Journal of Inflammation Research

Dovepress
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ORIGINAL RESEARCH

Erbin Regulates Tissue Factors Through Ras/Raf Pathway in Coagulation Disorders in Sepsis

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Background: Sepsis, as a clinically critical disease, usually induces coagulation disorders. It has been reported that ERBB2 Interacting Protein (Erbin) is involved in the development of various inflammatory diseases, and macrophages are involved in the regulation of coagulation disorders in sepsis. However, the role of Erbin in coagulation disorders in sepsis and the relationship between Erbin and macrophage regulation of coagulation function are still unclear.

Methods: At the cellular level, macrophages were treated with lipopolysaccharide (LPS) or MEK inhibitor (PD98059), protein expression levels were detected by Western blot, co-immunoprecipitation (Co-IP), and immunofluorescence, mRNA expression levels were detected by quantitative real-time polymerase chain reaction (qPCR), and the concentration of tissue factor (TF) in cell supernatant was detected by enzyme linked immunosorbent assay (ELISA). At the animal level, the cecal ligation and perforation (CLP) model was constructed in mice, and the inflammatory response and coagulation disorder of mice were observed by hematoxylin-eosin (HE) staining, immunohistochemistry, ELISA, and automatic hemagglutination analyzer. The protein and mRNA expression level were detected by Western blot and qPCR. Pearson linear correlation analysis was used to analyze the correlation between the inflammation index and the coagulation function index.

Results: We confirmed that the Erbin is involved in the regulation of coagulation function by macrophages and plays a role in the coagulation disorder of sepsis. In vivo studies have shown that mice with Erbin deletion have more obvious enhanced coagulation function, and in vitro studies have shown that Erbin knockout mediated macrophage secretion of TF by activating the Ras/Raf pathway.

Conclusion: Erbin reduces the coagulation activation by inhibiting TF release from macrophages.

Keywords: Erbin, coagulation disorders, sepsis, tissue factor, macrophage

Introduction

Sepsis is a common complication of organic injury and trauma, as well as a life-threatening organ dysfunction caused by a dysregulated host response to infection[1–4]. The inflammatory response not only promotes the release of various inflammatory factors, but also activates inflammatory vesicles to induce the activation and release of tissue factor (TF), which in turn initiates coagulation activation, leading to organ dysfunction, and ultimately multi-organ failure and septic death.[5] Coagulation can be activated by both exogenous and endogenous pathways; the endogenous pathway, also known as the contact pathway, is activated by injury within the vascular system that releases factor XII, whereas the exogenous pathway, also known as the TF pathway, can be activated by trauma or infection by pathogens and the release of tissue factor.[6,7] When sepsis occurs, the body is in a hypercoagulable state due to the activation of the coagulation system by the release of a large number of inflammatory factors, which is followed by a large consumption of coagulation substances that puts the body in a hypocoagulable phase, and finally, secondary hyperfibrinolysis occurs.[8]

Received: 16 September 2024
Accepted: 6 January 2025
Published: 5 February 2025

The initiation of coagulation activation and subsequent thrombin production is caused by TF expression on activated monocytes and endothelial cells.[9] It has been shown that lipopolysaccharide (LPS) stimulates human monocytes and monocyte cell lines to induce TF expression in vitro.[10] Another study found that monocytes express TF mRNA in a human model of endotoxemia and that experimental low-dose endotoxemia in healthy subjects resulted in a 125-fold increase in tissue factor mRNA levels in blood monocytes.[11] These findings suggest that in sepsis, monocytes are the predominant cell type expressing TF. At the same time, TF plays an important role in the endogenous and exogenous coagulation pathway and is directly involved in driving and regulating the exogenous coagulation pathway and indirectly activating factor FIX affecting the endogenous coagulation pathway,[12,13] which leads to the formation of the TF: FVIIa complex, activation of FX and FIX, and subsequent generation of thrombin, fibrin deposition, and platelet activation.[14] TF has a heterogeneous tissue distribution, with high levels in highly vascularized organs, such as the lungs, brain, and placenta.[15] Lung injury is the most common complication of sepsis and is mainly characterized by dysfunction of the inflammatory response, including neutrophil consolidation and release of a variety of cytokines, accompanied by increased expression of TF, which causes coagulation disorders when released into the blood.[16,17] In most septic coagulation disorders, abnormalities in exogenous pathways predominate, thus TF is the main initiator of the coagulation cascade and plays a central role in the coagulation process.[18,19]

ERBB2 Interacting Protein (Erbin) is a multifunctional protein with the presence of many binding chaperones, which is expressed in most human tissues and is more abundant in brain, heart, kidney, muscle, and lung tissues.[20] A related study demonstrated that Erbin-deficient mice exhibited more severe intestinal barrier disruption and greater production of pro-inflammatory cytokines in acute colitis mice induced using dextran sulfate sodium salt (DSS).[21] In the study of Erbin-regulated signaling pathways, Some articles have reported that Erbin inhibits Nod2-dependent NF-kB activation and inflammatory cytokine secretion in cells, thereby attenuating the inflammatory response,[22] A study found that Erbin prevents sepsis-induced cardiomyopathy by inhibiting RIPK1-dependent necrotic apoptosis through activation of the PKA/CREB pathway,[23] It has also been shown that Erbin accelerates TFEB-mediated lysosomal biosynthesis and autophagy and attenuates sepsis-induced inflammatory response and organ damage,[24] Another study also demonstrated that Erbin prevents sepsis-associated encephalopathy by attenuating microglia focal death through the IRE1α/Xbp1s-Ca^{2+} axis.[25] Thus, Erbin plays an important role in the sepsis inflammatory response; however, sepsis coagulation disorders associated with the sepsis inflammatory response have not been reported in articles.

The Ras-Raf-MEK-ERK pathway is an important pathway for signaling downstream of epidermal growth factor receptor (EGFR), which consists of a tertiary cascade network of small G proteins, Rat sarcoma (Ras) and Raf-MEK-ERK, on the cell membrane,[16] activated Ras-GTP binds with high affinity to Raf-1 proto-oncogene (Raf-1) to translocate it to the cell membrane and activate it, and phosphorylation of activated Ras-1 activates mitogen-activated extracellular signal-regulated kinase (MEK), which then further phosphorylates and activates extracellular regulated protein kinases (ERK).[26] Huang et al reported that the expression of Erbin could downregulate the transcription of the ε-subunit gene of acetylcholine receptor (AchR), and the mechanism was that Erbin blocked the Ras-Raf-1 pathway by interacting with Ras, which in turn inhibited the phosphorylation of ERK, leading to the down-regulation of ERK kinase activity, which in turn led to the reduction of AchR expression.[18] Guha M et al identified a role for the MEK-ERK1/2 signaling pathway in LPS-induced TF expression in human monocytes by the mechanism of phosphorylation of ETS transcription factor (ELK1) by the MEK-ERK1/2 pathway inducing the expression of early growth response factor 1 (Egr-1) in LPS-stimulated monocytes and the subsequent expression of TF genes.[19]

Therefore, we hypothesized that Erbin may regulate TF expression through the Ras/Raf pathway, which in turn affects coagulation dysfunction in sepsis. In the present study, we found that Erbin knockdown exacerbated cecal ligation and perforation (CLP)-induced inflammatory response and promoted TF expression and coagulation activation by constructing a mouse sepsis model. Treatment of macrophages with LPS or MEK inhibitor (PD98059) revealed that Erbin knockdown promoted Ras/Raf pathway activation induced by LPS stimulation and increased macrophage TF expression.

Materials And Methods

Materials

SPF-grade male C57BL/6 mice and knockout mice were selected at 8–10 weeks of age, weighing 20–30 g. Wild-type mice (WT) were purchased from Wuhan Mouse Lai Bao Biotechnology Co. Ltd. and Erbin knockout mice (EKO) were

purchased from Wuhan Fenran Biotechnology Co. The mice were housed in an SPF-grade mouse house, where the temperature was maintained at 24±2°C, the humidity was suitable, and the mice were allowed to drink freely and provided with a standard laboratory diet. All animal care and experiments were conducted by the ethical codes established by the Wuhan University Animal Experimentation Committee (WQ20210298).

