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Tumor necrosis factor- α requires Ezrin to regulate the cytoskeleton and cause pulmonary microvascular endothelial barrier damage

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ABSTRACT

Acute respiratory distress syndrome (ARDS) is a rapidly progressive disease with unknown pathogenesis. Damage of pulmonary microvascular endothelial cells (PMVECs) caused by inflammatory storm caused by cytokines such as TNF- α is the potential pathogenesis of ARDS. In this study, we examined the role of ezrin and Rac1 in TNF- α -related pathways, which regulates the permeability of PMVECs. Primary rat pulmonary microvascular endothelial cells (RPMVECs) were isolated and cultured. RPMVECs were treated with rat TNF- α (0, 1, 10, 100 ng/ml), and the cell activity of each group was measured using a CCK8 kit. The integrity of endothelial barrier was measured by transendothelial resistance (TEER) and FITC-BSA flux across RPMVECs membranes. Pulldown assay and Western blot was used to detect the activity of RAS-associated C3 botulinum toxin substrate 1 (Rac1) and Ezrin phosphorylation. Short hairpin RNA (shRNA) targeting ezrin and Rac1 was utilized to evaluate the effect of RPMVECs permeability and related pathway. The effects of ezrin and Rac1 on cytoskeleton were confirmed by immunofluorescence. Our results revealed that active Rac1 was essential for protecting the RPMVEC barrier stimulated by TNF- α , while active ezrin could partially destroy the PMVEC barrier by reducing Rac1 activity and regulating the subcellular structure of the cytoskeleton. These findings may be used to create new therapeutic strategies for targeting Rac1 in the treatment of ARDS.

1. Introduction

ARDS is a rapidly progressive disease occurring in critically ill patients. The most common causes of ARDS are pneumonia, aspiration of liquid contents, septicemia, acute liver failure, and acute pancreatitis, which directly or indirectly induce lung injury (Matthay et al., 2020; Fowler 3rd et al., 2019; Yang et al., 2019). At the same time, the exact pathogenesis still remain unclear. Some studies have suggested that the accumulation of neutrophils and proinflammatory cytokines around pulmonary microvascular endothelial cells (PMVECs) may lead to the destruction of intercellular connections, microtubule activation, and actin cytoskeleton remodeling, cell contraction and formation of gaps, which then allows a large amount of protein and fluid to enter the pulmonary interstitium and alveolar cavity, resulting in severe hypoxic respiratory failure (Lefeng et al., 2018; Sercundes et al., 2016; Reiss et al., 2018). Therefore, it is of potential and far-reaching clinical significance to study the molecular mechanisms of inflammatory injury of PMVECs barrier.

Ezrin-Radixin-Moesin (ERM) protein family is a group of membrane-related proteins with high homology, composed of three functional domains: the N-terminal FERM domain [band four-point-one, ezrin, radixin, moesin homology domains], the extended, curly helix domain, and the

short C-terminal domain (Kawaguchi et al., 2017). As a member of ERM, Ezrin exists in two conformational states. At rest, it exists in the form of folded inactivation. When stimulated by an effective signal, the N-terminal and C-terminal of the fold separate from each other, resulting in the phosphorylation of threonine at site 567th in the molecular structure, and the connection with phosphatidylinositol diphosphate [phosphatidylinositol (4,5)-bisphosphate, PIP₂] on the surface of N-terminal cell membrane leads to the activation of ezrin. Through the FERM domain, membrane proteins and actin participate in a variety of transmembrane signal transductions, thus having an essential role in regulating cell morphology, growth, survival, adhesion, proliferation, migration, and other biological functions (Pelaseyed and Bretscher, 2018; Froese et al., 2018; Abdou et al., 2016).

Among the many proteins, actin-binding protein (cortex actin-binding protein, cortactin) has a crucial role in actin cytoskeleton regulation. A large number of studies have confirmed that the increased distribution of F-actin and Cortactin in the cortex often indicates cell expansion, enhanced cell-to-cell junction, and decreased monolayer permeability of endothelial cells (Shao et al., 2013; Adamson et al., 2012). It has also been confirmed that as a member of the RhoGTPase family, ras-related C3 botulinum toxin substrate 1 (Rac1) is essential in stabilizing the actin cytoskeleton and maintaining microvascular barrier

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function (Spindler et al. 2010; Li et al. 2017). Rac1 regulates the function of the cytoskeleton and intercellular junction mainly by promoting the formation of actin fiber bundles and the remodeling of junction complexes in the cortex (Fei et al. 2019; Shao et al. 2016). Nevertheless, the molecular mechanism of Rac1 involved in the regulation of PMVECs permeability under inflammatory conditions still remains unclear.

TNF- α is a component that promotes and drives the inflammation of many lung diseases and conditions, including ARDS (Kumar et al., 2017; Qin and Qiu, 2019). Our previous studies have found that TNF- α can induce F-actin rearrangement in PMVECs, increasing cell permeability (Shao et al., 2013). However, the specific molecular mechanism of TNF- α destroying PMVECs barrier function is still not clear. We speculated that Ezrin and Rac1 might have a role in TNF- α -related pathways, regulating the permeability of PMVECs. To address this hypothesis, we used TNF- α to treat PMVECs, detected the effects of Ezrin and Rac1, respectively, and determined the mechanism of Ezrin and Rac1 in TNF- α mediated endothelial barrier destruction.

