A Critical Role for Phosphatidylinositol (3,4,5)-Trisphosphate–Dependent Rac Exchanger 1 in Endothelial Junction Disruption and Vascular Hyperpermeability

Ram P. Naikawadi, Ni Cheng, Stephen M. Vogel, Feng Qian, Dianqing Wu, Asrar B. Malik, Richard D. Ye

Rationale: The small GTPase Rac is critical to vascular endothelial functions, yet its regulation in endothelial cells remains unclear. Understanding the upstream pathway may delineate Rac activation mechanisms and its role in maintaining vascular endothelial barrier integrity.

Objective: By investigating phosphatidylinositol (3,4,5)-trisphosphate–dependent Rac exchanger 1 (P-Rex1), one of the Rac-specific guanine nucleotide exchange factors previously known for G protein–coupled receptor signaling, we sought to determine whether Rac-guanine nucleotide exchange factor is nodal for signal integration and potential target for drug intervention.

Methods and Results: Using gene deletion and small interference RNA silencing approach, we investigated the role of P-Rex1 in human lung microvascular endothelial cells. Tumor necrosis factor α (TNF-α) exposure led to disruption of endothelial junctions, and silencing P-Rex1 protected junction integrity. TNF-α stimulated Rac activation and reactive oxygen species production in a P-Rex1–dependent manner. Removal of P-Rex1 significantly reduced intercellular adhesion molecule-1 expression, polymorphonuclear leukocyte transendothelial migration, and leukocyte sequestration in TNF-α–challenged mouse lungs. The P-Rex1 knockout mice were also refractory to lung vascular hyperpermeability and edema in a lipopolysaccharide-induced sepsis model.

Conclusions: These results demonstrate for the first time that P-Rex1 expressed in endothelial cells is activated downstream of TNF-α, which is not a G protein–coupled receptor agonist. Our data identify P-Rex1 as a critical mediator of vascular barrier disruption. Targeting P-Rex1 may effectively protect against TNF-α– and lipopolysaccharide-induced endothelial junction disruption and vascular hyperpermeability.

Key Words: acute lung injury ■ endothelial dysfunction ■ guanine nucleotide exchange factors ■ pulmonary edema ■ reactive oxygen species ■ small GTPases ■ vascular permeability

Vascular endothelial cells form the lining of blood vessels and separate the underlying tissue from circulating blood. Disruption of the endothelial barrier leads to increased vascular permeability to plasma proteins and inflammatory cells, resulting in edema as seen in acute lung injury and in its more severe form, acute respiratory distress syndrome.4 Vascular permeability can be transcellular or paracellular. Transcellular permeability involves the formation of transport vesicles, whereas paracellular permeability requires disruption of the adherens junctions between 2 adjacent endothelial cells. Vascular endothelial (VE)-cadherin is an endothelial-specific marker of adherens junctions and a determinant of integrity of endothelial junctions. Increased vascular permeability is associated with extravasation of leukocytes into the underlying tissue,4 and VE-cadherin has been proposed to play a role in leukocyte transmigration.

Several proinflammatory factors are released into the blood stream during an inflammatory response, among which thrombin, tumor necrosis factor α (TNF-α), interleukin-1β, and histamine are known to disrupt the endothelial barrier. TNF-α is one of the most commonly encountered proinflammatory cytokines in pathological conditions, such as sepsis.6 Elevated TNF-α levels are found in the bronchoalveolar lavage (BAL) fluid7 and plasma8 of patients with acute respiratory...
distress syndrome. TNF-α increases the permeability of pulmonary microvascular endothelial barrier and causes edema in animals. However, the mechanisms of TNF-α–induced endothelial barrier dysfunction are not clearly understood. Tyrosine phosphorylation of VE-cadherin, production of reactive oxygen species (ROS), and activation of the small GTPase Rac have been associated with TNF-α–induced endothelial barrier dysfunction.

