

# Spermine Enhances Cholesterol Effluence of Macrophages Through Caspase 2-Mediated Autophagy

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**Background:** Spermine (SPM) is known to play a role in regulating cholesterol efflux of macrophages, which is a critical anti-atherosclerotic pathway, but the underlying mechanism remains elusive. The deficiency of caspase 2 (*CASP2*), a target of SPM, can induce autophagy, which promotes cholesterol efflux in atherosclerosis. Herein, we aimed to explore whether SPM could regulate *CASP2*-mediated autophagy in the cholesterol efflux of macrophages.

**Methods:** Human THP-1 monocytes were induced into macrophages. After transfection and treatment with SPM or autophagy inhibitor 3-methyladenine (3-MA), the cholesterol uptake of THP-1 cell-derived macrophages and the lipid accumulation were examined using Dil-oxidized low-density lipoprotein (Dil-oxLDL) uptake assay and Oil Red O staining, respectively. The cholesterol efflux was measured by means of [<sup>3</sup>H]-cholesterol detection. Quantification of *CASP2* and factors related to autophagy and apoptosis was completed using Western blotting and quantitative reverse transcription polymerase chain reaction (qRT-PCR).

**Results:** SPM treatment boosted cholesterol efflux and prevented lipid accumulation of THP-1 cell-derived macrophages ( $p < 0.001$ ), without inducing cholesterol uptake of THP-1 cell-derived macrophages ( $p > 0.05$ ). SPM upregulated the expression of autophagy-related 5 (ATG5), light chain 3 (LC3) II/LC3 I, and B-cell lymphoma-2 (Bcl-2) while downregulating Sequestosome 1 (P62) and *CASP2* levels ( $p < 0.01$ ). Treatment with 3-MA reversed the effects of SPM on cholesterol efflux, lipid accumulation, and autophagy-related protein expression ( $p < 0.01$ ). Separately, *CASP2* overexpression offset the impacts of SPM on cholesterol efflux, lipid accumulation, and expressions of proteins related to autophagy and apoptosis ( $p < 0.05$ ).

**Conclusion:** SPM promotes autophagy to enhance the cholesterol efflux of THP-1 cell-derived macrophages by downregulating *CASP2* expression.

**Keywords:** spermine; macrophages; cholesterol efflux; autophagy; caspase 2

## Introduction

Featuring dyslipidemia and over-accumulation of cholesterol within the arterial intima, atherosclerosis is a lipid-induced inflammatory disease predominantly affecting large arteries, and is recognized as a risk factor for cardiovascular diseases (CVD) such as myocardial infarction and stroke, during which macrophages act as the central effector cells [1–3]. Macrophages promote the generation of foam cells and the formation of plaque arising from cholesterol ester accumulation, which is an attribute of atherosclerosis [4]. Thus, enhancing cholesterol removal from these cells, notably by increasing cholesterol efflux from macrophages, is of great importance to suppress lipid accumulation and plaque lipid build-up.

Present in a diverse range of organisms, spermine (SPM) is a type of polyamine essential for cell growth and plays crucial roles in physiological and pathological functions [5]. It has been reported that SPM reverses mem-

ory deficit in mice induced by lipopolysaccharide [6], enhances the acetylation of tubulins, and promotes the autophagic degradation of prion aggregates [7]. Besides, SPM can relieve the age-related cardiac function deterioration in rats [8], and mitigate colon cancer, prostate cancer, and breast cancer [5,9,10]. SPM has protective effects on CVD such as atherosclerosis by promoting the pyroptosis of macrophages [11], and assisting PDC-109 to induce efflux of cholesterol and choline phospholipids [12]. However, there is a dearth of research expounding on whether SPM affects the cholesterol efflux of macrophages.

Caspase 2 (*CASP2*), a kind of protease, functions as an initiator and effector of apoptosis and is instrumental in maintaining the genomic integrity and the normal configuration of the chromosome [13]. It has been demonstrated that *CASP2* downregulation activates the AMP-dependent kinase/Protein kinase B/mammalian target of rapamycin (AMPK/AKT/mTOR) and the AMPK/mitogen-activated protein kinase (MAPK) pathways to induce in-

complete autophagy [14], and the loss of *CASP2*-dependent apoptosis induces autophagy after mitochondrial oxidative stress erupts in murine cortical neurons [15]. Autophagy can promote cholesterol efflux to suppress the development of atherosclerosis [16]. In this study, prediction analyses through SwissTargetPrediction (<http://www.swisstargetprediction.ch/>) establish *CASP2* as a target of SPM, which prompted us to investigate whether SPM can regulate cholesterol efflux of macrophages through *CASP2*-mediated autophagy.

THP-1 monocytes are commonly used as a precursor for macrophage conversion because of their ability to mimic the process of macrophage differentiation, and their high morphological and biological resemblance to primary monocytes [17]. Therefore, in the current study, we cultured human THP-1 monocytes-derived macrophages to explore the effects of SPM treatment and *CASP2* regulation on the cholesterol efflux of macrophages, with the primary aim of confirming whether SPM regulates the cholesterol efflux of macrophages through *CASP2*-mediated autophagy.

## Materials and Methods

### Cell Culture

Human THP-1 monocytes (CL-0233) from Procell (Wuhan, China) were cultured in a THP-1-specific medium (CM-0233, Procell, Wuhan, China). The cultured cells were routinely tested for mycoplasma contamination, and the cells were authenticated using short tandem repeat (STR) detection. The THP-1 cells were induced into macrophages by incubating, at a preset condition (37 °C, 5% CO<sub>2</sub>), in 160 nM phorbol 12-myristate 13-acetate (PMA; P6741, Solarbio, Beijing, China) for 24 h and then in RPMI-1640 medium (PM150110, Procell, Wuhan, China) containing 10% fetal bovine serum (FBS; 164210, Procell, Wuhan, China) and 1% penicillin-streptomycin solution (PB180120, Procell, Wuhan, China) for 12 h [18]. Afterward, the derived macrophages were collected for transfection or drug treatment.

### Cell Transfection

*CASP2* overexpression plasmid constructed using the pcDNA3.1 vector (VT1001) was obtained from YouBio (Hunan, China), and the negative control used in this experiment was the plasmid containing blank pcDNA3.1 vector. The sequence information for *CASP2* (NM\_001224.5:89-1027) overexpression is provided in **Supplementary File 1**. For transfection, THP-1 cell-derived macrophages were grown in 6-well plates to 80% confluence, followed by 48-h transfection of *CASP2* overexpression plasmid or control plasmid using 3 μL Lipo6000 Transfection Reagent (C0526, Beyotime, Shanghai, China).

