ORIGINAL RESEARCH

LPA$_2$ Alleviates Septic Acute Lung Injury via Protective Endothelial Barrier Function Through Activation of PLC-PKC-FAK

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Background: Increased endothelial permeability of pulmonary vessels is a primary pathological characteristic of septic acute lung injury (ALI). Previously, elevated lysophosphatidic acid (LPA) levels and LPA$_2$ (an LPA receptor) expression have been found in the peripheral blood and lungs of septic mice, respectively. However, the specific role of LPA$_2$ in septic ALI remains unclear.

Methods: A lipopolysaccharide (LPS)-induced model of sepsis was established in wild-type (WT) and global LPA$_2$ knockout ($Lpar2^{-/-}$) mice. We examined mortality, lung injury, assessed endothelial permeability through Evans blue dye (EBD) assay in vivo, and transendothelial electrical resistance (TEER) of mouse lung microvascular endothelial cells (MLMECs) in vitro. Enzyme-linked immunosorbent assay (ELISA), histopathological, immunofluorescence, immunohistochemistry, and Western blot were employed to investigate the role of LPA$_2$ in septic ALI.

Results: $Lpar2$ deficiency increased vascular endothelial permeability, impaired lung injury, and increased mortality. Histological examination revealed aggravated inflammation, edema, hemorrhage and alveolar septal thickening in the lungs of septic $Lpar2^{-/-}$ mice. *In vitro*, loss of $Lpar2$ resulted in increased permeability of MLMECs. Pharmacological activation of LPA$_2$ by the agonist DBIBB led to significantly reduced inflammation, edema and hemorrhage, as well as increased expression of the vascular endothelial tight junction (TJ) protein zonula occludens-1 (ZO-1) and claudin-5, as well as the adheren junction (AJ) protein VE-cadherin. Moreover, DBIBB treatment was found to alleviate mortality by protecting against vascular endothelial permeability. Mechanistically, we demonstrated that vascular endothelial permeability was alleviated through LPA-LPA$_2$ signaling via the PLC-PKC-FAK pathway.

Conclusion: These data provide a novel mechanism of endothelial barrier protection via PLC-PKC-FAK pathway and suggest that LPA$_2$ may contribute to the therapeutic effects of septic ALI.

Keywords: septic ALI, LPA, vascular endothelial permeability, LPA$_2$, lung microvascular endothelial cells

Introduction

Septic acute lung injury (ALI) is a potentially lethal medical condition, involves excessive accumulation of alveolar fluid in the pulmonary capillaries, refractory hypoxemia and respiratory embarrassment, resulting from disruption of the lung endothelial barrier integrity.[1] Currently, ALI patients are treated with nitric oxide, mechanical ventilation, extracorporeal membrane oxygenation, and steroid therapy. However, significant advances in ALI recognition and management have been made, treatment options remain limited to enable substantial improvement in survival.[2] Pulmonary vascular endothelial cells are vital constituents of the pulmonary capillary barrier and form a semi-permeable barrier at the interface between the blood in circulation and nearby tissues. The impairment of the endothelial barrier results in heightened vascular permeability, thus thought to be an important factor in septic ALI.[3] When endothelial injury occurs, the release of inflammatory mediators disrupts the integrity of the vascular endothelial barrier which contribute to high

mortality in septic ALI.[4] Since increased pulmonary vascular permeability is the main pathophysiological characteristic of ALI, targeting endothelial barrier dysfunction is a potential approach to treat septic ALI.

Tight junctions (TJs) and adherens junction (AJs) are essential in facilitating cell-cell attachment and regulating endothelial permeability, therefore critical during ALI development.[5,6] AJs common in capillary veins, consist of adhesion complexes comprised of α-, β-, and P120-catenin together with VE-cadherin. VE-cadherin has been shown to mediate the homeostasis of pulmonary fluids and integrity of endothelial junctions which expressed only in endothelial cells.[7] TJs form a continuous intercellular network, which create a barrier that prevents pathogens from penetrating the epithelium.[8] TJs consist of occludin and claudins which interact with intracellular zonulas (ZOs, including ZO-1, ZO-2, and ZO-3).[9] ZO-1 is expressed on pulmonary vascular endothelial cells, interacts with almost all claudin transmembrane proteins, and regulates endothelial AJs and endothelial barrier formation. Claudin-5 is observed primarily in vascular endothelial cells.[10]

The extracellular lipid mediator lysophosphatidic acid (LPA) engages in signaling via six G-protein-coupled receptors (LPA_1 - LPA_6) to regulate multiple physiological function.[11] Several reports have demonstrated that LPA is involved in stabilizing endothelial barrier function and reducing vascular endothelial permeability,[12,13] as well as stimulating angiogenesis.[14–16] We have previously shown that LPA_2 promotes vascular endothelial homeostasis and heart regeneration following myocardial infarction, which shown to play a critical part in the regulation of vascular endothelial barrier function.[17] Furthermore, LPA_2 has been shown to have over $100 \times$ affinity for LPA than other LPA receptors.[18,19]

LPA_2 is a G-protein coupled receptor (GPCR) that acts through three different pathways: $G\alpha_{12/13}$-Rho, $G\alpha_{q/11}$-PLC-PKC and $G\alpha_{i/o}$-PI3K or Ras-MAPK. Studies have shown that LPA promotes E-cadherin expression in HBEPCs through the PKC-FAK pathway.[20] Thus, we found that LPA promotes VE-cadherin expression in MLMECs. However, the role of LPA_2 in mediating endothelial permeability during septic ALI is unknown, and it is not clear whether the protective effect of LPA_2 occurs during PLC-PKC-FAK mediated endothelial barrier dysfunction.

Materials and Methods
Animals
Global LPA_2 knockout ($Lpar2^{-/-}$) mice were bred into a Balb/c background for at least ten generations. Wild-type (WT) and $Lpar2^{-/-}$ mice (male, aged 8–10 weeks) were used and reared in the Fuwai Hospital Animal Experimental Center (Chinese Academy of Medical Sciences) in a specific pathogen free (SPF) animal room under a 12 h light-dark cycle at 22–26°C were given water libitum. The study was carried out in accordance with the "Regulation to the Care and Use of Experimental Animals" of the Beijing Council on Animal Care study (1996). The experiments were approved by Fuwai Hospital Animal Care and Use Committee.

Sepsis Model
The sepsis model was induced by intraperitoneal injection of lipopolysaccharide (LPS) (10mg/kg, *Escherichia coli* serotype 055: B5, L2880; Sigma-Aldrich, USA) in male WT and $Lpar2^{-/-}$ mice. An equivalent volume of phosphate-buffered saline (PBS) was intraperitoneally injected as the control group. Survival rate was recorded every 4 h after LPS injection for a 72 h period.

Lung Injury Score and Histological Examination
At 24 h after LPS treatment, lung tissue was isolated and fixed in 4% paraformaldehyde. Then embedded in paraffin and cut into 5 μm sections. After staining with hematoxylin eosin (HE), histopathological alterations were assessed using light microscopy. The lung injury score was determined following previously criteria.[21]

	Score = 0	Score = 1	Score = 2	Score=3	Score =4
Inflammation	Absent	Mild	Moderate	Severe	Very severe
Edema and hemorrhage	None	<10%	10–30%	30–50%	>50%
Alveolar septal thickening	<15 μm	15–30 μm	30–45 μm	45–60 μm	>60 μm

Bronchoalveolar Lavage Fluid (BALF) Measurement

Mice were anesthetized with isoflurane after administration of LPS or PBS 24 h, and BALF was collected. Subsequently, BALF was centrifuged 15 min at 1500 g, and quantification of IL-6 (ab222503, Abcam, USA), CXCL15 (ab193720, Abcam, USA) and TNF-α (ab208348, Abcam) was performed with the corresponding ELISA kits within the supernatants. BCA protein assay Kit (cat. No. 23225; Thermo Fisher Scientific, USA) was used to assess the total protein level in the BALF.

