Supervillin Contributes to LPS-induced Inflammatory Response in THP-1 Cell-derived Macrophages

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Abstract—Supervillin (SVIL) is an actin-binding and membrane-associated protein, which belongs to villin/gelsolin family. It has been reported that SVIL was involved in the regulation of macrophages’ movement and lipopolysaccharide (LPS) increased the SVIL mRNA expression in neutrophils, but the underlying mechanisms remain unknown. This work investigated the underlying molecular mechanisms of LPS regulating SVIL expression in macrophages and hence the possible role of SVIL in LPS-induced inflammation. We found that in THP-1-derived macrophages, LPS obviously increased SVIL mRNA and protein expression. Inhibition of TLR4 by Resatorvid (Res) remarkably reversed the LPS-induced SVIL expression. Additionally, inhibition of ERK1/2 signaling pathway (by U0126 or GDC-0994) and NF-κB (by BAY) significantly reduced the LPS-induced SVIL expression. Interestingly, down-regulation of SVIL by SVIL-specific shRNAs significantly attenuated the expression of IL-6, IL-1β & TNF-α induced by LPS at both mRNA and protein levels. Furthermore, we also observed that SVIL knockdown decreased the proportion of cells in G2/M phase and increased the proportion of cells in S & G0-1 phase of THP-1 derived macrophages, but did not influence the cell viability. Taken together, we demonstrated that LPS induced the expression of SVIL via activating TLR4/NF-κB and ERK1/2 MAPK pathways, and SVIL participated in the inflammatory response of LPS-induced IL-6, IL-1β and TNF-α upregulation in macrophages.

KEY WORDS: LPS; SVIL; TLR4; macrophages; inflammation

INTRODUCTION

As the first vital barrier for preventing cardiovascular diseases in the human cardiovascular system, macrophages respond to a variety of pathophysiological changes through transforming different functional phenotypes [1]. Macrophages are critically involved in all stages during the development and progression of atherosclerosis, a chronic inflammatory disorder [2–4]. The persistent inflammatory state of macrophages leads to the accumulation of fragments and apoptotic cells, promoting the deterioration of atherosclerotic plaques [5]. Various factors contribute to the occurrence of macrophage inflammation in atherosclerotic plaque, and the infection of various bacteria is also one of the important factors [5, 6]. Lipopolysaccharide (LPS), a key component in the
outer membrane of Gram-negative bacteria, stimulates macrophages to produce pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, leading to atherosclerotic development [7].

Supervillin (SVIL), a member of villin/gelsolin family, is an actin-binding and membrane-associated protein participating in cell proliferation, motility, and signal transduction etc. [8, 9]. Current research mostly focuses on investigating the role of SVIL in cancers and muscular tissue [10, 11]. SVIL was found to enter into the nucleus to participate in the intracellular signal transduction and early myogenesis of myoblasts [12, 13]. Recent evidence indicated that SVIL was a component of podosome which bound to actin isoforms and myosin regulators contributing to regulation of podosome turnover, migration and invasion in primary human macrophages [14, 15]. However, the knowledge about the functions of SVIL in macrophage is still limited. mRNA expression of SVIL was once reported to be elevated by LPS stimulation in human neutrophils [16]. It is far from clear whether and how SVIL participates in macrophage inflammatory response. As far as we know, the specific mechanism of LPS’s SVIL-increasing effect has not been reported yet, and there is no more evidence of what role SVIL plays after LPS stimulation. It might be speculated that SVIL could also response to this LPS stimulation and participate in the subsequent pro-inflammatory process in macrophage.

Toll-like receptor 4 (TLR4) is a definite receptor of LPS, which belongs to pattern recognition receptor (PRR) and triggers all the responses to LPS stimulation [17, 18]. The mitogen-activated protein kinase (MAPK) signal molecules (ERK1/2, JNK, P38) are activated as the downstream of TLR4 after LPS stimulation [19–21], leading to activation of NF-κB and hence the expression of inflammatory factors [22, 23]. In smooth muscle cells, SVIL has been identified as an ERK scaffolding protein, which contributed to the regulation of ERK binding, activation, and release from the signaling complex [24, 25]. SVIL also activated EGFR-ERK1/2 and raised the motility in Hela cells [26]. In natural killer (NK) cells, SVIL was necessary for KIR2DL1-mediated ERK activation [27]. SVIL promoted liver cancer cell migration and invasion via the activation of the RhoA/ROCK-ERK/p38 pathway under hypoxia [28]. Above all, these reports highly suggest that SVIL may be closely related to TLR4-MAPK-NF-κB pathway and hence participate in macrophage inflammation response.