### Cell Treatment

The cells were divided into two categories, depending on the treatment plan. For treatment with SPM alone, THP-1 cell-derived macrophages were exposed to 5 μmol/L SPM (S8310, Solarbio, Beijing, China) for 24 h [11]. For co-treatment with 3-methyladenine (3-MA) and SPM, the cells were initially pretreated with 10 mM 3-MA (IM0190, Solarbio, Beijing, China) for 6 h [19] and then exposed to SPM (5 μmol/L).

### Quantitative Reverse Transcription Polymerase Chain Reaction

After transfection or drug treatment, *CASP2* mRNA level in the THP-1 cell-derived macrophages was quantified by means of quantitative reverse transcription polymerase chain reaction (qRT-PCR). Firstly, total RNA was extracted from the cells using TriQuick Reagent (R1100, Solarbio, Beijing, China), and then cDNA synthesis with Hifair II 1st Strand cDNA Synthesis Kit (11119ES60, YEASEN, Shanghai, China) was performed. Subsequently, the cDNA was mixed with *CASP2* primers and SYBR Green Master Mix (11203ES03, YEASEN, Shanghai, China), prior to the amplification using QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). *CASP2* mRNA expression was measured using the 2<sup>-ΔΔCT</sup> method [20]. The sequences of the primers used are as follows:

*CASP2* forward: 5'-AGCTGTTGTTGAGCGAATTGT-3', reverse: 5'-AGCAAGTTGAGGAGTTCCACA-3'; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) forward: 5'-GGAGCGAGATCCCTCCAAAT-3', reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'.

### Dil-Oxidized Low-Density Lipoprotein Uptake Assay

Following transfection or drug treatment, THP-1 cell-derived macrophages were exposed to 10 μg/mL Dil-oxidized low-density lipoprotein (Dil-oxLDL) for 4 h using a Dil-oxLDL uptake assay (20609ES76, YEASEN, Shanghai, China) [18]. For visualization, the images of cell fluorescence after the assay were captured under a DM2500 fluorescence microscope (Leica, Wetzlar, Germany) at 200× magnification.

### Oil Red O Staining

Following transfection or drug treatment, THP-1 cell-derived macrophages were fixed with 4% paraformaldehyde (P1110, Solarbio, Beijing, China) for 10 min, and subjected to washing with double-distilled water, incubated with 60% isopropanol (I434894, Aladdin, Shanghai, China) for 5 min, and staining in Oil Red O solution (G1260, Solarbio, Beijing, China) for 10 min. After being rinsed with 60% isopropanol and double-distilled water, the cells were stained with Mayer's hematoxylin solution (G4070, Solarbio, Beijing, China) for 3 min. After a round of washing

with double-distilled water, the stained cells were observed and imaged under DMi8 S optical microscope (Leica, Wetzlar, Germany) at 400 $\times$  magnification.

#### Cellular Cholesterol Efflux Determination

Cholesterol efflux of THP-1 cell-derived macrophages, which had been transfected with vector or treated, was examined. Briefly, prior to cellular cholesterol efflux determination, the transfected/treated macrophages were cultivated with 0.2  $\mu$ Ci/mL [ $^3$ H]-cholesterol (NET139250UC, PerkinElmer, Waltham, MA, USA) at 37  $^{\circ}$ C for 24 h [21]. After being rinsed with phosphate-buffered saline (PBS; P1022, Solarbio, Beijing, China), the cells were cultured in RPMI-1640 medium containing 0.1% bovine serum albumin (BSA; A8010, Solarbio, Beijing, China) overnight to equilibrate the [ $^3$ H]-cholesterol content. Next, the [ $^3$ H]-cholesterol in culture media and cells was measured using MicroBeta2 Microplate Counter (PerkinElmer, Waltham, MA, USA). The cholesterol efflux was calculated using the following formula: cholesterol efflux (%) = [ $^3$ H]-cholesterol in total media / ([ $^3$ H]-cholesterol in total cell + [ $^3$ H]-cholesterol total media)  $\times$  100.

#### Western Blotting

Total protein was isolated from the transfected/treated THP-1 cell-derived macrophages using RIPA lysis solution (20-188, Merck, St. Louis, MO, USA), and subsequently, protein concentration was determined using a QuantiPro BCA detection kit (QPBCA; SigmaAldrich, St. Louis, MO, USA). The protein samples were denatured through a 5-min incubation with the loading buffer (P1040, Solarbio, Beijing, China) at 100  $^{\circ}$ C. Next, the denatured protein samples were subjected to electrophoresis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (P1200, Solarbio, Beijing, China) for 2 h. The separated proteins were then transferred to the polyvinylidene difluoride (PVDF) membrane (IPVH00010, Merck, St. Louis, MO, USA). The membrane was then blocked with 5% skim milk for 2 h, and then incubated with relative primary antibodies (Abcam, Cambridge, UK) overnight at 4  $^{\circ}$ C; the primary antibodies included those targeting autophagy-related 5 (ATG5; ab108327, 1:5000, 32 kDa), Sequestosome 1 (P62) (ab109012, 1:10,000, 62 kDa), light chain 3 (LC3; ab192890, 1:2000, 14/16 kDa), CASP2 (ab179520, 1:1000, 51 kDa), B-cell lymphoma-2 (Bcl-2; ab32124, 1:1000, 26 kDa), Bcl-2 associated X (BAX; ab32503, 1:5000, 21 kDa), caspase-3 (ab32351, 1:5000, 32 kDa), cleaved caspase-3 (ab2302, 1:500, 17 kDa), and GAPDH (ab8245, 1:8000, 37 kDa). Later, the membrane was incubated with solutions containing secondary antibodies such as goat-anti rabbit IgG (ab6721, 1:20,000) and goat-anti mouse IgG (ab6789, 1:10,000). The protein expression was measured using a ChemiDoc MP chemiluminescence detector (Bio-Rad, Hercules, CA, USA) with Immobilon Western HRP substrate (WBKLS0050, Merck, St. Louis, MO, USA).

