GAB1 attenuates lipopolysaccharide-mediated endothelial dysfunction via regulation of SOCS3

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Abstract. Endothelial dysfunction is a crucial pathogenetic mechanism for sepsis. GRB2-associated binder 1 (GAB1) alleviates sepsis-induced multi-organ damage; however, to the best of our knowledge, its function in endothelial dysfunction in sepsis remains unclear. HUVECs were induced by lipopolysaccharide (LPS) to simulate endothelial cell injury under sepsis. Cell transfection was conducted to achieve GAB1 overexpression or suppressor of cytokine signaling 3 (SOCS3) knockdown. The expression levels of GAB1 and SOCS3 were detected by reverse transcription-quantitative PCR and western blotting. Cell viability, apoptosis and migration were assessed using Cell Counting Kit-8, TUNEL and wound healing assays, respectively. The production of cytokines and nitric oxide (NO) was detected using commercial kits. The interaction between GAB1 and SOCS3 was confirmed using a co-immunoprecipitation assay. GAB1 was downregulated in LPS-induced HUVECs. However, GAB1 overexpression significantly mitigated LPS-induced cell viability decrease and apoptosis in HUVECs, accompanied by upregulation of Bcl2 expression, and downregulation of Bax and cleaved caspase-3 expression. GAB1 also inhibited the production of pro-inflammatory cytokines and increased NO level, increased the levels of endothelial NO synthase (eNOS) and phosphorylated (p)-eNOS, and promoted migration in LPS-induced HUVECs. However, SOCS3 knockdown partially weakened the effects of GAB1 overexpression on cell viability, apoptosis, inflammation, p-eNOS, eNOS expression and NO levels in LPS-induced HUVECs. In addition, GAB1 and SOCS3 regulated Janus kinase 2 (JAK2)/STAT3 signaling in LPS-induced HUVECs. In conclusion, GAB1 exerted a protective effect against LPS-induced endothelial cell apoptosis, inflammation and dysfunction by modulating the SOCS3/JAK2/STAT3 signaling pathway.

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Introduction

Sepsis is a systemic inflammatory response syndrome caused by a dysregulated host response to infection, and can progress to multi-organ dysfunction and even death, which has become the leading cause of mortality in critically ill patients (1,2). It is estimated that ~30 million individuals suffer from sepsis and >6 million patients die from sepsis worldwide annually, causing a large burden to the global health care system (3). It has been reported that, in 2020, the incidence of sepsis among intensive care unit patients in 44 hospitals in China was up to 20.6%, and the mortality rate was as high as 35.5% (4). There is currently no targeted and effective therapeutic strategy for sepsis (5). Therefore, in-depth studies to understand the pathogenesis of sepsis and identify effective prevention and treatment strategies are of significant importance.

The pathogenesis of sepsis involves highly complex and integrated responses, including the host immune response, circulatory abnormalities, endothelial dysfunction and organ-organ crosstalk (6). Previous studies have demonstrated that the vascular endothelium is the main target of pathogens, microbial toxins or endogenous danger signals, and thus, sepsis-induced endothelial dysfunction is considered as an important pathogenetic mechanism for the development of sepsis (6,7).

The GRB2-associated binders (GABs) are a highly conserved class of scaffolding proteins, including GAB1, GAB2 and GAB3 (8). GABs are involved in signal transduction, mainly through the activation of the classical signaling pathways SH2 domain-containing tyrosine phosphatase 2/RAS/ERK and PI3K/AKT, and through the coupling between membrane receptors and signaling proteins, thereby regulating a series of biological responses, such as cell proliferation, angiogenesis and the inflammatory response (8). As the most widely distributed and abundant member of the GAB family, GAB1 has received widespread attention due to its biological functions. It has been demonstrated that GAB1 is aberrantly downregulated in patients with sepsis, and upregulation of GAB1 expression can alleviate sepsis-induced lung injury and renal injury by inhibiting apoptosis, oxidative stress and inflammatory responses, and thus, GAB1 is considered to be

a key regulator of sepsis (9-11). In addition, existing evidence has also revealed the important regulatory role of GAB1 in the expression of key transcription factors for endothelial homeostasis and vascular cell adhesion molecule-1, the production of proinflammatory cytokines to endothelium-associated neovascularization and the inflammatory response (12). However, to the best of our knowledge, whether GAB1 is involved in endothelial dysfunction-associated pathogenetic mechanisms for sepsis remains unclear.

Therefore, the present study aimed to explore the biological role of GAB1 in sepsis-mediated endothelial dysfunction, as well as its potential mechanism of action, offering novel insights for developing endothelium-specific therapies for the treatment of sepsis.

Materials and methods

Cell culture and treatment. HUVECs (cat. no. iCell-h110; iCell Bioscience, Inc.) were incubated with specific culture medium (cat. no. iCell-h110-001b; iCell Bioscience, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 1% endothelial cell growth supplements (ECGS; iCell Bioscience, Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) in a humified incubator with 5% CO_2 at 37˚C. To simulate endothelial cell injury under sepsis conditions, HUVECs were exposed to 2.5, 5 and 10 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA) for 24 h at 37˚C. LPS at the concentration of 10 μg/ml was utilized in the following functional experiments.

Reverse transcription-quantitative PCR. Total RNA was isolated from HUVECs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), followed by detection of its purity and concentration using a NanoDrop ND-1000 spectrophotometer. Total RNA (1 μg) was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's instructions. Subsequently, quantitative PCR was conducted with the application of Power SYBR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on the ABI 7500 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling program was as follows: 10 min at 95˚C; 40 cycles of 2 sec at 95˚C; 20 sec at 60˚C and 10 sec at 70˚C. The following primer sequences were used: GAB1 forward, 5'-ACCACCA CGACAACATTCCA-3' and reverse, 5'-CGCTGGCTTGAC TTTTCTGT-3'; suppressor of cytokine signaling 3 (SOCS3) forward, 5'-ATCCTGGTGACATGCTCCTC-3' and reverse, 5'-GGCACCAGGTAGACTTTGGA-3'; and GAPDH forward, 5'-CAGGAGGCATTGCTGATGAT-3' and reverse, 5'-GAA GGCTGGGGCTCATTT-3'. The relative expression levels of the target gene were calculated using the $2^{-\Delta\Delta Cq}$ method (13), and GAPDH served as the internal control.