In this study, we identified that LPS markedly upregulated the expression of SVIL in macrophage via activating TLR4/NF-κB and ERK1/2 MAPK signaling pathway. The deficiency of SVIL significantly attenuated the LPS-induced expression of IL-6, IL-1β and TNF-α in macrophages. This novel finding helps elucidate the involvement of SVIL in LPS-induced inflammation and hence gain a new insight into some inflammatory conditions like atherosclerosis.

**MATERIALS AND METHODS**

**Reagents**

Lipopolysaccharide (LPS, *Escherichia coli* 0111:B4) and phorbol 12-myristate 13-acetate (PMA, P8139) were purchased from Sigma-Aldrich (Saint Louis, USA). Lipofectamine TM 2000 transfection reagent (11668030) and TRIzol Reagent (10296028) were purchased from Invitrogen (California, USA). Annexin V-Alexa Fluor 647/PI Apoptosis Assay Kit (FMSAV647-100, FcMACS, Nanjing, China) was used to detect cell apoptosis. HiScript II Q-RT SuperMix for qPCR (R222-01) and ChamQ SYBR qPCR Master Mix (without ROX) (Q321-02/03) were purchased from Vazyme (Nanjing, China). TLR4 inhibitor Resatorvid (Res, S7455), NF-κB inhibitor BAY11-7082 (BAY, S2913), MEK1/2 inhibitor U0126 (S1102), P38 inhibitor SB203580 (SB, S1076), JNK inhibitor SP600125 (SP, S1460) were acquired from Selleck Chemicals Company (Houston, USA). ERK1/2 inhibitor Ravoxertinib (GDC-0994, HY-15947) was acquired from Med-ChemExpress (New Jersey, USA). Antibodies against NF-κB (8242S), p-NF-κB (3033S), p38 (8690S), p-p38 (4511S), ERK (4695S), p-ERK (4370S), JNK (9252S), p-JNK (4668S) were purchased from Cell Signaling Technologies (Boston, USA). Antibodies to β-Tubulin (10094-1-AP) and GAPDH (60004-1-Ig) were purchased from Proteintech (Chicago, USA). Anti-SVIL (HPA020138) was purchased from Sigma-Aldrich (Saint Louis, USA). The anti-rabbit secondary HRP-conjugated antibody (YFSA02) for western blot was purchased from Yifeixue Bio Tech (Nanjing, China). The anti-mouse secondary HRP-conjugated antibody (33201ES60) was purchased from Yeasen Biotech Company (Shanghai, China).
Cell culture

THP-1 (human monocytic cells) cell line was obtained from Zhong Qiao Xin Zhou Biotechnology Company (Shanghai, China). The THP-1 cells were grown in suspension in RPMI 1640 medium (Gibco, Suzhou, China) containing 10% fetal bovine serum (FBS, Procell, Wuhan, China), 1% penicillin-streptomycin (Invitrogen, California, USA) and 0.05 mM 2-mercaptoethanol (Sigma, Saint Louis, USA), and cultured in a 5% CO₂ incubator with humidified atmosphere at 37 °C. During the experiment, 100 ng/ml PMA induced THP-1 cells to differentiate from monocytes into macrophages. After attachment of the bottom surface, PMA-contained medium were removed and cells were washed with D-Hanks. Then, cells were starved with serum-free RPMI 1640 medium for 24 h. The macrophages were treated with drugs according to the needs of experiments.

Real-time PCR (RT-PCR)

Total RNA was extracted from treated THP-1-derived macrophages by using TRizol reagent. For cDNA synthesis, HiScript II Q-RT superMix was applied to reverse transcription reaction. The cDNA was used for qRT-PCR with SYBR green under standard reaction condition by Bio-Rad CFX Connect System (Bio-Rad, Hercules, CA, USA). The primers for SVIL, IL-1β, IL-6, TNF-α, GAPDH were provided by GENERAY (Shanghai, China) and the sequence were shown in Table 1.

