1 Yes associated protein (YAP) signaling regulates
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3 lipopolysaccharide-induced tissue factor
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5 expression in human endothelial cells
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Background. Sepsis-induced acute lung injury (ALI) is characterized by fibrin deposition, which indicates the local activation of coagulation. Tissue factor (TF), expressed in the pulmonary microvasculature, acts as a critical initiator of blood coagulation and ALI in sepsis. However, the molecular mechanism of LPS-induced TF expression in endothelial cells has not been determined. In this study, we implicate the Rho-associated protein kinase (ROCK)/Yes associated protein (YAP)/early growth response (Egr-1) signaling pathway in LPS-induced TF expression in vitro and in sepsis-induced ALI in vivo.

Methods. Human umbilical vein endothelial cells (HUVECs) incubated with LPS were pretreated with or without the ROCK inhibitor Y-27632, a YAP small interfering RNA (siRNA) and an Egr-1 siRNA. ROCK, YAP and Egr-1 signaling-induced protein expression was investigated by Western blot. The LPS-induced activation of YAP was analyzed by an immunofluorescent assay. Furthermore, we intratracheally injected YAP siRNA to assess septic ALI in mice by hematoxylin and eosin staining.

Results. LPS rapidly induced ROCK activation and increased TF expression in ECs. LPS caused YAP shuttling into the nuclei of ECs and combined with Egr-1 via the activation of ROCK. Furthermore, the LPS-mediated TF expression increase was prevented by ROCK inactivation, YAP knockdown and Egr-1 depletion, suggesting that LPS-induced TF expression is closely associated with the ROCK/YAP/Egr-1 signaling pathway in ECs. Finally, an intratracheal injection of YAP siRNA relieved lung injury in septic mice.

Conclusion. This study not only suggests that ROCK/YAP/Egr-1 signaling regulates TF expression after stimulation with LPS in ECs, but it also indicates that LPS-induced activation of YAP signaling plays an important role in septic ALI in mice. Our findings provide a new insight into the pathogenic mechanism of TF expression, which is closely linked to septic ALI, and YAP signaling is considered to be a novel target for therapeutic intervention under septic conditions.

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2 Sepsis is a serious disease characterized by the considerable derangement of
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4 pro-inflammatory and coagulation responses, which induces the dysfunction of
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6 multiple organs and lethal clinical syndromes, including acute lung injury (ALI) and
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8 acute respiratory distress syndrome (ARDS).¹ ALI, characterized by pulmonary
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10 edema, is induced by widespread intravascular and intra-alveolar fibrin deposition.²
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12 Tissue factor (TF) plays an important role in systemic bacterial toxin-mediated
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14 inflammation and the coagulation cascade in ALI.³ The activation of TF-dependent
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16 coagulation results in the formation of thrombin and the deposition of fibrin in the
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18 lung micro-vasculature.⁴ The inhibition of TF interrupts the inflammatory escalation
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20 in pulmonary tissue.⁵ The TF-dependent coagulation pathway is also extensively
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22 activated in patients with ARDS.⁶ Treatment of baboons with an anti-TF antibody
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24 prevents sepsis-induced lung injury.⁷ These findings suggest that the blockade of TF
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26 expression in the lung may be a therapy for septic ALI.
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30 Lipopolysaccharide (LPS), the major component of the outer surface of
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32 gram-negative bacteria, has been implicated in the pathogenesis of vascular EC
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34 injury and in TF expression.⁸⁻¹³ However, the underlying molecular mechanism has
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36 not been determined. Thus, this study aims to define the mechanisms by which LPS
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38 induces TF expression in ECs and to clarify the role of the TF-associated signaling
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40 pathway in sepsis-induced ALI in vivo.
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43 Under normal conditions, TF is not expressed in intravascular cells, such as
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45 endothelial cells and monocytes, but its expression can be induced after incubation
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47 with lipopolysaccharide, which plays an important role in the induction of the
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49 coagulation cascade.^{14,15} Although stimuli induce TF expression by activating
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51 transcription factors, such as nuclear factor κ B (NF- κ B) and early growth response
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53 (Egr-1) under certain pathological conditions,¹⁶ the signaling events upstream of
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55 these transcription factors remain poorly understood. Transcription factor activity is
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57 mediated by many transcriptional co-activators and co-repressors.^{17,18} Some studies
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revealed that Yes kinase-associated protein (YAP), a transcriptional co-activator, binds to Egr-1 to regulate target gene expression in irradiated prostate carcinoma cells.¹⁹ However, whether the activation of YAP/Egr-1 signaling induces TF expression in LPS-stimulated endothelial cells is not clear.