Rac is a monomeric GTPase of ≈21 kDa. In endothelial cells, Rac activation downstream of G protein–coupled receptors (GPCRs), such as the thrombin receptor protease-activated receptor-1, induces reannealing of endothelial junctions during endothelial barrier repair phase. However, it was also reported that introduction of a constitutively activated Rac led to endothelial barrier dysfunction. Similar findings were reported in endothelial cells stimulated with vascular endothelial growth factor and platelet-activating factor. In phagocytes, Rac is known for its role in nicotinamide adenine dinucleotide phosphate oxidase (Nox) activation and superoxide production. Genetic deletion or silencing of the Rac gene showed Rac-specific involvement in endothelial barrier function. Using reverse transcriptase polymerase chain reaction, P-Rex1 transcript was found in 3 different types of endothelial cells tested, including HLMVECs, human pulmonary artery endothelial cells, and human umbilical vein endothelial cells.

Methods

Endothelial Expression of P-Rex1 and Its Role in the Regulation of Endothelial Barrier Function

P-Rex1 was originally identified in neutrophils and neurons, and its function outside these cells remains unclear. We examined P-Rex1 expression in endothelial cells and its potential involvement in endothelial barrier function. Using reverse transcriptase polymerase chain reaction, P-Rex1 transcript was found in 3 different types of endothelial cells tested, including HLMVECs, human pulmonary artery endothelial cells, and human umbilical vein endothelial cells. The expression level of P-Rex1 protein in these endothelial cells was comparable with that in bone marrow–derived macrophages.

To assess the role of P-Rex1 in endothelial barrier function, HLMVECs were treated with P-Rex1–specific siRNA to reduce P-Rex1 expression. Sc-siRNA was used as a negative control. An ≈80% reduction in P-Rex1 protein level was obtained. The siRNA-transfected cells were then subjected to measurement of changes in transendothelial electric resistance (TER) after TNF-α stimulation, which increases vascular permeability. As expected, the control (sc-siRNA–transfected) cells showed a decrease in TER culminating 4 to 5 hours after stimulation, suggesting barrier disruption. In comparison, P-Rex1 siRNA–transfected cells showed much less barrier disruption (Figure 1A). Based on the quantification of barrier disruption against absolute resistance values (Figure 1B), P-Rex1 is a necessary component for TNF-α–induced loss of TER, which reflects endothelial cell barrier dysfunction. TNF-α–induced barrier dysfunction is not a consequence of endothelial cell apoptosis, because the majority of transfected HLMVECs remained healthy after TNF-α treatment in DNA fragmentation assay (Online Figure II).

Fluorescent imaging analysis was conducted to examine barrier dysfunction of HLMVECs, characterized by intercellular gap formation (Figure 1C). The endothelial adherens junctions were detected with an anti–VE-cadherin antibody.
After stimulation with TNF-α, sc-siRNA–transfected endothelial cells displayed discontinuities between neighboring cells (marked with arrows). In comparison, HLMVECs receiving P-Rex1 siRNA showed minimal alteration of barrier integrity. The area of interendothelial gaps was quantified (Figure 1D), and the changes were significant (*P*<0.05). Thus, data from both TER and imaging analysis support a role for P-Rex1 in TNF-α–induced disruption of endothelial barrier.

**P-Rex1 Is Essential for TNF-α–Induced Rac Activation**

Recent studies have shown that TNF-α, at concentrations that induce opening of interendothelial junctions, activates the small GTPase Rac.16 However, the GEF responsible for TNF-α–induced Rac activation in endothelial cells remains unidentified. To explore the signal transduction pathway downstream of TNF-α stimulation, we examined possible involvement of P-Rex1 in Rac activation.

TNF-α induced a rapid and transient Rac activation in HLMVECs, which peaked within 1 minute and continued for 2 minutes before it began to decrease (Figure 2A). Based on Rac-GTP pull-down assay, TNF-α induced up to an 8-fold increase in Rac activation compared with unstimulated cells (Figure 2B). In HLMVECs receiving P-Rex1 siRNA, Rac activation was significantly reduced, indicating that P-Rex1...
is required for TNF-α–induced Rac activation. In addition, the Rac inhibitor NSC23766 prevented TNF-α–induced endothelial permeability as measured by TER (Online Figure III). We also transfected HLMVECs with a dominant-negative Rac (T17NRac) to exclude the nonspecific effects of NSC23766. The dominant-negative Rac ablated TNF-α–induced barrier dysfunction (Online Figure IV). These results strongly suggest that Rac is required for TNF-α–induced endothelial barrier dysfunction and P-Rex1 is an essential Rac GEF regulating TNF-α–induced Rac activation.