Protein isolation and western blots analysis

The proteins were obtained from treated cells, which were lysed by RIPA buffer, which contained PMSF (1%), protease (10%) inhibitor and phosphatase (10%) inhibitor (Roche). and the concentration of protein was assessed by BCA (Bicinchoninic Acid) method. 30 µg of protein lysate was added to each lane and separated by SDS-PAGE, then transferred electrically to polyvinylidene fluoride (PVDF) membranes (Millipore). Then the PVDF membranes were blocked with 5% non-fat dry milk or BSA in TBST solution for 90 min and incubated with primary antibodies overnight at 4 °C. Membranes were washed with TBST and incubated with HRP-conjugated secondary antibodies for 1 h. After washed with TBST, membranes were added with SuperBright Subpico ECL substrate and visually observed by Chemiluminescence gel imaging system (SYNGENE). The Band densities were quantified by GELPRO4 software. The dilution ratio of primary antibody as follow: SVIL (1:500), GAPDH (1:5000), Tubulin (1:5000), p-ERK1/2 (1:1000), p-P38 (1:1000), p-JNK (1:1000), p-NF-κB (1:1000), ERK1/2 (1:1000), P38 (1:1000), JNK (1:1000), NF-κB (1:1000).

RNA interference

siRNA was used to knockdown SVIL genes. There were three specific sequences for SVIL. The scrambled NC sequence (siNC) and siRNA sequences against SVIL (siSVIL-1554, siSVIL-1882 and siSVIL-3970) were shown in Table 2. Lipofectamin 2000 transfection reagent was used for cell transfection. siNC and specific siRNAs were added to each well at a final concentration of 100 nM in serum free RPMI 1640 medium for 6 h and then the medium was changed back to complete RPMI 1640 medium. Cells were collected to assess the efficiency of knockdown by RT-PCR and Western blot after 48 h transfection.

Table 1  primer sequences for RT-PCR

| Name   | Sequences                             |
|--------|---------------------------------------|
|        | Forward primer (5’→3’) | Reverse primer (5’→3’)         |
| SVIL   | TTTCCAGCCTCGTCAACTCTCA               | CGTCATCTACTGCCATAACCC          |
| IL-1β  | GAAATGATGGCCTATTTACATGGCCA           | GTAGTTGGTTGCGAGATGCTAG         |
| IL-6   | GCTCTGCTTGTTCTTCTCACA                | AATCATGCTGGTCTTGTGGAG          |
| TNF-α  | AGGACACCATGAGCAGCTGAAGC              | AAGGAGAGGAGGGCTGAGAAACAG       |
| GAPDH  | CGCTGAGATCGTGGAGTC                 | GCTGATGATCTTAGGGCTGTGTC        |
**Lentiviral encapsulation and infection**

The sequences of shRNAs targeting SVIL (shSVIL-1882 and shSVIL-3970) and a scrambled shRNA (shNC) were designed and constructed by Gene Pharma (shanghai, China), all sequences were shown in Table 3. The shRNAs (shNC or shSVIL) and Lentiviral plasmids were packaged in HEK293FT cells with Lipofectamine 2000 and then lentiviral particles containing shRNA (shNC or shSVIL) were produced.

THP-1 cells were infected with Lentiviruses containing shNC or shSVIL by polybrene (8 μg/ml), then the infected cells were selected by puromycin (1 μg/ml) for about 2 weeks. Cells expressing green fluorescence were also differentiated by PMA and were used in subsequent experiments.

**EILSA**

Cells were treated with indicated conditions and the supernatants were collected by centrifuging for 20 min at the speed of 2000 g. According to the manufacturer’s protocols, secreted IL-1β, IL-6 and TNF-α in cell culture supernatants were analyzed with relevant ELISA kits (YIFEIXUE BIO TECH, Nanjing, China).

**Flow cytometry analysis**

The treated THP-1 Cells were harvested and stained with the Annexin V-FITC and PI to detect cell apoptosis. Briefly, the harvested cells were resuspended in 500 μl binding buffer (1x) and incubation with 5 μl Annexin V-FITC and 10 μl PI for 15 min in the dark, then cell apoptosis was detected by flow cytometry (BD FACS Calibur, NY) according to the manufacturer’s instructions.

Cell cycle distribution changes were also measured by flow cytometry (BD FACS Calibur, NY). The cells were collected and fixed with 75% ethanol and stored at 4 ℃. Then, cell cycle was detected by flow cytometry according to the manufacturer’s instructions.