2. Materials and methods

2.1. Main reagents

Rat TNF- α was purchased from PeproTech (New Jersey, USA). Ezrin and p-Ezrin (567Thr) antibodies were bought from ThermoFisher (Waltham, USA). Rac1 monoclonal antibodies were obtained from Abcam Company (Cambridge, UK). Cortactin antibody, rhodamine-labeled cyclopetide, and FITC-BSI were purchased from Sigma (St. Louis, Missouri, USA). 8-PCPT-20-OMethyl-cAMP (O-Me-cAMP) was from Tocris (Bristol, UK). Rabbit IgG antibody and mouse IgG antibody labeled with horseradish peroxidase (HRP) were acquired from Wuhan Bost Biological Engineering Co. Ltd. (Wuhan, China). Transwell transparent polyester membrane cell incubator (12-pore, 12 mm diameter, aperture 0.4 μ m) was from Costar (Washington, D.C., USA). The Cell Counting Kit-8 (CCK-8) was from Beibo Biological Reagent Co. Ltd. (Shanghai, China). Rac1 activation assay kits were from Abcam (Cambridge, UK). High glucose Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and trypsin were purchased from HyClone Laboratory (Salt Lake City, USA).

2.2. Isolation and culture of primary rat PMVECs (RPMVECs)

Primary RPMVECs were isolated and cultured according to the mature techniques in our laboratory (You et al., 2010; Wang et al. 2015). A total of 30 healthy male SD rats weighing 90–120 g were purchased from the Animal Experimental Center of Anhui Medical University. After intraperitoneal injection of pentobarbital sodium to full anesthesia, the rats were sacrificed, the pleura was removed, and the subpleural lung tissue without large blood vessels was separated and cut into small pieces, and placed in a culture flask. DMEM containing 15% FBS was added to the culture flask, which was placed in the incubator (37 °C, 5% CO₂) for 60 h. The tissue mass was removed, and the culture medium was changed every 3 days. All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Anhui Medical University institutional animal care and conducted according to the AAALAC and the IACUC guidelines.

2.3. Cell viability assay

Cell viability was analyzed using a CCK-8 kit. After cell counting, PMVECs (1×10^4 cells per well) were plated in 96-well plate and incubated overnight in a humidified atmosphere containing 5% CO₂/95% air at 37 °C. Cells were then exposed to gradually increased concentration of TNF- α (0, 1, 10, 100 ng/ml) for 2 h, after which 10 μ l of sterile CCK8 was added to each well, and cells were incubated for another 3 h at 37 °C. The absorbance of each group was detected at 450 nm by Microplate Reader (Enspire, PerkinElmer, USA), and a column chart was drawn to reflect the activity of cells.

2.4. Infection of shRNA lentivirus

Lentiviruses containing shRNA-Rac1 (shRac1) and shRNA-Ezrin (shEzrin) were purchased from GenePharma Pharmaceutical Technology Co. Ltd. (Shanghai, China) and were used to regulate the expression of Rac1 and Ezrin. PMVECs, grown to > 90% fusion in a culture flask, were inoculated in a six-well plate. On the third day of culture, the medium was replaced with the medium containing 15% fetal bovine serum, 100 μ l lentivirus, and 5 mg/ml polybrene. Then, 48–72 h after transfection, the protein was photographed by a fluorescence microscope, and the protein was collected for Western blot analysis to verify the silencing effect of Rac1 and Ezrin.

2.5. Measurements of transendothelial electrical resistance (TEER)

RPMVECs (2×10^5 /cm²) were inoculated on Matrigel-coated transwell polyester membranes (Costar, USA). The cells were cultured for > 48 h until the cell monolayer was formed. The cells were incubated with different concentrations of TNF- α or 200 mmol/l O-Me-cAMP for a certain period. The TEER of the cells at different time points was measured by epithelial volt ohmmeter and STX-2 electrode (EVOM, USA).

2.6. Assessment of FITC-BSA flux across RPMVEC membranes

When the TEER value was stable, and confluency with stable junctions was observed under the microscope, the cells were starved with DMEM containing 1% fetal bovine serum for 12 h and rinsed with PBS for 3 times. According to the experimental scheme, different stimulating factors act on the cells. The concentration of FITC-BSA 0.1 ml in the upper chamber was 0.1 mg/ml, and the same concentration of BSA0.6 ml was added in the lower chamber. After incubation in a 5% CO₂ incubator at 37 °C for 2 h, 0.1 ml samples were collected from the lower chamber and added into 96-well plates. The excitation wavelength of the luciferase-labeling instrument was set to 488 nm, and the fluorescence intensity of the absorbed samples was measured.

2.7. Rac1 activation assays

The active form of intracellular Rac1 (Rac1-GTP) was determined by a commercial kit. After stimulation with TNF- α , the PMVECs were lysed with the lysis buffer, and the immobilized PAK1-PBD was used to capture Rac1-GTP by pull-down experiment. The levels of Rac1-GTP and total Rac1 were analyzed by Western blot.

2.8. Immunofluorescence

RPMVECs (1×10^4) were treated with TNF- α or O-Me-cAMP for a certain period. Cells were then fixed with 4% paraformaldehyde for 15 min, blocked with 0.3% TritonX 100 and 5% goat serum in PBS for 1 h, and incubated with 1:200 anti-Cortactin antibody at 4 °C overnight. Consequently, cells were stained with rhodamine-Phalloidin (1:400) and cy3 (1:400) combined with F-actin and cortactin, respectively. The nucleus was stained with 0.5 mg/ml DAPI for 30 min. All images were obtained under a fluorescence microscope (Axio Observer3, ZEISS, Germany).

2.9. Western blot

PMVECs (2×10^6 cells/cm²) were collected, and the protein was extracted and quantified according to the manufacturer's instructions. A 15%, 12% separation gel, and 5% concentrated gel were prepared for protein loading and electrophoresis. The target protein was then transferred to the PVDF membrane by semi-dry method, sealed for 2 h, incubated with primary antibody at 4 degrees overnight, and with the secondary antibody for 1 h after Tris-buffered saline with Tween 20 (TBST) washing. After washing the PVDF film again with TBST, the gel imaging system (AI600RGB, GE, USA) was used to develop the film, and

the gray value of the band was analyzed by ImageJ software.