Vascular Permeability Assessment

Vascular endothelial permeability was examined as previously described.[22] Vascular leakage was assessed by injecting with 20 mg/kg Evans blue dye (EBD) through the tail vein 30 min prior to lungs collection. EBD was extracted from lung tissue samples by incubating with formamide at 55°C for 48 h. The EBD solution's absorbance was determined at 620nm-740 nm by spectrophotometry.

Immunofluorescence Staining

Tissue sections were baked at 68°C for 45 min, followed by deparaffinization and dehydration. The samples were boiled in sodium citrate (pH 6.0, 10 mmol/L) for 2 min for antigen retrieval. Samples then permeabilized by incubating in Triton X-100 (0.1%) for 10 min. Next, the samples were blocked in normal goat serum for 1 h at room temperature.[23] Sections were incubated with primary antibodies against VE-cadherin (1:200; ab205336, Abcam, USA), claudin-5 (1:200; ab15106, Abcam, USA), ZO-1 (1:200; ab216880, Abcam, USA) and CD31 (1:200, MA1-26196, Thermo Fisher, USA) overnight at 4°C, followed incubated with Alexa Fluor-488 (1:500; A-11001, Invitrogen, USA) or Alexa Fluor-594 (1:500; A-11012, Invitrogen, USA) secondary antibodies for 1 h at room temperature. Slides were counterstained with DAPI, coverslipped and fluorescent images captured using a laser-scanning confocal microscope (SP8, Leica).

Immunohistochemical Staining

Tissue slices were prepared as described previously.[24] After dewaxing, antigen was extracted with citric acid buffer and samples were permeabilized with Triton X-100 (0.1%) for 10 min. The samples were then treated with 30% H_2O_2 to inhibit endogenous peroxidase activity. Subsequently, the samples were incubated with normal goat serum (10%) and subsequently with primary antibodies targeting VE-cadherin (1:200; ab282277, Abcam, USA), claudin-5 (1:200; ab15106, Abcam, USA) and ZO-1 (1:200; ab216880, Abcam, USA), at 4°C overnight. Samples were rewarmed at 37°C for 30 min, incubation with reaction enhancer solution was performed for 20 min at 37°C, followed by incubation with HRP-conjugated secondary antibodies for 30 min at 37°C. Diaminobenzidine (DAB) solution was added and incubated at room temperature and the nuclei were stained with hematoxylin. The stained sections were observed under a light microscope.

Isolation of Mouse Lung Microvascular Endothelial Cells (MLMECs)

MLMECs were isolated from 6–8 week mice by serial immunoselection using a purified rat anti-mouse CD31 antibody coupled with Dynabeads M-450 sheep anti-rat IgG as previously described.[25] Briefly, lungs were removed from mice and washed with high-glucose DMEM containing 20% FBS. Next, the lung tissue was digested with collagenase I solution (2000 U/mL, 15 mL, C1639, Sigma, USA) in PBS for 45 min at 37°C, minced 15 times with a 20-gauge needle. The cell suspension was then passed through a 70 μm nylon filter then centrifuged for 8 min at 1300 g and 4°C. Primary MLMECs were plated in tissue culture flasks (T-25) precoated with human fibronectin (2.5 g/mL in aseptically filtered PBS, ECM001, Invitrogen, USA) and cultured at 37°C in a CO_2 incubator.

MLMECs Treated with the Inhibitor

MLMECs were cultured on the luminal side of filters (0.4 μm pore size, Corning, USA) in gelatin-coated 12-well plates until confluent. They were then cultured in endothelial serum-free medium for 12 h and exposed to LPS (1μg/mL) for 24 h, then corresponding inhibitor and LPA (10μM) cultured for 20 h. Fluorescein isothiocyanate (FITC)-dextran fluorescein was used to confirm the permeability. FITC-dextran (1 mg/mL; 78331, Sigma, USA) was added to the upper

compartments of the transwell cultures. A microplate reader was used to measure the values for absorbance of the lower chamber solution at 492 nm (excitation) and 520 nm (emission) wavelengths.

Western Blot Analysis

The left lungs of mice from each treatment group were stored in liquid nitrogen. After homogenization of tissue samples, protein extraction was conducted with an extraction reagent supplemented with protease inhibitors. The protein concentration was determined with a BCA Protein Assay Kit (23225, Thermo Fisher Scientific, USA). Proteins were separated with 10% SDS-PAGE, then transferred to PVDF membranes. The membranes were subsequently blocked in 5% BSA for 1 h, incubated overnight at 4°C with primary rabbit polyclonal antibodies anti-VE-cadherin (1:1000; ab205336, Abcam, USA), anti-ZO-1 (1:1000; ab216880, Abcam, USA), anti-claudin-5 (1:1000; 35–2500, Thermo Fisher, USA), PKCδ (1:1000; ab182126, Abcam, USA), PKCζ (1:1000; ab108970, Abcam, USA), FAK (1:1000; ab40794, Abcam, USA), and p-FAK (1:1000; ab81298, Abcam, USA). The next day membranes were incubated with secondary antibodies, visualization was performed with SuperSignal West Pico Plus Substrate (34577, Thermo Fisher Scientific, USA). Protein band intensity was determined in ImageJ software.

Statistical Analysis

GraphPad Prism 8 and SPSS 21.0 software were used for statistical analysis. The data are expressed as mean ± SD. One-way ANOVA, together with Tukey's test for multiple comparisons after the variance homogeneity test, was used to assess significant differences among groups. Differences were considered statistically significant when $p < 0.05$.

Results

Lpar2 Deficiency Decreases Survival and Aggravates LPS-Induced Septic ALI in Mice

The effects of LPA_2 on LPS-induced sepsis were examined in WT and *Lpar2*$^{-/-}$ mice injected with LPS or PBS intraperitoneally. The survival rate was monitored every 4 h for 72 h. The survival rates of the WT and *Lpar2*$^{-/-}$ mice following LPS treatment were 70.8% and 41.7%, respectively (Figure 1A). Organs were removed from the LPS-induced septic mice 12 h later to examine the extent of organ damage. The lung tissue of LPS-*Lpar2*$^{-/-}$ mice displayed obvious edema and congestion, with hemorrhagic spots covering the surface of the lung tissue, an abundance of secretions and an outflow of pink fluid. In addition, lung injury was more severe in *Lpar2*$^{-/-}$ mice (Figure 1B). HE staining revealed that mice in the LPS-*Lpar2*$^{-/-}$ group experienced substantially exacerbated inflammatory cell infiltration, structural damage, alveolar interstitial edema, and alveolar wall thickening (Figure 1C-1). The lung injury score exceeded in the LPS-*Lpar2*$^{-/-}$ group than that observed in the LPS-WT group (Figure 1C-2). Furthermore, the BALF of LPS-*Lpar2*$^{-/-}$ mice exhibited a significant increase in neutrophil level (Figure 1D), as well as elevated level of IL-6 (Figure 1E-1), TNF-α (Figure 1E-2), and CXCL15 (Figure 1E-3). These results collectively indicate the crucial protective role of LPA_2 in septic ALI.