**Cell viability assay**

Cell viability was measured by Cell Counting Kit-8 (CCK-8, Yeasen Biotech Company, Shanghai, China) according to the instructions of the manufacturer. Briefly, the cells were seeded into 96-well plates at a density of 1 x 10^4 cells/well and incubated for 24 h at 37 ℃. Then, cells were treated with indicated drugs and time. Next, 10 μl CCK-8 solution was added into each well and incubated

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**Table 2** siRNA sequences

| Name  | Sequences            | Sense (5' → 3') | Antisense (5' → 3') |
|-------|----------------------|-----------------|---------------------|
| siNC  | UUCUCCGAACGUGUCACGUTT| ACGUGACACGUUUCGAGAATT |
| siSVIL-1554 | CCAGACAAUUGGGCAATTT | UUUGCCCUAUUUGUCGTT |
| siSVIL-1882 | GCAAGUGCCACUGACUAAATT | UAUAGUCAGUGCCACUUGCTT |
| siSVIL-3970 | GGUAGUGAAGUUACGUAUTT | AUACGUAAACUUACUACCTT |

**Table 3** shRNA sequences

| Name  | Sequences            | Sense (5' → 3') | Antisense (5' → 3') |
|-------|----------------------|-----------------|---------------------|
| shNC  | GATCCGGTTCTCCGAACGTGCATTCAAGAGAACGTTCCGAGAACCTTTTTTGT | AATTCAAAAAAGTTCTCCGAACGTTCCGACGCTTATCTTGAACGAGGACGCTTCCGAGGAACCG |
| shSVIL-1882 | GATCCGCAATGCGCCACTGACTATATTCAAGAGATATAGTCAGTGCCACTTTTG | AATTCAAAAAAGGCAATGCGCCACTGACTATATTCAAGAGATATAGTCAGTGCCACTTTTGG |
| shSVIL-3970 | GATCCCGGATTTGATTTTACGTATTTTCGAAGAATAGCAAAATCCTACTACCTTTTGT | AATTCAAAAAAGGGAAGTGAGTTCGTTACGTATTTTCGAAGAATAGCAAAATCCTACTACCTTTTGT |
for 2.5 h. The absorbance was measured by microplate reader (Thermo, USA) at the wavelength of 450 nm.

Statistical analysis

The data was analyzed with GraphPad Prism 5.0 by two-tailed t-test (comparison of two groups) or one-way analysis followed by Turkey tests (comparison of multiple groups). *p < 0.05 was considered to be statistically significant. In our study, each experiment was repeated more than three times respectively.

RESULTS

LPS induced SVIL expression in THP-1 macrophages

Here, we detected the mRNA and protein expression levels of SVIL in THP-1-derived macrophages after LPS stimulation. LPS treatment significantly induced the mRNA expression (Fig. 1a) and protein expression (Fig. 1b) of SVIL in a dose-dependent manner and the concentration (100 ng/ml) of LPS was selected for following experiments. As shown in Fig. 1c and d, LPS treatment induced the highest mRNA expression level of SVIL at 3 h and the protein expression reached the peak at 9 h. These results indicated that LPS stimulation could significantly enhance the expression of SVIL in macrophages.

TLR4/NF-κB mediated LPS-induced SVIL expression in macrophages

TLR4 is the most important cell surface receptor downstream of LPS [29, 30]. However, whether TLR4 participates in LPS-induced expression of SVIL is not known yet. Here, Resatorvid (Res), a specific inhibitor of TLR4, was used to detect whether it could inhibit the expression of SVIL induced by LPS in macrophages. As shown in Fig. 2a and b, LPS led to a significant increase of SVIL expression in macrophages.

![Fig.1 LPS induced SVIL expression in THP-1 macrophages. THP-1 cells were seeded in 6-well plates and differentiated into macrophages by PMA (100 ng/ml). The cells were treated with LPS at indicated concentration and time after differentiation and starvation. a and b The macrophages were treated with LPS at concentrations ranging from 0 to 100 ng/ml. Results and statistical graphs of mRNA a and protein b expressions were represented. c and d THP-1 macrophages were treated with LPS (100 ng/ml) for the indicated times, mRNA c and protein d expressions were detected by RT-PCR and western blot respectively. *p < 0.05, **p < 0.01, ***p < 0.001. * versus control group. Data were expressed as the means ± S.E.M. of more than three independent experiments and a representative result was shown.](image-url)
Supervillin Contributes to LPS-induced Inflammatory Response in THP-1 Cell-derived...

mRNA expression level, and the protein expression level was also increased remarkably. LPS-induced SVIL expression was obviously suppressed by pretreatment of Res at both mRNA (Fig. 2a) and protein (Fig. 2b) levels.