#### Cell Counting Kit (CCK-8) Assay

Cell viability was detected using a CCK-8 Assay Kit (40203ES76, YEASEN, Shanghai, China). In a 96-well plate, 100  $\mu$ L of cell suspension was inoculated per well. CCK-8 solution (10  $\mu$ L) was added to each well. The plates were then incubated in the incubator for 1–4 h. The absorbance at 450 nm was measured by the microplate reader (iMark Microplate Absorbance Reader, Bio-Rad Laboratories, Hercules, CA, USA). The relative cell viability rate was determined using the following formula: relative cell viability (%) = [A (dosed) – A (blank)]/[A (0 dosed) – A (blank)]  $\times$  100, A: absorbance.

#### Statistical Analysis

The collected data were analyzed by one-way analysis of variance (ANOVA) using GraphPad 8.0 software (GraphPad Software, Inc., San Diego, CA, USA). Tukey's test was applied for post hoc comparisons. Statistical data are expressed as mean  $\pm$  standard deviation.  $p < 0.05$  was considered statistically significant.

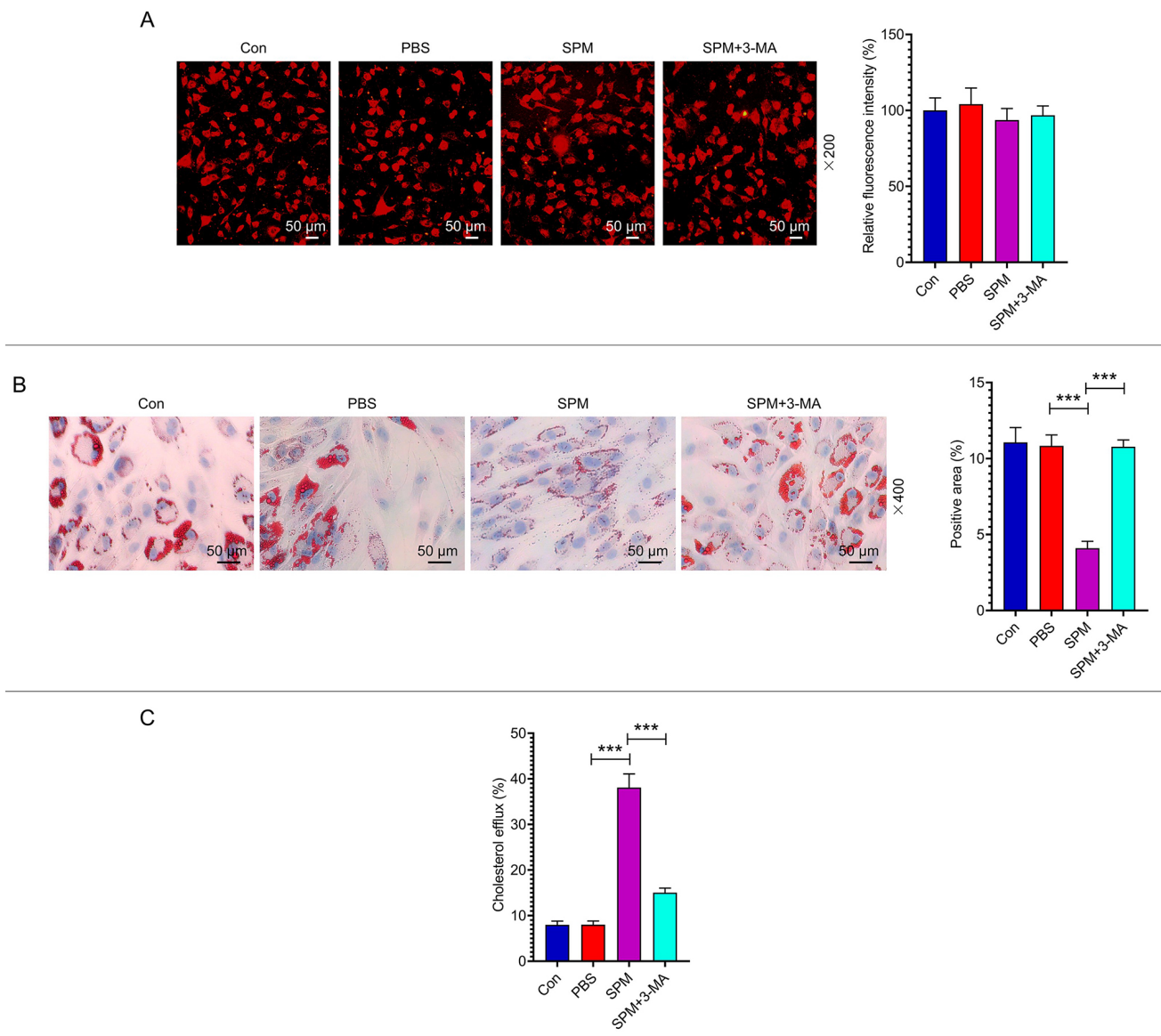
## Results

#### Impact of SPM and 3-MA Treatment on Cholesterol Efflux, Lipid Accumulation and Cholesterol Uptake in Macrophages

The role of SPM in cholesterol uptake was determined. As depicted in Fig. 1A, there was no statistical difference in cell fluorescence intensity among the groups, indicating that neither SPM nor 3-MA affected the cholesterol uptake of THP-1 cell-derived macrophages. Meanwhile, the lipid accumulation was decreased by SPM treatment (Fig. 1B,  $p < 0.001$ ) but increased by 3-MA pretreatment in THP-1 cell-derived macrophages (Fig. 1B,  $p < 0.001$ ). Therefore, we further detected the cholesterol efflux of the cells. As illustrated in Fig. 1C, SPM treatment enhanced the cholesterol efflux of THP-1 cell-derived macrophages ( $p < 0.001$ ), which was offset by 3-MA pretreatment ( $p < 0.001$ ). These findings revealed that SPM resulted in elevated cholesterol efflux and reduced lipid accumulation, which are effects distinctive in autophagy, without apparently impacting cholesterol uptake in macrophages.