2.10. Statistical analysis

SPSS20.0 was used to analyze the data. The results were expressed by the mean ± standard deviation ($\bar{x} \pm s$). The pairwise comparison of multiple variables was analyzed by one-way ANOVA, and the independent sample *t*-test was used to compare the variables between the two groups. A $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. TNF- α increases the permeability of primary RPMVECs

In the present study, PMVECs were identified by binding with lectin from BSI (FITC-BSI) staining and cell purity was > 90% (Supplemental Fig. S1). We confirmed previous results that TNF- α reduced the TEER of PMVECs in a time-dependent and dose-dependent manner without affecting cell viability (Fig. 1A). Next, we measured the TEER of cells in each group under specific intervention conditions. Compared to the control group, 1–100 ng/ml TNF- α decreased the TEER in PMVECs in a time-dependent manner (Fig. 1B and C). Furthermore, FITC-BSA flux increased in a concentration-dependent manner (Fig. 1D). These data indicated that the TNF- α could damage the barrier and increase the permeability of PMVECs. The most significant effect was observed at 100 ng/ml TNF- α , which was selected for further experiments.

3.2. TNF- α activates Ezrin in a time-dependent manner

To elucidate the effect of TNF- α on phosphorylation of Ezrin at its critical COOH-terminal threonine, phospho-specific Ezrin antibody (Thr567) was utilized to evaluate threonine phosphorylation of Ezrin by Western Blot. Treatment with 100 ng/mL TNF- α induced a significant increase in Ezrin phosphorylation in a time-dependent manner without affecting the total Ezrin expression, which increased after 15 min, reached maximum levels by 2 h, and remained elevated for at least 8 h (Fig. 2).

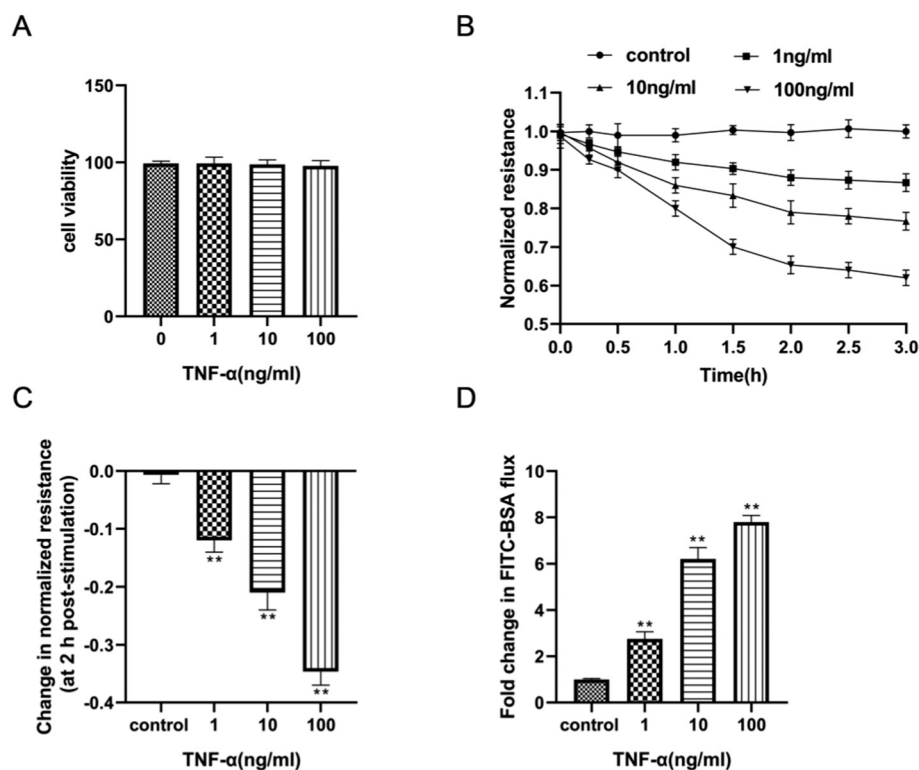


Fig. 1. TNF- α increases the MVECs permeability in a concentration- and time-dependent manner. (A) PMVECs were treated with mouse TNF- α (0, 1, 10, 100 ng/ml), and the cell activity of each group was measured using a CCK8 kit. (B–D) TEER (B, C) and FITC-BSA (D) analysis after treating PMVECs with TNF- α for 2 h. Results were expressed as mean ± standard deviation. N = 6, ** $p < 0.001$ vs untreated control.

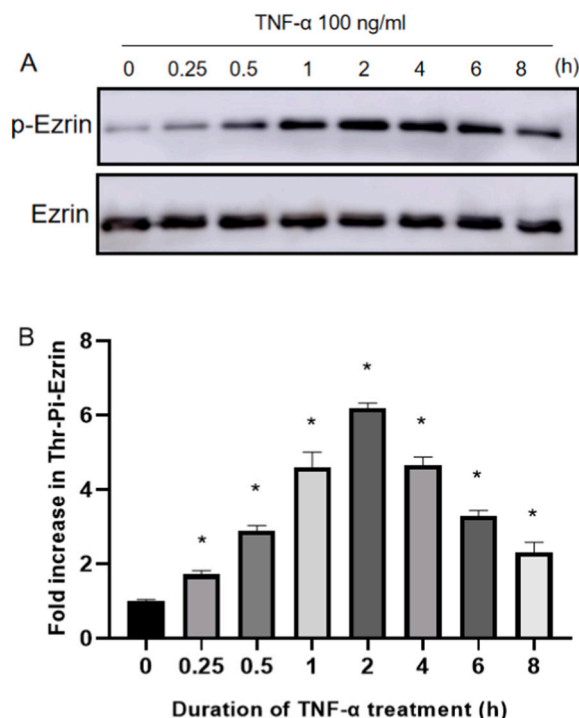


Fig. 2. TNF- α induces time-dependent changes in the threonine phosphorylation of Ezrin in PMVECs. (A, B) Time-dependent changes in threonine phosphorylation (A) and relative expressions of Ezrin (B) induced by 100 ng/ml TNF- α . Values were presented as the mean ± SD. N = 6, * $p < 0.01$ vs. the beginning time.