As the above data show, LPS caused a significant up-regulation in the mRNA level of SVIL via TLR4 (Figs. 1a, c and 2a). As known, NF-κB is a key downstream nuclear transcription signaling effector of the TLR4 pathway [31, 32]. Hence, we speculated that NF-κB was involved in the regulation of SVIL by LPS. As shown in Fig. 2c, LPS stimulation increased the phosphorylation level of NF-κB, which could be significantly reversed by Res. To investigate whether NF-κB participated in LPS-induced SVIL expression, NF-κB inhibitor BAY were used and significant inhibition of LPS-induced NF-κB phosphorylation was observed (Fig. 2d). As expected, pretreatment of the cells with BAY reversed LPS-induced SVIL mRNA (Fig. 2e) and protein expression (Fig. 2f). Therefore, these results suggested that TLR4/NF-κB mediated LPS-induced SVIL expression in macrophages.

![Fig. 2](image)

**Fig. 2** TLR4/NF-κB pathway mediated LPS-induced SVIL expression in macrophages. THP-1 macrophages were pretreated with or without TLR4 inhibitor Res (10 μM) for 1 h prior to LPS stimulation for 3 h or 9 h. **a** The cell samples collected at 3 h were used to detect the SVIL mRNA expression by RT-PCR. And cells collected at 9 h were used to detect the SVIL protein level by western blot. **b** The phosphorylation level of NF-κB was detected by western blot. **c** Cells were pretreated with or without NF-κB inhibitor BAY (10 μM) for 1 h prior to LPS stimulation. **d** BAY decreased LPS-induced phosphorylation of NF-κB. The SVIL mRNA e and protein expressions f were measured by RT-PCR and western blot respectively. *P < 0.05, **P < 0.01, ***P < 0.001. * versus control group or the left group below the horizontal line. Data were expressed as the means ± S.E.M. of more than three independent experiments and a representative result was shown. ns: no statistical significance.
Fig. 3 LPS induced SVIL expression via ERK1/2 MAPK in THP-1 macrophages. 

(a) Cells were pretreated with or without U0126 (the MEK and thus ERK1/2 inhibitor, 10 μM), SP600125 (JNK inhibitor, 10 μM) and SB203580 (P38 inhibitor, 10 μM) for 1 h prior to LPS stimulation, the SVIL mRNA expressions were detected by RT-PCR. 

(b) Cells were pretreated with or without TLR4 inhibitor Res (10 μM) for 1 h prior to LPS stimulation, the phosphorylation of ERK1/2 was detected by western blot. The phosphorylation level of ERK1/2 c, SVIL protein expression d and the phosphorylation level of NF-κB e were measured by western blot after cells pretreated with or without U0126 for 1 h prior to LPS stimulation. *P < 0.05, **P < 0.01, ***P < 0.001. * versus control group or the left group below the horizontal line. Data were expressed as the means ± S.E.M. of more than three independent experiments and a representative result was shown. ns: no statistical significance.
Supervillin Contributes to LPS-induced Inflammatory Response in THP-1 Cell-derived…

Although inhibition of NF-κB could reverse the up-regulation of SVIL induced by LPS, the reversion was not complete (Fig. 2e and 2f). Thus, we wondered if there were other signaling pathways involved in this process. Many studies demonstrated that TLR4 activation can activate MAPK signal pathway and initiate intracellular response [33–35]. In order to verify whether MAPK family was involved in the regulation of SVIL by LPS, we pretreated cells with MEK and thus ERK1/2 inhibitor U0126, JNK inhibitor SP or P38 inhibitor SB, respectively, before LPS stimulation. Interestingly, only U0126 inhibited LPS-induced mRNA expression of SVIL, while SP and SB did not.

**ERK1/2 MAPK mediated LPS-induced SVIL expression in macrophages**

Although inhibition of NF-κB could reverse the up-regulation of SVIL induced by LPS, the reversion was not complete (Fig. 2e and 2f). Thus, we wondered if there were other signaling pathways involved in this process. Many studies demonstrated that TLR4...

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**Fig. 4** GDC-0994 inhibited LPS-induced SVIL up-regulation and phosphorylation of ERK1/2 and NF-κB. Cells were pretreated with or without ERK1/2 inhibitor GDC-0994 (5 μM) for 1 h prior to LPS stimulation. a The phosphorylation of ERK1/2 and NF-κB were detected by western blot. b The SVIL mRNA expression levels were detected by RT-PCR. c The SVIL protein expression levels were measured by western blot. *P < 0.05, **P < 0.01, ***P < 0.001. * versus control group or the left group below the horizontal line. Data were expressed as the means ± S.E.M. of more than three independent experiments and a representative result was shown. ns: no statistical significance.
reverse this efficiency of LPS (Fig. 3a). We also used the ERK1/2 specific inhibitor, GDC-0994. It was observed that GDC-0994 significantly down-regulated LPS-induced SVIL mRNA expression (Fig. 4b). Therefore, the following research mainly focused on the ERK1/2 MAPK signaling pathway.