Lpar2 Deficiency Augments Lung Vascular Endothelial Permeability

Endothelial barrier function has been associated with the pathogenesis of septic ALI. To explore whether LPA_2 affects endothelial permeability during septic ALI, we measured endothelial permeability of WT mice and *Lpar2*$^{-/-}$ mice in vivo and in vitro, respectively. Endothelial permeability in vivo assessed using the wet-to-dry ratio (W/D), protein level in BALF and EBD extravasation in lung tissue. In vitro, an electric cell-substrate impedance sensing (ECIS) system was used to determine the transendothelial electrical resistance (TEER) in LPS-treated MLMECs.[26] The W/D ratio was found to be significantly elevated in the lungs of LPS-*Lpar2*$^{-/-}$ mice (Figure 2C). Similarly, enhanced EBD leakage was observed in *Lpar2*$^{-/-}$ mice (Figure 2A and B). Consistent with our W/D ratio data, significantly higher level of total BALF protein were observed in LPS-*Lpar2*$^{-/-}$ mice (Figure 2D). In addition, increased permeability of LPS-*Lpar2*$^{-/-}$ than LPS-WT in MLMECs (Figure 2E). Finally, LPS exposure led to decreased TEER level in *Lpar2*$^{-/-}$ MLMECs (Figure 2F and G). Together, our findings suggest a protective role of LPA_2 in mediating endothelial barrier integrity following LPS treatment.
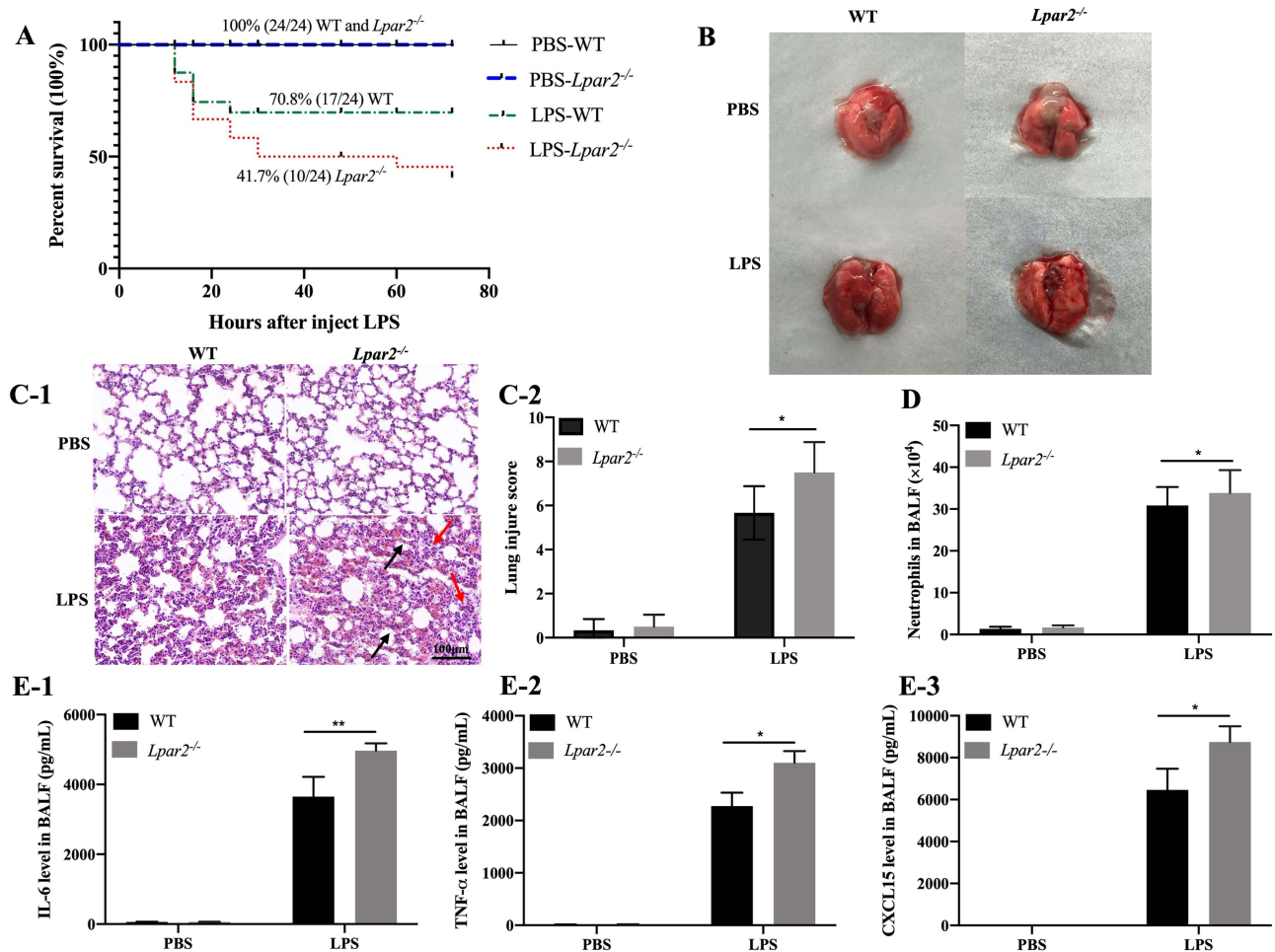
Figure 1 *Lpar2* deficiency increases the mortality, pulmonary damage and inflammation in LPS-induced mice. (**A**) Survival of WT and *Lpar2*⁻ᐟ⁻ mice challenged with LPS (n = 24). (**B**) Representative images of the lung tissue in WT and *Lpar2*⁻ᐟ⁻ mice. (**C-1**) Representative images of HE-stained lung tissue. Red arrows indicate inflammatory cell infiltration, black arrows indicate thickening of the alveolar walls. Scale bar, 100 μm. (**C-2**) The lung injury area was quantified by assessing the histological scores for each treatment group (n = 6). (**D**) Total neutrophil levels in the BALF (n = 6). (**E1–3**) CXCL15, TNF-α and IL-6 levels in the BALF (n = 6), *$p < 0.05$ and **$p < 0.01$ vs the LPS-WT group.

Lpar2 Deficiency Decreases VE-Cadherin, ZO-1, and Claudin-5 Expression in Septic ALI Mice

To further investigate whether LPA₂ regulates the expression of VE-cadherin, ZO-1, and claudin-5 and then affect the endothelial permeability, we detected the three proteins by Western blot, immunofluorescence and immunohistochemical. Our findings demonstrated a significant downregulation of VE-cadherin, ZO-1, and claudin-5 expression in the LPS-*Lpar2*⁻ᐟ⁻ group with the results of Western blot (Figure 3B). Our immunofluorescence (Figure 3A1–A4) and immuno-histochemical (Figure 3C1 and C2) data were consistent with Western blot results. The results indicated that LPA₂ preserving vascular integrity and mitigating increased vascular permeability through the restoration of VE-cadherin, ZO-1, and claudin-5 expression in septic ALI mice.