Sepsis Model

Mice were anesthetized by intraperitoneal injection of 1% sodium pentobarbital, and the cecum was isolated after opening the abdomen. Ligation was performed with a sterile 4-gauge silk thread at the midpoint between the ileocecal valve and the terminus of the cecum, followed by puncture of the cecum wall through the cecum using a 20 G sterile needle, and finally, the incision was closed, and resuscitation was performed with a subcutaneous injection of 1 mL of 0.9% saline. Mice in the sham-operated group underwent the same procedure but without ligation and puncture of the cecum.

Hematoxylin and Eosin (H&E)

Mouse lung tissues were stained with hematoxylin and eosin using conventional staining methods.

Cells Culture And Treatment

Establishment of Erbin knockout Raw264.7 cells (TIB-71, ATCC) ($Erbin^{-/-}$ cells): using CRISPER/CAS9 gene editing technology, the Erbin-sgRNA sequence of Pgl3-U6-sgRNA-PGK-puromycin-vector was designed: forward sequence: 5′-CACCGTCTCCTCGTAGACAGCGACA-3′, reverse sequence: 5′-AAACTGTCGCTGTCTACGAGGAGAC-3′, and co-transfected Raw264.7 cells with pST1374-NLS-flag-linker-Cas9 system.

Raw264.7 cells and $Erbin^{-/-}$ cells were cultured in complete medium (Gibco, America) containing 10% fetal bovine serum (FBS) (Biology, China), 100 units/mL penicillin, and 100 mg/L streptomycin (Biosharp, China) in a humidified environment at 37°C, 5% CO_2. Bone marrow progenitor macrophages (BMDM) in the WT group were recorded as $Erbin^{fl/fl}$ and those in the EKO group were recorded as $Erbin^{KO}$, and BMDM were collected from the femurs of $Erbin^{fl/fl}$ and $Erbin^{KO}$ mice in complete medium (Gibco, USA) containing 10% fetal bovine serum (FBS) (Biology, China), 100 units/mL penicillin and 100 mg/L streptomycin (Biosharp, China), and M-CSF (20 ng/mL) in complete medium (Gibco, America), 37°C, 5% CO_2 in a humidified environment.

The Macrophage cell activation model was established using LPS (E. coli 0111:B4, L2630, Sigma), cells were stimulated with LPS in the intervention group, MEK-ERK signaling pathway activation was blocked using MEK inhibitor (PD98059) (MCE, America), and PBS was added as a reference in the control group.

Coagulation 4 Items and ELISA

Plasma extraction method: Before blood extraction, 3.8% sodium citrate anticoagulant (purchased from Shanghai Yuanye Biologicals) was added to 1.5 mL EP tubes according to the ratio of 1:9, and the tubes were wetted by shaking up and down, and the eyeballs of the mice were clipped to extract 0.5–1 mL of blood, which was put on ice for half an hour, and then centrifuged for 10–15 min at 4°C with a flow rate of 3000 r/min, and the upper layer of the blood plasma was extracted. The absorbance of the samples at 450 nm was measured by a microplate reader; plasma prothrombin time (PT), activated partial thromboplastin time (APTT), prothrombin time (TT), and fibrinogen (FIG) were determined by a RAC-1830 automatic hemagglutination analyzer.

Lung Tissue Homogenates and Cell Supernatants

Mouse lung tissue was weighed and cut into pieces and loaded into EP tubes, the tissue homogenizer was pre-cooled in advance and then the samples were added for full homogenization, after full homogenization, it was placed in a centrifuge at 4 °C and centrifuged for 10–15 min (2500–3500 rpm/min), and the supernatant was placed into new EP tubes, and the TF was detected in the homogenates of mouse lung tissue using ELISA kits (HYCEZMBIO), thrombin-antithrombin complex (TAT) and plasminogen activator inhibitor 1 (PAI-1) levels in mouse lung tissue homogenate; collect the cell supernatant from 6-well plate, put it in EP tube, then put it into 3000 rpm/min, 4 °C,

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10 min centrifuge, take the supernatant and put it into a new EP tube, ELISA kit (HYCEZMBIO) was used to detect the level of TF in the cell supernatant.

Reverse Transcription Real-Time Quantitative Polymerase Chain Reaction (RT q - PCR)

Total RNA was extracted from mice and cells with TRIZOL Reagent (Vazyme, China). RNA was reverse transcribed to complementary DNA using ABScript III RT Master Mix (Abclonal, China). Real-time fluorescence quantitative PCR assay was performed according to the manufacturer's instructions. The expression of IL-6, tumor necrosis factor-α (TNF-α) and TF, Egr-1, tissue-type plasminogen activator (t-PA), and fibrinogen α-chain (FGA) were analyzed using β-actin gene as an internal control. Gene expression was quantified by the $^{2\text{-}\Delta\Delta Ct}$ method. The primer sequences for all the genes in order are: β-actin Forward 5′-AGACCTTCAACACCCCAG-3′, Reverse 5′-CACGATTTCCCTCTCAGC-3′, IL-6 Forward 5′-TAGTCCTTCCTACCCCAATTTCC-3′, Reverse 5′-TTGGTCCTTAGCCACTCCTTC-3′, TNF-α Forward 5′-? −3′, Reverse 5′-? −3′, TF Forward 5′-AACCCACCAACTATACCTACACT-3′, Reverse 5′-GTCTGTGAGGTCGCACTCG-3′, Egr-1 Forward 5′-TCGGCTCCTTTCCTCACTCA-3′, Reverse 5′- CTCATAGGGTTGTTCGCTCGG-3′, t-PA Forward 5′-TGACCAGGGAATACATGGGAG-3′, Reverse 5′-GTCTGCGTTGGCTCATCTCTG-3′, FGA Forward 5′-CAGGATGAAAGGGTTGATTGACG-3′, Reverse 5′-GCGAAGTCCCCTCTCAAATACT-3′.

Western Blot

Total protein content was extracted from mouse lung tissues or cultured cells using RIPA lysis buffer (Beyotime, China) containing PMSF (Servicebio, China) and phosphatase inhibitor (Bioprimacy, China). The protein concentration in the supernatant was determined by BCA protein assay (BioSharp, China). An equal amount of proteins was supersampled and transferred to PVDF membrane by electrophoresis and membrane transfer (Thermo Fisher Scientific, China). The PVDF membrane was then blocked with 5% BSA at room temperature for 2 h. The membrane was incubated with primary antibodies against Erbin (NBP2-56104, Novus), Ras (HY-P80298, MCE), Fibrin (FIB) (MABS2155, Merck), p-ERK1/2 (AP0472, Abclonal), ERK1/2 (A22447, Abclonal), p-ELK1 (AP0033, Abclonal), and p-ELK1 (A22447, Abclonal). Abclonal), p-ELK1 (AP0033, Abclonal), ELK1 (A19046, Abclonal), Egr-1 (A2722, Abclonal), TF (A4395, Abclonal), β-actin (GB12001-100, Servicebio) and others. Antibodies were incubated at 4 °C overnight. The membrane was washed three times with TBST on the following day, and then the corresponding enzyme secondary antibody was added and incubated at room temperature for 1 h. After washing, the membrane was developed and exposed using an ECL chemiluminescence developer. The grayscale value of the bands was analyzed by Image J software, and the ratio of the grayscale value of the target protein bands to the grayscale value of the internal reference β-actin bands was calculated to reflect the expression level of the target protein.

Immunofluorescence

For cells, they were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.5% Triton X-100 for 20 min. After being closed with 5% BSA for half an hour at room temperature, the cells were incubated with the indicated primary antibodies TF (ET1611-95, HuaBio), Egr-1 (A2722, Abclonal) overnight at 4 °C. After three washes with PBST, the cells were incubated with fluorescent secondary antibodies ABflo® 488-conjugated (AS053, Abclonal) and ABflo ® 594-conjugated (AS039, Abclonal) for 1 h. Cell nuclei were stained with DAPI (Beyotime, China) containing an anti-fluorescence quencher, and images were recorded with an Olympus fluorescence inverted microscope. For lung tissues, formalin-fixed, paraffin-embedded lung tissue sections were used for immunohistochemistry, sections were dewaxed to aqueous-antigen repair-3% hydrogen peroxide treatment, closed using 5% BSA, primary antibody FIB (MABS2155, Merck) was incubated overnight at 4 °C, and the secondary antibody was incubated for 1 h at room temperature to develop the color, and then the nuclei were stained using hematoxylin, and finally dehydrated, transparent, sealed and microscopically examined to record images.