As shown in Fig. 3b, LPS stimulation increased the phosphorylation of ERK1/2 and this effect could be reversed by Res. U0126 and GDC-0994 markedly inhibited LPS-induced ERK1/2 phosphorylation (Figs. 3c and 4a). Western blot analysis showed that SVIL protein expression induced by LPS was also
Supervillin Contributes to LPS-induced Inflammatory Response in THP-1 Cell-derived...

significantly down-regulated by U0126 and GDC-0994 (Figs. 3d and 4c). Additionally, U0126 and GDC-0994 could partially inhibit LPS-induced NF-κB phosphorylation (Figs. 3e and 4a). These results indicated that ERK1/2 MAPK was at least partially involved in the regulation of SVIL by LPS.

SVIL Knockdown efficiency in THP-1 cells

To further investigate the role of SVIL in LPS-induced macrophage inflammatory response, and according to the previous SVIL-specific siRNAs (siSVIL-1554, siSVIL-1882 and siSVIL-3970) interference sequences in our laboratory (Fig. 5a), we selected the two (siSVIL-1882 and siSVIL-3970) most efficient sequences to design SVIL-targeted shRNAs (shSVIL-1882 and shSVIL-3970) to construct stable transfected cell lines. Expression of LV(H1/GFP&Puro)-shNC and LV(H1/GFP&Puro)-shSVILs were confirmed by detection of green fluorescent protein (GFP)-fluorescence in cells (Fig. 5b). Then, we detected the mRNA expression (Fig. 5c) and protein

Fig.6 The influence of SVIL knockdown on cell cycle, cell vitality & apoptosis. a The representative flow cytometry images showed the proportion of cells in different phases of cell cycle, G1, G2 and S. b The cell percentage in each phase of different treated cells was shown as scatter plot graph. c Cell vitality was measured by CCK8 assay and the statistical data was shown as histogram. d The representative apoptotic images detected by flow cytometry were selected and the percentage of apoptotic cell was shown as histogram. *P < 0.05, **P < 0.01, ***P < 0.001. * versus control group or the left group below the horizontal line. Data were expressed as the means ± S.E.M. of more than three independent experiments and a representative result was shown. ns: no statistical significance.
expression (Fig. 5d) of SVIL in the transfected cells. As shown, the data indicated that SVIL was significantly knocked down by shRNAs.

The influence of SVIL knockdown on cell cycle, cell vitality and apoptosis

The effects of SVIL depletion on the THP-1 viability was detected by cell flow cytometry and CCK-8 assay. Cell cycle was evaluated by measuring DNA content. As shown in Fig. 6a and b, PMA significantly reduced the proportion of cells in G0-1 and S phases, while the proportion of cells in G2/M phases was increased remarkably in shNC THP-1 cells. After PMA stimulation, the vitality of cells was significantly inhibited compared with the control group (Fig. 6c). These results demonstrated that PMA significantly inhibited the proliferation of THP-1 cells in the process of inducing THP-1 to differentiate into macrophages (Fig. 5b).
As shown in Fig. 6a and b, without PMA stimulation, SVIL ablation decreased the proportion of cells in G2/M phase, while the proportion of cells in S phase with no significant difference. After THP-1 cells were induced to differentiate into macrophages by PMA, the knockdown of SVIL caused similar changes in G0/1 and G2/M phases, and significantly increased the proportion of cells in S phase. However, CCK8 data showed that SVIL knockdown did not affect the cell viability compared with control group with or without PMA incubation (Fig. 6c). As shown in Fig. 6d, SVIL depletion also could not induce apoptosis of THP-1 cells.