YAP is a key effector of the Hippo pathway, which is conserved from *Drosophila* to mammals and serves as an important mechanism responding to extracellular environmental stimuli.^{20,21} YAP is the crucial transcriptional co-activator of the Hippo pathway that is mainly kept inactive in the cytoplasm through serine phosphorylation by LATS.²² When YAP is activated, it shuttles into the nucleus and combines with various transcription factors to regulate different biological functions.^{19,23,24} Several upstream regulators receive chemical or mechanical signals to regulate the transcriptional activity of YAP.²⁵ For instance, RhoA induces the nuclear retention and activation of YAP protein.²⁶ Interestingly, our previous research indicated that LPS induced the hyper-permeability of monolayer endothelial cells by activating RhoA.²⁷ Therefore, we hypothesized that RhoA/Rho-associated protein kinase (ROCK) signaling is involved in LPS-induced TF expression via the translocation of YAP and the activation of Egr-1 in ECs. Elucidation of this pathway may be of immense significance in the design of therapies that regulate the hyper-coagulation and inflammation cascades that lead to septic ALI.

In the present study, we showed that LPS-induced activation of Egr-1 via ROCK/YAP signaling contributed to TF expression in HUVECs. Intratracheal injection of in vivo interference reagents of YAP protected the septic mice from ALI. Our findings give us a novel insight into the pathogenic mechanisms of LPS-induced TF expression, which may be a new therapeutic method for ALI under septic conditions.

MATERIALS AND METHODS

Cell culture and reagents. Human umbilical vein endothelial cells (HUVECs) cell line were purchased from ScienCell Research Laboratories (Carlsbad, CA). HUVECs were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen, Gibco Cell Culture, San Jose, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂, and the medium was changed at 48-hours intervals. LPS and Y-27632 were purchased from Sigma-Aldrich (St. Louis, MO). The anti-YAP rabbit monoclonal antibody (mAb) and the anti-TF mouse monoclonal antibody (mAb) were obtained from Abcam (USA). The anti-phospho-MYPT1 (Thr696) rabbit mAb, anti-phospho-YAP (Ser127) rabbit monoclonal antibody (mAb) and anti-rabbit immunoglobulin-G-HRP-linked antibody were from Cell Signaling Technologies (Danvers, Mass). The anti-Egr-1 mouse mAb and goat anti-mouse immunoglobulin-G-HRP-linked antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Alexa Fluor 568-labeled donkey anti-rabbit secondary antibody and the ProLong Gold Anti-fade Mountant with DAPI were obtained from Invitrogen Life Science.

ALI model and pulmonary histopathology. Male C57BL/6 mice (8-10 weeks old, 20-25 g) were purchased from the Experimental Animal Center of Ruijin Hospital, Shanghai, China. All of the mice were provided with standard laboratory food and water and housed in accordance with institutional animal care policies. The animals were acclimatized to the laboratory conditions (25 °C, 12 h/12 h light/dark, 50% humidity, and ad libitum access to food and water) for one week prior to experimentation. Previously, the mice were injected with YAP siRNA (Invitrogen, s76160) or Control siRNA in the trachea at a dose of 3 mg/kg for 24 hours, and then, an intraperitoneal injection with LPS (30 mg/kg, dissolved in (phosphate-buffered saline) PBS) was given to induce murine endotoxemia (Control

groups received PBS). After 24 hours, the mice were anesthetized with 1% sodium pentobarbital (40 mg/kg). The lungs were quickly removed, and the right lower lung was fixed in 10% paraformaldehyde. The paraformaldehyde-fixed lobe of the right lung was embedded in paraffin and cut into 5 μ m sections. Hematoxylin-eosin staining was performed using standard protocols. The slides of each group were assessed under high power fields and evaluated for intra-alveolar and interstitial neutrophil accumulation, alveolar edema, alveolar hemorrhage and alveolar wall thickening.