Western blotting. Total protein was isolated from HUVECs using RIPA lysis buffer (Thermo Fisher Scientific, Inc.), followed by quantification of the protein concentration using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Subsequently, equal amounts of protein (40 μg/lane) were separated by 10% SDS-PAGE, and transferred onto PVDF membranes (MilliporeSigma). Membranes were then blocked with 5% skimmed milk at room temperature for 2 h, and probed with primary antibodies against GAB1 (1:1,000; cat. no. ab59362; Abcam), Bcl2 (1:1,000; cat. no. ab32124; Abcam), Bax (1:1,000; cat. no. ab32503; Abcam), cleaved caspase-3 (1:1,000; cat. no. 9661; Cell Signaling Technology, Inc.), caspase-3 (1:5,000; cat. no. ab32351; Abcam), endothelial nitric oxide (NO) synthase (eNOS; 1:1,000; cat. no. ab199956; Abcam), phosphorylated (p-)eNOS (1:1,000; cat. no. ab215717; Abcam), SOCS3 (1:1,000; cat. no. ab16030; Abcam), p-Janus kinase 2 (JAK2) (1:1,000; cat. no. ab32101; Abcam), JAK2 (1:5,000; cat. no. ab108596; Abcam), p-STAT3 (1:1,000; cat. no. 9131; Cell Signaling Technology, Inc.), STAT3 (1:2,000; cat. no. 4904; Cell Signaling Technology, Inc.) and GAPDH (1:2,500; cat. no. ab9485; Abcam) at 4˚C overnight. On the following day, after three washes with TBS with 10% Tween-20, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG antibody (1:2,000; cat. no. ab6721; Abcam) at room temperature for 2 h. Finally, blots were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.), and the band intensity was semi-quantified using ImageJ software (version 1.8.0; National Institutes of Health).

Cell transfection. The full-length coding sequence of GAB1 was cloned into the pcDNA3.1 vector (Sangon Biotech Co., Ltd.) to construct a GAB1-overexpressing vector (Ov-GAB1), and the empty pcDNA3.1 vector acted as the negative control (Ov-NC). Small interfering RNA (siRNA) targeting SOCS3, including siRNA-SOCS3-1 (sense, 5'-CCUGGUGGGACG AUAGCAACC-3'; antisense, 5'-GGACCACCCUGCUAU CGUUGG-3') and siRNA-SOCS3-2 (sense, 5'-AACAAGUUC CGUUGGAAAGUU-3'; antisense, 5'-UUGUUCAAGGCA ACCUUUCAA-3'), were also obtained from Sangon Biotech Co., Ltd., and non-targeting siRNA acted as the negative control (siRNA-NC; sense, 5'-UUCUCCGAACGUGUCACG UTT-3'; antisense, 5'-ACGUGACACGUUCGGAGAATT-3'). Upon achieving 60-70% confluence, HUVECs were transfected with 50 nM SOCS3 siRNA, 50 nM siRNA-NC, 10 μg Ov-GAB1 or 10 μg Ov-NC at 37˚C using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. In brief, the aforementioned vectors and the Lipofectamine® 3000 reagent were separately diluted in Opti-MEM. Subsequently, the two dilutions were mixed for 20 min, and then added to each well. Cells were incubated with the mixture for 6 h before the medium was changed. After 48 h, the transfection efficiency was determined via reverse transcription-quantitative PCR and western blotting as aforementioned and were used for subsequent experiments.

Cell viability assay. Cell viability was assessed using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) assay according to the manufacturer's instructions. In brief, HUVECs were inoculated into 96-well plates ($5x10^3$ cells/well) and cultured at 37˚C with 5% CO_2. Cells were treated with LPS (2.5, 5 and 10 μg/ml) for 24 h at 37˚C, and 10 μM CCK-8 solution was added to each well for an additional incubation for 2 h. Finally, the absorbance at 450 nm of each well was detected using a microplate reader. Relative cell viability (%) was calculated as follows: [Treated optical density (OD) A_{450}-blank ODA_{450}]/(control ODA_{450}-blank ODA_{450}) x100%.

TUNEL assay. The apoptotic cells were assessed using a One Step TUNEL Detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. In brief, cells were fixed with 4% paraformaldehyde for 5 min at room temperature and then incubated with 0.3% Triton X-100 for 5 min at room temperature. After washing with PBS, cells were incubated with TUNEL reagent for 60 min at 37˚C in the dark. Finally, cells were stained with 1 mg/ml DAPI solution (Invitrogen; Thermo Fisher Scientific, Inc.) for 10 min in the dark at room temperature, washed with PBS and mounted in glycerol. Images were captured in three randomly selected fields of view using an inverted fluorescence microscope (Olympus Corporation). The cell apoptosis rate (%) was calculated as follows: Number of apoptotic positive cells/total number of cells.

Measurement of cytokine concentrations and NO levels. The culture medium was harvested and centrifuged at 500 x g for 5 min at 4˚C, and the supernatant was collected. The concentrations of the inflammatory cytokines, including TNF-α, IL-1β and IL-6, in the supernatant were detected using their corresponding commercial ELISA kits (TNF-α, cat. no. PT518; IL-1β, cat. no. PI305; IL-6, cat. no. PI330; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The absorbance at 450 nm was detected using a microplate reader. The levels of NO in the culture medium were detected using a commercial kit (cat. no. BC1475; Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's protocol, and the absorbance at 550 nm was detected using a microplate reader.

Wound healing assay. HUVECs were inoculated into 6-well plates and cultured at 37˚C with 5% CO_2. Upon reaching 100% confluence, a wound was generated using a 200-μl sterile micropipette tip. Cells were washed with PBS to remove the scratched cells, and incubated with serum-free medium for 24 h. Images at 0 and 24 h were captured under an inverted light microscope (Olympus Corporation). The relative migration rate (%)=(wound width at 0 h-wound width at 24 h)/wound width at 0 h x100.

Co-immunoprecipitation (Co-IP) assay. The interaction between GAB1 and SOCS3 was confirmed using a Co-IP assay. In brief, the total protein was extracted using RIPA lysis buffer (Thermo Fisher Scientific, Inc.) and the supernatant was collected after centrifugation at 13,000 x g for 10 min at 4˚C. The lysed protein samples (500 μg) were then incubated with 2 μg anti-IgG, IP-indicated antibodies against GAB1 (1:100; cat. no. ab133486; Abcam), SOCS3 (1:30; cat. no. ab280884; Abcam), and untreated proteins as an input control. The mixtures were incubated with 50 μg Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc.) at 4˚C for 6 h. After the IP reaction, agarose beads were centrifuged at 1,000 x g for 3 min at 4˚C to the bottom of the tube. The supernatant was then carefully absorbed, and the agarose beads were washed three times with 1 ml lysis buffer. Subsequently, the immunoprecipitated protein complex was boiled and denatured, and western blotting with the anti-GAB1 and anti-SOCS3 antibodies was carried out as aforementioned to detect the precipitated protein.

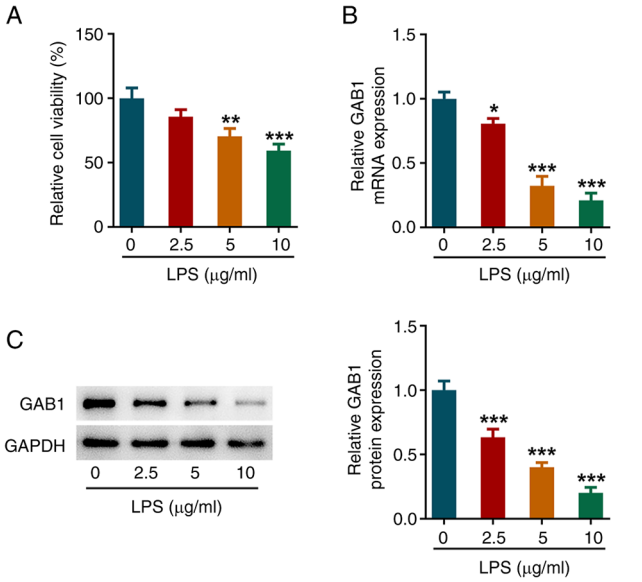

Figure 1. GAB1 is downregulated in LPS-treated HUVECs. HUVECs were exposed to 0, 2.5, 5 and 10 μg/ml LPS for 24 h. (A) Cell viability was assessed using a Cell Counting Kit-8 assay. (B) mRNA expression levels of GAB1 were examined using reverse transcription-quantitative PCR. (C) Protein expression levels of GAB1 were detected using western blotting. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 μg/ml LPS. GAB1, GRB2-associated binder 1; LPS, lipopolysaccharide.