#### Enhanced Autophagy and Repression of Macrophage Apoptosis After SPM Treatment

We evaluated the expression of autophagy-related proteins to help delineate the effect of SPM on macrophage autophagy. As shown in Fig. 2A, SPM downregulated P62 level while upregulating ATG5 level and LC3 II/LC3 I ratio in THP-1 cell-derived macrophages ( $p < 0.01$ ); all of these effects could be offset by 3-MA ( $p < 0.01$ ). In addition, CASP2 expression was diminished by SPM treatment ( $p < 0.001$ ; Fig. 2B), which, however, was not influenced by 3-MA treatment (Fig. 2B). Subsequently, SPM promoted cellular viability ( $p < 0.001$ ; Fig. 2C). In the anal-

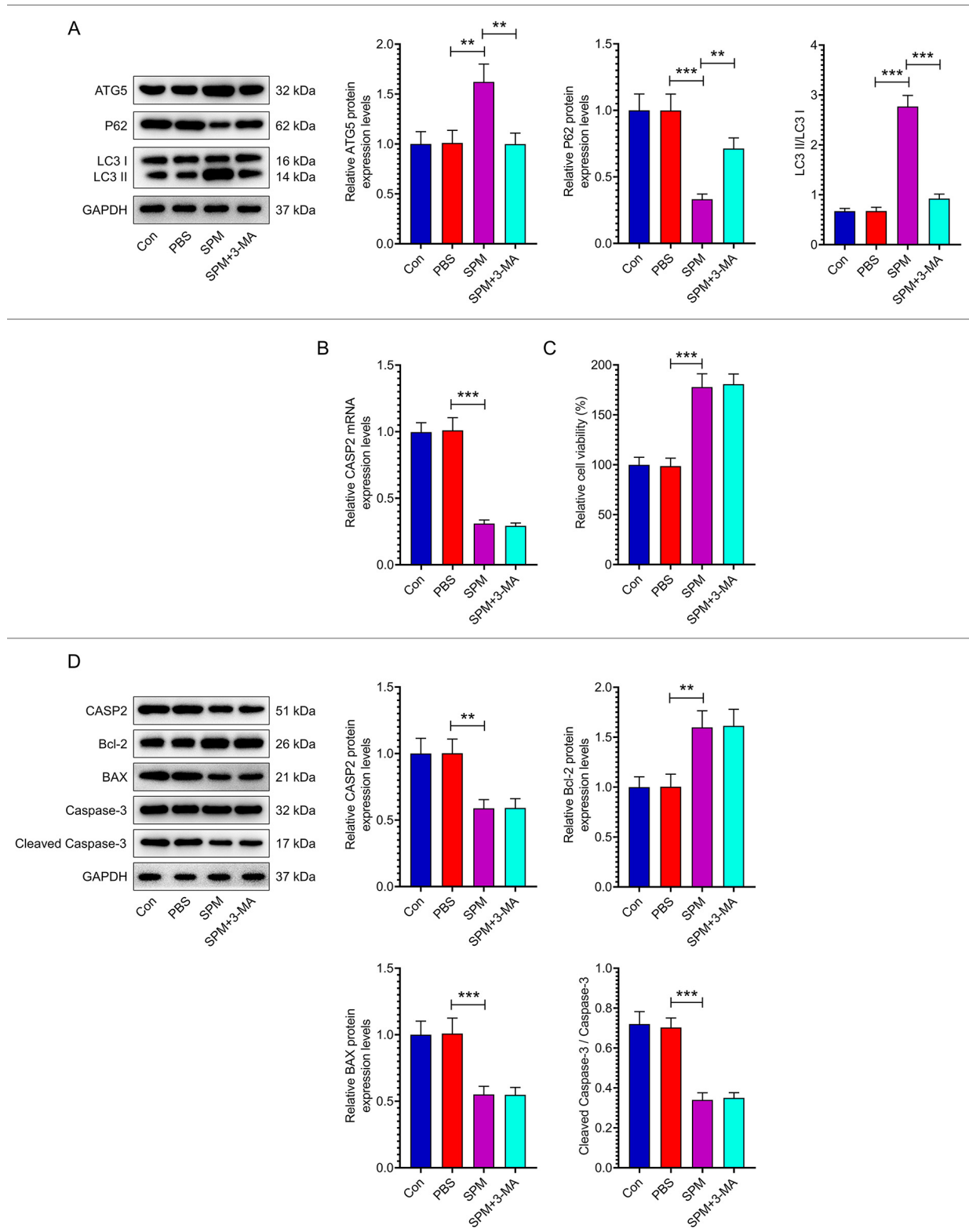


**Fig. 1. Elevated cholesterol efflux and reduced lipid accumulation but minimally altered cholesterol uptake profile in macrophages after SPM treatment, which were reversible by 3-MA treatment.** (A) Fluorescent images of THP-1 cells-derived macrophages treated with SPM for 24 h or pretreated with 3-MA for 6 h and the corresponding quantification of cholesterol uptake measured by Dil-oxLDL uptake assay (200× magnification). (B) Images of cells stained with Oil Red O and the corresponding quantification of lipid accumulation (400× magnification). (C) Measurement of cholesterol efflux in cells, measured through [<sup>3</sup>H]-cholesterol detection. \*\*\**p* < 0.001. N = 3. SPM, spermine; 3-MA, 3-methyladenine; Dil-oxLDL, Dil-oxidized low-density lipoprotein; PBS, phosphate-buffered saline; Con, Control.

ysis of apoptosis-related protein expression, we identified that the SPM treatment led to a reduction of CASP2, BAX, and cleaved caspase-3/caspase-3 levels (*p* < 0.01; Fig. 2D) and elevated Bcl-2 level (*p* < 0.01; Fig. 2D) in the THP-1 cell-derived macrophages. Also, these effects instigated by SPM treatment were not found to be impacted by 3-MA (Fig. 2C,D). Taken together, these findings indicated that SPM promotes autophagy but suppresses macrophage apoptosis.

#### *Effect of SPM on Cholesterol Efflux and Lipid Accumulation in Macrophages, and Its Role on CASP2 Regulation*

To further verify whether the effects of SPM on THP-1 cell-derived macrophages are mediated through regulating CASP2, we assessed the expression of CASP2 in the transfected macrophages. The results showed that CASP2 level was upregulated in the macrophages after CASP2 overexpression by plasmid transfection (*p* < 0.001; Fig. 3A). We



**Fig. 2. Enhanced autophagy and suppressed macrophage apoptosis after SPM treatment.** (A) Expression of autophagy-related factors in THP-1 cell-derived macrophages treated with SPM (24 h) or pretreated with 3-MA (6 h) measured by Western blotting. (B) *CASP2* mRNA expression levels measured by qRT-PCR. (C) Cell viability was measured by CCK-8 assay. (D) Expression of apoptosis-related proteins measured by Western blotting. GAPDH was used as the internal control. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .  $N = 3$ . *CASP2*, caspase 2; ATG5, autophagy-related 5; qRT-PCR, quantitative reverse transcription polymerase chain reaction; CCK-8, Cell Counting Kit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC3, light chain 3; Bcl-2, B-cell lymphoma-2; BAX, Bcl-2 associated X.