Several molecules were examined to exclude several possibilities that might have affected the outcome of our experiments. GEF-H1 is not only a Rac GEF but also a Rho GEF and has been implicated in TNF-α–induced epithelial barrier integrity. To determine whether it plays a role in our experiments, we used siRNA to knock down GEF-H1 in HLMVECs and performed TER. Our results indicate that, unlike P-Rex1, GEF-H1 removal did not reverse barrier dysfunction induced by TNF-α (Online Figure V). We also considered potential involvement of Rho, known to be responsible for endothelial barrier dysfunction. To exclude the involvement of Rho downstream of the TNF-α and P-Rex1 pathway, we performed Rho pull-down assay as detailed in the Methods section. The absence of P-Rex1 did not alter Rho activation (Online Figure VI). Therefore, although TNF-α has the ability to activate Rho, it does not require P-Rex1.

**TNF-α–Induced ROS Production Is P-Rex1–Dependent**

Nox has been implicated in TNF-α–induced endothelial barrier dysfunction. However, it is unclear how TNF-α regulates ROS production. In phagocytes, Rac is required for Nox activation, leading to ROS production. Therefore, we determined whether P-Rex1 is involved in endothelial ROS production through Rac activation. HLMVECs plated on gelatin-coated glass dishes were treated with either sc-siRNA or P-Rex1 siRNA and then stimulated with TNF-α. ROS production was measured by dihydrorhodamine 123. As shown in Figure 3A and quantified in Figure 3B, siRNA-mediated silencing of P-Rex1 led to a significant reduction in TNF-α–stimulated ROS production. Diphenyleneiodonium, a flavocytochrome inhibitor, diminished TNF-α–induced ROS production, suggesting that inducible activation of the Nox is required (Figure 3C and 3D). Similarly, NSC23766 reduced ROS production, supporting the notion that Rac is involved in TNF-α–induced Nox activation in HLMVECs (Figure 3C and 3D). We next determined whether suppression of ROS production could alter the integrity of the HLMVEC monolayer. Diphenyleneiodonium-treated cells were refractory to TNF-α–induced decrease in TER (Figure 3E and 3F). These results demonstrate a correlation between endothelial P-Rex1 and TNF-α–induced ROS production, leading to a loss of barrier function.

**Silencing P-Rex1 Prevents TNF-α–Induced Src Activation and VE-Cadherin Phosphorylation**

We examined the role of P-Rex1 in regulating TNF-α–induced tyrosine phosphorylation of VE-cadherin because this has been reported to be critical to the loss of vascular integrity. In sc-siRNA–transfected HLMVECs, TNF-α treatment led to phosphorylation of VE-cadherin at 10 minutes and peaked at 20 minutes (Figure 4A and 4B). TNF-α–induced VE-cadherin phosphorylation was diminished in HLMVECs transfected with P-Rex1 siRNA (Figure 4A and 4B), suggesting that VE-cadherin was phosphorylated in a P-Rex1–dependent manner. It was reported that the Src family protein tyrosine kinases undergo ROS-dependent phosphorylation that is required for VE-cadherin phosphorylation. To test whether P-Rex1 is required for Src activation downstream of TNF-α stimulation, HLMVECs transfected with sc-siRNA or P-Rex1 siRNA were stimulated with TNF-α for the indicated time points, and phosphorylation of c-Src at Tyr416 was determined. HLMVECs receiving P-Rex1 siRNA displayed >70% reduction in phosphorylation of Tyr416 at 5 and 10 minutes compared with cells transfected with sc-siRNA (Figure 4C and 4D), indicating that P-Rex1 is required for TNF-α–induced Src activation.