Fig. 8 SVIL depletion inhibited LPS-induced expression of inflammatory cytokines. The shNC and shSVIL (shSVIL-1882 and shSVIL-3970) THP-1 cells were differentiated to macrophages and stimulated with or without LPS. The SVIL expression a in shNC and shSVIL (shSVIL-1882 and shSVIL-3970) THP-1 macrophages with or without LPS stimulation were measured. The mRNA expression of inflammatory cytokines IL-1β b, IL-6 c and TNF-α d were detected by RT-PCR. The secretion of inflammatory cytokines IL-1β e, IL-6 f and TNF-α g were detected by ELISA. *P < 0.05, **P < 0.01, ***P < 0.001. * versus control group or the left group below the horizontal line. Data were expressed as the means ± S.E.M. of more than three independent experiments and a representative result was shown. ns: no statistical significance.
SVIL depletion inhibited LPS-induced expression of inflammatory cytokines

LPS stimulation induces pro-inflammatory responses of macrophages and promotes the production of inflammatory cytokines, such as IL-1β, IL-6 and TNF-α [7, 21]. Moreover, SVIL has been identified as an interacting protein of Sphingosine kinase 1 (sphk1) [36], and sphk1 is reported to involve in macrophage inflammation induced by LPS [37, 38]. Therefore, it was of interest to examine if SVIL depletion could change the regulation of LPS-induced pro-inflammatory responses in THP-1 macrophages. Indeed, LPS significantly induced the expression of inflammatory cytokines, TNF-α, IL-6 and IL-1β (Fig. 7a-i). The mRNA expression of all the three inflammatory cytokines induced by LPS could be inhibited by Res (Fig. 7a-c), BAY (Fig. 7d-f) and U0126 (Fig. 7g-i).

Importantly, knockdown of SVIL produced opposite effects of LPS. As shown in Fig. 8a, LPS significantly increased the expression of SVIL in shNC macrophages, while the same stimulation condition could not induce the SVIL expression in shSVIL macrophages. Interestingly, we found that the depletion of SVIL in macrophages significantly reversed the mRNA expression (Fig. 8b-d) and secretion (Fig. 8e-g) of inflammatory factors, IL-1β, IL-6 & TNF-α induced by LPS stimulation.

DISCUSSION

Since TLR4 was firstly identified as the receptor of LPS in 1998, various studies have shown that it could induce numerous intracellular variation in response to LPS [39]. The production of inflammatory factors in macrophages was one of the important change [40]. And our data showed that LPS increased SVIL expression in macrophages, which was consistent with Morozumin T’s report that LPS elevated SVIL mRNA expression in human neutrophils [16]. Therefore, in the process of regulating SVIL expression, TLR4 was very much likely the major receptor in response to LPS stimulation. As expected, our data showed that the inhibition of TLR4 with Res could significantly reverse this SVIL-inducing effect of LPS, indicating that TLR4 played a critical role in LPS-regulated SVIL expression in macrophages. Moreover, BAY, an identified antagonist of NF-κB blocked the LPS-induced SVIL up-regulation, suggesting that NF-κB (downstream of TLR4) contributed to LPS-induced SVIL expression in macrophages. To our knowledge, it is the first report that LPS-induced SVIL expression is partly mediated by TLR4/NF-κB pathway.

Furthermore, we also investigated the role of TLR4-mediated protein kinases activation, MAPKs, in LPS-induced SVIL change. MAPKs are responsible for cellular responses induced by LPS in the production of proinflammatory cytokines [31, 41]. It is well known that MAPKs activate a variety of transcription factors, and most of the MAPK pathways regulate the inflammatory responses in conjunction with NF-κB pathway [42, 43]. Our data indicated that inhibition of ERK1/2 significantly reversed the LPS-induced expression of SVIL, suggesting that SVIL was a downstream molecule of ERK1/2. However, this result seems to be inconsistent with the conclusions of other studies, that is, SVIL is the upstream molecule of ERK1/2 [26–28]. Other researches showed that SVIL upregulated phosphorylation of ERK to increase the velocity of cancer cell translocation [26] and promoted epithelial-mesenchymal transition and metastasis of hepatocellular carcinoma in hypoxia via activation of ERK pathway [28]. Interestingly, we also observed that SVIL silence down-regulated the phosphorylation of ERK1/2 (data not shown), which is consistent with the report by Chen X et al. [28]. The paradoxical phenomenon makes us suppose that the correlation between SVIL and ERK is not just a one-way upstream or downstream relationship, but a mutual adjustment relationship. Other report pointed out that SmAV (smooth muscle archvillin, a smooth muscle specific isoform of SVIL) served as a ERK scaffolding protein and provided a mechanism for regulating ERK binding, activation, and release from the signaling complex [24]. Thus, it is reasonable to believe that LPS-induced ERK1/2 activation promoted the expression of SVIL, and the SVIL could recombine with ERK1/2 to further enhance the signal transduction of ERK1/2. The complicated relationship between SVIL and ERK1/2 seems to be in a feedback loop. However, more specific mechanism needs to be further investigated. In addition, inhibition of ERK1/2 suppressed LPS-induced NF-κB phosphorylation, indicating that ERK1/2 contributed to LPS-induced SVIL expression perhaps at least partly through NF-κB pathway. Taken together, our research provided a certain basis for exploring the relationship between SVIL and ERK.