LPA Has a Key Role in Promoting Expression of VE-Cadherin

Previous studies have shown that treatment of human bronchial epithelial cells with LPA promotes the cell-cell connection between E-cadherin/c-Met.[20] Thus, we hypothesized that LPA may enhance the lung endothelial barrier function of septic mice. As shown in Figure 4A1 and A2, treatment with LPA increased the TEER of LPS-stimulated MLMECs. Western blot analysis revealed that expression of VE-cadherin markedly increased following LPA treatment
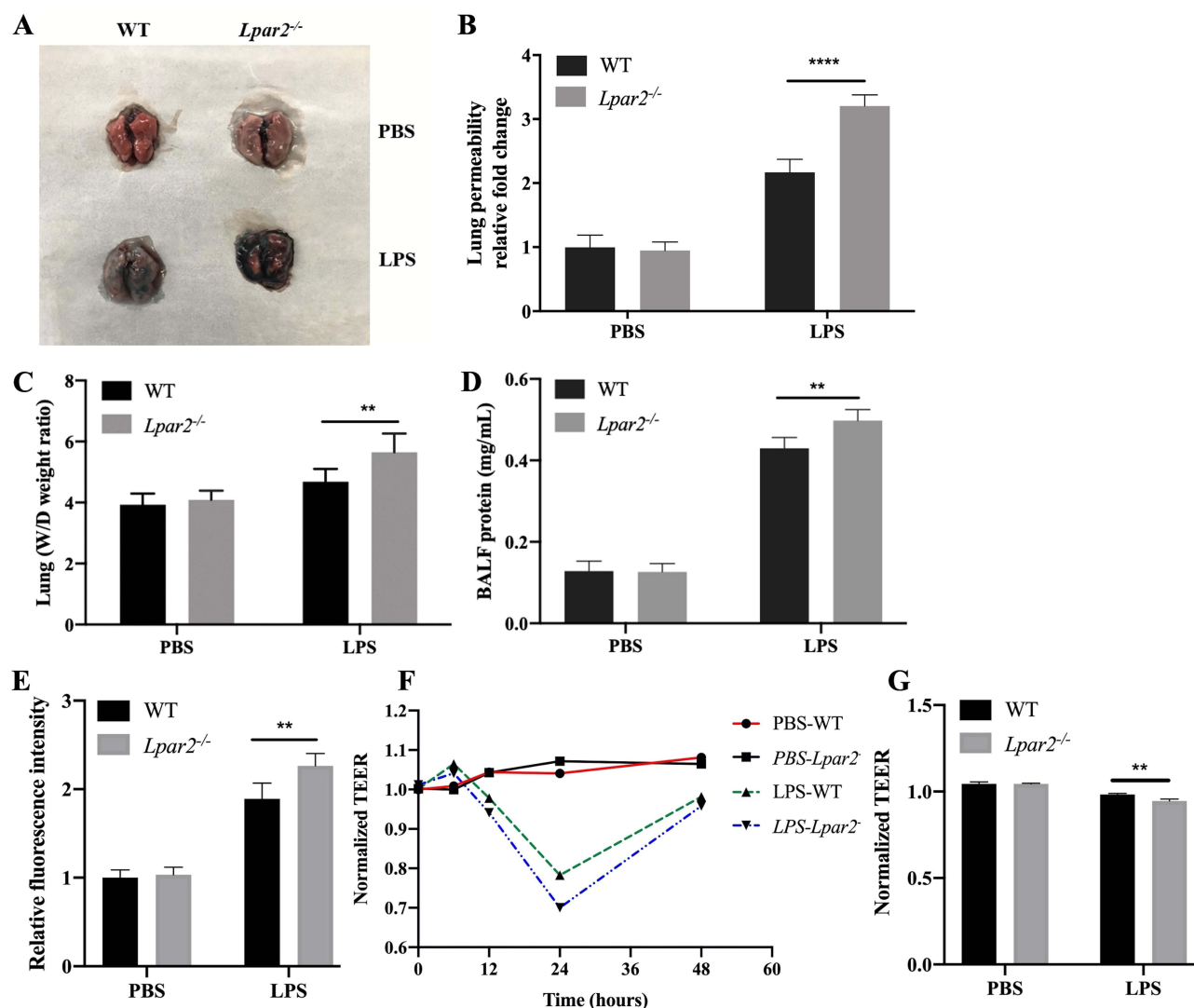
Figure 2 *Lpar2* deficiency increases LPS-induced vascular endothelial permeability in mice lung tissue and MLMECs. (**A**) Representative images showing WT and *Lpar2*$^{-/-}$ mice lung tissue. (**B**) The vascular permeability of mouse lung tissue was quantified using the EBD assay (n = 6). (**C**) Quantification of mice pulmonary W/D ratio (n = 6). (**D**) Total BALF protein (n = 8). (**E**) The vascular permeability of MLMECs (n=3). (**F**) The permeability of MLMECs was assessed by measuring the TEER. (**G**) The statistical analysis of TEER in 24h, **$p < 0.01$ and ****$p < 0.0001$ vs LPS-WT group.

of LPS-treated cells compared to the LPS alone treatment group (Figure 4B1 and B2). Taken together, our findings suggest that LPA reduces the permeability of MLMECs, thereby protecting endothelial barrier integrity.

LPA-LPA$_2$ Promotes VE-Cadherin Expression Through the PLC-PKC-FAK Pathway

LPA$_2$ is a G-protein coupled receptor (GPCR) that acts through three different pathways: G$\alpha_{12/13}$-Rho, G$\alpha_{q/11}$-PLC and G$\alpha_{i/o}$-PI3K or Ras-MAPK (Figure 5A). We demonstrated that inhibition of PLC, PKC and FAK diminished the expression of VE-cadherin and increased permeability of MLMECs compared to MLMECs treated with LPS alone (Figure 5B). To further confirm that LPA promotes VE-cadherin expression in MLMECs through the PLC-PKC-FAK pathway, MLMECs were treated with PLC, PKC and FAK inhibitors and the expression of downstream signaling proteins was examined through Western blot. As shown in Figure 5C1 and C2, PKCδ, PKCζ, and pFAK/FAK expression levels were decreased following treatment with PLC inhibitors. Treatment with PKC inhibitors led to decreased VE-cadherin and pFAK/FAK expression levels (Figure 5D1 and D2), while VE-cadherin expression was reduced following treatment with FAK inhibitors (Figure 5E1 and E2). These results suggest that LPA-LPA$_2$ may mediate the expression of VE-cadherin and endothelial cell permeability via the PLC-PKC-FAK pathway.
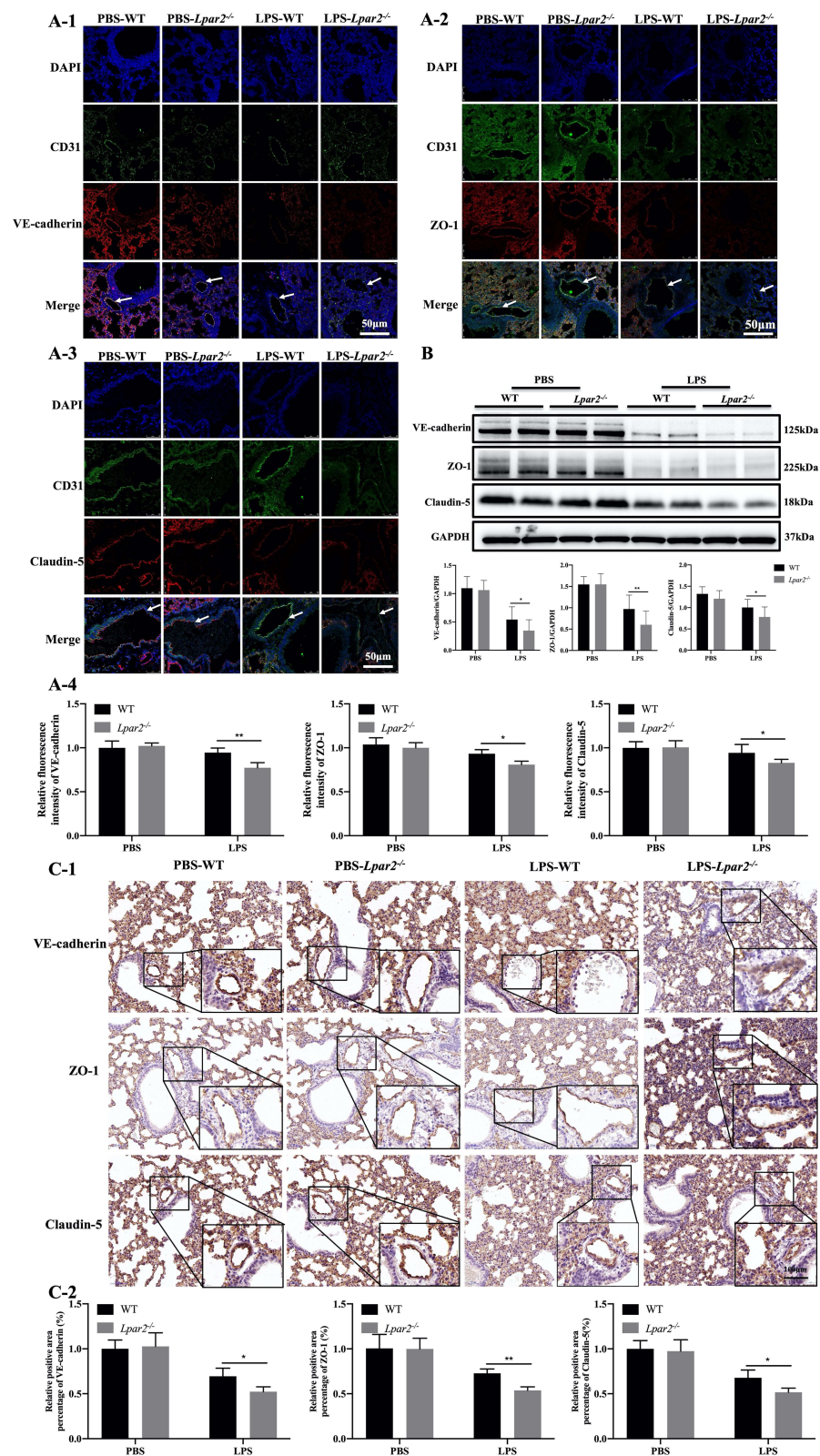
Figure 3 *Lpar2* deficiency leads to a decrease in AJs and TJs in mice lung tissue. (**A1–A3**) Representative images showing immunofluorescence staining in mice lung tissue. White arrows indicate expression of VE-cadherin, ZO-1, and claudin-5 in vascular endothelium. Scale bar, 50 μm. (**A4**) Relative fluorescence intensity of the images in (**A1–A3**). (**B**) Representative Western blot and quantification of protein expression level (n = 6). (**C-1**) Representative images showing immunohistochemical staining in paraffin-embedded pulmonary tissue section. Scale bar, 100 μm. (**C-2**) The percentage of positively stained area, *p <0.05 and **p <0.01 vs LPS-WT group.
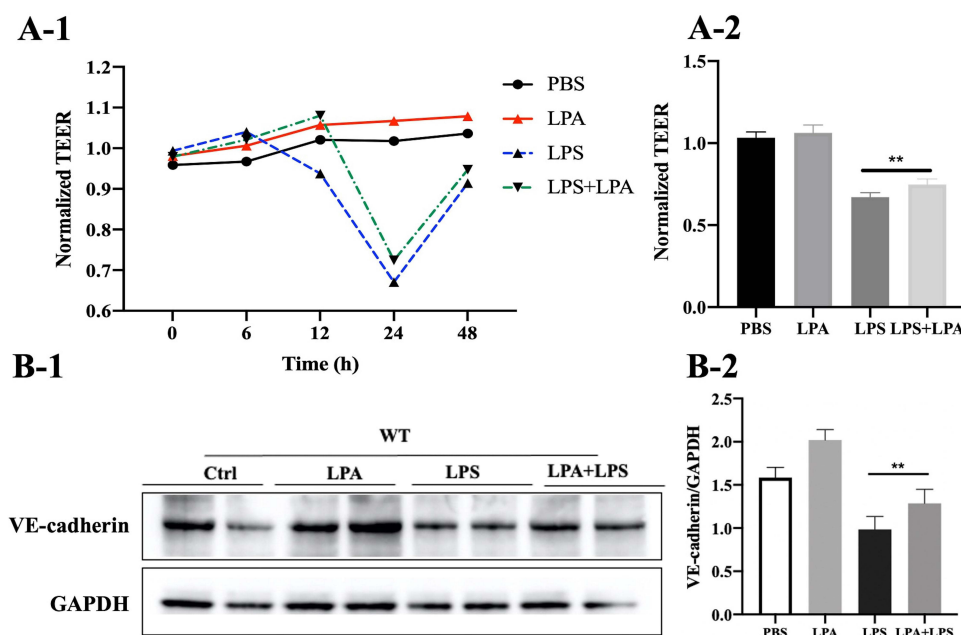
Figure 4 LPA facilitates expression of VE-cadherin in MLMECs. (**A-1**) The effects of LPA on the transmembrane resistance of MLMECs were examined by measuring the TEER. (**A-2**) The statistical analysis of TEER in 24h. (**B-1**) Western blot analysis to assess the effects of LPA treatment on expression of VE-cadherin. (**B-2**) Quantitative analysis of the Western blot data (n = 6), **$p < 0.01$ vs LPS group.