Transfection with YAP and Egr-1 siRNA in vitro. Stealth RNA interference duplexes against human YAP were designed and synthesized by Gene-pharma Technologies (Shanghai, China). YAP small interfering RNA (siRNA) molecules were transfected individually into HUVECs at 50 nmol/L using Lipofectamine 3000 (Carlsbad, CA) according to the manufacturer's instructions. Duplex siRNA were constructed against sequences coding for YAP positions (5'-CUG CCA CCA AGC UAG AUA ATT -3' and 5'-UUA UCU AGC UUG GUG GCA GTT -3'). A scrambled, negative control siRNA (5'-UUC UCC GAA CGU GUC ACG UTT-3' negative control-F and 5'-ACG UGA CAC GUU CGG AGA ATT-3' negative control-R) was also included. Egr-1 siRNA (h) (Santa Cruz, CA, sc-29303) is a target-specific 19-25 nt siRNA designed to knock down Egr-1 expression. The ability of the RNA interference molecules to knock down target protein expression was analyzed 48 hours after transfection by Western blot analysis. The transfections of the FITC-labeled nonspecific siRNA showed that the transfection efficiency was up to 85% in the HUVECs.

Western blot assay. HUVECs were seeded in 6-well plates. After adhesion, the cells were washed twice with cold PBS and lysed for 30 minutes in pre-chilled PIPA + PMSF (100:1). Then, the lysed liquid was transferred to 1.5 mL tubes. After centrifugation at 12,000 rpm for 15 minutes, the supernatants were collected as total cellular protein extracts, and the protein concentration was measured using the Bradford method (Pierce BCA Protein Assay Kit, No. 23225; Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. After resuspending the

lysates in sodium dodecyl sulfate sample buffer, the protein extracts were heated at 99°C for 5 minutes. The whole cellular protein extracts were separated by 6–12% sodium dodecyl sulfate poly-acrylamide gel electrophoresis according to the molecular weight of the target protein. The proteins were electro-transferred to the an Immobilon-P Transfer Membrane (Millipore, Billerica, MA) by wet transfer. The membranes were blocked in Tris-buffered saline (TBS) and Tween 20 containing 5% non-fat milk at room temperature for 1 hour and then incubated with antibodies to the desired proteins (YAP, TF, P-YAP (S127) and P-MYPT1 dilutions were 1:1000; the Egr-1 was 1:500) overnight at 4°C, washed in TBS and Tween 20 three times for 10 minutes each, and incubated with the appropriate HRP-linked secondary antibodies (1:2000). The signal intensities were normalized by β -actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal controls. Finally, the specific protein bands on the membranes were developed with Western Blotting Luminal Reagent (Millipore, Billerica, MA) according to the manufacturer's instructions.

Immunofluorescence staining and image analysis. For the YAP protein staining, the HUVECs were grown to 90% density in Millicell EZ SLIDE 8-well glass slides (Millipore, No. PEZGS0816) and treated with LPS in the presence or absence of Y-27632 for the indicated time. The monolayers were fixed with 4% paraformaldehyde for 30 minutes, washed, and then blocked in PBS containing 10% bovine serum albumin and 0.1% Triton X-100 for 30 minutes. The monolayers were incubated with the anti-YAP rabbit monoclonal antibody (1:100) overnight at 4°C and then the Alexa Fluor 568-labeled donkey anti-rabbit secondary antibody (1:250) for 1 hour. Before the immunofluorescent analysis, anti-fade mountant with DAPI was added, and the fluorescence images of YAP on the glass coverslips were captured by microscopy (Nikon, Tokyo, Japan).