3.3. TNF- α inactivates Rac1 in a time-dependent manner

Rac1 maintains cellular barrier function through a variety of mechanisms. To investigate the effect of TNF- α on Rac1 expression, PMVECs were stimulated with TNF- α (100 ng/ml), and the expression

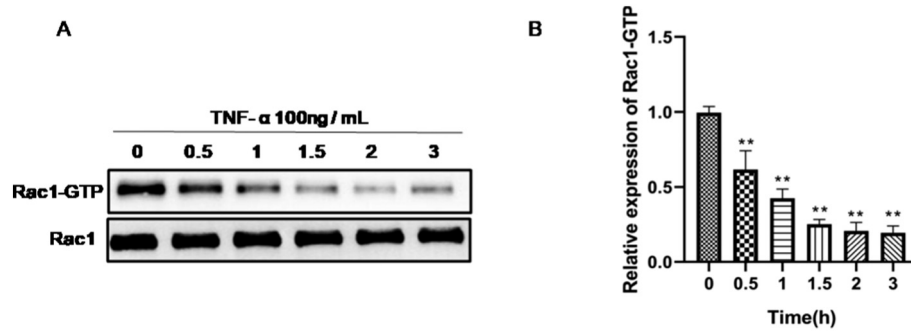


Fig. 3. TNF- α inactivates Rac1 in a time-dependent manner. (A) Time-dependent decrease in Rac1-GTP induced by 100 ng/ml TNF- α . (B) Relative expressions of Rac1 after treating cells with 100 ng/ml TNF- α . Values were presented as the mean \pm SD. N = 6, **p < 0.001 vs. the beginning time.

of Rac1 in cells at different time points was determined by western blot. As shown in Fig. 3, the TNF- α decreased Rac1 activity in a time-dependent manner.

3.4. Rac1 participates in the regulation of Ezrin phosphorylation induced by TNF- α

To explore the signaling mechanism of Ezrin phosphorylation, we used Rac1-specific shRNA (shRac1). The results showed that the threonine phosphorylation of Ezrin was increased after transfecting PMVECs with shRac1 alone. ShRac1 combined with TNF- α significantly increased Ezrin threonine phosphorylation in PMVECs (Fig. 4).

3.5. Ezrin silencing alleviates the damage of the PMVEC barrier caused by TNF- α

The following experiments were used to determine the role of Ezrin in TNF- α -induced RPMVECs barrier destruction. ShRNA targeting Ezrin was used to inhibit the expression of Ezrin (Fig. 5A and B) but not the expression of Moesin or Radixin (Fig. 5C and D), after which changes in TEER and FITC-BSA permeability of cells were measured. The results showed that Ezrin silencing could partially recover the decrease of TEER and the increase of FITC-BSA permeability induced by TNF- α exposure (Fig. 5E and F), which indicated that Ezrin silencing could reduce the damage of PMVECs barrier induced by TNF- α .

The distribution of F-actin in the cortical region and cortactin on the membrane is one of the key factors for maintaining the endothelial barrier. Next, we examined the effect of Ezrin on cytoskeleton rearrangement. As shown in Fig. 6, after transfecting cells with control-shRNA or shEzrin, F-actin was evenly arranged and distributed all over the cell (more on the cell membrane), while cortactin was mainly found in the cytoplasm. When PMVECs were exposed to TNF- α , a large number of F-actin gathered and formed stress fibers in the cytoplasm, and the level of cortactin on the cell membrane decreased. PMVECs pretreated with shEzrin reversed F-actin rearrangement and cortactin redistribution induced by TNF- α exposure (Fig. 7). These results were consistent with the immunofluorescence analysis. Therefore, we inferred that Ezrin was essential in the destruction of the PMVEC barrier mediated by TNF- α partly by inducing cytoskeleton remodeling.

3.6. Ezrin silencing increased the expression of active Rac1 in RPMVECs

Rac1 is an important regulator of actin cytoskeleton dynamics that has a key role in maintaining endothelial integrity. After pre-incubation with O-me-cAMP, a specific agonist of Rac1, we found that O-me-cAMP partially restored the decrease of TEER and the increase of FITC-BSA permeability induced by TNF- α (Fig. 8A and B), and decreased the vascular permeability induced by TNF- α . Moreover, a Western blot analysis indicated that Ezrin inhibition promotes the expression of active rac1 and significantly reduces the decrease of rac1-GTP expression

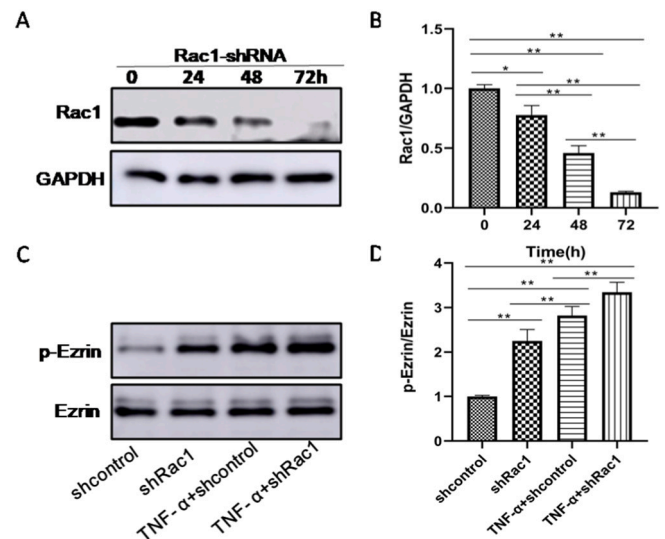


Fig. 4. Rac1 inhibition and TNF- α stimulation (100 ng/ml) together increase threonine phosphorylation of Ezrin in PMVECs. (A, B) After the transfection of PMVECs with shRNA targeting Rac1, the expression of Rac1 was significantly inhibited, and relative expression was assessed by Western blotting. (C, D) The expressions of p-Ezrin and normal Ezrin in different groups were assayed by Western Blotting. Values were presented as the mean \pm SD. N = 6, *p < 0.01, **p < 0.001.

induced by TNF- α exposure (Fig. 8C and D). These results suggested that both TNF- α and Ezrin could inhibit the activity of Rac1.