LPA$_2$ Activation Reduces LPS-Induced Mice Lung Damage and Mortality

To determine whether activation of LPA$_2$ alleviated the lungs damage and mortality in LPS-induced septic mice, we next administered DBIBB, a specific agonist of LPA$_2$, followed by intraperitoneal injection of LPS to WT mice. We found that DBIBB treatment significantly reduced mortality with a survival rate of 79.2% in LPS + DBIBB mice compared to 58.3% in LPS mice (Figure 6A). Furthermore, DBIBB treatment resulted in alleviation of edema and congestion of the lung tissue, as well as a reduction in bleeding spots on the surface of the lung tissue and exudate secretions (Figure 6B). Histopathological analysis of the lung tissue revealed that DBIBB ameliorated alveolar interstitial edema, alveolar wall thickening and inflammatory cell infiltration (Figure 6C-1). Finally, the LPS + DBIBB group displayed a reduced lung injury score compared to the LPS group (Figure 6C-2). Together, these findings highlight the protective role of LPA$_2$ in septic ALI.

Activation of LPA$_2$ Decreases Vascular Endothelial Permeability in Septic ALI

Next, we examined whether activation of LPA$_2$ alleviates endothelial barrier dysfunction in septic ALI using the W/D ratio and EBD. We found that DBIBB treatment substantially reversed the LPS-induced increased vascular permeability (Figure 7A). Similarly, the EBD concentration was significantly lower in the LPS + DBIBB group than the LPS group, suggesting that DBIBB treatment significantly weakened lung tissue permeability (Figure 7B). In addition, DBIBB treatment led to a significant reduction in W/D ratio values compared to LPS-treated mice (Figure 7C). Together, our findings suggested that treatment with an LPA$_2$ agonist reduced endothelial barrier dysfunction in septic ALI mice.