Immunoprecipitation. To verify the interaction between the YAP and Egr-1 protein, the HUVECs were lysed in 100 μ l of RIPA+PMSF (1:100) with a protease inhibitor cocktail (1:100). The EC extracts were incubated with the YAP rabbit antibody (1:100) overnight followed by incubation for 2 hours with 30 μ l

1 protein A agarose beads (Santa Cruz Biotechnology) according to the
2 manufacturer's protocol. The precipitates were washed three times with lysis buffer
3 on ice and then probed with the anti-Egr-1 mouse antibody (1:500) using a Western
4 blotting assay.
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10 **Statistical analysis.** All of the results are expressed as the means $\pm$ SEM of
11 at least three independent experiments. The differences between groups were
12 compared using Student's t test by SPSS software, and significance was accepted at
13 $P < 0.05$.
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21 **RESULTS**

22 **LPS induces the expression of TF and ROCK signaling activation in**
23 **HUVECs.** HUVECs were stimulated with different concentrations of LPS (0-1
24 $\mu\text{g/ml}$) for 6 hours. TF protein expression increased with an increased LPS
25 concentration, and the top of the curve was at a concentration of 1 $\mu\text{g/ml}$ compared
26 with the control group (Fig 1, A). HUVECs were then incubated with LPS (1 $\mu\text{g/ml}$)
27 at the different indicated times. The Western blot showed that LPS increased TF
28 expression in time-dependent manner and peaked at 6 hours after LPS stimulation
29 (Fig 1, B). To analyze the ROCK activity of the ECs after incubation with LPS, we
30 checked the production of MYPT1 phosphorylation (P-MYPT1), which serves as a
31 substrate of ROCK.²⁸ LPS (1 $\mu\text{g/ml}$) mediated MYPT1 phosphorylation after 1 hour
32 in the ECs (Fig 1, C). Furthermore, we pretreated the ECs with different
33 concentrations of Y-27632 (the specific inhibitor of ROCK) (0, 5 and 10 μM) for 1
34 hour before LPS stimulation (1 $\mu\text{g/ml}$, 6 hours). We found that 10 μM Y-27632
35 effectively blocked the activity of ROCK in LPS-stimulated HUVECs (Fig 1, D).
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55 **LPS induces the activation of the transcriptional co-activator YAP.**
56 HUVECs were incubated with LPS (1 $\mu\text{g/ml}$) at different times. LPS increased the
57 expression of total YAP, and YAP expression peaked at 12 hours, which was similar
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1 to TF expression (Fig 2, A). YAP phosphorylation causes the cytoplasmic retention
2 of YAP and blocks its activity.²⁹ To address whether YAP is activated by LPS in ECs,
3 YAP phosphorylation on Ser127 was detected. Figure 2B indicates that YAP
4 phosphorylation was reduced after LPS (1 µg/ml) stimulation, and this impact was
5 notable in 6 hours. These data were further supported by immunofluorescence
6 staining analysis of YAP. Figure 2C and D showed that after stimulation with LPS
7 for 6 hours, YAP protein (red) shuttled from the cytoplasm to the nucleus in the ECs.
8 Subsequently, we transfected YAP siRNA into HUVECs, and the YAP knockdown
9 was confirmed by Western blot (Fig 2, E).

21 **ROCK signaling regulates LPS-induced YAP activity.** ROCK signaling
22 mediates the proliferation of human corneal endothelial cells by activating YAP.³⁰ In
23 the present study, we further exploited the underling roles of ROCK signaling on
24 LPS-induced YAP activation. We pretreated HUVECs with Y-27632 (10 µM) for 1
25 hour and then incubated with the cells LPS (1 µg/ml) for 6 hours to examine the
26 change in YAP protein. We found that the increase in total YAP protein expression
27 by LPS was attenuated by pretreating the HUVECs with Y-27632 (Fig 3, A).
28 Meanwhile, the LPS-induced reduction of YAP phosphorylation was reversed by
29 Y-27632 (Fig 3, B). Similarly, the effects of Y-27632 on LPS-induced YAP
30 activation were further confirmed by immunofluorescence staining analysis.
31 Compared to the control group, the LPS group displayed increased YAP (red) in the
32 EC nuclei and less YAP in the cytoplasm. However, pretreatment with Y-27632
33 effectively decreased the LPS-induced nuclear retention of YAP in HUVECs (Fig 3,
34 C).

54 **Role of the ROCK/YAP pathway in LPS-induced TF expression.** To
55 further investigate the underlying roles of ROCK/YAP signaling on TF expression
56 in ECs after stimulation of LPS, we incubated ECs with Y-27632 (10 µM) for 1
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1 hour and then added LPS (1 μ g/ml). Y-27632 reduced LPS-induced TF expression
2 compared to the untreated ECs (Fig 4, A). In addition, the HUVECs were
3 transfected with YAP siRNA or Control siRNA for 6 hours and then treated with
4 medium or LPS (1 μ g/ml) for 6 hours. YAP silencing effectively reduced
5 LPS-induced TF expression compared to the ECs transfected with the Control
6 siRNA (Fig 4, B).

