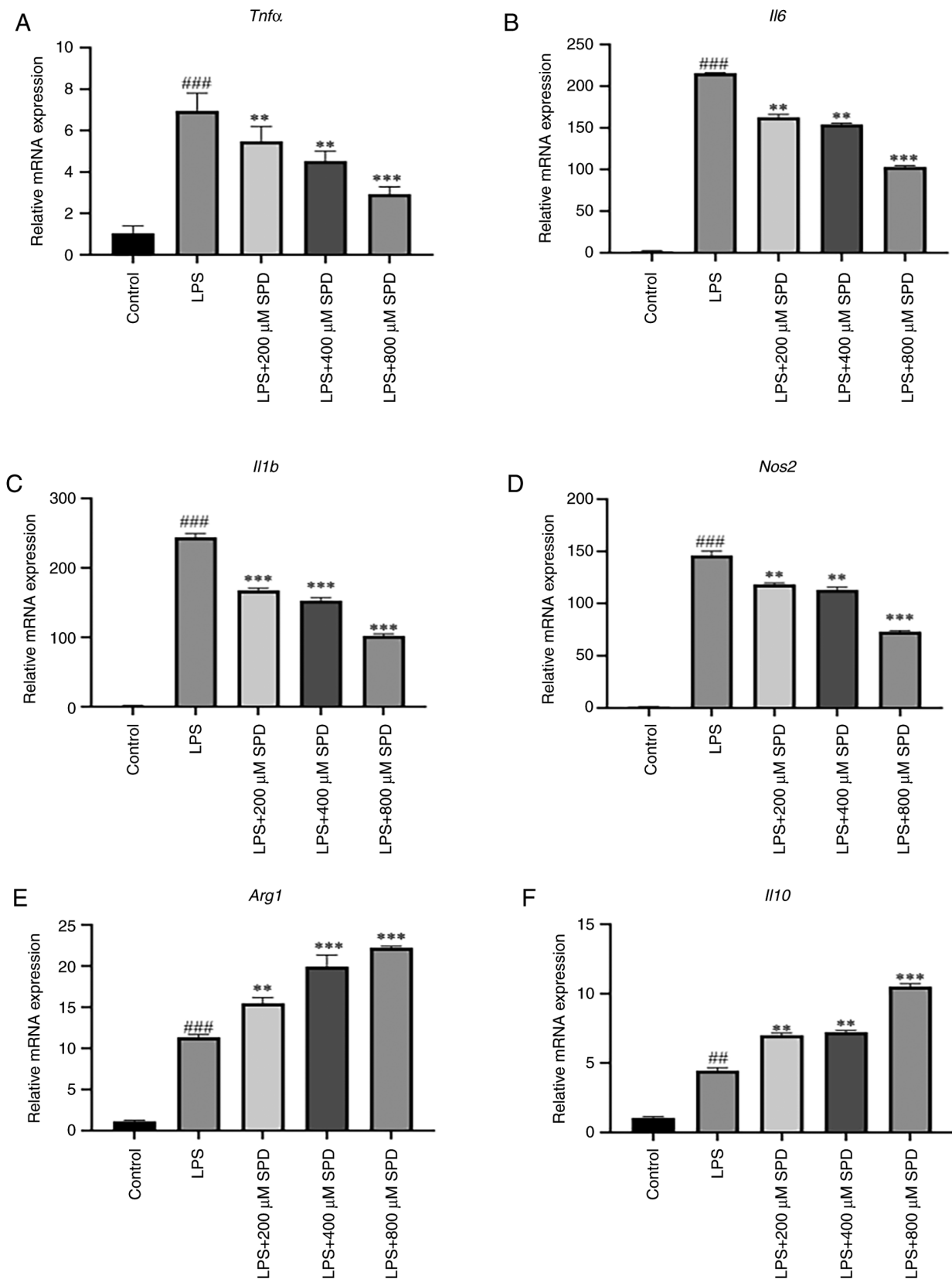


Figure 3. Effect of SPD on LPS-induced inflammatory gene expression in RAW 264.7 cells. Expression levels of (A) *Tnfa*, (B) *Il6*, (C) *Il1b*, (D) *Nos2*, (E) *Arg1* and (F) *Il10* (n=3; mean  $\pm$  SD; one-way ANOVA with Tukey's post-hoc test). ###P<0.001 and ##P<0.01 vs. control group; \*\*\*P<0.001 and \*\*P<0.01 vs. LPS group. LPS, lipopolysaccharide; SPD, spermidine; *Nos2*, nitric oxide synthase; *Arg1*, arginase-1.