LPA$_2$ Activation Promotes Expression of VE-Cadherin, ZO-1, and Claudin-5

VE-cadherin, ZO-1, and claudin-5 expression decreased after LPS treatment compared to PBS treatment. DBIBB treatment led to a notable increase expression of VE-cadherin, ZO-1, and claudin-5, as evidenced by the results obtained from immunofluorescence (Figure 8A1–A4), Western blot (Figure 8B) and immunohistochemical (Figure 8C1 and C2) assays. Thus, our results suggested that drug activation of LPA$_2$ can protect against endothelial barrier dysfunction and decrease vascular endothelial permeability, thereby playing an important protective role in septic ALI.
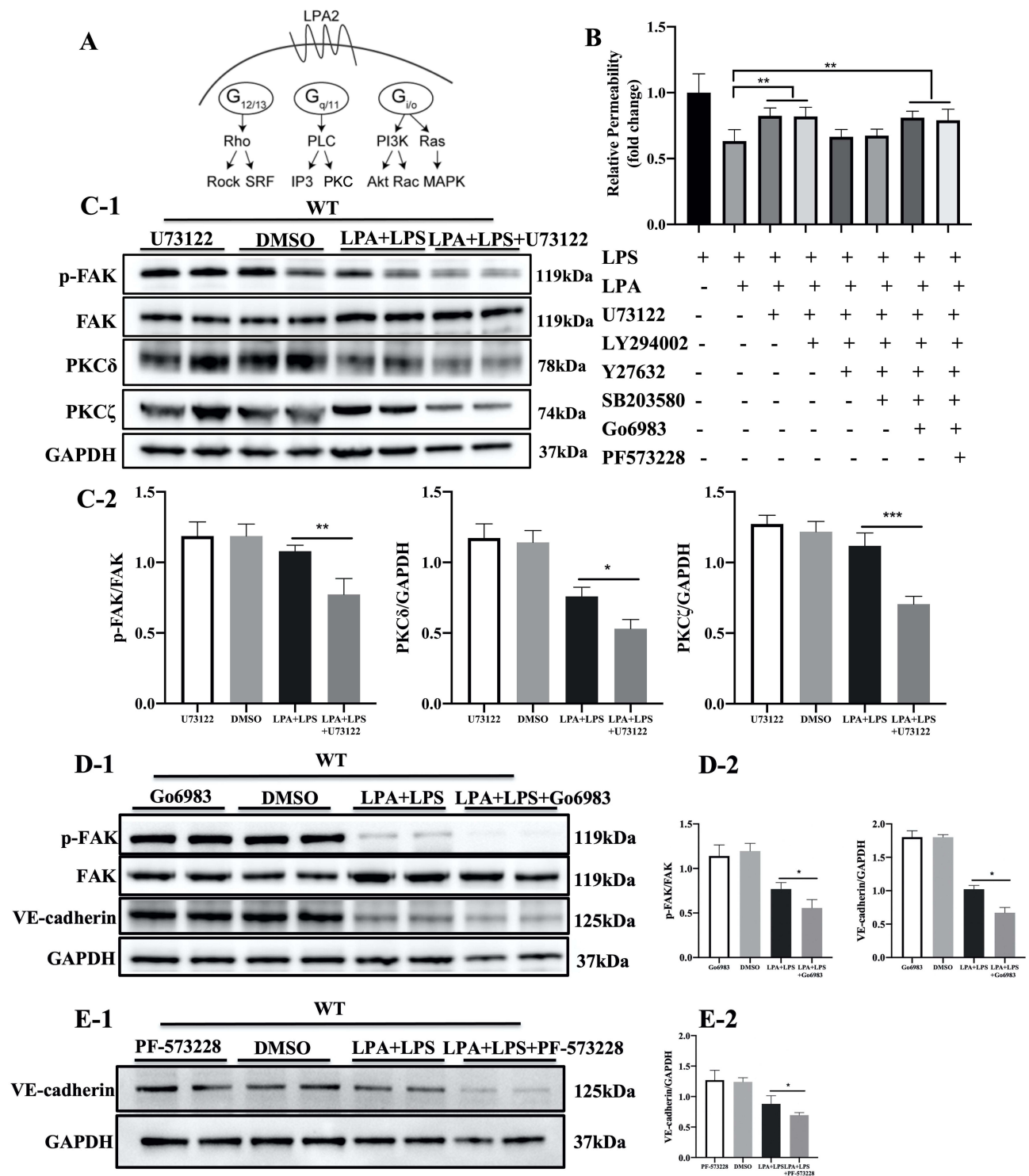
Figure 5 LPA-LPA$_2$ promotes VE-cadherin expression through the PLC-PKC-FAK signaling pathway in MLMECs. (**A**) Potential signaling pathways associated with LPA$_2$. (**B**) MLMECs were treated with various pathway inhibitors (LPA$_2$ inhibitor H2L518636, PLC inhibitor U73122, PI3K inhibitor LY294002, ROCK inhibitor Y27632, MAPK inhibitor SB203580, PKC inhibitor Go6983, FAK inhibitor PF-573228), and their effects on expression of VE-cadherin were analyzed by Western blot (n=6). (**C-1**) MLMECs were treated with the PLC inhibitor, and Western blot was performed to assess changes in the corresponding signaling pathway molecule. (**C-2**) Quantitative analysis of the Western blot data in (**C-1**) (n=6). (**D-1**) Expression levels of p-FAK, FAK and VE-cadherin were detected following treatment with PKCδ/PKCζ inhibitors. (**D-2**) Quantitative analysis of the Western blot data in D-1 (n=6). (**E-1**) Expression levels of VE-cadherin in MLMECs were assessed following treatment with the FAK pathway inhibitor. (**E-2**) Quantitative analysis of the Western blot data in E-1 (n=6), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs LPA+LPS group.
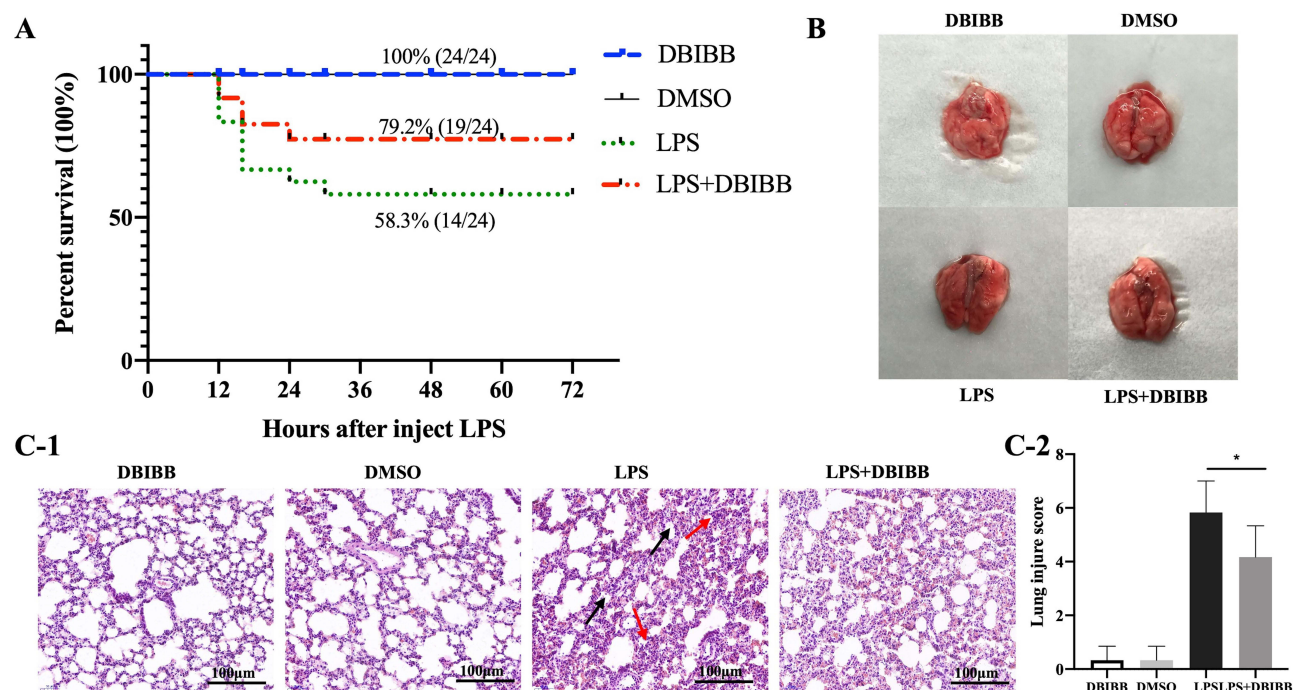
Figure 6 LPA$_2$ reduces lung injury and mortality in LPS-treated mice. (**A**) Mice survival in all treatment groups (n = 24). (**B**) Representative images showing the mice lung tissue from each treatment group (n = 24). (**C-1**) Representative HE staining of the mice lung tissue for each treatment group. Red arrows indicate inflammatory cell infiltration, black arrows indicate thickening of the alveolar walls. Scale bar, 100 μm. (**C-2**) Quantification of the lung injury area was assessed by histological scores (n = 6), *$p < 0.05$ vs LPS group.
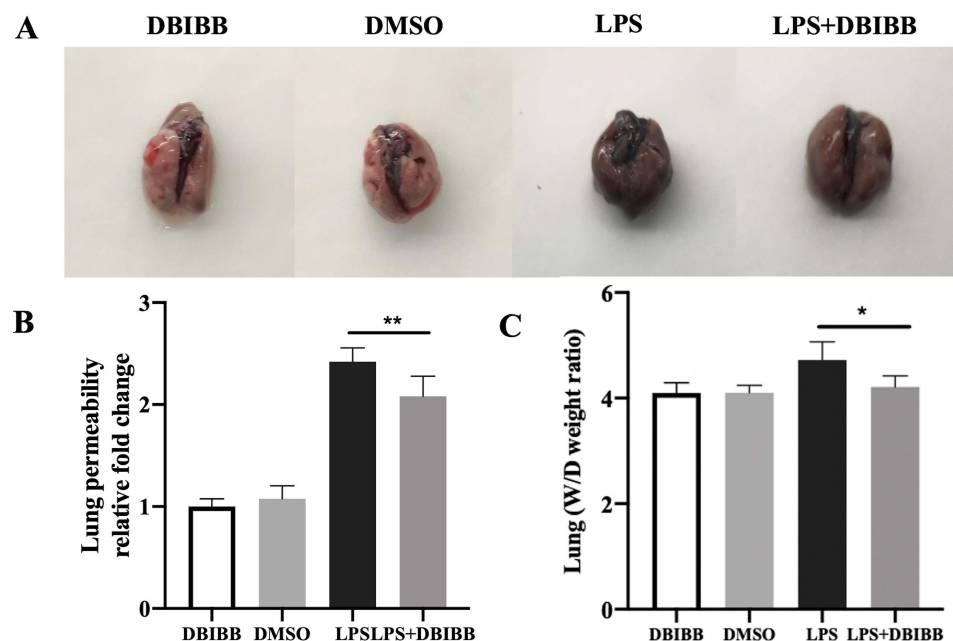

Figure 7 LPA$_2$ protects against increased endothelial permeability in septic mice. (**A**) Representative images showing pulmonary tissue in LPS-induced WT and *Lpar2*$^{-/-}$ mice. (**B**) Vascular permeability was assessed in mice lung tissue using the EBD assay (n = 6). (**C**) Quantification of W/D ratio in mice lung tissue (n = 6), *$p < 0.01$ and **$p < 0.05$ vs LPS group.