3.7. Down-regulation of Rac1 eliminates the protective effect of Ezrin silence

To confirm whether Rac1 activity was directly related to Ezrin-induced barrier injury, we co-transfected RPMVECs with shEzrin and shRac1, then treated with TNF- α , and finally evaluated changes in cell permeability. As shown in Fig. 9, Ezrin silencing reduced the damage of the PMVEC barrier caused by TNF- α , while the addition of shRac1 could partially resist shEzrin's barrier protection function, which was manifested by the decrease of TEER and the increase of FITC-BSA permeability. In addition, shRac1 aggravated the reshaping of the cytoskeleton and the loss of cortex cortactin.

4. Discussion

As a typical clinical emergency and critical illness, ARDS has a high mortality rate and is still lacks effective treatment. The lung injury in ARDS is mainly caused by the dysfunction of RPMVECs, which are triggered by systemic inflammation (Lefeng et al. 2018; Potey et al. 2019). Therefore, it is crucial to explore the signaling pathways related

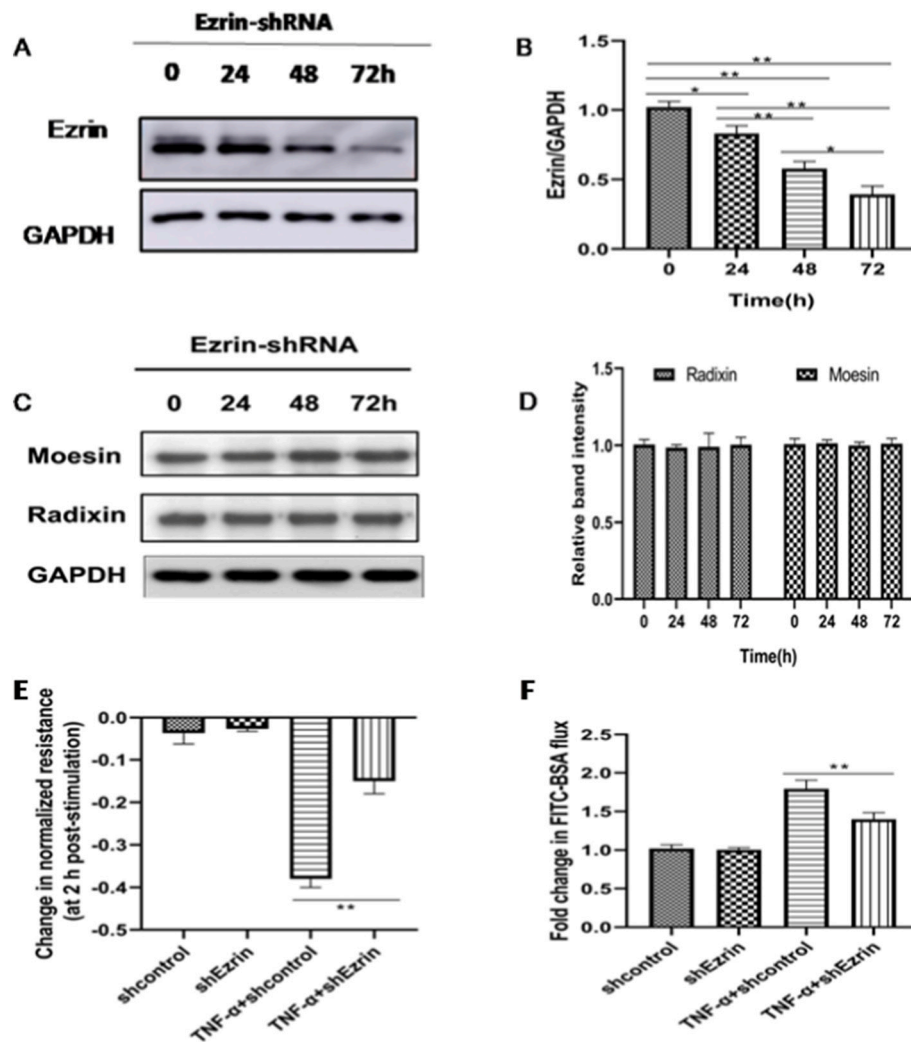


Fig. 5. Ezrin silencing can reduce the increase of PMVECs permeability induced by TNF- α . (A, B, C, D) The expression changes of Ezrin, Moesin and Radixin in PMVECs transfected with shRNA transfection by Western blotting. (E) Ezrin silencing can reduce the decrease of TEER-induced by TNF- α . (F) Ezrin silencing can partially reverse the increase of FITC-BSA permeability induced by TNF- α . Values were presented as the mean \pm SD. N = 6, *p < 0.01, **p < 0.001.

to ARDS, which, in turn, may affect the function of RPMVECs. This study described the effects of Ezrin and Rac1 on the permeability of PMVEC monolayers in an inflammatory environment mediated by TNF- α . We found that TNF- α increases PMVEC permeability in a dose- and time-dependent manner. In addition, the intracellular Ezrin threonine phosphorylation increased with the prolongation of TNF- α stimulation time, thus suggesting that Ezrin has a vital role in mediating TNF- α -induced endothelial barrier dysfunction. By silencing Rac1 and Ezrin with shRNA, our data suggested that Rac1 and Ezrin could regulate each other and participate in mediating TNF- α PMVECs monolayer permeability increase by regulating the cytoskeleton.