numerous types of immune cells contribute to the development and progression of synovitis, among which macrophages are important. The enhanced pro-inflammatory capacity of

macrophages is associated with their hyperactivation (5,28). They subsequently secrete TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , and drive inflammation by engaging other immune cells, thereby

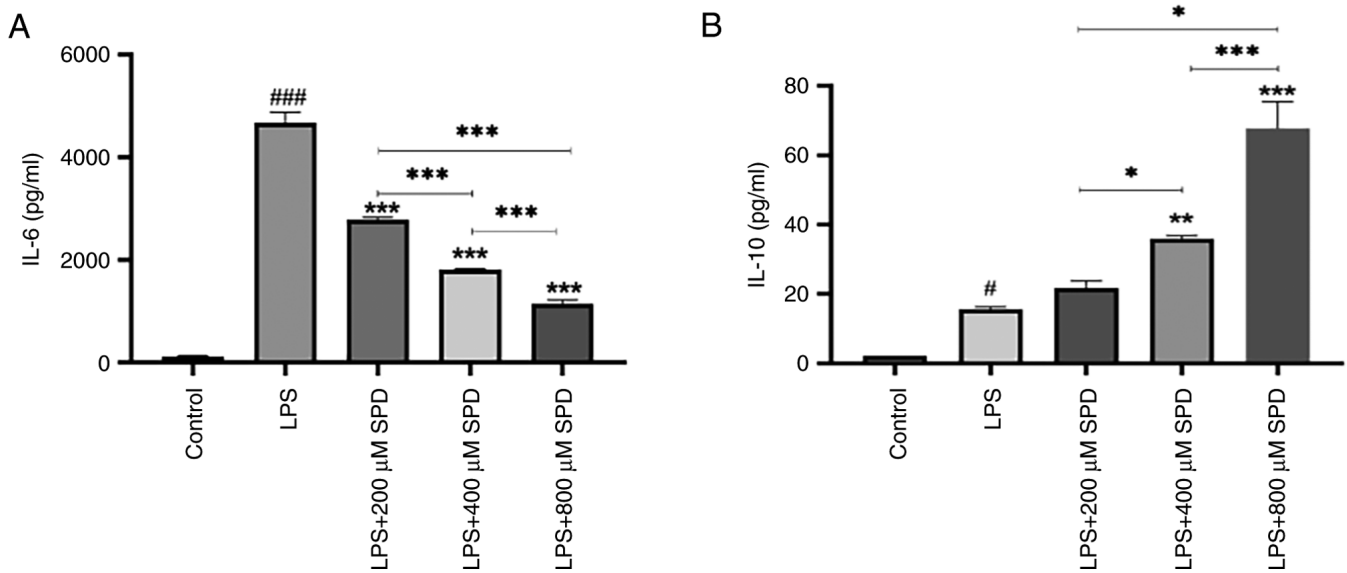


Figure 4. Influence of SPD on LPS-induced cytokine secretion in RAW 264.7 cells. Levels of (A) IL-6 and (B) IL-10 (n=3; mean ± SD; one-way ANOVA with Tukey's post-hoc test). ###P<0.001 and #P<0.05 vs. control group; \*\*\*P<0.001, \*\*P<0.01 and \*P<0.05 vs. LPS group or as indicated by lines between specific columns. LPS, lipopolysaccharide; SPD, spermidine.

activating fibroblasts and promoting T-cell polarization (5). Activated macrophages include M1 and M2 phenotypes, with M1 macrophages being the primary type present in patients with RA (29). Previous studies have highlighted the key pathogenic effect of M1-type macrophages in RA (6,20). Specifically, an imbalance between M1 and M2 macrophages is one of the principal drivers of synovial inflammation progression in RA (29). Therefore, macrophages are considered potentially novel therapeutic targets for RA (30). This potential has also been demonstrated by altered macrophage numbers and inflammatory product levels in the synovial membrane of patients with RA (20). Following macrophage activation by LPS, a number of signaling pathways are activated, including the NF-κB pathway (31). In addition, macrophage activation is tightly controlled by specific signaling pathways such as NF-κB to maintain immune homeostasis (32).