Bioinformatics tools. By searching for 'GAB1' and selecting 'Homo sapiens' in Biogrid version 4.4.232 (https://thebiogrid.org/) and entering 'GAB1' as the protein ID in the FpClass (http://dcv.uhnres.utoronto.ca/FPCLASS/ppis/) database (threshold value>0.25), the interaction between GAB1 and SOCS3 was predicted.

Statistical analysis. All data are presented as the mean ± standard deviation. GraphPad Prism software 9.0 (Dotmatics) was used to perform statistical analysis. All experiments were independently repeated in triplicate. One-way ANOVA followed by Tukey's post hoc test was performed to compare the differences among groups. P<0.05 was considered to indicate a statistically significant difference.

Results

GAB1 is downregulated in LPS-challenged HUVECs. HUVECs were exposed to 2.5, 5 and 10 μg/ml LPS for 24 h to construct an *in vitro* model of sepsis-induced endothelial cell injury. As shown in Fig. 1A, cell viability was significantly reduced following treatment with 5 and 10 μg/ml LPS. Furthermore, the mRNA and protein expression levels of GAB1 were significantly decreased following LPS exposure in a concentration-dependent manner (Fig. 1B and C).

GAB1 mitigates LPS-induced cell viability decrease and apoptosis in HUVECs. To explore the regulatory role of GAB1 underlying sepsis-mediated endothelial cell injury, a gain-of-function experiment was performed. As shown in Fig. 2A and B, compared with those in the Ov-NC group, both the mRNA and protein expression levels of GAB1 were significantly increased in the Ov-GAB1 group. Subsequently,

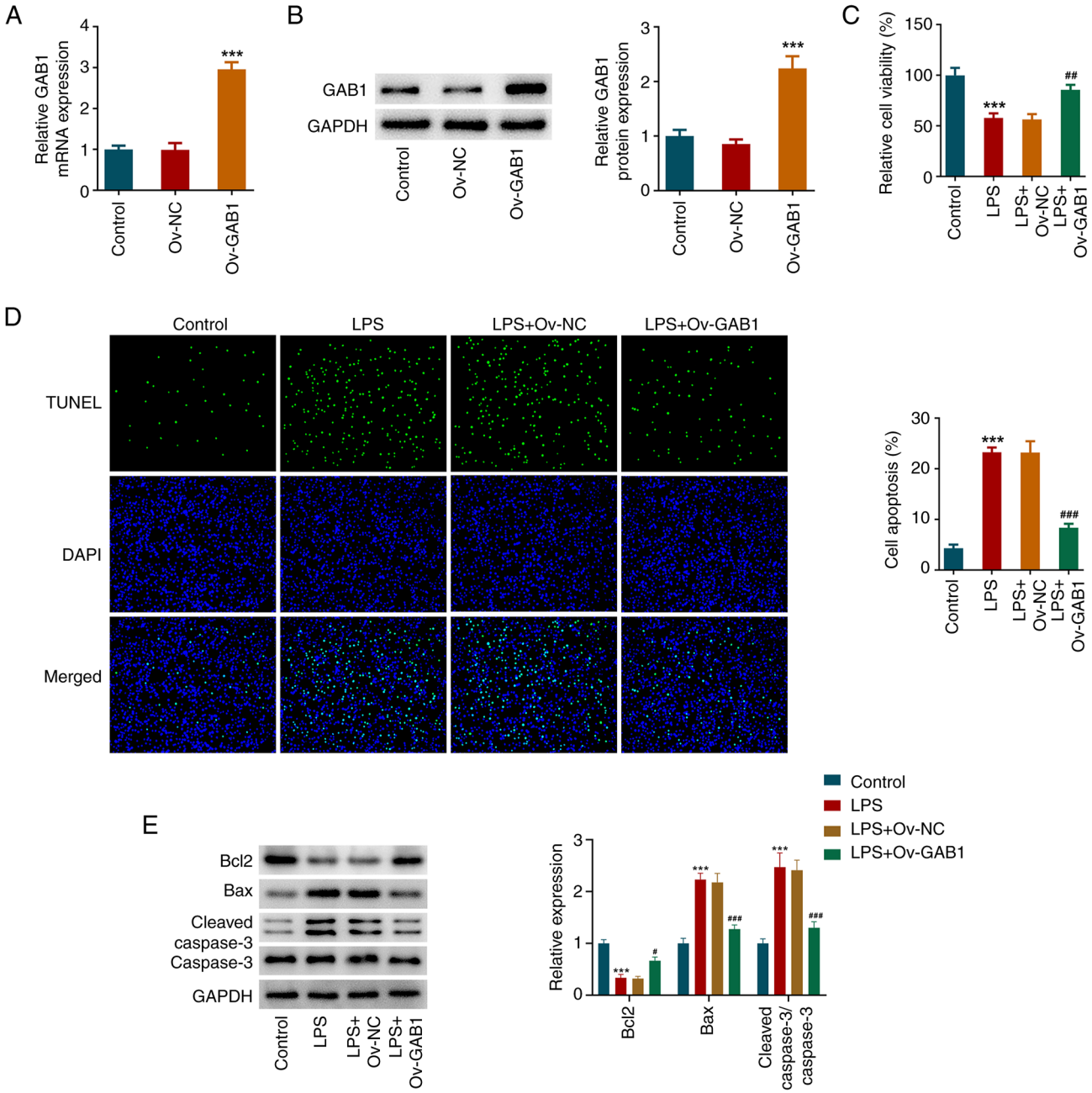

Figure 2. GAB1 mitigates LPS-induced cell viability decrease and apoptosis in HUVECs. (A) HUVECs were transfected with Ov-NC or Ov-GAB1, and the mRNA expression levels of GAB1 were detected using reverse transcription-quantitative PCR. (B) Protein expression levels of GAB1 were detected using western blotting. ***P<0.001 vs. Ov-NC. Un-transfected HUVECs (LPS group) and transfected HUVECs (LPS + Ov-NC and LPS + Ov-GAB1 groups) were exposed to LPS for 24 h. HUVECs without transfection and LPS induction served as the control group. (C) A Cell Counting Kit-8 assay was performed to assess cell viability. (D) A TUNEL assay was conducted to examine cell apoptosis; magnification, x200. (E) Expression levels of apoptosis-related proteins were detected using western blotting. ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS + Ov-NC. GAB1, GRB2-associated binder 1; LPS, lipopolysaccharide; Ov-GAB1, GAB1-overexpressing vector; Ov-NC, scramble pcDNA3.1 vector.