Co-Immunoprecipitation (CO-IP)

The cells in the culture dish were transferred to a 1.5 mL EP tube, centrifuged at 3000 r/min for 5 min and the supernatant was discarded, IP lysate (Beaverbio, China) and PMSF (Servicebio, China) were added, fully lysed and then encapsulated into new EP tubes according to the ratio of IP-IgG-Input 6:3:1. In the IP group, primary antibody Erbin (NBP2-56104, Novus) was added, IgG antibody (AC005, Abclonal) was added to the IgG group, and protein samples were made directly from the Input group, and the IP group and IgG group were placed on a rotary shaker at 4 °C overnight. ProteinA/G was added to the IP group and IgG group respectively, and the magnetic beads were washed with IP lysate, and then placed on a 4 °C rotary shaker for 4–6 h. Finally, the magnetic beads were washed again, and the protein samples were resuspended with IP lysate in the IP group and the IgG group respectively, and validated by Western Blot.

Statistical Analysis

Data analysis and graph construction were performed using GraphPad Prism 8.0. Kaplan-Meier analysis was applied to assess the probability of survival, and all data were expressed as mean ± SD. When multi-sample means were compared and the variances of the groups were aligned, one-way ANOVA was used for comparison between groups. Pearson linear correlation analysis was used for correlation analysis, and the difference was considered to be statistically significant at $P<0.05$.

Results

Erbin Knockout Exacerbates Inflammatory Response and Reduces Survival in Septic Mice

To assess how Erbin deficiency affects the inflammatory response to sepsis, we constructed a mouse model of CLP 8 h-induced sepsis, which was found to be lethargic, with reduced activity, slowed movement, loss of fur, and increased periocular secretions after surgery. The 7-day mouse survival curves showed that all WT and EKO mice had a 100% survival rate at 7 days after sham surgery. The survival rate was significantly lower in the CLP group compared to the Sham group, with a 7-day survival rate of approximately 55% (11 out of 20 mice survived). In addition, Erbin knockout further increased the mortality rate of septic mice, with a survival rate of only 25% (5 out of 20 mice survived) (Figure 1A). HE staining of lung tissue sections from WT mice showed that CLP 8h-induced inflammatory responses in lung tissues manifested as thickening of inter-alveolar septa, alveolar edema, some erythrocytes exuded into the intra-alveolar and interstitial spaces, and inflammatory cells infiltrated into interstitial and interalveolar spaces, in contrast to EKO mice, which showed further exacerbation of lung tissue inflammatory response (Figure 1B). After 8h of CLP modeling, mRNA expression of IL-6 and TNF-α was upregulated in lung tissues of EKO mice relative to WT mice (Figure 1C and D); plasma was taken from the mice to detect IL-6 concentration using ELISA, and it was found that IL-6 concentration was elevated in the CLP group compared to the Sham group, and it was further increased after Erbin knockdown (Figure 1E). Overall, Erbin knockdown exacerbated the inflammatory response and decreased survival in septic mice.

Erbin Knockout Promotes Coagulation Activation in Septic Mice

Extensive activation of the coagulation cascade is important for the occurrence of sepsis-related organ dysfunction. Therefore, we assessed the coagulation function of mice by using the RAC-1830 fully automated hemagglutination analyzer to detect the indicators of the four plasma coagulation items (PT, APTT, TT, and FIB) and by using ELISA to detect the coagulation indicators of TF, PAI-1, and TAT. It was found that after 8 h of CLP, EKO mice had prolonged plasma PT, APTT, TT time and increased FIB concentration relative to WT group mice (Figure 2A–D), and coagulation indices, such as TF, PAI-1, and TAT, in plasma and lung tissues of the EKO group mice were also significantly higher relative to WT group mice (Figure 2E–I). These results suggest that Erbin knockdown promotes coagulation activation in septic mice.

Erbin Knockdown Promotes LPS Stimulation-Induced Activation of the Ras/Raf Pathway and Increases Macrophage TF Expression

TF is an important component of the exogenous coagulation pathway, expressed on macrophages and monocytes, and is thought to play a central role in coagulation initiation plays an important role in coagulation regulation.[27] To investigate

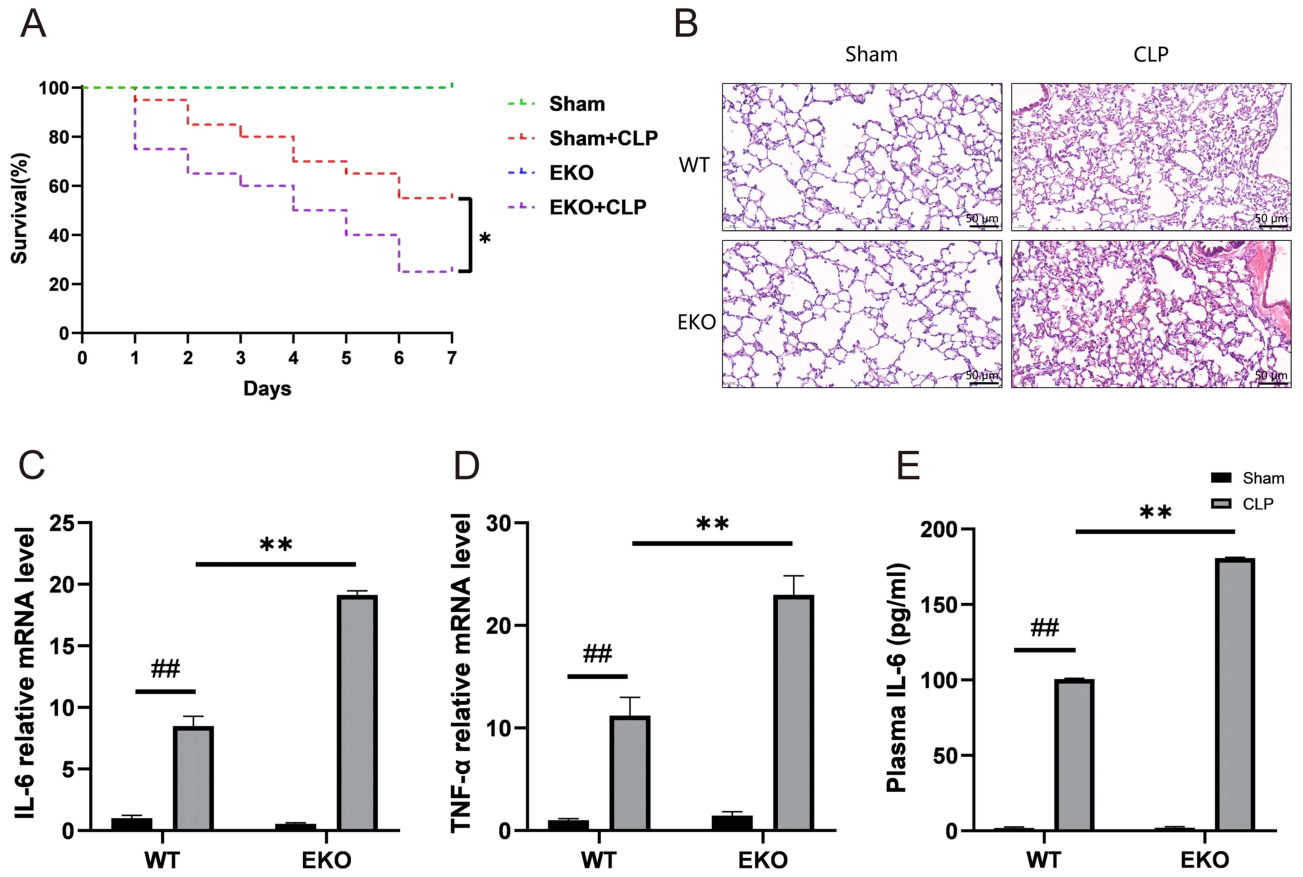

Figure 1 Erbin knockout exacerbates the inflammatory response and reduces survival in septic mice. (**A**) 7-day survival of mice (20 per group; Kaplan-Meier survival analysis). (**B**) Lung tissues of mice were taken for HE staining. (**C** and **D**) mRNA expression levels of IL-6 and TNF-α in lung tissues were detected by using qPCR. (**E**) Plasma IL-6 concentration in mice was detected by using ELISA. Graphs show mean ± standard deviation (mean ± SD), and the data shown (**C-E**) are representative of three independent experiments. $^{\#\#}P<0.01$ for the WT+Sham group compared with the WT+CLP group; $^{*}P<0.05$, $^{**}P<0.01$ for the WT+CLP group compared with the EKO+CLP group.