LPS-induced inflammatory RAW 264.7 cell models have been utilized to screen anti-inflammatory drugs *in vitro* (33). Notably, LPS upregulates CD86, a specific marker of M1-type macrophages (34). Consistent with these findings, in the present study, the increase in CD86 protein levels following LPS activation demonstrated its ability to induce the M1 phenotype in RAW 264.7 cells.

SPD, a trivalent cationic polyamine compound present in eukaryotic cells, particularly sperm (18), interacts with nucleic acids, proteins, ATP and other polyanions to regulate autophagy and apoptosis, foster lipid metabolism, enhance anti-inflammatory and antioxidant activities, and augment mitochondrial metabolism and respiration (17). Furthermore, SPD reduces inflammation-induced damage in the body by suppressing the production of inflammatory cytokines (35).

SPD treatment reduces joint swelling and synovitis in mice with collagen-induced arthritis (20). In the present study, flow cytometry revealed that LPS facilitated the conversion of RAW 264.7 cells into M1-type macrophages, whereas SPD treatment suppressed this effect and promoted the conversion

of RAW 264.7 cells into M2-type macrophages. Thus, SPD may exert its anti-inflammatory effects by suppressing the LPS-driven transition of RAW 264.7 cells to the M1 phenotype. However, a limitation of the present study was the lack of SPD-only control groups, which prevented the exclusion of potential direct effects of SPD on macrophage polarization or NF-κB signaling in resting cells. Future studies should aim to include these controls to further clarify the basal regulatory effects of SPD.

Macrophages are the primary TNF-α- and IL-6-secreting cells in the joints of patients with RA. TNF-α and IL-6 are central components of the RA synovial cytokine network, stimulating osteoclast synthesis, causing bone and cartilage degradation and damage, and inducing the release of other pro-inflammatory mediators, leading to exacerbation of the inflammatory response (36). The degree of release of inflammatory factors is an important indicator of the severity of inflammation. In the present study, LPS stimulated RAW 264.7 cells, which increased the mRNA levels of *Tnf*, *Il6*, *Il1b* and *Nos2*, and enhanced IL-6 production, similar to the findings of Jeong *et al* (19) and Huang *et al* (37). By contrast, SPD markedly diminished IL-6 production in RAW 264.7 cells by downregulating *Il6* expression at the mRNA level without inducing cytotoxicity. This was consistent with previous findings in mice with collagen-induced arthritis (20). In addition, SPD increased the levels of M2 macrophage-related factors (*Arg1* and *Il10*) and promoted the secretion of IL-10, with the most notable effect at 800 μM. This suggests that SPD may attenuate the LPS-induced inflammatory response in macrophages by inhibiting the secretion of pro-inflammatory factors and promoting the secretion of the anti-inflammatory cytokine. The present study only detected *Tnf* and *Il1b* mRNA levels and their secretion levels remain unclear. Differences between transcriptional and secretory levels may exist and future studies should aim to supplement ELISA to further demonstrate these results.