failed to find statistical differences in cell fluorescence intensity among the groups, which indicated that SPM treatment and *CASP2* regulation did not affect cholesterol uptake in THP-1 cell-derived macrophages (Fig. 3B). Meanwhile, SPM treatment led to dampened lipid accumulation in THP-1 cell-derived macrophages (Fig. 3C,  $p < 0.001$ ), which could be reversed by *CASP2* overexpression (Fig. 3C,  $p < 0.001$ ). Furthermore, we observed that the cholesterol efflux of THP-1 cell-derived macrophages was enhanced after SPM treatment ( $p < 0.001$ ; Fig. 4A), but it was diminished as a result of *CASP2* overexpression in SPM-treated macrophages ( $p < 0.001$ ; Fig. 4A). These results indicated that SPM facilitates cholesterol efflux and attenuates lipid accumulation in macrophages by regulating *CASP2* expression.

### *CASP2-Mediated Potentiation of Autophagy and Suppression of Macrophage Apoptosis After SPM Treatment*

The final part of the study focuses on determining the expression of autophagy- and apoptosis-related proteins. As shown in Fig. 4B, SPM treatment reduced the P62 level, but increased the ATG5 expression level and LC3 II/LC3 I ratio in THP-1 cell-derived macrophages ( $p < 0.01$ ); these changes in expression could be counteracted through *CASP2* overexpression ( $p < 0.05$ ). Besides, we found that SPM treatment promoted cell viability ( $p < 0.001$ ; Fig. 4C), decreased *CASP2*, *BAX*, and cleaved caspase-3/caspase-3 expression ( $p < 0.001$ ; Fig. 4D), and increased *Bcl-2* level ( $p < 0.05$ ; Fig. 4D) in the THP-1 cell-derived macrophages. Similarly, those effects instigated by SPM could be neutralized via *CASP2* overexpression ( $p < 0.05$ ; Fig. 4C,D). These data implied that SPM potentiates autophagy and suppresses macrophage apoptosis by mediating *CASP2*.

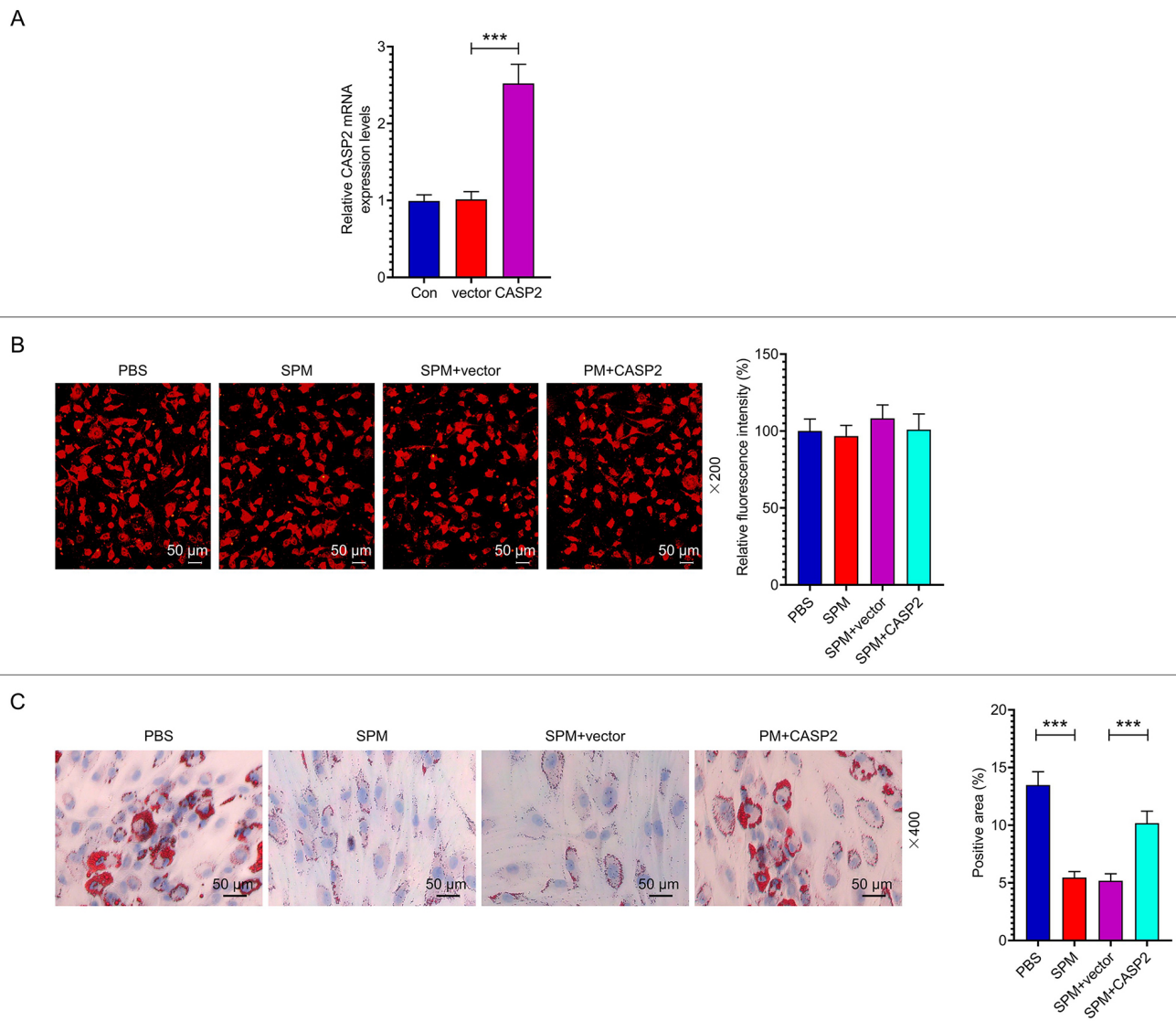
## Discussion

In the present research, SPM was discovered to promote cholesterol efflux and autophagy, and mitigate the lipid accumulation in THP-1 cell-derived macrophages. Besides, the impacts of SPM on cholesterol efflux and lipid accumulation were found to be elicited by autophagy consequent to *CASP2* downregulation. Our research provides evidence that SPM facilitates autophagy to enhance cholesterol efflux of THP-1 cell-derived macrophages by downregulating *CASP2* expression, providing a novel therapeutic agent for atherosclerosis.