Discussion

Herein, we provided evidence of a protective role of LPA$_2$ in mitigating LPS-induced septic ALI by regulating the integrity of the endothelial barrier. We showed that *Lpar2* deficiency increased vascular endothelial permeability of lungs in vivo,
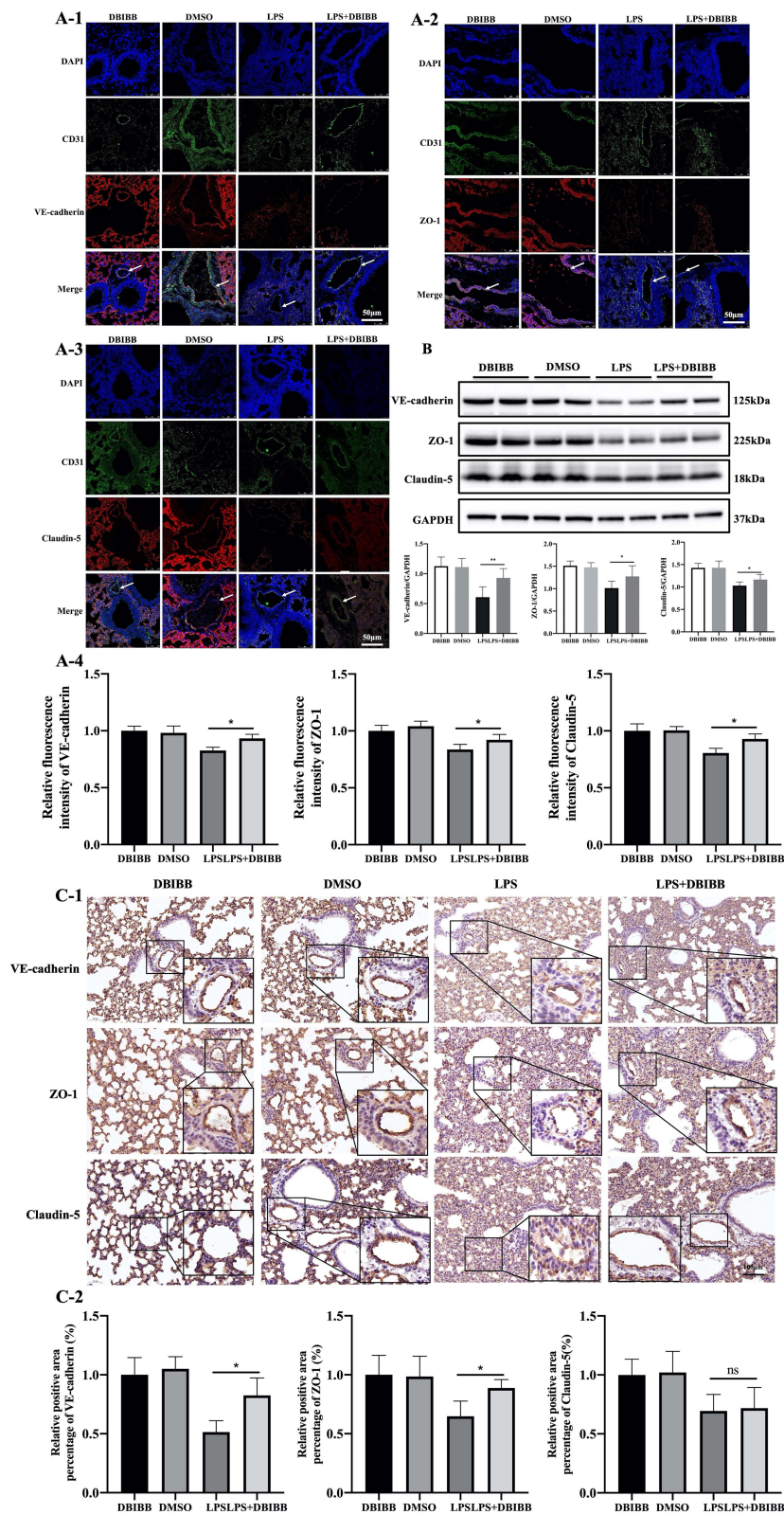
Figure 8 DBIBB treatment leads to an increase in AJs and TJs in septic mice. (**A1–A3**) Representative immunofluorescent staining images in lung sections. White arrows indicate expression of VE-cadherin, ZO-1, and claudin-5 in vascular endothelium. Scale bar, 50 μm. (**A4**) Relative fluorescence intensity of the images in (**A1–A3**). (**B**) Representative Western blot and corresponding densitometric quantification (n = 6). (**C-1**) Representative immunohistochemical images in paraffin-embedded lung sections. Scale bar, 100 μm. (**C-2**) The percentage of positively stained areas, *p <0.05 and **p <0.01 vs LPS group.

while activation of LPA$_2$ with DBIBB in vitro promoted VE-cadherin expression through the PLC-PKC-FAK pathway. Together, our findings suggested that pharmacological activation of LPA$_2$ with DBIBB could mitigate septic ALI.

The endothelial barrier function plays a critical role in modulating septic ALI, elevated endothelial permeability has been found in sepsis.[27] Previous studies have reported a 6.2-fold increased of LPA$_2$ in the carotid artery following ligation.[28,29] Previously, we have shown that LPA$_2$ is upregulated in myocardial infarction mice, and that the elevated LPA$_2$ levels were mainly due to increased LPA$_2$ expression on vascular endothelial cells.[17] These findings are of particular interest because they highlight a potential underlying role of LPA$_2$ in the pathogenesis of septic ALI. However, the precise mechanism of LPA$_2$ in ALI etiopathogenesis during sepsis remains unclear.

Endothelial cells are critical in the maintenance of vascular homeostasis. An excessive increase in vascular permeability leads to vascular leakage, which causes an increase in blood cells and plasma proteins entering the interstitium, and ultimately organ failure and death. Here, we demonstrated that *Lpar2* deficiency resulted in significantly higher mortality and impaired lung injury, as well as increased vascular endothelial permeability in the lungs of septic mice. Thus, our findings indicated that LPA$_2$ had a significant effect on endothelial barrier function. Similarly, using primary MLMECs isolated from WT and *Lpar2$^{-/-}$* mice, we demonstrated that loss of *Lpar2* led to an increase in endothelial permeability in vitro measured by the fluorescence permeability assay and TEER. Together, our data suggested that *Lpar2* deficiency may aggravate mortality and septic ALI through disruption of the endothelial barrier function, which leads to increased vascular endothelial permeability. Recent studies have revealed an important role of *Lpar2* in allergic lung inflammation through negative regulation of dendritic cell activation, as well as evidence of a protective role for LPA$_2$ in the lung.

LPA is involved in maintaining the function of the endothelial barrier through mediation of multiple pathological and physiological functions. Recently, elevated LPA level have been reported in patients with acute myocardial infarction, as well as acute coronary syndrome.[30,31] Herein, we observed that septic ALI increased level of LPA, thus indicating that there is a conserved mechanism between the two species. Our studies have demonstrated that LPA exerts its protective effect on septic ALI by acting through LPA$_2$ to regulate endothelial permeability. Sumitomo et al demonstrated that LPA protected the integrity of the endothelial cell barrier,[32,33] consistent with our findings. Similarly, LPA has been shown to promote lung and corneal epithelial barrier integrity.[20] In contrast, high concentrations of LPA have been found to increase vascular endothelial permeability after LPA exposure.[34,35] These discrepancies may be due to differences between the studies in terms of diseases, organs and LPA concentration.