**Signaling Mechanism of TNF-α–Induced P-Rex1 Activation**

P-Rex1 resides in the cytosol of resting neutrophils and translocates to membrane on cell activation. We determined whether P-Rex1 is translocated to plasma membrane in TNF-α–stimulated endothelial cells. HLMVECs were stimulated with TNF-α for 0, 1, and 2 minutes, and P-Rex1 in the membrane

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**Figure 2. Role for phosphatidylinositol (3,4,5)-trisphosphate–dependent Rac exchanger 1 (P-Rex1) in tumor necrosis factor (TNF)-α–induced Rac activation.** A, Scrambled (sc) and P-Rex1 small interference RNA (siRNA)–transfected human lung microvascular endothelial cells were serum starved and stimulated with 25 ng/mL of TNF-α for 1, 2, and 5 minutes. Rac pull-down with glutathione S-transferase–p21 activated kinase binding domain was performed, and active Rac1 was detected by Western blotting. Total Rac1 indicates loading control. B, Bar graph showing quantification of Western blotting from 3 experiments. Data shown are mean±SEM. Fold change is calculated based on the ratio of active Rac1 to total Rac1. **P<0.01.
and cytosolic fractions was determined. P-Rex1 underwent membrane translocation as early as 1 minute and continued to increase at 2 minutes (Figure 5A–5C). We also used an additional approach to test the membrane translocation of P-Rex1. HLMVECs were unstimulated or stimulated with TNF-α for 2 minutes. The cells were then permeabilized, fixed, and incubated with anti–P-Rex1 and an Alexa fluor 488–conjugated secondary antibody. Images acquired by confocal microscopy showed accumulation of P-Rex1 to the membrane periphery after stimulation (Figure 5D). Pretreatment of HLMVECs with the PI3K inhibitor LY294002 prevented membrane translocation of P-Rex1 (Figure 5D), suggesting that it is PI3K-dependent in TNF-α–stimulated HLMVECs.

Earlier studies have shown that P-Rex1 activation downstream of GPCRs requires both PI3K and the βγ subunits of heterotrimeric G proteins. However, TNF-α is not a GPCR agonist and how a non-GPCR activates P-Rex1 remains unclear. To determine whether Gβγ subunits are involved in TNF-α signaling, we pretreated HLMVECs with Gβγ modulator II (Gallein; 3′,4′,5′,6′-tetrahydroxyspirol[isobenzofuran-1(3H),9′-(9H)xanthen]-3-one), which inhibits conformational changes of Gβγ subunits and blocks Gβγ-dependent activation of PI3K and Rac in human leukemia-60 cell line (HL-60) cells. Pretreatment with Gallein did not affect TNF-α–induced membrane translocation of P-Rex1 (Figure 5D, lower panels), whereas it significantly inhibited thrombin-induced calcium
mobilization in HLMVECs (Figure 5E and 5F). These findings suggest that Gβγ is not indispensable in TNF-α-induced P-Rex1 activation, but PI3K is necessary. The requirement of PI3K for P-Rex1 activation in TNF-α–stimulated endothelial cells was also confirmed when HLMVECs pretreated with the PI3K inhibitor LY294002 displayed reduced Rac activation by >50% (Online Figure VIIA and VIIB).

**P-Rex1 Knockout Mice Display Reduced Lung Vascular Permeability and Edema**

To determine an in vivo function of P-Rex1 in acute lung injury, wild-type (WT) and P-Rex1 knockout mice were instilled intratracheally with TNF-α. Changes in lung vascular permeability were evaluated based on the accumulation of Evans blue albumin (EBA) after tail vein injection. Significantly less accumulation of EBA was seen in the lungs of mice lacking P-Rex1, compared with WT controls (Figure 6A and 6B). The in vivo role of P-Rex1 in lung edema was also evaluated based on lung wet-to-dry weight ratio after intratracheal instillation of TNF-α. Again, the P-Rex1 knockout mice had significantly less edema compared with WT controls (Figure 6C). These results demonstrate a critical role for P-Rex1 in the dynamic regulation of lung vascular permeability in vivo.

Lipopolysaccharide-induced sepsis is a clinically relevant model of acute lung injury. We tested a potential role for P-Rex1 in this model where barrier dysfunction contributes to the pathological changes. WT and P-Rex1 knockout mice were intraperitoneally injected with lipopolysaccharide or PBS for 6 hours, which produced septic signs such as decreases in leucocyte count and platelet count (Online Figure XI). The lungs were subjected to Kf,c measurements (Figure 6D) and EBA dye leakage measurement (Figure 6E). Both the Kf,c and EBA data showed that P-Rex1 knockout mice have significantly less lung microvascular capillary filtration and EBA leakage, indicating a role for P-Rex1 in lipopolysaccharide-induced barrier dysfunction during sepsis.