Egr-1 is implicated in LPS-induced ROCK/YAP/TF signaling activation.

7 HUVECs were incubated with LPS (1 μ g/ml) at the different indicated times.
8 The Western blot revealed that Egr-1 was abundantly expressed after 1 hr of LPS
9 stimulation (Fig 5, A). Subsequently, the HUVECs were transfected with Egr-1
10 siRNA, and Egr-1 depletion was confirmed by Western blot (Fig 5, B). Consequently,
11 LPS-induced TF expression was blocked by transfection of Egr-1 siRNA at 6 hours
12 (Fig 5, C). We performed co-immunoprecipitation experiments to verify whether
13 Egr-1 interacts with YAP in LPS-stimulated ECs. After a 1 hr LPS incubation, the
14 interaction between the two proteins increased (Fig 5, D). In addition, we
15 investigated the role of ROCK/YAP on LPS-induced Egr-1 expression in HUVECs.
16 As shown in Fig 5, E and F, pretreatment with Y-27632 (10 μ M) and YAP siRNA
17 down-regulated LPS-induced Egr-1 expression compared with the control group.

Inhibition of YAP signaling ameliorates LPS-induced ALI in mice.

18 To further determine whether the LPS-induced activation of YAP signaling is
19 required for sepsis-induced ALI in vivo, the effects of YAP siRNA on LPS-induced
20 lung histological changes were examined in this study. After intratracheal delivery
21 of YAP siRNA (3 mg/kg), the YAP protein in the lung homogenate was quantified
22 by Western blotting (Fig 6, A). Compared with the control group (Fig 6, B), the
23 right lung tissues of the mice in the LPS group exhibited obvious pathological
24 changes, including alveolar wall thickening, an abundant infiltration of

inflammatory cells and interstitial edema (Fig 6, C). However, these changes were ameliorated by pretreatment with YAP siRNA (Fig 6, D).

DISCUSSION

ALI and ARDS are common complications of gram-negative sepsis and septic shock in which TF is regarded to be the primary initiator.⁶ TF expression in the lung microvasculature regulates the LPS-induced hypercoagulation of ALI.³¹ Some studies have suggested that administration of TF-associated coagulation pathway inhibitors or directly blocking the expression of TF could attenuate lung injury and fibrinogen degradation products.^{7,32} However, the mechanisms of LPS-induced expression of TF are not well understood. Our present study demonstrates that the ROCK/YAP/Egr-1 signaling pathway plays an important role in LPS-induced TF expression in ECs, and the blockade of YAP protein effectively ameliorates sepsis-induced ALI.

In septic conditions and other critical illnesses, the lungs are the frequently affected organ. The subsequent ALI and ARDS are the key reasons for death in patients with sepsis.³³⁻³⁵ The hypercoagulation in sepsis is primarily driven by TF.³⁶ TF modulates and elicits the inflammation cascade by driving the coagulation-inflammation cycle.³⁷⁻³⁹ More importantly, the exaggerated pro-coagulant state and inflammatory reaction in the pulmonary vasculature disrupts the alveolo-capillary membrane and leads to vascular leakage.⁴⁰ These data provide evidence that the sepsis-induced expression of TF is the leading cause of ALI. Although many previous studies have accumulated data on the effects of LPS on EC barrier disruption in sepsis-induced ALI,⁴¹⁻⁴³ this study mainly focused on the LPS-induced signaling pathway of coagulopathy in the vascular endothelium.