It remains unclear which LPA receptors are involved in mediating key events associated with vascular endothelial permeability in the lung. Although knockdown of LPA$_1$ was found to mitigate pulmonary damage and fibrosis, treatment with an LPA$_1$ antagonist unexpectedly exhibited an antifibrotic effect, resulting in increased lung endothelial barrier permeability.[36] LPA has been shown to act through LPA$_6$ to stimulate the generation of actin stress fibers, as well as enhance the capillary endothelial permeability of brain cells.[37] Previously, we described a role for LPA$_2$ in maintaining vascular endothelial homeostasis following cardiac ischemia.[17] Thus, these studies indicate that different LPA receptors mediate various function of endothelial cells. Herein, we employed *Lpar2$^{-/-}$* mice to determine the effects of LPA$_2$ on endothelial permeability. Our results indicate that LPA stimulation facilitated the expression of VE-cadherin, ZO-1, and claudin-5 through LPA$_2$.

Studies have indicated that LPA$_2$ acts through G$\alpha_{12/13}$-Rho, G$\alpha_{q/11}$-PLC and G$\alpha_{i/o}$-PI3K or Ras-MAPK pathways. We found that LPA$_2$ acts mainly through the PLC-PKC pathway in septic ALI. On the other hand, LPA promotes E-cadherin expression in HBEPCs through the PKC-FAK pathway.[20] In our study LPA-LPA$_2$ promotes expression of VE-cadherin in MLMECs. Therefore, we hypothesized that LPA-LPA$_2$ promoted the expression of VE-cadherin through PLC-PKC-FAK pathway, and our conjecture was confirmed by employing the PLC, PKC and FAK inhibitors separately. In summary, the protective effect of LPA$_2$ occurs during PLC-PKC-FAK pathway mediated endothelial barrier dysfunction in septic ALI.

DBIBB, an agonist of LPA$_2$, has been shown to mitigate gastrointestinal radiation syndrome by promoting survival of the intestinal crypt, enterocyte multiplication and decreasing apoptosis.[19] In addition, DBIBB has been observed to participate in maintaining vascular endothelium homeostasis and heart regeneration following myocardial infarction.[17] In the current study, DBIBB was found to reduce endothelial permeability in LPS-induced septic ALI mice. Thus, our findings suggest that LPA$_2$ is a key mediator during septic ALI, consistent with the gastrointestinal radiation syndrome study.[19] Interestingly, S1P$_1$, which is highly homologous to the LPA receptor family, has been shown to maintain endothelial function. Furthermore, FTY720, agonist of S1P$_1$, is considered to be a potential drug target for 4 FDA-approved
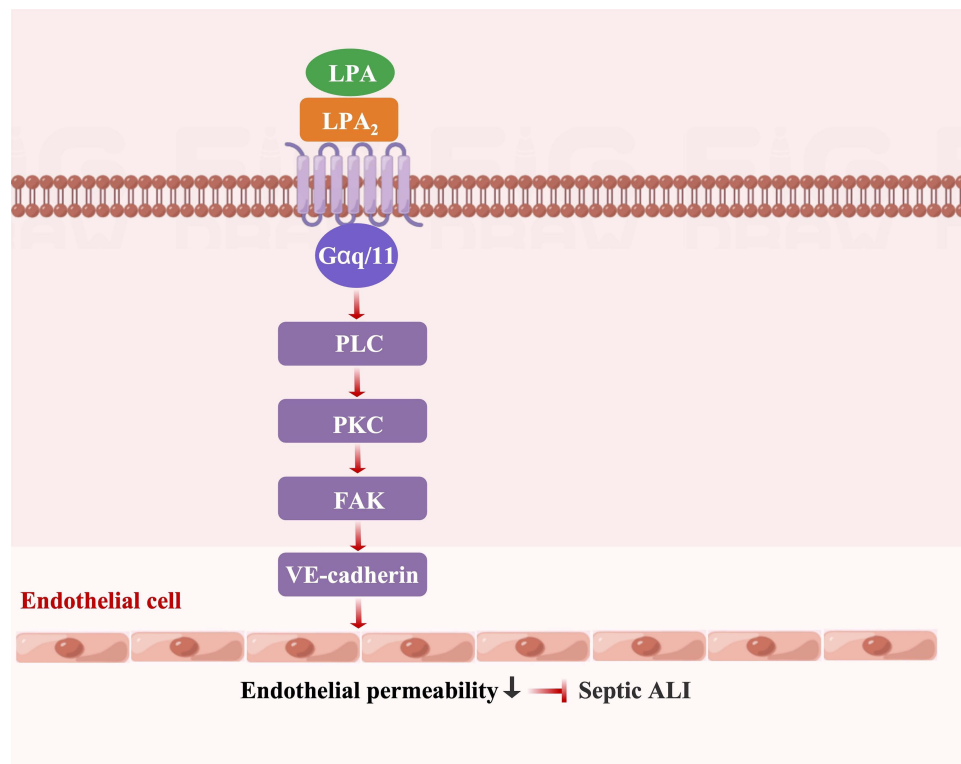
Figure 9 The protective role of LPA$_2$ against septic ALI is mediated through the PLC-PKC-FAK signaling pathway. LPA acts through LPA$_2$ to promote VE-cadherin expression through the PLC-PKC-FAK signaling pathway, thereby attenuating endothelial permeability, and alleviating sepsis-induced lung impairment.

medicines,[38] Since no obvious adverse accidents have been found in small-scale human trials as a target.[39,40] It is likely that targeting LPA$_2$ may also be a potential clinical therapeutic strategy for the treatment of septic ALI.

The innovation of this study is the first time elucidate LPA$_2$ regulates the expression of VE-cadherin, ZO-1 and claudin-5 to alleviate ALI and thus reduce the mortality of mice, which may find a new pathway for the regulation of septic ALI. This study has several limitations. Administration of LPS and cecum ligation and puncture (CLP) are the most commonly used in sepsis research. In our study, we identified the role for LPA$_2$ in a LPS model of sepsis-induced ALI to assure a better consistency, which is considered a more highly controlled and standardized model. We only whereas its role in other sepsis models (such as CLP) requires further study. In addition, our study showed that LPA$_2$ mediates lung injury through PLC-PKC-FAK dependent endothelial barrier pathway. However, multiple associated proteins such as ZO-1 and claudin-5, could lead to protect endothelial barrier.[41] It is unclear LPA$_2$ specifically through which pathway regulates ZO-1 and claudin-5. Further investigations are required to have a better understanding of the precise mechanisms in the future.

In summary, our data reveal for the first time a novel, selective and causal role of LPA$_2$ in protecting against endothelial barrier disruption, lung impairment, and mortality in LPS-mediated sepsis model in vivo, and in mediating the endothelial barrier in LPS-induced MLMECs in vitro. The potential protective role of LPA$_2$ results from activation of the PLC-PKC-FAK pathway, as well as restoration of the endothelial AJs and TJs proteins VE-cadherin, ZO-1 and claudin-5. In consistent, activation of LPA$_2$ with DBIBB protects against endothelial barrier function and improve ALI in sepsis. These findings suggest that targeting LPA$_2$ and its downstream signaling pathways may prove to be an innovative therapeutic strategy for the treatment of septic ALI.

Conclusion

In conclusion, the present study provides a novel mechanism of endothelial barrier protection and suggest that LPA$_2$ may contribute to the clinical therapeutic effects of septic ALI. Mechanisms may be involved that LPA- LPA$_2$ signaling alleviate vascular endothelial permeability through the PLC-PKC-FAK pathway (Figure 9).

Ethical Statement

Acknowledgments

Disclosure

The authors declare no conflicts of interest associated with this work.

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