SPM has a pivotal role in a plethora of physiological and pathological processes [5]. The regulatory effects of SPM on CVD have been widely validated in studies. For example, administration of exogenous SPM has been found to mitigate hyperglycemia-induced myocardial injury through suppressing Golgi stress [22], and attenuate diabetic cardiomyopathy by inhibiting reactive oxy-

gen species (ROS)-p53 mediated calcium-sensitive receptor downregulation [23]. Additionally, SPM relieves the age-related cardiac function deterioration of rats [8], and prevents cardiac aging by enhancing mitochondrial biogenesis and function [24]. A metabolomics study showed that SPM level is downregulated in the plasma of atherosclerosis rats [25], and SPM can protect against atherosclerosis by promoting the pyroptosis of macrophages [11]. Notably, reducing lipid accumulation in macrophages presents a potent strategy for treating atherosclerosis [4]. Besides, SPM assists PDC-109 to induce cholesterol efflux and choline phospholipids [12]. Considering the findings above, we infer that the protective effects of SPM on atherosclerosis are mediated by modulating the lipid accumulation in macrophages. Herein, we discovered that in response to SPM treatment, the lipid accumulation of THP-1 cell-derived macrophages was impeded. Cellular lipid accumulation is a consequence of heightened cholesterol absorption and/or decreased cholesterol efflux [16]. We found that SPM treatment had no influence on cholesterol uptake while promoting the cholesterol efflux of THP-1 cell-derived macrophages, confirming our speculation that SPM offers protection against atherosclerosis by increasing the cholesterol efflux of macrophages.

Autophagy, a highly conserved eukaryotic cellular recycling process, is instrumental for cellular survival and maintenance through the degradation of cytoplasmic organelles, macromolecules and proteins, as well as recycling of breakdown products [26]. Thus, the dysfunction of autophagy contributes to the development of multiple diseases including atherosclerosis [27]. Accumulating evidence proved that autophagy induction is a promising strategy for atherosclerosis treatment [28], and this has been specifically verified in several studies: for instance, quercetin hampers the progression of atherosclerosis by augmenting Mammalian Ste20-like kinase 1 (MST1)-mediated autophagy [29]; araloside C modulates the polarization of the macrophage and mitigates atherosclerosis by promoting Silent information regulator 1 (Sirt1)-mediated autophagy [30]; and Long noncoding RNAs (lncRNA) FA2H-2 downregulation aggravates atherosclerosis due to the inhibited autophagy flux [31]. At the molecular level, the anti-atherosclerotic effect of autophagy is mediated through the modulation of cholesterol efflux [16]. SPM-induced autophagy can suppress the pro-inflammatory response of macrophages and further ameliorate acute liver injury [32]. In this research, we discovered that SPM upregulates ATG5 and LC3 II/LC3 I levels, while downregulating P62 level, suggesting that SPM promotes autophagy in THP-1 cell-derived macrophages. Furthermore, we corroborated autophagy as a crucial mechanistic pathway in the alteration of cholesterol efflux and lipid accumulation by employing 3-MA treatment. As expected, the 3-MA treatment reversed the influence of SPM on increasing cholesterol efflux and decreasing lipid accumulation of THP-1 cell-



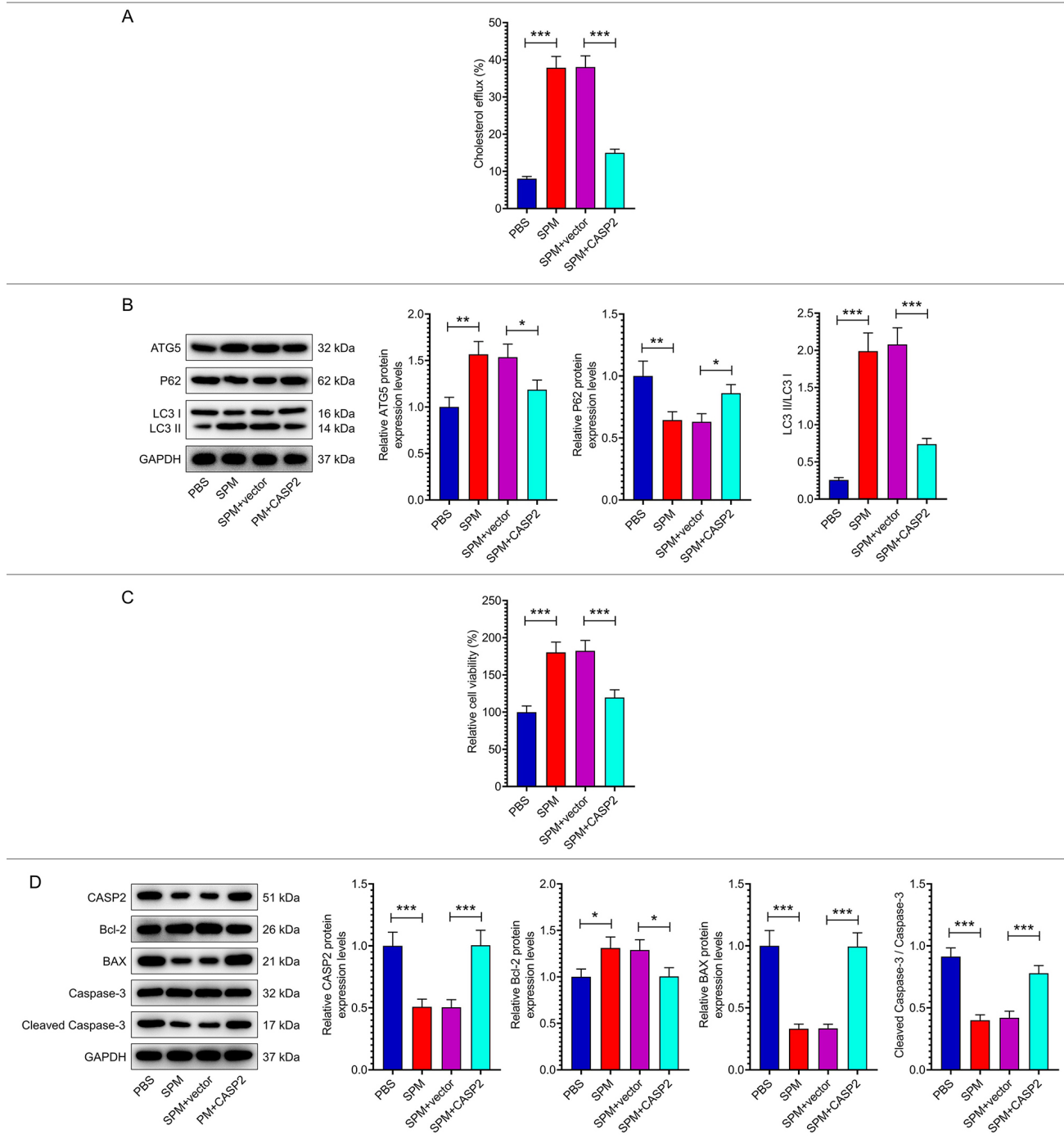
**Fig. 3. CASP2-mediated enhancement of lipid accumulation in macrophages after SPM treatment, but minimally altered cholesterol uptake.** (A) CASP2 expression level in THP-1-induced macrophages transfected with CASP2 overexpression plasmid, measured by qRT-PCR. (B) Fluorescence images of THP-1 cell-derived macrophages transfected with CASP2 overexpression plasmid and treated with SPM as well as the corresponding quantification of cholesterol uptake measured by Dil-oxLDL uptake assay (200 $\times$  magnification). (C) Results of Oil Red O staining to depict lipid accumulation in the cells, and corresponding measurement of areas positively stained by Oil Red O dye (400 $\times$  magnification). \*\*\* $p < 0.001$ . N = 3.