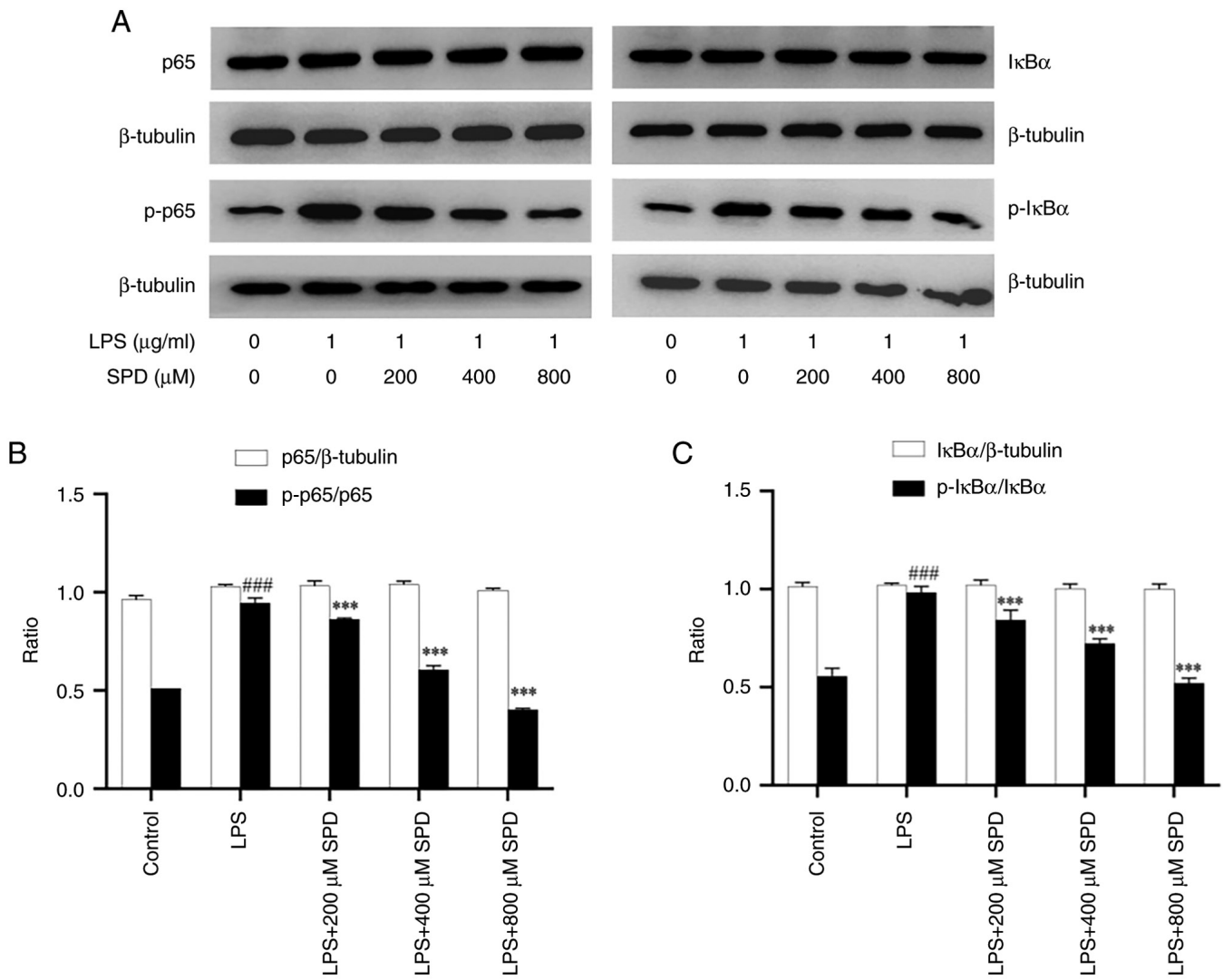


Figure 5. Effect of SPD on LPS-induced expression of NF- $\kappa$ B signaling pathway proteins in RAW 264.7 cells. (A) Representative western blotting images, and relative expression levels of (B) p-p65/p65 and (C) p-I $\kappa$ B $\alpha$ /I $\kappa$ B $\alpha$  (n=3; mean  $\pm$  SD; one-way ANOVA with Tukey's post-hoc test). ###P<0.001 vs. control group; \*\*\*P<0.001 vs. LPS group. LPS, lipopolysaccharide; SPD, spermidine; p-, phosphorylated.