In the present study, LPS up-regulated the expression of TF protein in dose- and time-dependent manners in HUVECs. These observations are consistent with previous reports in which authors demonstrated increased TF activity after

1 incubation with LPS in ECs.^{9,13,16} In human vascular smooth muscle cells, a specific
2 RhoA inhibitor prevents low density lipoprotein (LDL)-induced TF
3 overexpression.⁴⁴ Pretreating human coronary artery endothelial cells (HCAEC)
4 with a ROCK inhibitor blocks TF expression.⁴⁵ In addition, TF can be induced by
5 thrombin through the activation of Rho/Rho-kinase signaling.⁴⁶ To further
6 characterize the role of ROCK in LPS-induced TF expression, we further analyzed
7 ROCK activity in LPS-stimulated HUVECs by examining MYPT1 phosphorylation.
8 We found that LPS rapidly induced the activation of ROCK, and the trend was
9 similar to that of TF expression. Furthermore, TF expression was depressed after
10 pretreating with Y-27632 in the ECs exposed to LPS. These results suggested that
11 LPS induced TF expression in the ECs via the activation of ROCK signaling. Our
12 previous findings showed that LPS also induced the release of inflammation factors,
13 the breakdown of the endothelial barrier and stress fiber formation via ROCK
14 signaling in ECs.^{27,47} These facts indicate that RhoA/ ROCK is substantially
15 implicated in cardiovascular disorders and is related to endothelial function.⁴⁸⁻⁵⁰

16 To regulate cellular function, extracellular signals transmit to the nuclei of cells
17 via the activation of ROCK/YAP signaling.⁵¹⁻⁵³ However, it remains unknown
18 whether ROCK/YAP signaling is involved in LPS-induced endothelial injury and
19 TF expression. LPS rapidly induces stress fiber formation, IL-6 expression, the
20 activation of protease activated receptor 1 (PAR1) and the disruption of adherens
21 junctions in ECs.^{27,54-57} Interestingly, other studies indicated that changes of the
22 above-mentioned proteins activate YAP by decreasing phosphorylation and
23 increasing nuclear localization.⁵⁸⁻⁶¹ In light of the indirect correlation between LPS
24 and YAP, we further analyzed the kinetics of YAP after stimulating with LPS in
25 HUVECs. We found that LPS stimulation reduced the phosphorylation of YAP and
26 mediated the translocation of YAP into the nuclei of ECs. These data suggested that
27 LPS directly induced the activation of YAP and its retention in EC nuclei. These
28 observations are analogous to previous reports in which authors showed that dextran
29 sulphate sodium (DSS) induced inflammatory bowel disease and increased
30 intestinal permeability via the activation of NF- κ B and the translocation of YAP to
31 the nucleus.⁶² Because YAP activation regulates the activities of several

transcription factors involved in inflammation and coagulation, including AP-1 and Egr-1^{19,63}, LPS and DSS-induced activation of YAP may trigger an explosion of inflammatory mediators and coagulation via activation of related transcription factors. However, we also observed that LPS increased the total endogenous YAP level in ECs. YAP phosphorylation on Ser 127 promotes its binding to 14-3-3 protein in the cytoplasm, inducing YAP protein degradation.⁶⁴ In addition, the activation of YAP signaling forms a positive feedback loop in some pathological states.⁶⁵ So YAP accumulation in HUVECs may be due to the fact that LPS-induced YAP dephosphorylation and activation forms a positive feedback loop, which reduces YAP protein degradation and induces its synthesis. Subsequently, the increase of endogenous YAP may bind different transcription factors and be involved in a cascade-like reaction of coagulation and inflammation under septic conditions.

To investigate whether YAP protein is downstream of ROCK in ECs exposed to LPS, we analyzed the effect of the specific ROCK inhibitor Y-27632 on YAP expression in LPS-stimulated ECs. Previous reports demonstrated that ROCK activation regulated the function of YAP dependently or independently of the Hippo/LATS cascade.^{66,67} Consistent with the previous studies, we also observed that the inactivation of ROCK failed to accumulate YAP in the nuclei of LPS-stimulated HUVECs. Furthermore, Y-27632 decreased the total YAP volume in the LPS-incubated ECs. These results indicate that ROCK inhibition may not only block YAP shuttling into the nucleus but also induce the interaction between YAP and 14-3-3, resulting in the cytoplasmic retention of YAP in HUVECs, which promotes its degradation.²² Our data suggested that ROCK signaling was implicated in the LPS-induced activation of YAP in HUVECs, and the ROCK/YAP pathway might play an important role in the disordered homeostasis of LPS-induced EC injury.