derived macrophages, suggesting that SPM promotes autophagy to enhance the cholesterol efflux of macrophages.

CASP2 is a kind of protease that is associated with the development of atherosclerosis, and its inhibition drastically suppresses macrophage death induced by triglyceride whose accumulation affects the development of atherosclerosis [33]. In addition, CASP2 downregulation activates the AMPK/AKT/mTOR and the AMPK/MAPK pathways to induce incomplete autophagy [14], and the loss of CASP2-dependent apoptosis induces autophagy after mitochondrial oxidative stress erupts in murine cortical neurons [15]. In the present study, CASP2 was found to be a target of SPM,

which acts to downregulate the expression of CASP2 in THP-1 cell-derived macrophages. Accordingly, we surmised that the effect of SPM on promoting autophagy in macrophages may be mediated by targeting CASP2. In this study, we found that CASP2 overexpression reverses the influence of SPM on increasing cholesterol efflux and autophagy and decreasing lipid accumulation and apoptosis of THP-1 cell-derived macrophages, confirming that the effect of SPM in macrophages is regulated by CASP2-mediated autophagy.

In this study, there are some limitations. Firstly, the present study only investigated the expression of genes in-



**Fig. 4. *CASP2*-mediated increased levels of cholesterol efflux and autophagy, as well as suppressed macrophage apoptosis, following SPM treatment.** The THP-1 cell-derived macrophages used in these experiments had been transfected with *CASP2* overexpression plasmid and treated with SPM for 24 h. (A) Levels of cholesterol efflux in the cells were measured via [<sup>3</sup>H]-cholesterol detection. (B) Expression levels of autophagy-related proteins measured by Western blotting. (C) Cell viability was detected using CCK-8 assay. (D) Expression levels of apoptosis-related proteins were detected using Western blotting. GAPDH was used as an internal control. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001. N = 3.

involved in the autophagy pathway, and did not expand the microscopy experiments involving the laser confocal microscope or transmission electron microscope to deeply investigate the impact of SPM on autophagy. Secondly, a

preliminary test on the SPM concentration on cultured cells was not conducted to determine the most appropriate concentration for cell pretreatment. Further, this study also did not attempt to unravel the effects of different interventions

on macrophage function. Lastly, no *in vivo* experiments were conducted to verify the *in vitro* results obtained.

## Conclusion

In summary, SPM is a potential anti-atherosclerotic agent, which promotes autophagy to enhance cholesterol efflux of THP-1 cell-derived macrophages by downregulating *CASP2* expression.

## Availability of Data and Materials

The analyzed data sets generated during the study are available from the corresponding author upon reasonable request.

## Author Contributions

YYY and YFM designed the research study; YQC, JZ performed the research; DHC, XLL collected and analyzed the data. All authors have been involved in drafting the manuscript. All authors contributed significantly to editorial changes of important content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

Not applicable.

## Acknowledgment

Not applicable.

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## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.24976/Descov.Med.202537196.78>.

## References

[1] Xie H, Zhuang Q, Mu J, Sun J, Wei P, Zhao X, *et al.* The relationship between lipid risk score and new-onset hypertension in a prospective cohort study. *Frontiers in Endocrinology*. 2022; 13: 916951.