In unstimulated cells, the NF- $\kappa$ B dimer and I $\kappa$ B protein associate tightly to form a complex that resides in the cytoplasm of inactive cells. When cells are stimulated by LPS, the I $\kappa$ B protein is phosphorylated and the NF- $\kappa$ B dimer is no longer inhibited. NF- $\kappa$ B enters the nucleus under the guidance of the nuclear localization sequence, where it interacts with specific DNA sequences and participates in the control of gene transcription, thereby encoding multiple functions, such as inflammation, cell survival and cell division (38,39). In the present study, LPS-activated phosphorylation of p65 and I $\kappa$ B $\alpha$  was elevated in RAW 264.7 macrophages. SPD decreased LPS-activated phosphorylation of p65 and I $\kappa$ B $\alpha$  without altering total p65 and I $\kappa$ B $\alpha$  protein levels. Overall, SPD mediated the NF- $\kappa$ B pathway by preventing the phosphorylation of I $\kappa$ B- $\alpha$ . The present results also suggest that deactivation of the NF- $\kappa$ B pathway by SPD may downregulate pro-inflammatory gene expression. NF- $\kappa$ B activation occurs rapidly (within minutes to hours) and the single time-point measurement at 24 h in the present study may neglect the early dynamic changes of the pathway. The 24 h time-point was chosen to align

with the detection of macrophage polarization and cytokine secretion, which are key downstream events of NF- $\kappa$ B signaling in inflammatory responses. The present study did not perform nuclear/cytosolic fractionation or immunofluorescence to directly confirm p65 nuclear translocation, which further limits the depth of the NF- $\kappa$ B mechanism analysis. Although the present data imply that the potential anti-inflammatory effect of SPD is achieved by blocking the NF- $\kappa$ B pathway, the precise mechanism by which SPD influences the production of cell factors that activate macrophages warrants further investigation.

In conclusion, the present study established that SPD exerts potent anti-inflammatory effects on RAW 264.7 macrophages. Specifically, SPD decreased the mRNA expression levels of pro-inflammatory mediators, thereby reducing the synthesis of inflammatory factors in LPS-activated RAW 264.7 cells. These effects were associated with the suppression of LPS-induced activation of the NF- $\kappa$ B signaling pathway, as evidenced by the inhibition of p65 and I $\kappa$ B $\alpha$  phosphorylation. Therefore, these findings indicate the potential of SPD as a natural drug for the treatment of RA.

## Acknowledgements

Not applicable.

## Funding

The present study was supported by the Natural Science Foundation of Hunan Provincial Department of Science and Technology (grant no. 2025JJ90184), the Key Project of Hunan Provincial Health Commission (grant no. C202311000138), the Key Cultivation Project of Renshu Fund of Hunan Provincial People's Hospital (grant no. RS2022A14) and the Natural Science Foundation of Changsha Science and Technology Bureau (grant no. kq2403140).

## Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

## Authors' contributions

HY and FP conceptualized the present study. HY, FP, YFZ and YZZ established the methodology. FP, ZYL, SXW, YMP and YZZ conducted the present investigation. HY verified the experimental design and data analysis. FP, YMP and SXW performed the data analysis, and FP, SXW and ZYL provided the reagents. ZYL and FP prepared the figures and tables. FP and HY conducted the formal analysis. HY, FP and YFZ conducted data curation. FP wrote the original draft. HY and YFZ wrote, reviewed and edited the manuscript. HY supervised the study, conducted project administration and acquired the funding. HY and FP confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