whether Erbin is involved in LPS-stimulated TF production in macrophages, we selected Raw264.7 cells and Erbin$^{-/-}$ cells for the study. After 3h of LPS 1μg/mL stimulation, Western blot results showed that the expression of TF was increased in Raw264.7 cells, and relative to Raw264.7 cells, Erbin$^{-/-}$ TF expression was further increased in Raw264.7 cells, suggesting that Erbin knockdown promoted LPS-induced TF production in macrophages. To further determine whether the high expression of TF in macrophages was related to the activation of the Ras/Raf pathway, relevant pathway proteins were detected in LPS-stimulated cells, and elevated levels of p-ERK, p-ELK1, and Egr-1 proteins were found in Raw264.7 cells. Erbin knockdown resulted in further elevated expression of these proteins in Erbin$^{-/-}$ cells, indicating that Erbin knockdown activated the Ras/Raf pathway (Figure 3A–F). At the level of mRNA expression of proteins, we analyzed and compared the expression of TF mRNA and Egr-1 mRNA, and found that the mRNA expression of both proteins was up-regulated in Raw264.7 cells after LPS stimulation, and the expression was further up-regulated in Erbin$^{-/-}$ cells, suggesting that Erbin knockdown up-regulated the expression of TF, Egr-1 mRNA in macrophages (Figure 3G and H). Similarly, we obtained similar results in cellular immunofluorescence experiments to detect TF and Egr-1 expression in macrophages by immunofluorescence. In the untreated group, we found low fluorescent expression of TF and Egr-1 proteins in macrophages, which increased after administration of LPS stimulation, with a more pronounced increase in fluorescent expression of TF and Egr-1 proteins in Erbin$^{-/-}$ cells relative to Raw264.7 cells (Figure 3I and J). We obtained similar results when using ELISA to detect TF concentration in the supernatant of macrophage secretion, which was elevated in the cell supernatant after administration of LPS stimulation, and further elevated in the supernatant of Erbin-/- cells relative to Raw264.7 cells (Figure 3K).

To further verify whether there is an interaction between endogenous Erbin and Ras, which in turn affects the downstream pathway protein expression, we carried out immunoprecipitation experiments for further verification. Raw264.7 cells were

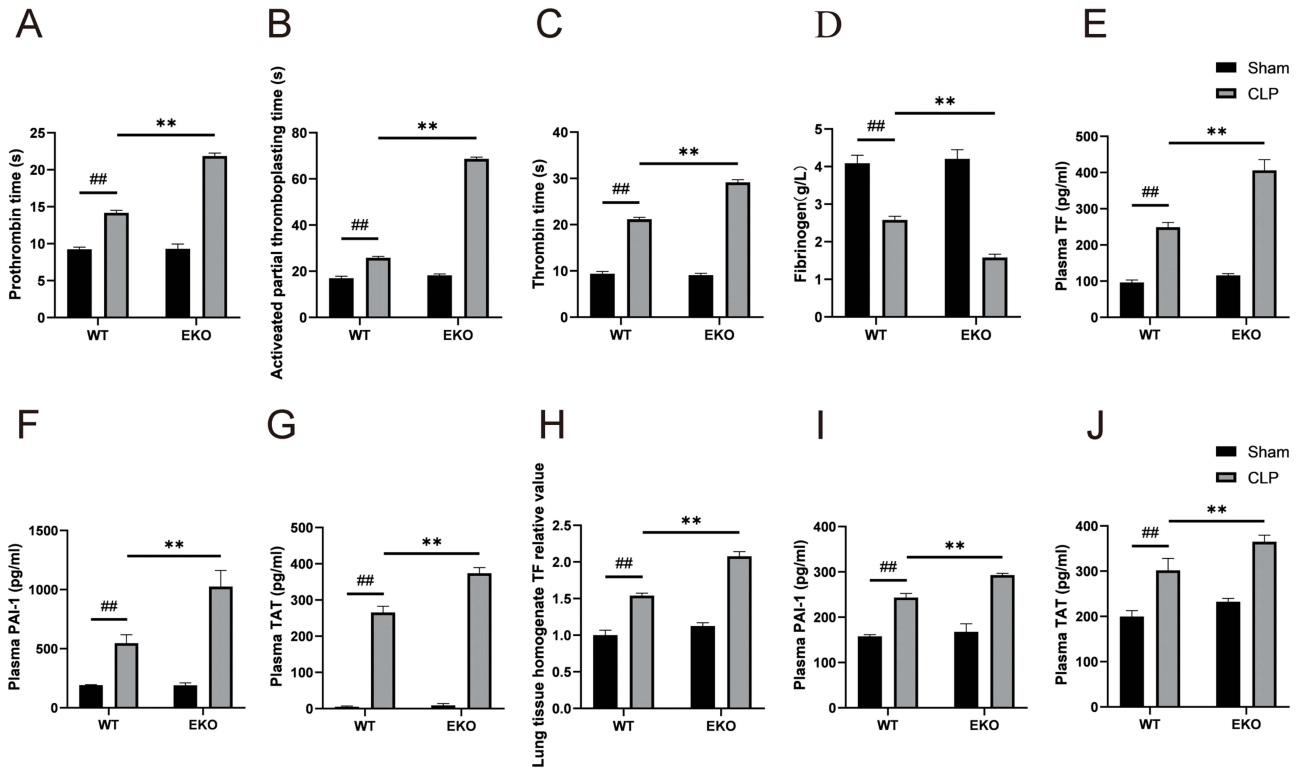

Figure 2 Erbin knockdown promotes coagulation activation in septic mice. (**A-D**) Plasma coagulation tetrapartite PT, APTT, TT, and FIG were detected in mice using a fully automated hemagglutination analyzer. (**E-J**) ELISA was performed to detect the levels of TF, PAI-1, and TAT in the plasma and lung tissues of mice. Graphs show mean ± standard deviation (mean ± SD), and the data shown (**A-J**) are representative of three independent experiments. $^{\#\#}P<0.01$ for the WT+Sham group compared with the WT +CLP group; $^{**}P<0.01$ for the WT+CLP group compared with the EKO+CLP group.

selected for the study, cell lysates were prepared and incubated with Ras antibody, IgG antibody was used as a negative control, and the obtained immunocomplexes were subjected to Western blot with Erbin and Ras antibodies, and Input protein was used as a positive control, and the results showed that Erbin was present in the immunocomplexes, which indicated that endogenous Erbin and Ras might interact or be in the same complex (Figure 3L).

These results suggest that Erbin knockdown promotes LPS stimulation-induced activation of the Ras/Raf pathway and increases macrophage TF expression.

PD98059 Inhibits Ras/Raf Pathway Activation and Reduces Macrophage TF Expression Upregulation Induced by LPS and Erbin Knockout

From the above cell experiment, we found that Erbin knockdown promotes LPS stimulation-induced activation of the Ras/Raf pathway and increases macrophage TF expression. To further investigate whether the increased macrophage TF expression is related to ERK signaling, we chose to block ERK signaling using PD98059 and observed macrophage TF expression. Therefore, we chose Raw264.7 cells and BMDM as our study subjects, and pretreated them with PD98059 (10 μM) or DMSO for 22 h, respectively, and then stimulated them with LPS 1 μg/mL for 3 h. Western blot results showed that p-ERK expression increased in Raw264.7 cells after LPS stimulation, and was decreased after using PD98059, while Erbin expression did not change significantly, indicating that PD98059 significantly inhibited ERK signaling but did not affect Erbin expression, suggesting that Erbin may be located upstream of ERK signaling. The expression of p-ERK increased in Raw264.7 cells after LPS stimulation and decreased after using PD98059, while the expression of Erbin did not change significantly, indicating that PD98059 significantly inhibited ERK signaling but did not affect the expression of Erbin, suggesting that Erbin may be located in the upstream of ERK signaling. Similarly, TF expression was increased in Raw264.7 cells after LPS stimulation, while Erbin$^{-/-}$ cell TF expression was further increased relative to Raw264.7 cells, and decreased after administration of PD98059, suggesting that PD98059 inhibits