- [2] Yin Y, Xie Z, Chen D, Guo H, Han M, Zhu Z, *et al.* Integrated investigation of DNA methylation, gene expression and immune cell population revealed immune cell infiltration associated with atherosclerotic plaque formation. *BMC Medical Genomics*. 2022; 15: 108.
- [3] Björkegren JLM, Lusis AJ. Atherosclerosis: Recent developments. *Cell*. 2022; 185: 1630–1645.
- [4] Tabas I, Bornfeldt KE. Macrophage Phenotype and Function in Different Stages of Atherosclerosis. *Circulation Research*. 2016; 118: 653–667.
- [5] Peng Q, Wong CYP, Cheuk IWY, Teoh JYC, Chiu PKF, Ng CF. The Emerging Clinical Role of Spermine in Prostate Cancer. *International Journal of Molecular Sciences*. 2021; 22: 4382.
- [6] Frühauf PKS, Ineu RP, Tomazi L, Duarte T, Mello CF, Rubin MA. Spermine reverses lipopolysaccharide-induced memory deficit in mice. *Journal of Neuroinflammation*. 2015; 12: 3.
- [7] Phadwal K, Kurian D, Salamat MKF, MacRae VE, Diack AB, Manson JC. Spermine increases acetylation of tubulins and facilitates autophagic degradation of prion aggregates. *Scientific Reports*. 2018; 8: 10004.
- [8] Zhang H, Wang J, Li L, Chai N, Chen Y, Wu F, *et al.* Spermine and spermidine reversed age-related cardiac deterioration in rats. *Oncotarget*. 2017; 8: 64793–64808.
- [9] Tsujinaka S, Soda K, Kano Y, Konishi F. Spermine accelerates hypoxia-initiated cancer cell migration. *International Journal of Oncology*. 2011; 38: 305–312.
- [10] Avazzadeh R, Vasheghani-Farahani E, Soleimani M, Amanpour S, Sadeghi M. Synthesis and application of magnetite dextran-spermine nanoparticles in breast cancer hyperthermia. *Progress in Biomaterials*. 2017; 6: 75–84.
- [11] Qiu Y, Li L, Guo X, Liu J, Xu L, Li Y. Exogenous spermine inhibits high glucose/oxidized LDL-induced oxidative stress and macrophage pyroptosis by activating the Nrf2 pathway. *Experimental and Therapeutic Medicine*. 2022; 23: 310.
- [12] Singh BP, Saha I, Nandi I, Swamy MJ. Spermine and spermidine act as chemical chaperones and enhance chaperone-like and membranolytic activities of major bovine seminal plasma protein, PDC-109. *Biochemical and Biophysical Research Communications*. 2017; 493: 1418–1424.
- [13] Kopeina GS, Zhivotovsky B. Caspase-2 as a master regulator of genomic stability. *Trends in Cell Biology*. 2021; 31: 712–720.
- [14] Liu J, Wang H, Gu J, Deng T, Yuan Z, Hu B, *et al.* BECN1-dependent CASP2 incomplete autophagy induction by binding to rabies virus phosphoprotein. *Autophagy*. 2017; 13: 739–753.
- [15] Tiwari M, Lopez-Cruzan M, Morgan WW, Herman B. Loss of caspase-2-dependent apoptosis induces autophagy after mitochondrial oxidative stress in primary cultures of young adult cortical neurons. *The Journal of Biological Chemistry*. 2011; 286: 8493–8506.
- [16] Xiaolong L, Dongmin G, Liu M, Zuo W, Huijun H, Qiufen T, *et al.* FGF21 induces autophagy-mediated cholesterol efflux to inhibit atherogenesis via RACK1 up-regulation. *Journal of Cellular and Molecular Medicine*. 2020; 24: 4992–5006.
- [17] Nascimento CR, Rodrigues Fernandes NA, Gonzalez Maldonado LA, Rossa Junior C. Comparison of monocytic cell lines U937 and THP-1 as macrophage models for in vitro studies. *Biochemistry and Biophysics Reports*. 2022; 32: 101383.
- [18] Zhao ZW, Zhang M, Zou J, Wan XJ, Zhou L, Wu Y, *et al.* TIGAR mitigates atherosclerosis by promoting cholesterol efflux from macrophages. *Atherosclerosis*. 2021; 327: 76–86.
- [19] Huang C, Yu XH, Zheng XL, Ou X, Tang CK. Interferon-stimulated gene 15 promotes cholesterol efflux by activating autophagy via the miR-17-5p/Beclin-1 pathway in THP-1 macrophage-derived foam cells. *European Journal of Pharmacology*. 2018; 827: 13–21.
- [20] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the

- comparative C(T) method. *Nature Protocols*. 2008; 3: 1101–1108.
- [21] Lin XL, He XL, Zeng JF, Zhang H, Zhao Y, Tan JK, *et al*. FGF21 increases cholesterol efflux by upregulating ABCA1 through the ERK1/2-PPAR $\gamma$ -LXR $\alpha$  pathway in THP1 macrophage-derived foam cells. *DNA and Cell Biology*. 2014; 33: 514–521.
- [22] Fan YQ, Wang YH, Shao YY, Shao XT, Hu J, Xu CQ, *et al*. Exogenous spermine alleviates myocardial injury induced by hyperglycemia by inhibiting golgi stress. *Zhongguo Ying Yong Sheng Li Xue Za Zhi*. 2019; 35: 385–389. (In Chinese)
- [23] Wang ZP, Che Y, Zhou H, Meng YY, Wu HM, Jin YG, *et al*. Corosolic acid attenuates cardiac fibrosis following myocardial infarction in mice. *International Journal of Molecular Medicine*. 2020; 45: 1425–1435.
- [24] Wang J, Li S, Wang J, Wu F, Chen Y, Zhang H, *et al*. Spermidine alleviates cardiac aging by improving mitochondrial biogenesis and function. *Aging*. 2020; 12: 650–671.
- [25] Zhang F, Jia Z, Gao P, Kong H, Li X, Chen J, *et al*. Metabonomics study of atherosclerosis rats by ultra fast liquid chromatography coupled with ion trap-time of flight mass spectrometry. *Talanta*. 2009; 79: 836–844.
- [26] Parzych KR, Klionsky DJ. An overview of autophagy: morphology, mechanism, and regulation. *Antioxidants & Redox Signaling*. 2014; 20: 460–473.
- [27] Miao J, Zang X, Cui X, Zhang J. Autophagy, Hyperlipidemia, and Atherosclerosis. *Advances in Experimental Medicine and Biology*. 2020; 1207: 237–264.
- [28] Shao BZ, Han BZ, Zeng YX, Su DF, Liu C. The roles of macrophage autophagy in atherosclerosis. *Acta Pharmacologica Sinica*. 2016; 37: 150–156.
- [29] Cao H, Jia Q, Yan L, Chen C, Xing S, Shen D. Quercetin Suppresses the Progression of Atherosclerosis by Regulating MST1-Mediated Autophagy in ox-LDL-Induced RAW264.7 Macrophage Foam Cells. *International Journal of Molecular Sciences*. 2019; 20: 6093.
- [30] Luo Y, Lu S, Gao Y, Yang K, Wu D, Xu X, *et al*. Araloside C attenuates atherosclerosis by modulating macrophage polarization via Sirt1-mediated autophagy. *Aging*. 2020; 12: 1704–1724.
- [31] Guo FX, Wu Q, Li P, Zheng L, Ye S, Dai XY, *et al*. The role of the LncRNA-FA2H-2-MLKL pathway in atherosclerosis by regulation of autophagy flux and inflammation through mTOR-dependent signaling. *Cell Death and Differentiation*. 2019; 26: 1670–1687.
- [32] Zhou S, Gu J, Liu R, Wei S, Wang Q, Shen H, *et al*. Spermine Alleviates Acute Liver Injury by Inhibiting Liver-Resident Macrophage Pro-Inflammatory Response Through ATG5-Dependent Autophagy. *Frontiers in Immunology*. 2018; 9: 948.
- [33] Lim J, Kim HK, Kim SH, Rhee KJ, Kim YS. Caspase-2 mediates triglyceride (TG)-induced macrophage cell death. *BMB Reports*. 2017; 50: 510–515.