the normal HUVECs and GAB1-overexpressing HUVECs were exposed to LPS for 24 h, and the CCK-8 assay showed that GAB1 overexpression partly counteracted LPS-induced cell viability decrease in HUVECs (Fig. 2C). In addition, it was observable from the TUNEL assay that LPS stimulation caused an elevation of apoptosis in HUVECs, which was partly abolished by GAB1 overexpression (Fig. 2D). The downregulated protein expression levels of Bcl2 and the upregulated protein levels of Bax and cleaved caspase-3 in the LPS group compared with the control group further confirmed the high apoptosis rate of LPS-exposed HUVECs. However, these changes were partly weakened by GAB1 overexpression (Fig. 2E), suggesting that GAB1 could partly inhibit LPS-induced apoptosis in HUVECs.

GAB1 mitigates LPS-induced inflammation and endothelial dysfunction in HUVECs. An ELISA revealed that, after LPS exposure, the concentrations of TNF-α, IL-1β and IL-6 in the culture medium of HUVECs were notably elevated, which were all decreased in the LPS + Ov-GAB1 group (Fig. 3A-C).

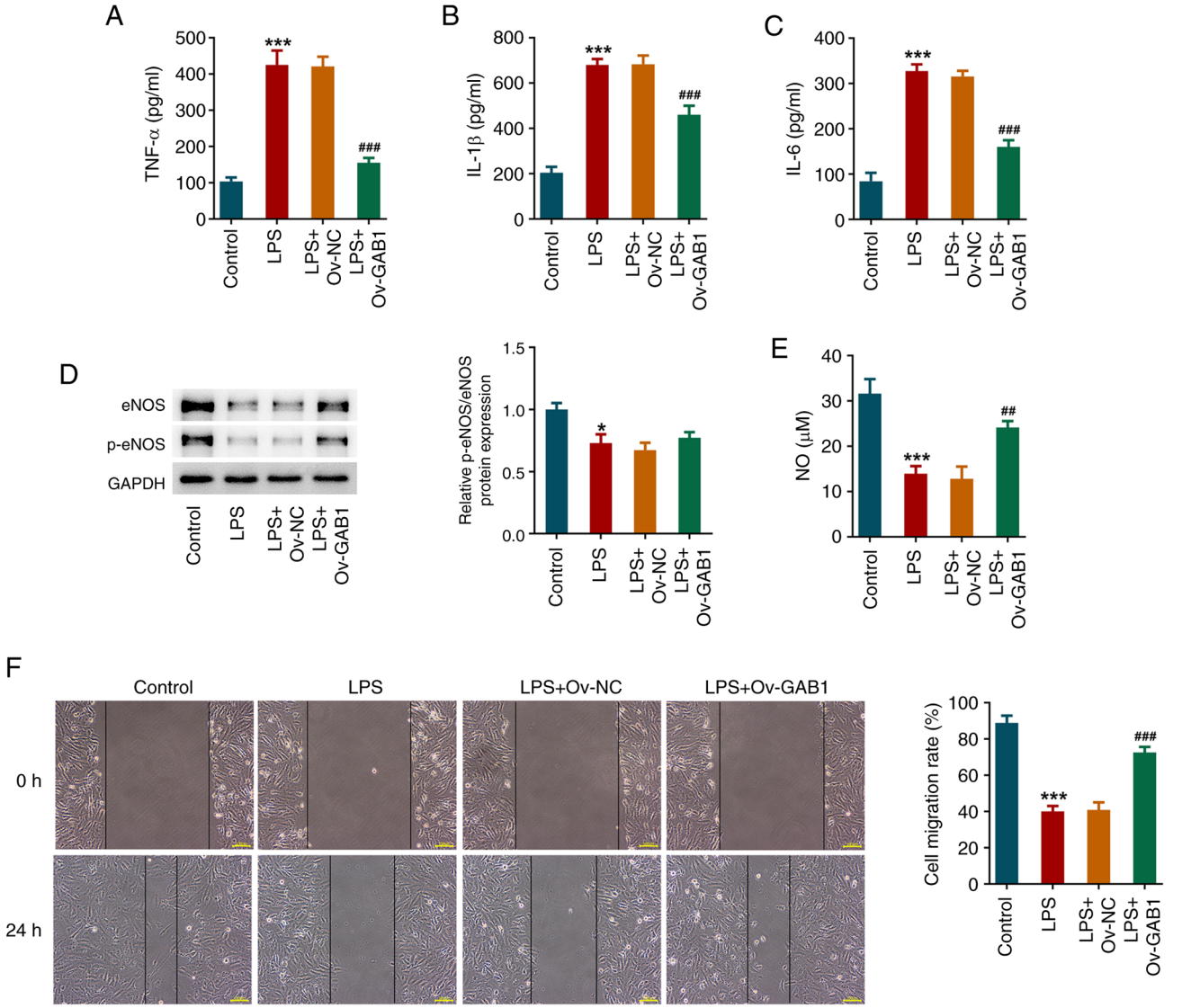

Figure 3. GAB1 mitigates LPS-induced inflammation and endothelial dysfunction in HUVECs. HUVECs with or without transfection were exposed to LPS for 24 h. HUVECs without transfection and LPS induction served as the control group. Concentrations of (A) TNF-α, (B) IL-1β and (C) IL-6 in the culture medium of HUVECs were detected using ELISA kits. (D) Protein levels of eNOS and p-eNOS were examined using western blotting. (E) NO levels in the culture medium of HUVECs were examined. (F) Cell migration was assessed using a wound healing assay. Scale bar, 100 μm. *P<0.05 and ***P<0.001 vs. control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS + Ov-NC. eNOS, endothelial NO synthase; GAB1, GRB2-associated binder 1; LPS, lipopolysaccharide; NO, nitric oxide; Ov-GAB1, GAB1-overexpressing vector; Ov-NC, scramble pcDNA3.1 vector; p-, phosphorylated.

eNOS and p-eNOS levels were significantly reduced following LPS stimulation; however, GAB1 overexpression partly restricted this reduction (Fig. 3D). Overexpressing eNOS protein is an important approach to promote NO production (14). Accordingly, compared with that in the control group, the NO level in LPS-induced HUVECs was significantly decreased, while GAB1 overexpression notably elevated NO levels (Fig. 3E). In addition, the results of the wound healing assay revealed that LPS induction significantly weakened the migration of HUVECs, which was partly counteracted by GAB1 overexpression (Fig. 3F).