- McInnes IB and Schett G: Pathogenetic insights from the treatment of rheumatoid arthritis. *Lancet* 389: 2328-2337, 2017.
- Smolen JS, Aletaha D, Barton A, Burmester GR, Emery P, Firestein GS, Kavanaugh A, McInnes IB, Solomon DH, Strand V and Yamamoto K: Rheumatoid arthritis. *Nat Rev Dis Primers* 4: 18001, 2018.
- Calabresi E, Petrelli F, Bonifacio AF, Puxeddu I and Alunno A: One year: Pathogenesis of rheumatoid arthritis. *Clin Exp Rheumatol* 36: 175-184, 2018.
- Roszkowski L and Ciechomska M: Tuning monocytes and macrophages for personalized therapy and diagnostic challenge in rheumatoid arthritis. *Cells* 10: 1860, 2021.
- Cutolo M, Soldano S, Gotelli E, Montagna P, Campitiello R, Paolino S, Pizzorni C, Sulli A, Smith V and Tardito S: CTLA4-Ig treatment induces M1-M2 shift in cultured monocyte-derived macrophages from healthy subjects and rheumatoid arthritis patients. *Arthritis Res Ther* 23: 306, 2021.
- Paoletti A, Rohmer J, Ly B, Pascaud J, Rivière E, Seror R, Le Goff B, Nocturne G and Mariette X: Monocyte/macrophage abnormalities specific to rheumatoid arthritis are linked to miR-155 and are differentially modulated by different TNF inhibitors. *J Immunol* 203: 1766-1775, 2019.
- Tu J, Hong W, Guo Y, Zhang P, Fang Y, Wang X, Chen X, Lu S and Wei W: Ontogeny of synovial macrophages and the roles of synovial macrophages from different origins in arthritis. *Front Immunol* 10: 1146, 2019.
- Wynn TA and Vannella KM: Macrophages in tissue repair, regeneration, and fibrosis. *Immunity* 44: 450-462, 2016.
- Huen SC and Cantley LG: Macrophages in renal injury and repair. *Annu Rev Physiol* 79: 449-469, 2017.
- Mosser DM and Edwards JP: Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8: 958-969, 2008.
- Meng XM, Mak TSK and Lan HY: Macrophages in renal fibrosis. *Adv Exp Med Biol* 1165: 285-303, 2019.
- Han C, Yang Y, Sheng Y, Wang J, Zhou X, Li W, Guo L, Zhang C and Ye Q: Glaucocalyxin B inhibits cartilage inflammatory injury in rheumatoid arthritis by regulating M1 polarization of synovial macrophages through NF- $\kappa$ B pathway. *Aging (Albany, NY)* 13: 22544-22555, 2021.
- Fan J, Feng Z and Chen N: Spermidine as a target for cancer therapy. *Pharmacol Res* 159: 104943, 2020.
- Madeo F, Hofer SJ, Pendl T, Bauer MA, Eisenberg T, Carmona-Gutierrez D and Kroemer G: Nutritional aspects of spermidine. *Annu Rev Nutr* 40: 135-159, 2020.
- Hofer SJ, Liang Y, Zimmermann A, Schroeder S, Dengjel J, Kroemer G, Eisenberg T, Sigrist SJ and Madeo F: Spermidine-induced hypusination preserves mitochondrial and cognitive function during aging. *Autophagy* 17: 2037-2039, 2021.
- Ni YQ and Liu YS: New insights into the roles and mechanisms of spermidine in aging and age-related diseases. *Aging Dis* 12: 1948-1963, 2021.
- Madeo F, Eisenberg T, Pietrocola F and Kroemer G: Spermidine in health and disease. *Science* 359: eaan2788, 2018.
- Liu R, Li X, Ma H, Yang Q, Shang Q, Song L, Zheng Z, Zhang S, Pan Y, Huang P, *et al*: Spermidine endows macrophages anti-inflammatory properties by inducing mitochondrial superoxide-dependent AMPK activation, Hif-1 $\alpha$  upregulation and autophagy. *Free Radic Biol Med* 161: 339-350, 2020.
- Jeong JW, Cha HJ, Han MH, Hwang SJ, Lee DS, Yoo JS, Choi IW, Kim S, Kim HS, Kim GY, *et al*: Spermidine protects against oxidative stress in inflammation models using macrophages and Zebrafish. *Biomol Ther (Seoul)* 26: 146-156, 2018.
- Yuan H, Wu SX, Zhou YF and Peng F: Spermidine inhibits joints inflammation and macrophage activation in mice with collagen-induced arthritis. *J Inflamm Res* 14: 2713-2721, 2021.
- Mathiesen CBK, Rudjord-Levann AM, Gad M, Larsen J, Seljelberg F and Pedersen AE: Cladribine inhibits secretion of pro-inflammatory cytokines and phagocytosis in human monocyte-derived M1 macrophages in-vitro. *Int Immunopharmacol* 91: 107270, 2021.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Bian H, Li F, Wang W, Zhao Q, Gao S, Ma J, Li X, Ren W, Qin C and Qi J: MAPK/p38 regulation of cytoskeleton rearrangement accelerates induction of macrophage activation by TLR4, but not TLR3. *Int J Mol Med* 40: 1495-1503, 2017.
- Yang X, Chang Y and Wei W: Emerging role of targeting macrophages in rheumatoid arthritis: Focus on polarization, metabolism and apoptosis. *Cell Prolif* 53: e12854, 2020.
- Castejón ML, Alarcón-de-la-Lastra C, Rosillo MÁ, Montoya T, Fernández-Bolaños JG, González-Benjumea A and Sánchez-Hidalgo M: A new peracetylated oleuropein derivative ameliorates joint inflammation and destruction in a murine collagen-induced arthritis model via activation of the Nrf-2/Ho-1 antioxidant pathway and suppression of MAPKs and NF- $\kappa$ B activation/Ho-1 antioxidant pathway and suppression of MAPKs and NF- $\kappa$ B activation. *Nutrients* 13: 311, 2021.
- Zhao JM, Chen X, Cheng K, Shi Q and Peng K: Anserine and glucosamine supplementation attenuates the levels of inflammatory markers in rats with rheumatoid arthritis. *AMB Express* 10: 57, 2020.
- Tanaka Y: Rheumatoid arthritis. *Inflamm Regen* 40: 20, 2020.