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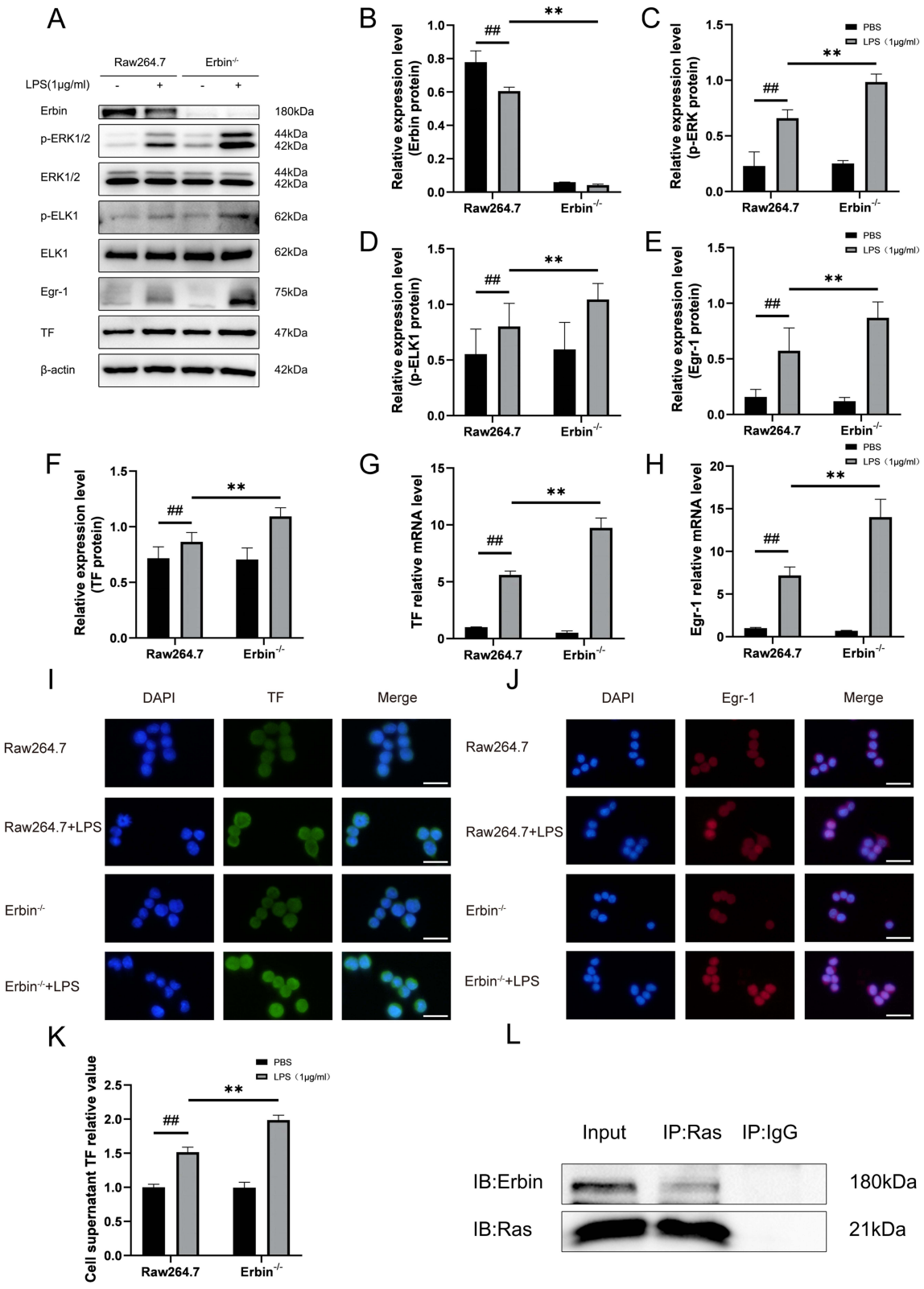

Figure 3 Erbin knockdown promotes LPS stimulation-induced activation of the Ras/Raf pathway and increases macrophage TF expression. (**A-F**) Western blot detected the expression levels of Erbin protein, TF protein, and Ras/Raf pathway protein in Raw264.7 cells and Erbin$^{-/-}$ cells. G-H qPCR detected the expression levels of Egr- 1, and TF mRNA expression levels in cells. (**I-J**) Fluorescence intensity of TF and Egr-1 in cells was observed by fluorescence inverted microscopy, showing representative images, TF: green; Egr-1: red; DAPI: blue, scale bar:50 μm. K ELISA to detect the concentration of TF in the supernatant secreted by macrophage cells. L CO-IP to detect the endogenous Erbin in the cell protein interactions with Ras protein. Graphs show mean ± standard deviation (mean ± SD), and the data shown (**B-H, K**) are representative of three independent experiments. $^{\#\#}P<0.01$ for the WT+Sham group compared with the WT+CLP group; $^{**}P<0.01$ for the WT+CLP group compared with the Erbin$^{-/-}$+CLP group.

LPS-induced TF production by macrophages and attenuates Erbin knockdown-promoted macrophage TF expression Effects. To further determine whether the reduced macrophage TF expression was related to the inhibition of Ras/Raf pathway activation by PD98059, the relevant pathway proteins were detected after LPS stimulation of the cells, and it was found that the levels of p-ERK, p-ELK1, and Egr-1 proteins were elevated in Raw264.7 cells, and that Erbin knockdown led to a further elevation of these proteins expression in Erbin$^{-/-}$ cells, after administration of PD98059, the levels of p-ERK, p-ELK1 and Egr-1 proteins were decreased in the cells, indicating that Erbin knockdown activated the Ras/Raf pathway (Figure 4A–F). Similarly, we obtained Western blot results in BMDM that were consistent with Raw264.7 cells (Figure 4G–L).