TNF- α is an essential inflammatory modulator involved in a variety of pathological processes such as inducing the accumulation of inflammatory cells, stimulating the production of inflammatory mediators, proliferation, and differentiation of injury and infected sites, as well as airway hyperreactivity and tissue remodeling (Sercundes et al. 2016; Reiss et al. 2020; Franca et al. 2018). In the present study, we found that TNF- α induced signal transduction *in vitro* pulmonary microvascular endothelial cells and regulated cytoskeleton and EC permeability, without affecting RPMVECs cells viability.

Ezrin is considered a dormant, inactive protein. It is activated by phosphorylation, which dissociates the intramolecular interaction between the N-terminal domain and the C-terminal domain, and makes the N-terminal domain interact with the membrane receptor complex, while

the C-terminal domain interacts with F-actin. The interaction between Ezrin and other proteins is regulated by the phosphorylation of multiple kinases in multiple domains (Yin et al., 2018). The most common Ezrin phosphorylation site is threonine 567 in the C-terminal domain (Gungor-Ordueri et al., 2015). Our results revealed that TNF- α stimulation increases the intracellular p-Ezrin (Thr567) in a time-dependent manner but does not affect the total intracellular Ezrin expression. In lung diseases, Ezrin has been confirmed to be related to the pathogenesis of asthma (Wu and Eickelberg, 2019). cAMP/PKA pathway induces contraction of airway smooth muscle cells and the production of cytokines by phosphorylating Ezrin. In the early stage of asthma, epidermal growth factor (EGF) can induce the phosphorylation of Ezrin, which connects CD44 and cortical actin cytoskeleton, and participates in the repair of bronchial epithelium (Yin et al., 2018). Currently, there are only few studies on lung injury and pulmonary infection. To further examine the connection of Ezrin and TNF- α stimulation *in vitro*, we transfected the PMVECs with shEzrin vector. Our finding revealed that the inhibition of Ezrin could partially alleviate the damage of the PMVEC barrier caused by TNF- α . Therefore, it can be inferred that activated Ezrin was involved in barrier dysfunction mediated by TNF- α .

Rac1, Cdc42, and Rap1 can help to maintain and stabilize the function of the microvascular endothelial cell barrier (Wojciak-Stothard and Ridley 2002; Wojciak-Stothard et al. 2005). In the past few years, the role of rac1 in the assembly of endothelial junctions and the increase of its

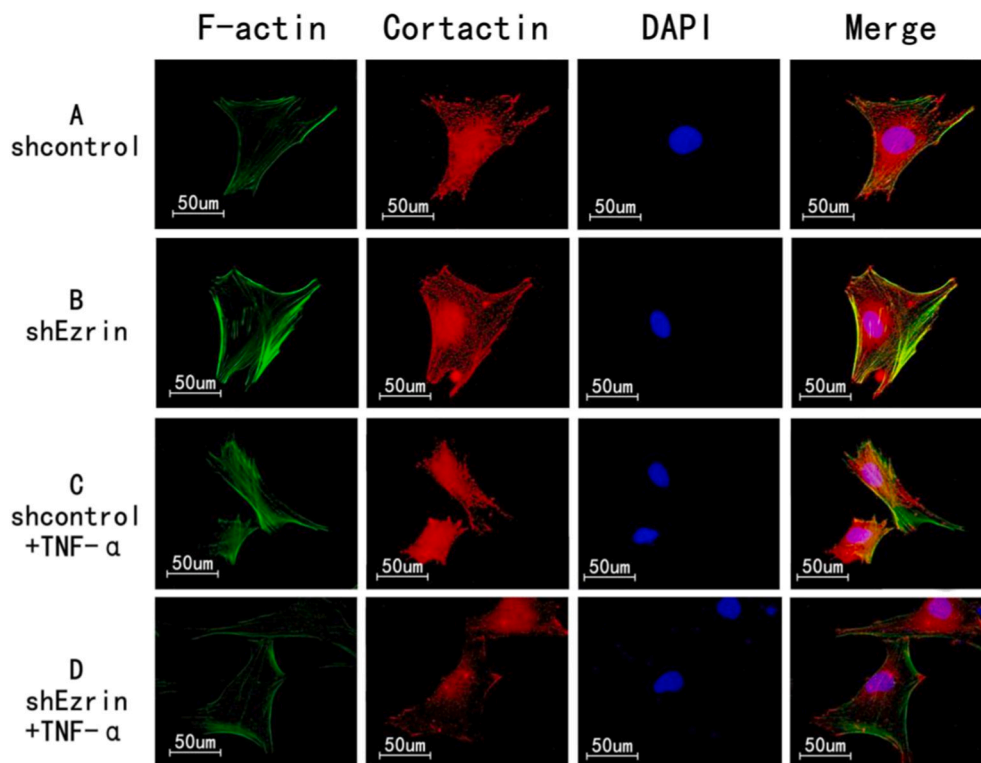


Fig. 6. The effects of the downregulation of Ezrin on the rearrangement of F-actin and cortactin distribution. (A) In control shRNA cells, F-actin was distributed in a uniform, thin filamentous pattern, primarily around the cell periphery. Cortactin was mainly distributed in the cytoplasm and a small part of the membrane. (B) The same situation was found in the cells of shEzrin group. (C) After 2 h of TNF- α stimulation in the control-shRNA group, F-actin gathered and transferred to the cell center; stress fibers were formed in the cytoplasm, and the distribution of cortactin around the cells was also decreased. (D) This phenomenon can be partially reversed by shEzrin.