**Role of Endothelial P-Rex1 in PMN Transmigration**

In acute lung injury, there is marked infiltration of PMNs and macrophages in the lungs. To determine infiltration of phagocytes into BAL fluid, WT and P-Rex1 knockout mice were instilled intratracheally with PBS or 0.5 μg of murine recombinant TNF-α. After 24 hours, mice were anesthetized and BAL fluid was collected. BAL fluid obtained from P-Rex1 knockout mice showed significantly less PMNs and macrophages compared with WT mice (Figure 7A and 7B). This result indicates that P-Rex1 is necessary for transendothelial migration of these leukocytes. In a parallel experiment, WT and P-Rex1 knockout mice were intratracheally injected with murine TNF-α for 24 hours followed by collection of lungs for histological analysis. Hematoxylin and eosin staining showed significantly less cellular infiltration and interstitial tissue thickening in P-Rex1 knockout mouse lungs compared with WT lungs (Online Figure VIII).

Because the knockout approach results in a loss of P-Rex1 in all tissues, we next determined the relative contribution of P-Rex1 in endothelial cells versus PMNs to the reduced PMN transendothelial...
migration of WT and P-Rex1−/− PMNs (a more detailed version of the experiment, with ligand controls included, is shown in Online Figure X). HLMVECs transfected with sc-siRNA or P-Rex1 siRNA were plated on 3-μm membrane pore inserts. PMNs were isolated concurrently from both WT and P-Rex1−/− mice and applied to the HLMVEC monolayer, which received sc-siRNA (Figure 7C, filled bars) or P-Rex1 siRNA (Figure 7C, open bars) and stimulated with TNF-α (25 ng/mL) for 4 hours. Eliminating P-Rex1 from the endothelial cells caused a significant reduction in PMN transmigration, which applies to both WT and P-Rex1−/− PMNs (Figure 7C). In comparison, removal of P-Rex1 from PMNs does not significantly impact cell migration in this experiment (ns, Figure 7C). Based on these findings, we concluded that endothelial P-Rex1 plays an important role in PMN transendothelial migration.

We have also taken an ex vivo approach to determine the effect of P-Rex1 in PMN transmigration into the lung tissue. Lungs from WT and P-Rex1 knockout mice were perfused to remove blood cells and then exposed to murine TNF-α. Freshly isolated PMNs from WT and P-Rex1−/− mice were radiolabeled with 111Indium oxine and perfused through WT and P-Rex1−/− lungs, or vice versa. As shown in Figure 7D, the P-Rex1−/− lungs showed significantly less radioactivity accumulation than WT lungs, suggesting that absence of P-Rex1 in lung tissue could significantly reduce PMN transmigration. In contrast, no significant difference in radioactivity accumulation was observed in WT lungs perfused with either WT PMNs or P-Rex1−/− PMNs (Figure 7D). These findings suggest that endothelial P-Rex1 is highly important in PMN transmigration into the lung tissue.

**P-Rex1 Is Important for TNF-α–Induced ICAM-1 Expression in Endothelial Cells**

In the above experiments, we observed that TNF-α treatment of HLMVECs is necessary for PMN transmigration. Several
coefficient (Kf,c), indicative of vascular leakiness, was determined. Ex vivo lung perfusion was performed, and capillary filtration was assessed for lung microvascular permeability. Mice were intratracheally instilled with PBS or lipopolysaccharide (LPS; 5 μg/kg body weight) for 6 hours and were assessed for lung microvascular permeability. Multiple means comparison was performed by the Tukey method. *P<0.05. **P<0.01.

Methods section. Multiple means comparison was performed by the Tukey method. *P<0.05. **P<0.01.