To gain further insight into the role of the ROCK/YAP signaling pathway in TF expression in LPS-induced ECs, we checked the expression of TF in LPS-stimulated HUVECs after transfection with YAP siRNA. Our data showed that the LPS-induced TF expression was reversed by YAP depletion in ECs. These intriguing observations indicate that ROCK/YAP signaling is required for

1 LPS-induced TF expression. However, which transcription factors bind to YAP and
2 are implicated in TF expression is not clear.
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4 Egr-1 is an important transcription factor that is responsible for the activation
5 of the TF promoter and is implicated in thrombosis and inflammation-induced
6 ALI.⁶⁸⁻⁷¹ In addition, YAP rapidly shuttles into the nucleus and binds Egr-1 to
7 promote downstream protein expression in irradiated prostate carcinoma cells.¹⁹
8 Consistent with these reports, our studies not only showed that LPS induced Egr-1
9 expression in ECs but also reported that LPS-induced TF expression was
10 significantly decreased by Egr-1 depletion. Interestingly, YAP and Egr-1 formed a
11 signaling complex after stimulation with LPS. The LPS-induced augment of the
12 Egr-1 protein was blocked by the depletion of YAP and the inhibition of ROCK.
13 These data indicated that the ROCK/YAP/Egr-1 signaling pathway plays an
14 important role in LPS-induced TF expression in HUVECs.
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28 Previous studies have revealed that TF blockade prevents ALI in baboons
29 under sepsis conditions.³³ Compared with WT mice, the lungs from Egr-1 null mice
30 demonstrated less TF activation and fibrin deposition.⁷² In addition, the suppression
31 of Egr-1 expression and activation reduced fibrin deposition, alveolar leukocyte
32 counts, lung vascular permeability and improved the gas exchange of the lung.^{73,74}
33 Tasaka et al⁴² also found that mice pretreated with Y-27632 showed attenuated
34 pulmonary edema and neutrophil emigration after intravenous injection of LPS. To
35 further understand the role of YAP in LPS-induced ALI in vivo, we used siRNA to
36 decrease the expression of YAP in the septic murine lung model. As a result, an
37 intratracheal injection of YAP siRNA protected against sepsis-induced ALI in mice.
38 These data indicate that LPS might induce endothelial injury-associated ALI
39 through the ROCK/YAP/Egr-1/TF signaling pathway in vivo. Our study not only
40 suggests that YAP, as a downstream effector, is essential for ROCK-mediated Egr-1
41 activation and TF synthesis after exposure to LPS, but it also highlights the
42 important role of the YAP protein in sepsis-induced ALI. In addition to intravascular
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1 thrombosis occurring in the injured lung under sepsis conditions, lung epithelial cell
2 injury and alveolar fibrin deposition also play critical roles in the pathogenesis of
3 ALI.⁷¹ In this regard, the protective role of YAP on sepsis-induced ALI may be due
4 to anticoagulation both in the vascular endothelium and in the lung epithelium. The
5 mechanism in the extravascular hypercoagulation and fibrin deposition in
6 sepsis-induced ALI needs to be studied further.

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13 In summary, our findings suggested that LPS induces TF expression via the
14 ROCK/YAP/Egr-1 signaling pathway in HUVECs and may induce endothelial
15 injury-associated ALI by YAP signaling in vivo. The depletion of YAP by siRNA in
16 ECs suppresses TF expression. An intratracheal injection of YAP siRNA protects
17 mice from septic ALI. Our studies identified YAP as a promising therapeutic target
18 for sepsis-induced ALI.
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Figure legends

Fig 1. LPS induces TF expression and ROCK activation in HUVECs. (A) TF was detected after stimulation with LPS (0, 0.1 and 1 µg/ml) for 6 hours, and a peak appeared at a concentration of 1 µg/ml. (B) The expression of TF was examined at different time points after LPS stimulation (1 µg/ml). (C) HUVECs were incubated with LPS (1 µg/ml) at the different indicated times and the phosphorylation of myosin associated phosphatase type 1 (P-MYPT1) was detected. (D) HUVECs were incubated with Y-27632 (0, 5 and 10 µM) for 1 hour followed by a 6-hour LPS stimulation (1 µg/ml), and the knockdown effect on P-MYPT1 was determined by Western blot. *P<0.05 versus negative control. #P<0.05 versus the corresponding LPS treatment group.