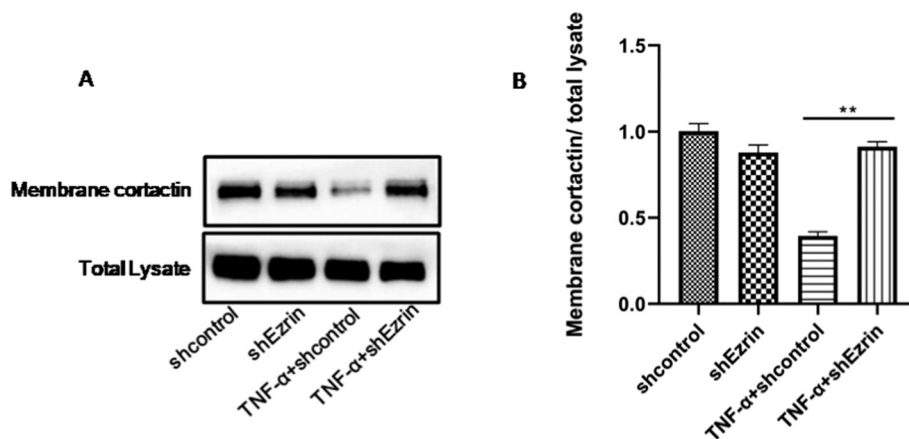


Fig. 7. The effect of shEzrin on the distribution of cortactin in cells. (A) TNF- α could reduce the expression of cortactin on the cell membrane, while shEzrin could reverse the effect of TNF- α . The related results were analyzed by Western blot (B).

activity in the process of junction formation have attracted a lot of attention (Beckers et al., 2010). Rac1 regulates cytoskeletal actin rearrangement through a variety of mechanisms (Hohmann and Dehghani, 2019; Gorovoy et al., 2005; Acevedo and Gonzalez-Billault, 2018). In this experiment, we used TNF- α to stimulate RPMVECs, and then measured the expression of Rac1 at different time points (0, 0.5, 1, 1.5, 2, and 3 h) using Western blot. TNF- α had no effect on the expression of total Rac1 protein, but the content of active Rac1 (Rac1-GTP) decreased gradually with time. By measuring the permeability of PMVEC monolayers preincubated with O-me-cAMP, a specific agonist of Rac1, we found that the up-regulation of Rac1 activity could reduce PMVEC hyperpermeability induced by TNF- α . Of note, O-me-cAMP was also reported to activate Rac1 via Epac/Rap1 signaling (Baumer et al., 2008). In the future, more refined studies are required to understand the complex interaction between Rac1, Epac and Rap1. ShEzrin increased the expression of active Rac1 and significantly reduced the decrease of intracellular Rac1 activity induced by TNF- α . When shRac1 was added to cells, the protective effect of shEzrin was weakened. Wójciak-Stothard et al. found that in human

umbilical vein endothelial cells (HUVECs) (Wójciak-Stothard et al., 1998), TNF- α can affect both RhoA and Rac1. Moreover, in HUVECs, TNF- α enhances hyperpermeability of HUVECs infected *Listeria monocytogenes* via RhoA signaling pathway (Lu et al., 2020). In this study, we showed that RhoA was activated by both TNF- α and sh-Rac1 in PMVEC (Supplemental Fig. S2). More interestingly, shRac1 led to rearrangement of the distribution of F-actin and cortactin in PMVEC (Supplemental Fig. S3), suggesting that RhoA signaling pathway may also be involved in regulating the increase of cell permeability induced by TNF- α . Besides finding that shEzrin activated Rac1, we also observed a significant increase in phosphorylated Ezrin induced by shRac1, i.e. Rac1 was not only the downstream signal target of Ezrin, but also the upstream regulatory factor of phosphorylation in the process of endothelial barrier destruction induced by TNF- α .

McKenzie et al. found that in human PMVEC, TNF- α can induce the activation of PKC to phosphorylate the C-terminal threonine of Ezrin, resulting in increased endothelial permeability (Koss et al., 2006). Our study further revealed that TNF- α could directly promote Ezrin phosphonic acid

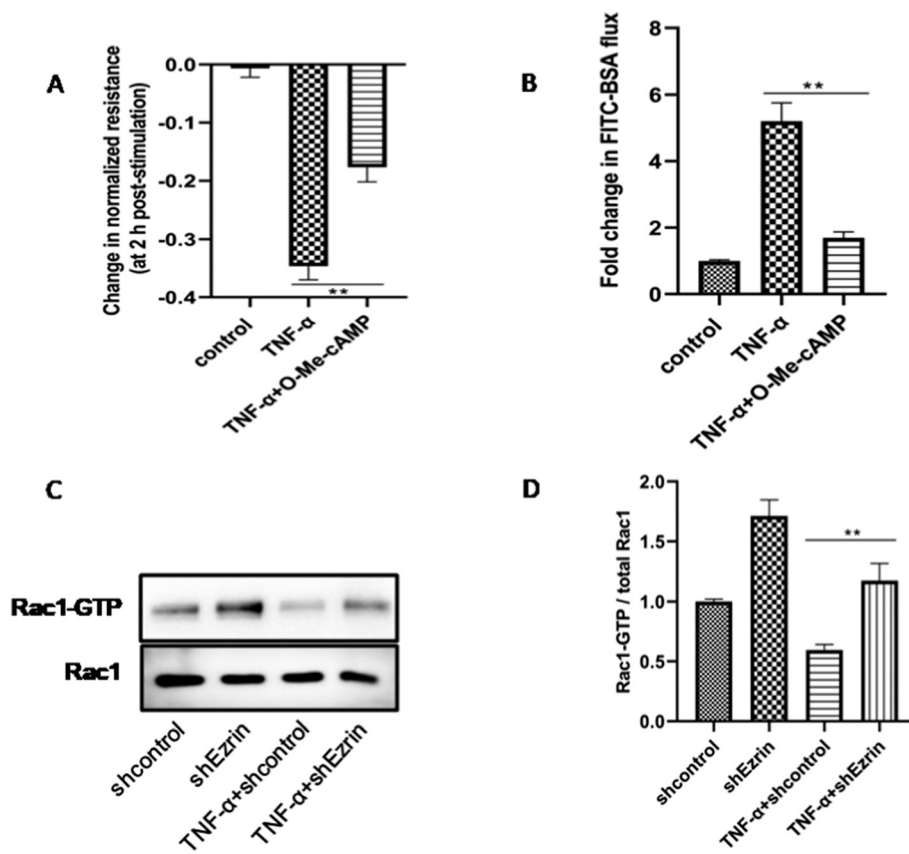


Fig. 8. Ezzrin inhibition increases the Rac1 activity in PMVECs. (A) O-Me-cAMP can reduce the decrease of TEER induced by TNF- α . (B) O-Me-cAMP can reduce the increase of FITC-BSA permeability induced by TNF- α . (C) Pull-down assay and Western blot were used to analyze the Rac1 activity after TNF- α stimulation and Ezrin inhibition and relative expressions as assessed by Western blotting (D).