Lung microvascular permeability and edema formation in wild-type (WT) and phosphatidylinositol (3,4,5)-trisphosphate–dependent Rac exchanger 1 (P-Rex1)−/− mice. A, WT and P-Rex1−/− mice were intratracheally instilled with PBS or tumor necrosis factor (TNF)-α (0.5 μg/mouse) for a total of 6 hours. At 5.5 hours, mice were intravenously injected with Evans blue albumin (EBA; 20 mg/kg body weight). Lungs were collected 30 minutes later for assessment of damage. Representative samples from a total of 5 experiments are shown. B, Quantification of EBA accumulated in the lung (n=5) homogenates was conducted as described in the Methods section. C, Lung wet weight to dry weight ratio was determined after intratracheal instillation of PBS or TNF-α (0.5 μg/mouse) for 6 hours. D, WT and P-Rex1−/− mice were intraperitoneally injected with PBS or lipopolysaccharide (LPS; 5 μg/kg body weight) for 6 hours and were assessed for lung microvascular permeability. Ex vivo lung perfusion was performed, and capillary filtration coefficient (Kf,c), indicative of vascular leakiness, was determined as described in the Methods section. n=3, where n is the number of mice that have undergone treatment in each group. E, WT and P-Rex1−/− mice were intraperitoneally given LPS or PBS as in D, and EBA injection was performed as in A. Quantification of dye accumulation in lungs was conducted as described in B. A total of 5 mice were used in each group. For bar graphs (B–D), data shown are mean±SEM, and statistical analysis was conducted as described in the Methods section. Multiple means comparison was performed by the Tukey method. *P<0.05. **P<0.01.

Discussion

The present study examines P-Rex1 expression in endothelial cells and its role in mediating TNF-α–induced increase in vascular endothelial permeability. Many new findings were made. (1) P-Rex1 is highly expressed in vascular endothelial cells and plays important roles in these cells. (2) Our results show for the first time that P-Rex1 can be activated by a non-GPCR, in this case the TNF receptor, in endothelial cells. (3) This study reaffirms a role for Rac in TNF-α–induced vascular endothelial dysfunction, which has been an unsettled issue. (4) P-Rex1 activation leads to ROS production in endothelial cells. (5) Endothelial P-Rex1 is important for PMN transmigration into the lung tissue. These findings are summarized schematically in a working model (Figure 8).

P-Rex1 Expression and Functions in Lung Vascular Endothelial Cells

In this model, P-Rex1 is a major Rho GEF downstream of TNF-α receptor in endothelial cells. Before this study, P-Rex1 is mainly known for its functions in the brain and in PMNs, where it was first discovered.26 Thus, the finding of P-Rex1 in various endothelial cells suggests that this Dbl family Rho GEF is more broadly expressed than previously thought. Our data demonstrate that P-Rex1 is expressed in vascular endothelial cells, and it mediates TNF-α–induced vascular permeability as well as PMN infiltration into the lung tissue. These functions of P-Rex1 require Rac activation, which leads to Nox-dependent ROS production, c-Src activation, and VE-cadherin phosphorylation in HLMVECs. Our in vitro results are corroborated by data from P-Rex1 knockout mice, which are refractory to TNF-α–induced increase in vascular permeability in the lungs as demonstrated by reduced edema. Collectively, these results demonstrate that endothelial P-Rex1 is critical to TNF-α signaling that leads to increased vascular endothelial permeability.

P-Rex1 Activation by a Non-GPCR

Our model places P-Rex1 downstream of the TNF-α receptor, whereas published reports depict P-Rex1 as a Rac-specific GEF activated by GPCRs.26 In endothelial cells, P-Rex1 can be activated by a GPCR.42 Our finding that P-Rex1 is activated by TNF-α is totally unexpected because TNF-α is not known to couple to G proteins. We observed rapid membrane translocation of P-Rex1 in endothelial cells, which is characteristic of its activation.40 It is also evident that TNF-α stimulates P-Rex1–dependent Rac activation in HLMVECs. Because the time course of Rac activation and P-Rex1 membrane translocation is consistent with that of
GPCR signaling, we examined the requirement for P-Rex1 activation in TNF-α-stimulated cells. A reported feature of P-Rex1 is its dependence on phosphatidylinositol (3,4,5)-trisphosphate and Gβγ for activation.26,27 Our results show that TNF-α-induced Rac activation is PI3K-dependent. However, we observed no effect for the Gβγ inhibitor Gallein to affect TNF-α-induced P-Rex1 membrane translocation, thus challenging the conventional view that Gβγ is required for P-Rex1 activation. It is notable that TNF-α signaling has not been associated with activation or transactivation of heterotrimeric G proteins, although TNF-α is known for its activation of PI3K,41 suggesting that TNF-α-induced phosphatidylinositol (3,4,5)-trisphosphate production might be sufficient to trigger P-Rex1 activation in HLMVECs.