Fig 2. YAP is activated by LPS and shuttles into nucleus of ECs. (A) The expression of endogenous YAP was examined at the indicated time points after LPS stimulation (1 μ g/ml) in HUVECs. (B) The phosphorylation of YAP was detected after LPS incubation (1 μ g/ml) at different time points. (C) HUVEC monolayers were incubated with medium or LPS (1 μ g/ml) for 6 hours, and the distribution of YAP (red) was analyzed by immunofluorescence. (D) IF analysis of YAP localization. The percentage of cells showing YAP translocation to nucleus was assessed by counting the number of (cytoplasmic + nuclear) cells. (E) The YAP knockdown effect was analyzed in HUVECs by Western blot. * P <0.05, ** P <0.01 versus the negative control. # P <0.05 versus the control small interfering RNA (siRNA) group.

Fig 3. Effect of ROCK signaling on the LPS-induced activation of YAP. (A) HUVECs were incubated with Y-27632 (10 μ M) for 1 hour before LPS (1 μ g/ml) stimulation, and the expression of total YAP protein was determined by Western blot. (B) ECs were pretreated with Y-27632 for 1 hour and then incubated with LPS for 6 hours, and the expression of P-YAP (S127) was detected by an immunoblot analysis. (C) HUVECs were treated with Y-27632 (10 μ M) and/or LPS (1 μ g/ml) before fixing and staining with the anti-YAP antibody. YAP (red) and DAPI (blue) were examined by immunofluorescence microscopy. * P <0.05 versus the negative control. # P <0.05 versus the corresponding LPS treatment group.

Fig 4. ROCK/YAP signaling pathway is involved in LPS-induced TF expression. (A) HUVECs were pretreated with Y-27632 (10 μ M) for 1 hour and then were exposed to LPS (1 μ g/ml) for 6 hours. The expression of TF was determined by Western blot. (B) HUVECs were transfected with YAP siRNA or control siRNA for 6 hours and then were subjected to LPS (1 μ g/ml) for 6 hours. The expression of TF was assessed by Western blot. * P <0.05 versus the negative control. # P <0.05 versus the corresponding LPS treatment group.

Fig 5. The role of Egr-1 in LPS-induced activation of ROCK/YAP/TF

1 signaling. (A) Expression of Egr-1 was examined at the indicated time points after
2 LPS stimulation (1 μ g/ml) in HUVECs. *P<0.05 versus the negative control. (B)
3 Egr-1 knockdown effect was analyzed in HUVECs by Western blot. #P<0.05 versus
4 the control siRNA group. (C) After transfecting with Egr-1 siRNA or Control
5 siRNA, the HUVECs were subjected to LPS (1 μ g/ml) for 6 hours and the
6 expression of TF was detected by Western blot. (D) After pretreating with Y-27632
7 (10 μ M) for 1 hour, the HUVECs were exposed to LPS (1 μ g/ml) for 1 hour. The
8 expression of Egr-1 was determined by Western blot. (E) HUVECs were transfected
9 with YAP siRNA or Control siRNA for 6 hours and then subjected to LPS (1 μ g/ml)
10 for 1 hour, and the expression of Egr-1 was assessed by Western blot. *P<0.05
11 versus the negative control. #P<0.05 versus the corresponding LPS treatment group.
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24 **Fig 6.** Loss of YAP ameliorates LPS-induced acute lung injury in mice. (A)
25 After delivering YAP and Control siRNA (3 mg/kg) into the trachea of the mice for
26 24 hours, the YAP protein concentrations in the lung tissues of the mice were
27 detected by immunoblotting. *P<0.05 versus the Control siRNA group. After
28 pretreating with a small interfering RNA, the mice were challenged by an
29 intraperitoneal injection of LPS or PBS for another 24 hours, and the right lungs
30 were perfused and stained with H&E for histological examination (200 $\times$). (B) The
31 histological appearance of the lungs from the Control group that received the
32 Control siRNA. (C) The histological changes of the lungs from the LPS group that
33 received the Control siRNA. (D) The histological appearance of the lungs from the
34 LPS group that received the YAP siRNA. (N=6-8 mice in each group).
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