SOCS3 knockdown partly weakens the impacts of GAB1 overexpression on cell viability, apoptosis, inflammation and endothelial function in LPS-induced HUVECs. The present study also attempted to identify GAB1-interacting proteins to explain its regulatory mechanisms. Based on Biogrid and FpClass database, it was found that there may be a protein-protein interaction between GAB1 and SOCS3, which was then verified using a Co-IP assay (Fig. 4A). SOCS3 expression was also downregulated in LPS-induced HUVECs, while GAB1 overexpression increased SOCS3 expression (Fig. 4B). Therefore, to understand the role of SOCS3 in GAB1-mediated endothelial function, HUVECs were transfected with siRNA-SOCS3-1/2 or siRNA-NC to knock down SOCS3. As shown in Fig. 4C and D, compared with those in the siRNA-NC group, the mRNA and protein expression levels of SOCS3 were significantly decreased in the siRNA-SOCS3-1 and siRNA-SOCS3-2 groups. Due to the superior transfection efficacy, siRNA-SOCS3-1 was used in the subsequent gain-of-function and loss-of-function experiments. As shown in Fig. 4E, HUVECs were transfected with Ov-GAB1 alone or co-transfected with siRNA-NC or siRNA-SOCS3, followed by LPS stimulation. The elevated cell viability caused by GAB1

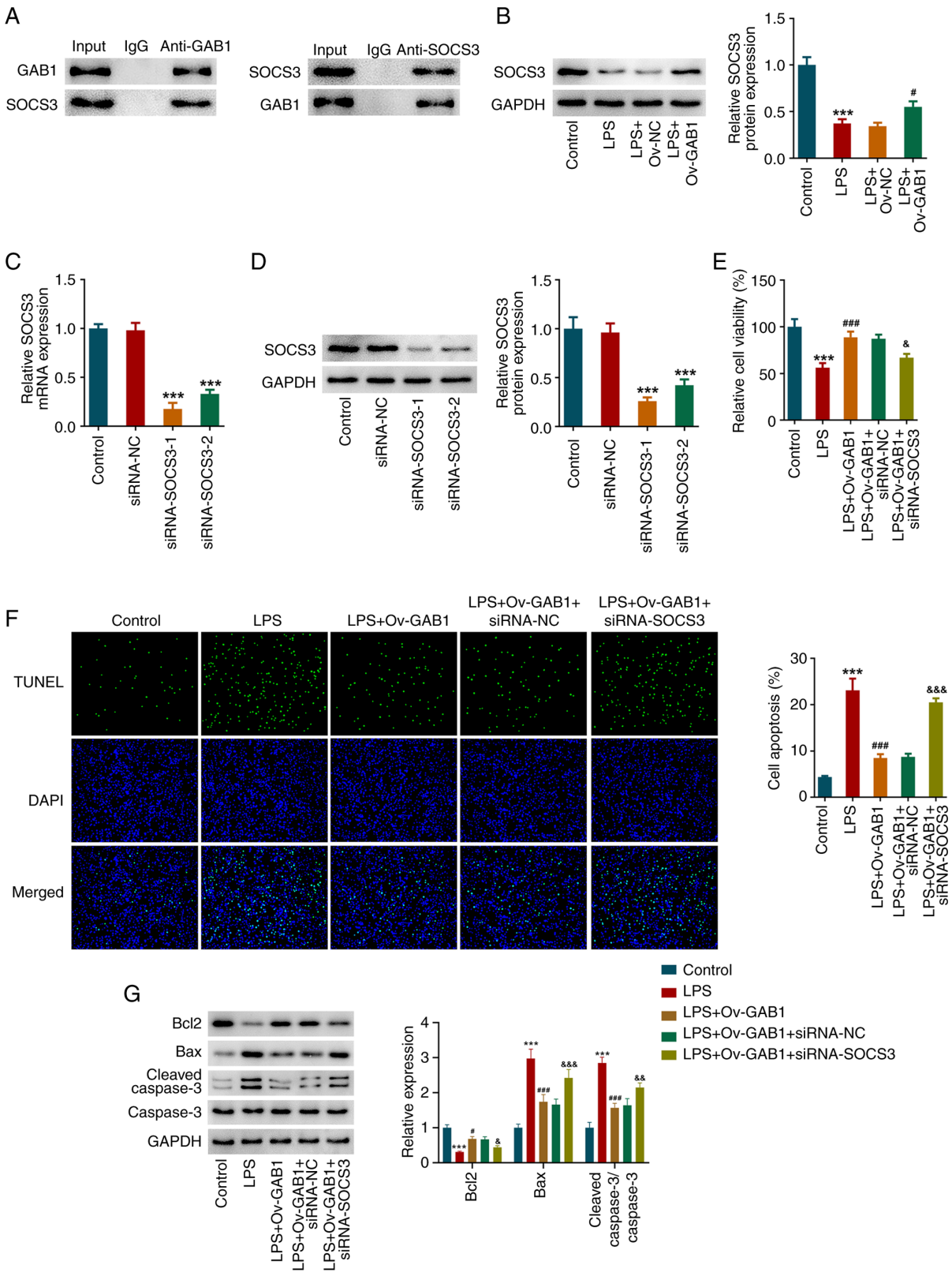

Figure 4. SOCS3 knockdown partially weakens the impacts of GAB1 overexpression on cell viability and apoptosis in LPS-induced HUVECs. (A) In HUVECs, a co-immunoprecipitation assay was performed to verify the protein-protein interaction between GAB1 and SOCS3. (B) HUVECs were transfected with Ov-NC or Ov-GAB1, followed by LPS stimulation. HUVECs without any treatment served as the control group. The protein expression levels of SOCS3 were detected using western blotting. ***P<0.001 vs. control; $^{\#}$P<0.05 vs. LPS + Ov-NC. (C) HUVECs were transfected with siRNA-SOCS3-1/2 or siRNA-NC to knock down SOCS3, and the mRNA levels of SOCS3 were detected using reverse transcription-quantitative PCR. (D) Protein expression levels of SOCS3 were detected using western blotting. ***P<0.001 vs. siRNA-NC. (E) HUVECs were transfected with Ov-GAB1 alone or co-transfected with siRNA-NC/siRNA-SOCS3, followed by LPS stimulation, and a Cell Counting Kit-8 assay was performed to assess cell viability. (F) A TUNEL assay was conducted to examine cell apoptosis; magnification, x200. (G) Expression levels of apoptosis-related proteins were detected using western blotting. ***P<0.001 vs. control; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. LPS; $^{\&}$P<0.05, $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001 vs. LPS + Ov-GAB1 + siRNA-NC. GAB1, GRB2-associated binder 1; LPS, lipopolysaccharide; Ov-GAB1, GAB1-overexpressing vector; Ov-NC, scramble pcDNA3.1 vector; siRNA-NC, scramble small interfering RNA; siRNA-SOCS3, small interfering RNA targeting SOCS3; SOCS3, suppressor of cytokine signaling 3.