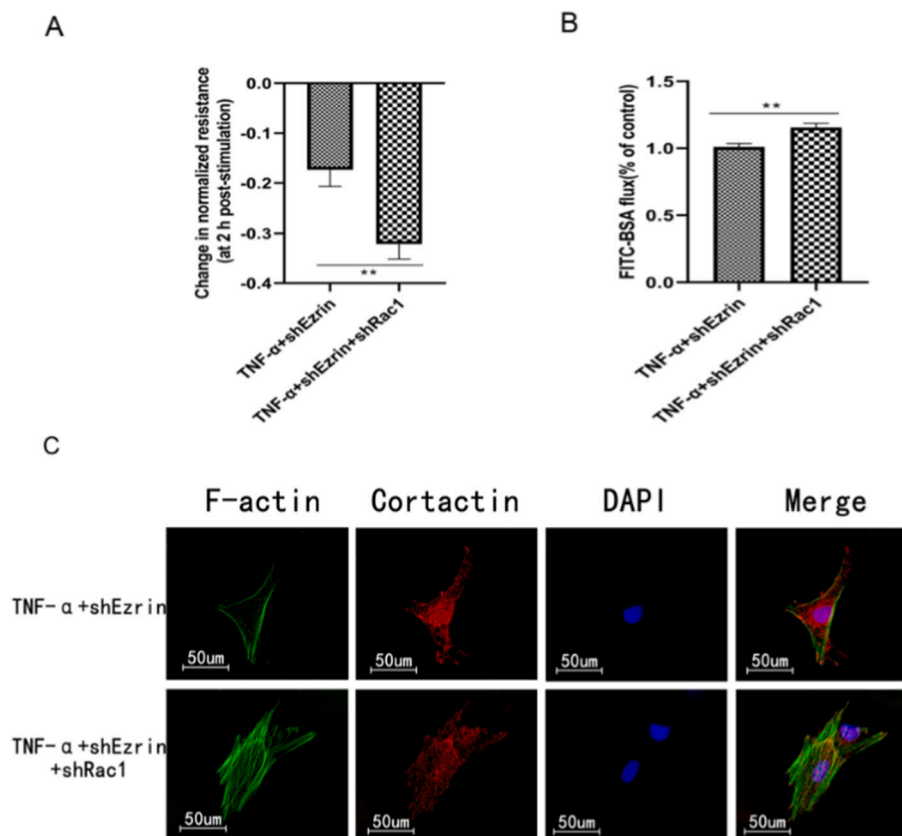


Fig. 9. Downregulation of Rac1 weakens the protective effect of Ezrin silence. (A, B) TEER and FITC-BSA permeability changed after Rac1 inhibition. (C) Simultaneous transfection of shEzrin and shRac1 can promote intracellular F-actin remodeling and cortactin redistribution.

and regulate endothelial barrier function by regulating the activity of Rac1. Adyshev et al. suggested that ERM differentially regulates S1P-induced changes in the cytoskeleton and permeability of lung EC cells, among which Radixin has the most significant role (Adyshev et al., 2011). Moesin and total ERM have a prominent role in thrombin-induced cytoskeleton rearrangement (Adyshev et al., 2013). Our results confirmed that Ezrin, as a member of ERM, is important for the cytoskeleton rearrangement of PMVEC cells induced by TNF- α . Moreover, Pujuguet et al. found that phosphorylated Ezrin activated Rac1 can further activate the Rho signal pathway and regulate the expression of E-cadherin in the cytoplasm and cell membrane (Pujuguet et al., 2003). This was not consistent with our results, according to which phosphorylated Ezrin inhibited Rac1 activation, likely because of different cell models.

The present study has a few limitations. First, all the experiments were conducted *in vitro* using rat PMVECs. Future studies should evaluate the effects of Ezrin silencing on PMVECs in animal models, and determine the effects of permeability and cytoskeleton remodeling in inflammatory human PMVECs. Secondly, the specific mechanism of p-Ezrin in regulating the increase of cell permeability induced by TNF- α is still not fully clear, especially the mechanism of the Ezrin-related signal transduction pathway. Also, potential cooperation between related signal transduction pathways needs to be further investigated, especially the crosstalk effect of ICAM-2 and Rac1-Ezrin signaling (Amsellem et al., 2014).

To sum up, our preliminary data suggest that Ezrin participates in the experimental phenomenon of TNF- α -induced increase of PMVEC permeability by regulating Rac1 activity and the process of regulating the subcellular structure of the cytoskeleton. ShEzrin did not completely reverse the barrier damage caused by TNF- α , which indicated that there are other mechanisms involved in the regulation of the downstream of the TNF- α pathway. It is worth mentioning that the activity of Rac1 can also negatively regulate the phosphorylation of Ezrin. These results enhance our understanding of the molecular mechanism of ARDS and the function of the pulmonary microvascular barrier.

CRedit authorship contribution statement

This paper has not been published elsewhere in whole or in part. All authors have read and approved the content, and agree to submit it for consideration for publication in your journal. There are no conflicts of interest involved in the article.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mvr.2020.104093>.

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