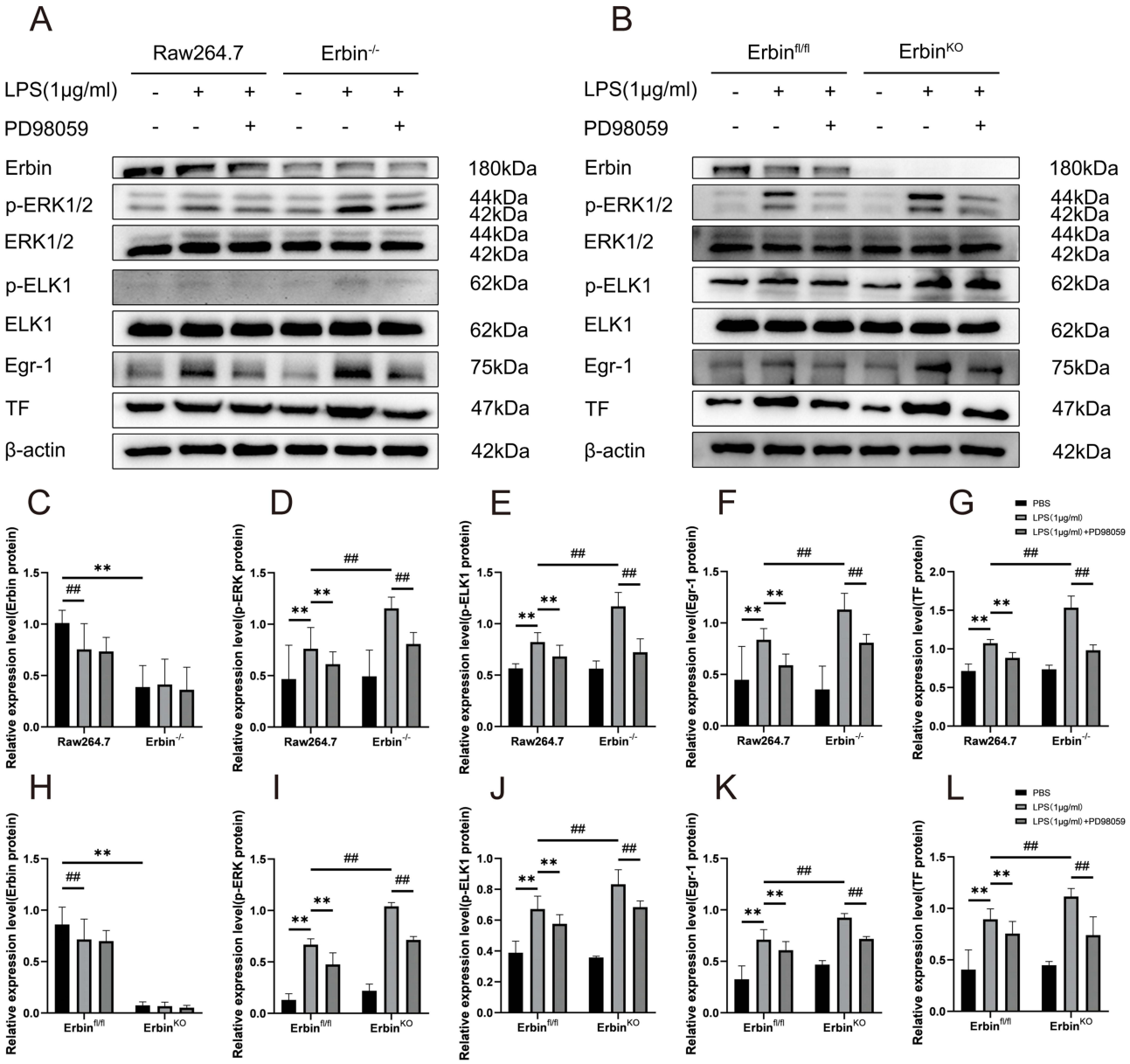

Figure 4 PD98059 inhibits Ras/Raf pathway activation and reduces the up-regulation of macrophage TF expression induced by LPS and Erbin knockdown. (**A**), C-G Western blot detects the expression levels of Erbin protein, TF protein, and Ras/Raf pathway protein in Raw264.7 cells and Erbin$^{-/-}$ cells. (**B**), H-L Western blot detection of the expression levels of Erbin protein, TF protein, and Ras/Raf pathway protein in Erbin$^{fl/fl}$ and Erbin^{KO}. Graphs show mean ± standard deviation (mean ± SD), and the data shown (**C-L**) are representative of three independent experiments. Raw264.7+PBS group compared with the Raw264.7+LPS group, $^{\#\#}P<0.01$, $^{**}P<0.01$; Raw264.7+PBS group compared with Erbin$^{-/-}$+PBS group, $^{**}P<0.01$; Raw264.7+LPS group compared with Raw264.7+LPS+PD98059 group, $^{**}P<0.01$; Raw264.7+LPS group compared with Erbin$^{-/-}$+LPS group, $^{\#\#}P<0.01$; Erbin$^{-/-}$+LPS group compared with Erbin$^{-/-}$+LPS+PD98059 group, $^{\#\#}P<0.01$. Erbin$^{fl/fl}$+PBS group compared with the Erbin$^{fl/fl}$+LPS group, $^{\#\#}P<0.01$, $^{**}P<0.01$; Erbin$^{fl/fl}$+PBS group compared with Erbin^{KO}+PBS group, $^{**}P<0.01$; Erbin$^{fl/fl}$+LPS group compared with Erbin$^{fl/fl}$+LPS+PD98059 group, $^{**}P<0.01$; Erbin$^{fl/fl}$ +LPS group compared with Erbin^{KO}+LPS group, $^{\#\#}P<0.01$; Erbin^{KO}+LPS group compared with Erbin^{KO}+LPS+PD98059 group, $^{\#\#}P<0.01$.

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Collectively, these results suggest that PD98059 inhibits Ras/Raf pathway activation and reduces the upregulation of macrophage TF expression induced by LPS and Erbin knockdown.

Erbin Knockdown Promotes Ras/Raf Pathway Activation in Septic Mice and Increases TF and FIB Expression in Lung Tissue

To verify whether Erbin could be involved in the expression of coagulation-related proteins in lung tissues, we selected WT mice and EKO mice for the study, and lung tissues were taken 8 h after CLP modeling. Immunohistochemistry results of lung tissues showed that there was almost no FIB precipitation in the lung tissues of WT mice and EKO mice in the Sham group, and the expression of FIB was increased in the lung tissues of WT mice after CLP, and the expression of FIB was further increased in the lung tissues of EKO mice relative to mice in the WT group, and these results suggested that Erbin knockdown upregulated the expression of FIB in lung tissues (Figure 5A).

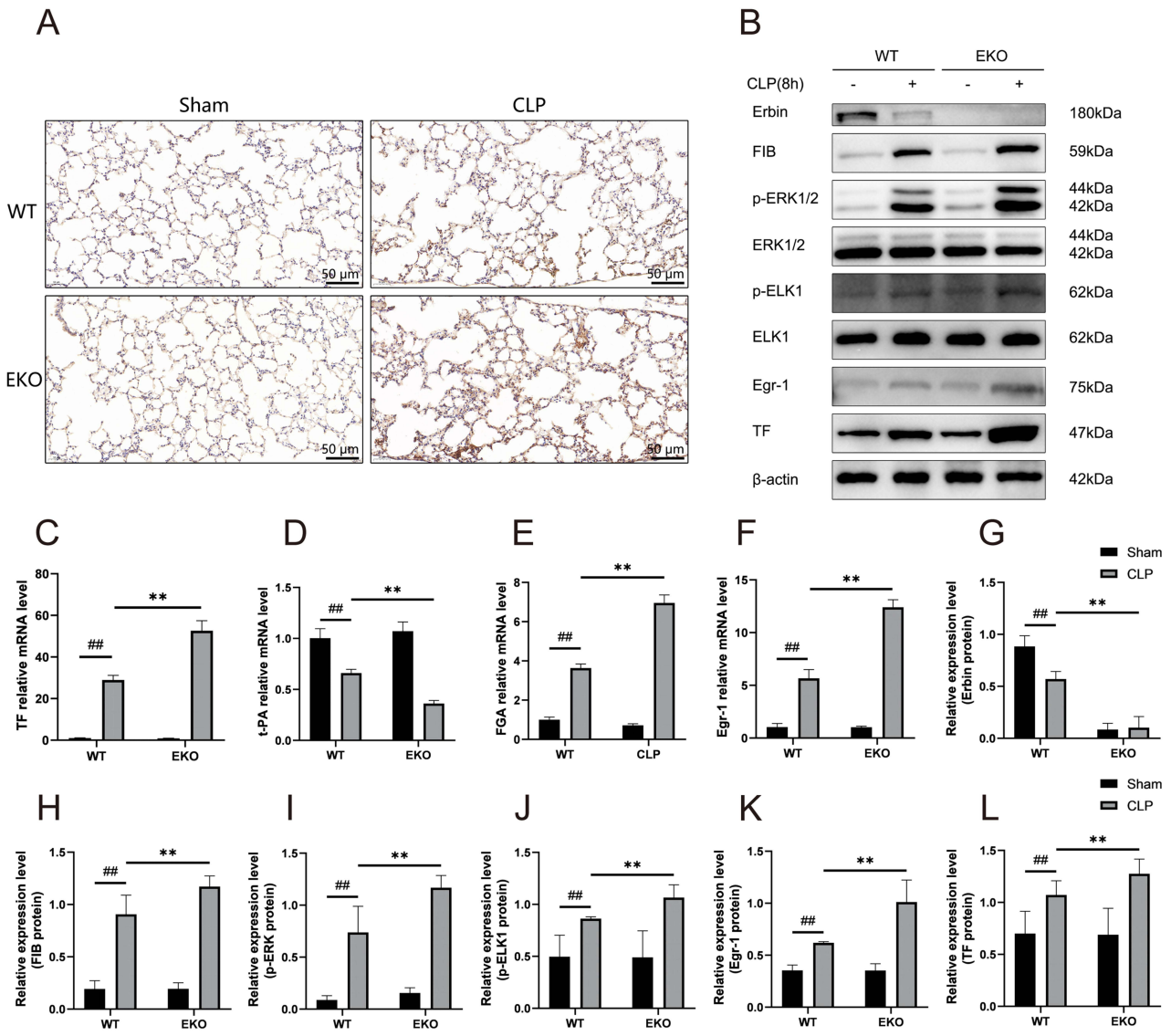

Figure 5 Erbin knockdown promotes the activation of the Ras/Raf pathway and increases the expression of TF and FIB in lung tissues of septic mice. (**A**) Mouse lung tissues were taken for immunohistochemical staining of FIB protein. C-F qPCR was performed to detect the expression levels of TF, t-PA, FGA, and Egr-1 mRNA in the lung tissues of mice. (**B**), G-L Western blot was performed to detect the expression levels of Erbin protein, TF protein, FIB protein, and Ras/Raf pathway proteins in mouse lung tissues. Tissues for Erbin protein, TF protein, FIB protein, and Ras/Raf pathway protein. Graphs show mean ± standard deviation (mean ± SD), and the data shown (**C-L**) are representative of three independent experiments. $^{\#\#}P<0.01$ for the WT+Sham group compared with the WT+CLP group; $^{**}P<0.01$ for the WT+CLP group compared with the EKO+CLP group.