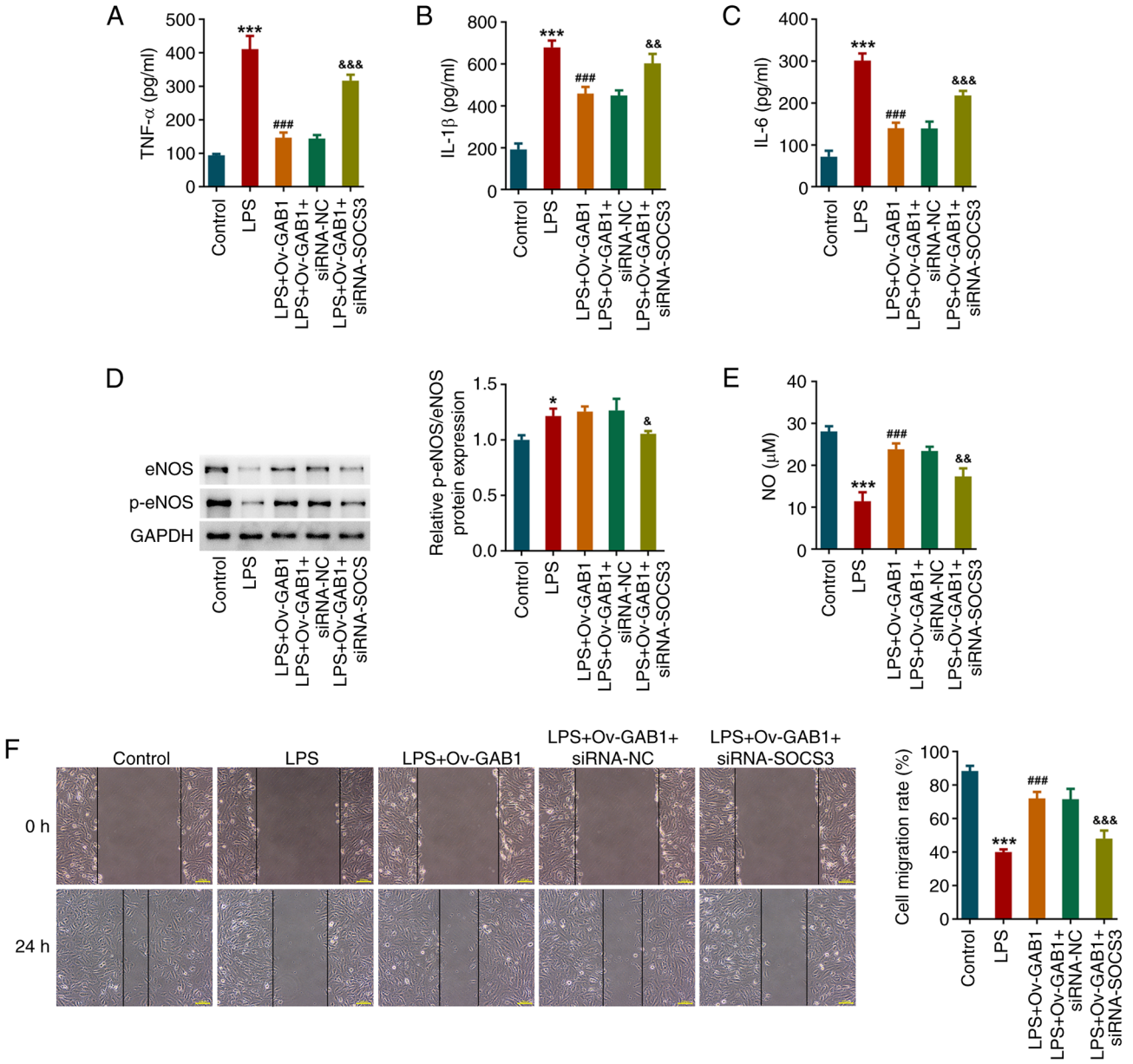

Figure 5. SOCS3 knockdown partially weakens the impacts of GAB1 overexpression on inflammation and endothelial function in LPS-induced HUVECs. Concentrations of (A) TNF-α, (B) IL-1β and (C) IL-6 in the culture medium of HUVECs were detected using ELISA kits. (D) Protein levels of eNOS and p-eNOS were examined using western blotting. (E) NO levels in the culture medium of HUVECs were examined. (F) Cell migration was assessed using a wound healing assay. Scale bar, 100 μm. *P<0.05 and ***P<0.001 vs. control; $^{\#\#\#}$P<0.001 vs. LPS; $^{\&}$P<0.05, $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001 vs. LPS + Ov-GAB1 + siRNA-NC. eNOS, endothelial NO synthase; GAB1, GRB2-associated binder 1; LPS, lipopolysaccharide; NO, nitric oxide; Ov-GAB1, GAB1-overexpressing vector; p-, phosphorylated; siRNA-NC, scramble small interfering RNA; siRNA-SOCS3, small interfering RNA targeting SOCS3; SOCS3, suppressor of cytokine signaling 3.

overexpression in LPS-exposed HUVECs was partly reduced following additional SOCS3 knockdown. Furthermore, the inhibitory effects of GAB1 overexpression on TUNEL-positive cells and the protein levels of Bcl2, Bax and cleaved caspase-3 in LPS-exposed HUVECs were significantly abolished by additional SOCS3 knockdown (Fig. 4F and G).

In addition, SOCS3 knockdown also weakened the anti-inflammatory activity of GAB1 overexpression in LPS-exposed HUVECs, as demonstrated by the upregulated levels of TNF-α, IL-1β and IL-6 in the LPS + Ov-GAB1 + siRNA-SOCS3 group compared with the LPS + Ov-GAB1 + siRNA-NC group (Fig. 5A-C). Furthermore, the increase in the protein levels of p-eNOS and eNOS, NO levels, and cell migration in LPS-exposed HUVECs following GAB1 overexpression was partly counteracted by SOCS3 knockdown (Fig. 5D-F).

GAB1 and SOCS3 regulate JAK2/STAT3 signaling in LPS-induced HUVECs. The changes in JAK2/STAT3 signaling underlying the regulation of the GAB1/SOCS3 axis in LPS-exposed HUVECs were also explored. As shown in Fig. 6, compared with those in the control group, the protein levels of p-JAK2 and p-STAT3 were significantly increased in the LPS group, indicating that LPS triggered the activation of JAK2/STAT3 signaling in HUVECs. However, the activation of JAK2/STAT3 signaling in LPS-exposed HUVECs was