SVIL is reported to be associated with cytokinesis and mainly play a regulatory role in earlier cytokinesis
Supervillin Contributes to LPS-induced Inflammatory Response in THP-1 Cell-derived...

by interacting with central spindle proteins and myosin II [44, 45], which in turn increases cell survival rate and promotes the cell proliferative activity [10, 46]; SVIL and its interactive partners coordinate actin and microtubule motor functions throughout the cell cycle [47]. Our data showed that SVIL deletion decreased the proportion of cells in G2/M phase which was consistent with the report that SVIL participated in regulation of cell cycle [45, 47]. However, after PMA-induced differentiation, the SVIL deletion significantly increased the proportion of cells in S phase while the CCK8 data did not change. It is reported that PMA inhibited cell proliferation and regulated the cell growth arrest via ERK pathway, which in turn decreased the proportion of cells in S phase [48]. Thus, it is reasonable to suspect that SVIL deletion could inhibit the activation of ERK and hence increase the proportion of S-phase. However, as we know, PMA-treated cells could not synthesize DNA and these cells could not be cycling [48]. Therefore, the total number of cells after PMA-induced differentiation did not change significantly, which may explain why the CCK-8 data remained unaltered.

As well known, TLR4/MAPK/NF-κB is one of the key pathways for LPS-induced inflammatory response and mediates the expression of inflammatory factors (IL-1β, IL-6 and TNF-α) [49]. There is a consistent trend in the expressions of SVIL and inflammatory cytokines after LPS stimulation, indicating that SVIL was potentially associated with LPS-induced inflammatory response in macrophages. Due to the lack of studies about the role of SVIL in inflammation, it was challenging but interesting to explore the involvement of SVIL in the regulation of LPS-induced macrophage inflammation. Interestingly, we found that after interfering with the expression of SVIL, the expressions of inflammatory cytokines (IL-6, IL-1β and TNF-α) in macrophages induced by LPS were significantly inhibited. These data suggested that SVIL played an important role in LPS-induced inflammation. However, the principle of how SVIL regulates the expression of inflammatory factors has not been further investigated here. Our speculation was that, as described above, SVIL could bind to ERK1/2, just like SmAV serving as a ERK scaffolding protein in smooth muscle cells [24], enhancing the phosphorylation of NF-κB and increasing the expression of IL-6, IL-1β and TNF-α. Likewise, SVIL-knockdown reduced the combination of SVIL and ERK1/2, thereby suppressing the activation and signal transduction of ERK1/2 and inhibiting the LPS-induced expression of these inflammatory cytokines.

In conclusion, this is the first study investigating the involvement of SVIL in LPS-induced macrophages inflammatory response. As depicted in Fig. 9, our current study clearly showed that LPS induced the mRNA and protein expressions of SVIL in macrophages via activating TLR4/NF-κB and ERK1/2 MAPK signaling pathway. Additionally, depletion of SVIL could significantly reverse the expression of inflammatory factors in

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**Fig. 9** Schematic diagram of SVIL involved in LPS-induced inflammatory response in macrophages. LPS induced SVIL expression by activating TLR4/NF-κB signaling pathway. ERK1/2 MAPK pathway also participated in this process as a downstream of TLR4. Elevated SVIL was involved in the production and release of inflammatory cytokines.
macrophages induced by LPS. The present study provides a basis for exploring the relationship between SVIL and inflammation, and suggests a clue that SVIL may be a potential target for the therapy of vascular inflammatory diseases such as atherosclerosis.

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AUTHORS’ CONTRIBUTIONS

Shengnan Li and Jun Zhou designed the study, and drafted the manuscript. Jun Zhou, Yuhui Que, Lihua Pan and Xu Li performed the experiments and analyzed the data. Lai Jin contributed essential reagents and revised the manuscript. Chao Zhu revised the manuscript. All authors read and approved the final manuscript.

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AVAILABILITY OF DATA AND MATERIALS

The materials and analyzed data sets generated during the current study are available from the corresponding author on reasonable request.

DECLARATIONS

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors have no competing interests to declare that are relevant to the content of this article.

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Supervillin Contributes to LPS-induced Inflammatory Response in THP-1 Cell-derived…

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