It was further explored whether Erbin could also increase TF expression in lung tissues by activating the Ras/Raf pathway. qPCR results showed that after 8h of CLP, compared with WT mice, the mRNA expressions of TF, FGA, and Egr-1 in lung tissue of EKO mice were up-regulated, while the mRNA expression of t-PA was down-regulated (Figure 5B–E); Western blot showed that TF protein and FIB protein levels were elevated in lung tissues of WT mice lung tissues, the levels of TF protein and FIB protein were elevated, and the levels of pathway-associated proteins p-ERK, p-ELK1, and Egr-1 were elevated, while the expression levels of these proteins were further elevated in EKO mice. It indicated that Erbin knockdown was involved in the activation of the Ras/Raf pathway in lung tissues and promoted the expression of TF and FIB in lung tissues (Figure 5F–L).

Overall, Erbin knockdown promotes Ras/Raf pathway activation in septic mice and increases TF and FIB expression in lung tissues.

Positive Correlation Between Coagulation Indices and Inflammatory Response Indices in a Sepsis Model

The relationship between coagulation dysfunction and inflammatory response in sepsis has been a hot research topic, Kang S et al reported that under the activation of the coagulation cascade, IL-6 increased the expression of TF in monocytes and promoted the formation of fibrin clots, indicating that the inflammatory factor IL-6 promotes the expression of some coagulation proteins under septic conditions.[28] To further verify whether the inflammatory mediator IL-6 is involved in the activation of the coagulation cascade reaction under the sepsis model, we performed Pearson linear correlation analysis between TF, PAI-1, TAT, and IL-6 in the plasma of each group of mice, and the results showed that TF, PAI-1, and TAT in the plasma of mice showed a high degree of positive correlation with IL-6, which is in agreement with the results of the proposal by Shi J et al that IL-1β expression downstream of NLRP3 inflammasome was positively correlated with TAT, PAI-1, and TF in the circulation,[29] suggesting that there is a positive correlation between the coagulation cascade and the inflammatory response in sepsis (Figure 6A–D).

Discussion

In the last two decades, sepsis has been defined as a microbial infection that produces systemic inflammation and a dysregulated immune response, which leads to organ damage and can cause fever (or hypothermia), tachycardia, tachypnea, and blood leukocyte changes.[30] Pathogen invasion or cellular damage in sepsis causes pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) to be recognized by pattern-recognition receptors (PRRs) of the immune system, which activate the immune system and cause inflammatory responses and coagulation system activation.[31] It has been suggested that coagulation disorders are a major factor in death in sepsis patients, ranging from mild thrombocytopenia to fatal disease. Sepsis-induced multiorgan failure develops through a pathway that includes diffuse activation of the endothelium by pro-inflammatory cytokines, leukocytes, and other proteins, and the activated endothelium becomes the cause of thrombosis, leading to microvascular thrombosis, as well as the inhibition of fibrinolysis, which leads to fibrin thrombosis, and promotes inflammatory responses as well.[13] Thus, we found that the activation of coagulation and inflammation is a necessary response for host defense during sepsis, whereas some pro-inflammatory cytokines, such as TNF-α, IL-1, 2, 6, and 8, cause neutrophil-endothelial cell adhesion, activate complement and coagulation cascades, and can lead to the generation of microthrombi.[32] The inflammatory response not only promotes the release of various inflammatory factors, but also activates inflammatory vesicles to induce the activation and release of TF, which in turn initiates coagulation activation, leading to organ dysfunction, and ultimately to multi-organ failure and septic death.[33] Thus, in the study of coagulation dysfunction in sepsis, we focused on the coagulation dysfunction caused by the inflammatory response and explored the potential mechanism that regulates the release of TF from the organism. CLP prepared a mouse model of the inflammatory response to sepsis, and lung tissues were selected as the tissue organs that were rich in the expression of TF and obvious in the expression of fibrin deposition. It was found that the concentration of IL-6 in the plasma of mice increased at 8 h after surgery, and the mRNA expression of IL-6 and TNF-α was up-regulated in the lung tissues, and HE staining suggested that the alveolar

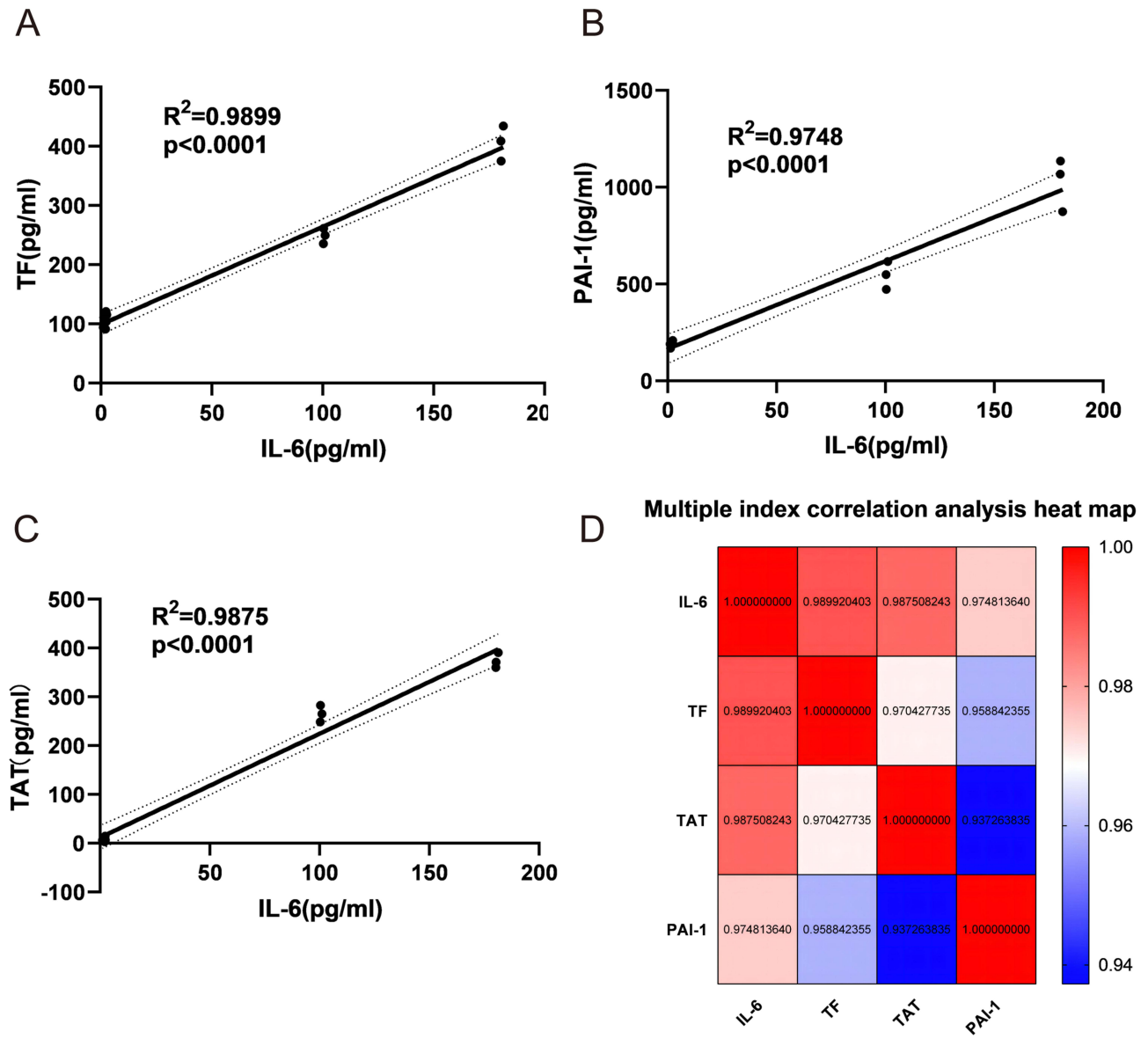

Figure 6 Positive correlation between coagulation indices and inflammatory response indices under the sepsis model. (**A-C**) mice showed a significant correlation between plasma TF, TAT, and PAI-1 concentrations and IL-6 concentrations. (**D**) Heatmap of multiple indices correlation analysis based on the results of Pearson linear correlation analysis. Dots indicate individual mice with data meeting the criteria of normal distribution and covariance for Pearson correlation analysis.

septum was widened, and a large number of erythrocytes and neutrophils were exuded, which indicated that the model of sepsis inflammatory response was successfully constructed.