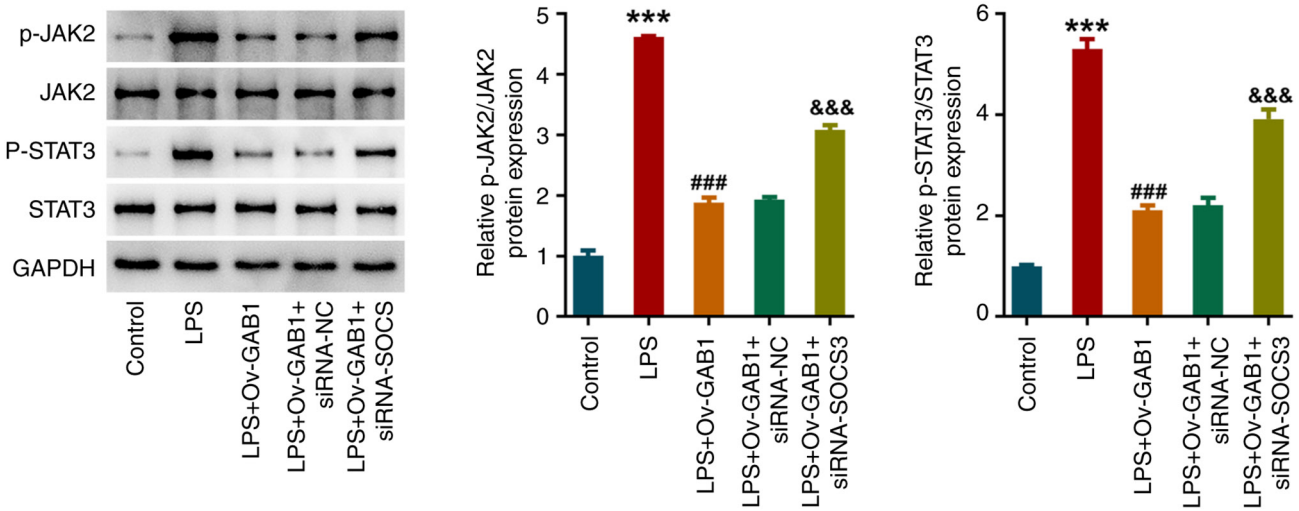

Figure 6. GAB1/SOCS3 regulates JAK2/STAT3 signaling in LPS-induced HUVECs. The levels of JAK2/STAT3 signaling-related proteins were detected using western blotting. ***P<0.001 vs. control; ###P<0.001 vs. LPS; &&&P<0.001 vs. LPS + Ov-GAB1 + siRNA-NC. GAB1, GRB2-associated binder 1; JAK2, Janus kinase 2; LPS, lipopolysaccharide; Ov-GAB1, GAB1-overexpressing vector; p-, phosphorylated; siRNA-SOCS3, small interfering RNA targeting SOCS3; siRNA-NC, scramble small interfering RNA; SOCS3, suppressor of cytokine signaling 3.

inhibited by GAB1 overexpression, which was then partly abolished by additional SOCS3 knockdown, suggesting that JAK2/STAT3 signaling might be involved in the regulation of the GAB1/SOCS3 axis in LPS-exposed HUVECs.

Discussion

The present study was undertaken to explore the protective role and mechanism of action of GAB1 in attenuating the endothelial dysfunction induced by LPS. The major findings of the present study were that GAB1 could mitigate cell viability decrease, apoptosis, inflammation and endothelial dysfunction in LPS-induced HUVECs, an *in vitro* cellular model stimulating sepsis-induced endothelial dysfunction, partly through upregulating SOCS3, accompanied by the involvement of JAK2/STAT3 signaling.

Endothelial cells, which cover the inner wall of blood vessels, are non-conventional immune cells of blood vessels and constitute the basic barrier between tissues and blood, serving a crucial role in the maintenance of the homeostasis of the internal environment of the body (15,16). During sepsis, endothelial cells are stimulated by a large number of pathogens and endotoxins, leading to endothelial cell activation and a gradual phenotypic shift toward pro-apoptosis, pro-inflammatory, pro-adhesion and pro-coagulant phenotypes, accompanied by the excessive release of pro-inflammatory factors and upregulation of endothelial adhesion molecules. This leads to impaired endothelial barrier function and an uncontrolled systemic inflammatory response, and ultimately results in multisystem organ dysfunction and failure (17,18). Therefore, protection of endothelial cells is one of the mechanisms in the current treatment of sepsis. In the present study, LPS-exposed HUVECs were used to mimic endothelial dysfunction in sepsis *in vitro* as previously proposed (19,20). It was demonstrated that LPS exposure resulted in the excessive production of pro-inflammatory cytokines and apoptosis of HUVECs, while GAB1 overexpression significantly hindered these changes. In addition, there was a reduction in p-eNOS and eNOS levels, and NO production in HUVECs in response to LPS exposure. Upregulation of eNOS protein is an important approach to promote NO production (14). NO was originally identified as a vasodilator, and the reduction of NO was regarded as one of the critical causes of endothelial dysfunction (21). Therefore, a moderate increase in NO production is of great importance for maintaining endothelial function. Accordingly, elevated p-eNOS and eNOS expression and NO production were observed following GAB1 overexpression in LPS-exposed HUVECs, indicating that GAB1 could attenuate LPS-mediated endothelial dysfunction, and might have a protective effect against sepsis.

SOCS3 is a novel intracellular regulator that negatively regulates the sustained activation of multiple cytokine-associated signaling pathways, which in turn participates in biological processes such as inflammation, oxidative stress, cell proliferation and apoptosis (22,23). SOCS3 has been shown to limit TNF-α and endotoxin-induced endothelial dysfunction by blocking essential autocrine IL-6 signaling in human endothelial cells (24). For example, upregulation of SOCS3 protein levels was able to inhibit IL-6 signaling and repair impairment of endothelial barrier function (25). This confirms that SOCS3 is a key component contributing to the inhibition of endothelial lesions during sepsis, and stabilizing endothelial SOCS3 could potentially be an effective measure against sepsis-induced multi-organ failure (25). Therefore, SOCS3 serves a protective role in sepsis-induced endothelial cell injury. Notably, in the present study, the protein-protein interaction between GAB1 and SOCS3 was verified, and GAB1 positively regulated SOCS3 expression. SOCS3 knockdown significantly weakened the inhibitory effects of GAB1 overexpression on LPS-mediated endothelial damage, further suggesting that the protective role of GAB1 against LPS-induced endothelial dysfunction was partly achieved via upregulation of SOCS3.

The JAK2/STAT3 signaling pathway is a common signaling pathway that regulates a variety of important biological behaviors such as cell proliferation, apoptosis, differentiation and inflammation (26). A previous study has shown that the development of sepsis is closely related to the persistent activation of the JAK2/STAT3 signaling pathway, and that modulation of the JAK2/STAT3 pathway can affect the course of sepsis and organ dysfunction (27). For example, melatonin alleviates sepsis-induced myocardial injury by regulating the JAK2/STAT3 signaling pathway (28), and eupatilin effectively reduces inflammation and coagulation dysfunction by inhibiting the JAK2/STAT3 signaling pathway, thereby reducing the progression of sepsis-induced lung injury (29). Consistently, in the present study, GAB1 overexpression significantly inhibited the JAK2/STAT3 signaling pathway in LPS-treated HUVECs, which may partly account for the protective effect of GAB1 against endothelial dysfunction. Further studies have revealed that SOCS3 is a critical negative regulator of the JAK/STAT3 signaling pathway (30,31). Therefore, the protective effect of GAB1 against LPS-induced endothelial dysfunction might be achieved via regulation of the SOCS3/JAK/STAT3 signaling pathway.

In conclusion, to the best of our knowledge, the present study was the first to reveal that GAB1 exerted significant ameliorative effects on LPS-induced endothelial cell apoptosis, inflammation and dysfunction by modulating the SOCS3/JAK2/STAT3 signaling pathway. The findings may provide preclinical data to support the use of GAB1 as a candidate gene in targeted therapy and drug development for the treatment of sepsis.