28. Li J, Hsu HC and Mountz JD: Managing macrophages in rheumatoid arthritis by reform or removal. *Curr Rheumatol Rep* 14: 445-454, 2012.
29. Tsubaki T, Arita N, Kawakami T, Shiratsuchi T, Yamamoto H, Takubo N, Yamada K, Nakata S, Yamamoto S and Nose M: Characterization of histopathology and gene-expression profiles of synovitis in early rheumatoid arthritis using targeted biopsy specimens. *Arthritis Res Ther* 7: R825-R836, 2005.
30. Tardito S, Martinelli G, Soldano S, Paolino S, Pacini G, Patane M, Alessandri E, Smith V and Cutolo M: Macrophage M1/M2 polarization and rheumatoid arthritis: A systematic review. *Autoimmun Rev* 18: 102397, 2019.
31. Han Y, Yuan C, Zhou X, Han Y, He Y, Ouyang J, Zhou W, Wang Z, Wang H and Li G: Anti-inflammatory activity of three triterpene from *Hippophae rhamnoides* L. in lipopolysaccharide-stimulated RAW264.7 cells. *Int J Mol Sci* 22: 12009, 2021.
32. Dorrington MG and Fraser IDC: NF- $\kappa$ B signaling in macrophages: Dynamics, crosstalk, and signal integration. *Front Immunol* 10: 705, 2019.
33. Byun J, Kim SK and Ban JY: Anti-inflammatory and anti-oxidant effects of Korean ginseng berry extract in LPS-activated RAW264.7 macrophages. *Am J Chin Med* 49: 719-735, 2021.
34. Liu L, Guo H, Song A, Huang J, Zhang Y, Jin S, Li S, Zhang L, Yang C and Yang P: Progranulin inhibits LPS-induced macrophage M1 polarization via NF- $\kappa$ B and MAPK pathways. *BMC Immunol* 21: 32, 2020.
35. Ma L, Ni L, Yang T, Mao P, Huang X, Luo Y, Jiang Z, Hu L, Zhao Y, Fu Z and Ni Y: Preventive and therapeutic spermidine treatment attenuates acute colitis in mice. *J Agric Food Chem* 69: 1864-1876, 2021.
36. Zhong Z, Sanchez-Lopez E and Karin M: Autophagy, NLRP3 inflammasome and auto-inflammatory/immune diseases. *Clin Exp Rheumatol* 34 (4 Suppl 98): S12-S16, 2016.
37. Huang P, Hong J, Mi J, Sun B, Zhang J, Li C and Yang W: Polyphenols extracted from *Enteromorpha clathrata* alleviates inflammation in lipopolysaccharide-induced RAW 264.7 cells by inhibiting the MAPKs/NF- $\kappa$ B signaling pathways. *J Ethnopharmacol* 286: 114897, 2022.
38. Barger SW, Moerman AM and Mao X: Molecular mechanisms of cytokine-induced neuroprotection: NF $\kappa$ B and neuroplasticity. *Curr Pharm Des* 11: 985-998, 2005.
39. Syama HP, Sithara T, Lekshmy Krishnan S and Jayamurthy P: *Syzygium cumini* seed attenuates LPS induced inflammatory response in murine macrophage cell line RAW264.7 through NF- $\kappa$ B translocation. *J Funct Foods* 44: 218-226, 2018.