The 4 items of coagulation (PT, APTT, TT, and FIG) are the 4 main indicators reflecting the function of exogenous pathway, endogenous pathway, and common pathway in the process of coagulation activation, of which PT is the indicator reflecting the exogenous coagulation pathway of the organism, APTT is the indicator reflecting the endogenous coagulation pathway of the organism, and TT and FIG reflect the fibrinolytic status.[34] TAT is a marker of thrombin generation, which can sensitively reflect the coagulation status in vivo, monitor the changes in the body's coagulation-anticoagulation, and have diagnostic significance in the pre-thrombotic stage and the early stage of thrombotic diseases;[35] PAI-1 is a single-chained globular glycoprotein, and tissue-type plasminogen activator of fibrinolytic enzymes and PAI-1 are the pair of key substances regulating the activity of fibrinolysis, which are both synthesized by the endothelial cells of the vasculature and released into the bloodstream.[36] These findings suggest that the 4 items of coagulation, TAT, and PAI-1 play important roles in coagulation function. In our study, the prolongation

of PT, APTT, and TT times, increased concentrations of TF, TAT, and PAI-1, and decreased concentration of FIG in the plasma of Erbin$^{-/-}$+CLP group of mice compared with the WT+CLP group suggests that Erbin knockout mice exhibit coagulation activation in the CLP model.

Bone marrow cells, especially monocytes/macrophages expressing TF, but not endothelial cells, contribute to pathologic coagulation during sepsis,[37] and Levi M et al suggested that the initiation of coagulation activation and the subsequent production of thrombin is caused by TF expression on activated monocytes/macrophages and endothelial cells.[38] In our study, Raw264.7 and BMDM showed increased TF expression in response to LPS stimulation, confirming the central role of macrophage-derived TF in coagulation initiation during sepsis. One study identified a role for the MEK-ERK1/2 mitogen-activated protein kinase (MAPK) pathway in the LPS-induced expression of TF and TNF-α genes in human monocytes, which are regulated by a variety of transcription factors, including nuclear factor (NF)-kB /Rel proteins and Egr-1.[39] Another study showed that Egr-1, one of the early growth response family of zinc-finger transcription factors, can be induced to be expressed by activated peripheral blood mononuclear cells and is involved in the regulation of TF and inflammatory mediators in sepsis models.[40] Relevant literature reported that PD98059, as a MEK-ERK pathway inhibitor, is involved in blocking ERK signaling and influencing extracellular signals to enter the intracellular to play biological roles.[41] Taken together, we hypothesized that macrophage TF production may affect the expression of the Ras/Raf signaling pathway by activating the MAPK pathway, which ultimately promotes the up-regulation of TF expression. Therefore, we detected the pathway-associated proteins after treating Raw264.7 and BMDM with LPS and PD98059, which found that LPS promotes the expression of TF by activating the Ras/Raf pathway, which in turn verified this conjecture. To further verify whether Erbin is involved in TF production by macrophages, we used LPS to stimulate Raw264.7 and BMDM, and after detecting the pathway-related proteins, we found that the expression of cellular pathway proteins was up-regulated and the production of TF was increased after Erbin knockdown, suggesting that Erbin is involved in the production of TF by macrophages; similarly, enhanced Egr −1, TF fluorescence expression was also enhanced after Erbin knockdown. Ras protein is an upstream protein of the MAPK pathway and is involved in activating downstream ERK proteins to conduct cellular signaling,[42] and the CO-IP results showed that endogenous Erbin is present in the same immune complex as Ras and is involved in inhibiting the activation of the Ras/Raf pathway. We, therefore, conclude that Erbin knockdown regulates TF expression by macrophages through activation of the Ras/Raf pathway, and these findings provide new insights into the mechanisms by which Erbin regulates TF expression and coagulation activation in sepsis.

To further verify the reliability of the above pathway in vivo in mice, we constructed a model of CLP for 8 h. Lung tissues were selected to observe the expression of Ras/Raf pathway proteins and coagulation-related proteins TF and FIB. The results showed that under the in vivo CLP model, the expression of coagulation-related proteins TF and FIB was up-regulated in lung tissues of EKO mice relative to WT mice, and the Ras/Raf pathway was significantly activated. Our study demonstrates that Erbin knockdown promotes the production of coagulation-associated proteins TF and FIB in mouse lung tissues through activation of the Ras/Raf pathway under the CLP model.

However, there are some limitations to our study. First, we did not backfill Erbin or overexpress Erbin in Erbin knockdown macrophages to observe whether macrophage production of TF was reduced or reversed the increase in macrophage TF induced by LPS stimulation. Second, the MAPK pathway is diverse, and the pathway we studied is one of them, it is not possible to rule out the existence of other pathways that affect macrophage TF production when they are involved in the regulation of a wide range of cellular signaling. Therefore, although the present study contributes to the understanding of the protective role of Erbin in coagulation disorders in sepsis, further experimental validation is needed.

Conclusion

Taken together, Erbin may ameliorate coagulation dysfunction in sepsis by interacting with Ras and inhibiting the activation of the Ras/Raf pathway, leading to a reduction in macrophage TF expression and suppression of coagulation activation (Figure 7). These results emphasize the importance of Erbin as a key pathological mechanism to ameliorate coagulation dysfunction in sepsis.

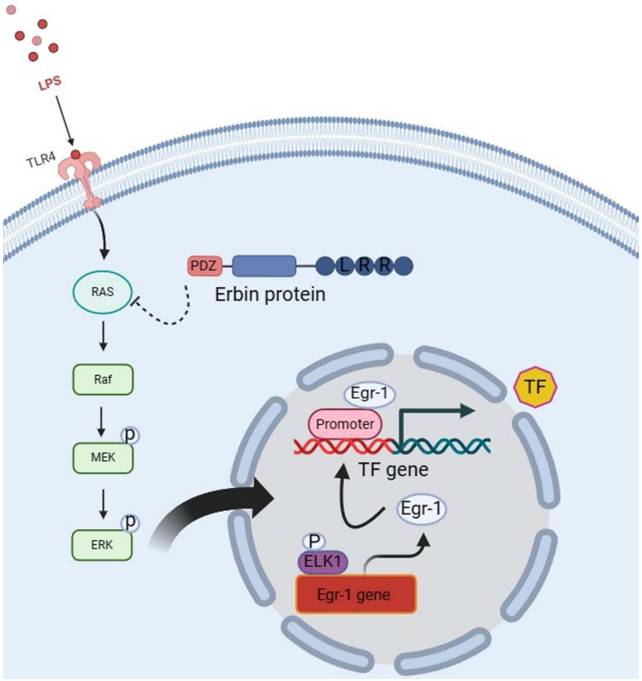

Figure 7 Erbin plays a role in coagulation inhibition in sepsis coagulation. CLP-induced sepsis activates macrophages. Under the stimulation of LPS, Erbin expression was down-regulated, accelerating the activation of the Ras/Raf pathway and inducing tissue factor expression in macrophages, and after Erbin knockdown Ras/Raf pathway is further activated and tissue factor expression in macrophages is further increased, which causes the exogenous coagulation pathway to be activated and the coagulation cascade reaction to occur.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments

We acknowledge the Scientific Research Center of Zhongnan Hospital of Wuhan University. This study was supported by the National Natural Science Foundation of China (Grant Nos. 82371370, 82172144, and 82372156). This paper has been uploaded to SSRN as a preprint: https://papers.ssrn.com/sol3/papers.cfm?abstract_id=4808666.

Disclosure

All the authors declare that they have no competing interests in this work.

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