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Availability of data and materials

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

Authors' contributions

GR designed the study. GR, RL, HM, GY, FD, CW, SC and XL conducted the experiments to collect data. RL, HM, GY and FD analyzed and interpreted the data. GR and RL wrote the manuscript. GR and RL confirm the authenticity of all the raw data. All authors 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

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, *et al*: The third international consensus definitions for sepsis and septic shock (sepsis-3). JAMA 315: 801-810, 2016.
2. Evans L, Rhodes A, Alhazzani W, Antonelli M, Coopersmith CM, French C, Machado FR, Mcintyre L, Ostermann M, Prescott HC, *et al*: Surviving sepsis campaign: International guidelines for management of sepsis and septic shock 2021. Crit Care Med 49: e1063-e1143, 2021.
3. Seymour CW, Kennedy JN, Wang S, Chang CCH, Elliott CF, Xu Z, Berry S, Clermont G, Cooper G, Gomez H, *et al*: Derivation, validation, and potential treatment implications of novel clinical phenotypes for sepsis. JAMA 321: 2003-2017, 2019.
4. Xie J, Wang H, Kang Y, Zhou L, Liu Z, Qin B, Ma X, Cao X, Chen D, Lu W, *et al*: The epidemiology of sepsis in Chinese ICUs: A national cross-sectional survey. Crit Care Med 48: e209-e218, 2020.
5. van der Poll T, van de Veerdonk FL, Scicluna BP and Netea MG: The immunopathology of sepsis and potential therapeutic targets. Nat Rev Immunol 17: 407-420, 2017.
6. Lelubre C and Vincent JL: Mechanisms and treatment of organ failure in sepsis. Nat Rev Nephrol 14: 417-427, 2018.
7. Vincent JL, Ince C and Pickkers P: Endothelial dysfunction: A therapeutic target in bacterial sepsis? Expert Opin Ther Targets 25: 733-748, 2021.
8. Gu H and Neel BG: The 'Gab' in signal transduction. Trends Cell Biol 13: 122-130, 2003.
9. Sun L, Zhu H and Zhang K: GAB1 alleviates septic lung injury by inhibiting the TLR4/NF-κB pathway. Clin Exp Pharmacol Physiol 49: 94-103, 2022.
10. Qiu N, Xu X and He Y: LncRNA TUG1 alleviates sepsis-induced acute lung injury by targeting miR-34b-5p/GAB1. BMC Pulm Med 20: 49, 2020.
11. Xiong Y, Wang Y, Tian H, Li Y, Xu Q and He Z: Circ-PRKCI alleviates lipopolysaccharide-induced human kidney 2 cell injury by regulating miR-106b-5p/GAB1 axis. J Cardiovasc Pharmacol 78: 523-533, 2021.
12. Higuchi K, Nakaoka Y, Shioyama W, Arita Y, Hashimoto T, Yasui T, Ikeoka K, Kuroda T, Minami T, Nishida K, *et al*: Endothelial Gab1 deletion accelerates angiotensin II-dependent vascular inflammation and atherosclerosis in apolipoprotein E knockout mice. Circ J 76: 2031-2040, 2012.
13. 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 402-408, 2001.
14. Kolluru GK, Siamwala JH and Chatterjee S: eNOS phosphorylation in health and disease. Biochimie 92: 1186-1198, 2010.
15. Aird WC: The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. Blood 101: 3765-3777, 2003.
16. Page AV and Liles WC: Biomarkers of endothelial activation/dysfunction in infectious diseases. Virulence 4: 507-516, 2013.
17. Joffre J, Hellman J, Ince C and Ait-Oufella H: Endothelial responses in sepsis. Am J Respir Crit Care Med 202: 361-370, 2020.
18. Li Z, Yin M, Zhang H, Ni W, Pierce RW, Zhou HJ and Min W: BMX represses thrombin-PAR1-mediated endothelial permeability and vascular leakage during early sepsis. Circ Res 126: 471-485, 2020.
19. Huang L, Li Y, Cheng Z, Lv Z, Luo S and Xia Y: PCSK9 promotes endothelial dysfunction during sepsis via the TLR4/MyD88/NF-κB and NLRP3 pathways. Inflammation 46: 115-128, 2023.
20. Zhao L, Hu J, Zheng P, Mi B, Chen Z, Zhao X, Wu J and Wang Y: PAR1 regulates sepsis-induced vascular endothelial barrier dysfunction by mediating ERM phosphorylation via the RhoA/ROCK signaling pathway. Int Immunopharmacol 124: 110992, 2023.

21. Janaszak-Jasiecka A, Siekierzycka A, Płoska A, Dobrucki IT and Kalinowski L: Endothelial dysfunction driven by hypoxia-the influence of oxygen deficiency on no bioavailability. Biomolecules 11: 982, 2021.
22. Carow B and Rottenberg ME: SOCS3, a major regulator of infection and inflammation. Front Immunol 5: 58, 2014.
23. Jin D, Liu Y, Sun F, Wang X, Liu X and He Z: Restoration of skilled locomotion by sprouting corticospinal axons induced by co-deletion of PTEN and SOCS3. Nat Commun 6: 8074, 2015.
24. Martino N, Ramos RB, Lu S, Leyden K, Tomaszek L, Sadhu S, Fredman G, Jaitovich A, Vincent PA and Adam AP: Endothelial SOCS3 maintains homeostasis and promotes survival in endotoxemic mice. JCI Insight 6: e147280, 2021.
25. Martino N, Bossardi Ramos R, Chuy D, Tomaszek L and Adam AP: SOCS3 limits TNF and endotoxin-induced endothelial dysfunction by blocking a required autocrine interleukin-6 signal in human endothelial cells. Am J Physiol Cell Physiol 323: C556-C569, 2022.
26. Jaśkiewicz A, Domoradzki T and Pająk B: Targeting the JAK2/STAT3 pathway-can we compare it to the two faces of the God Janus? Int J Mol Sci 21: 8261, 2020.
27. Yu Q, Wang H, Li X and Leng B: Effects of JAK2/STAT3 signaling pathway activation on sepsis-induced kidney injury. Minerva Med 113: 350-352, 2022.
28. Zhen G, Liang W, Jia H and Zheng X: Melatonin relieves sepsis-induced myocardial injury via regulating JAK2/STAT3 signaling pathway. Minerva Med 113: 983-989, 2022.
29. Lu Y, Li D, Huang Y, Sun Y, Zhou H, Ye F, Yang H, Xu T, Quan S and Pan J: Pretreatment with eupatilin attenuates inflammation and coagulation in sepsis by suppressing JAK2/STAT3 signaling pathway. J Inflamm Res 16: 1027-1042, 2023.
30. Durham GA, Williams JJL, Nasim MT and Palmer TM: Targeting SOCS proteins to control JAK-STAT signalling in disease. Trends Pharmacol Sci 40: 298-308, 2019.
31. Gao Y, Zhao H, Wang P, Wang J and Zou L: The roles of SOCS3 and STAT3 in bacterial infection and inflammatory diseases. Scand J Immunol 88: e12727, 2018.