A Role for Rac in Endothelial Barrier Dysfunction

In endothelial cells stimulated with GPCR agonists such as thrombin, a reversible endothelial barrier disruption occurs. Although there are multiple pathways for disrupting the endothelial barrier, Rac has been associated with reannealing of junctions in response to GPCR activation.18 Contrary to this view, there is evidence supporting a role of Rac in endothelial

Figure 7. Role for phosphatidylinositol (3,4,5)-trisphosphate–dependent Rac exchanger 1 (P-Rex1) in leukocyte transmigration. Wild-type (WT) and P-Rex1−/− mice were instilled intratracheally with PBS or 0.5 μg of murine recombinant tumor necrosis factor (TNF)-α. After 24 hours, mice were anesthetized, bronchoalveolar fluid (BAL) was collected, and total leukocytes, neutrophils, and macrophages were counted. The number of neutrophils (A) and macrophages (B) in the BAL was shown, using 3 mice per group. C, Transmigration of WT or P-Rex1−/− neutrophils across monolayers of human lung microvascular endothelial cells (HLMVECs). The endothelial cells were transfected with either scrambled small interference RNA (sc siRNA) or P-Rex1 siRNA, plated on gelatin-coated 3-mm pore filters, and treated with TNF-α (25 ng/mL) for 2 hours. The formyl peptide N-formyl-Met-Ile-Val-Ile-Leu (100 nmol/L) was placed in bottom wells. A detailed figure set including all controls is shown in Online Figure X. Data shown are means±SEM from 3 experiments. D, Neutrophil sequestration assay was performed using 111Indium oxine–labeled polymorphonuclear leukocytes (PMNs) from WT or P-Rex1−/− mice, perfused across WT or P-Rex1−/− mouse lungs. The retained radioactivity was expressed as ratio of tissue cpm to infusate cpm. Data shown are means±SEM from 3 separate experiments, with 3 mice in each group. Statistical analysis was done by 2-factor ANOVA to analyze group mean comparison. Multiple means comparison was performed by the Tukey method. *P<0.05; **P<0.01. ns indicates not significant.

Figure 8. Schematic model showing the involvement of phosphatidylinositol (3,4,5)-trisphosphate–dependent Rac exchanger 1 (P-Rex1) in tumor necrosis factor (TNF)-α–induced endothelial barrier dysfunction. See the Discussion section for a description of the role of P-Rex1 in TNF-α–induced Rac activation, reactive oxygen species (ROS) production, src-dependent tyrosine phosphorylation of vascular endothelial (VE)–cadherin, and intercellular adhesion molecule-1 (ICAM-1) expression. Nox indicates nicotinamide adenine dinucleotide phosphate oxidase; NF-κB, nuclear factor-κB; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PI3K, phosphoinositide 3-kinase; TNFR, tumor necrosis factor receptor.
barrier dysfunction. For instance, van Wetering et al.\(^5\) reported that expression of a constitutively active Rac in human umbilical vein endothelial cells could cause changes leading to increased vascular permeability. Rac has been implicated in TNF-\(\alpha\) and vascular endothelial growth factor–induced increase in vascular permeability.\(^16,20\) As shown in our model, GTP-bound Rac is required for TNF-\(\alpha\)-induced ROS production, and the Rac inhibitor NSC23766 abrogated TNF-\(\alpha\)-induced vascular endothelial permeability in HLMVEC. Similarly, dominant-negative RacT17N, when expressed in endothelial cells, prevented TNF-\(\alpha\)-induced endothelial barrier dysfunction. Our data support a role for Rac in TNF-\(\alpha\)-induced endothelial barrier dysfunction, which is mediated through P-Rex1 activation and ROS production. These findings connect a Rho GEF to previously reported functions of ROS in the regulation of vascular permeability.\(^13,19,44-46\)

**Endothelial P-Rex1 and PMN Transmigration**

Disruption of endothelial barrier is a triggering factor for infiltration of PMNs into tissues.\(^1-4\) We tested whether TNF-\(\alpha\)-induced disruption of endothelial barrier aggravates PMN infiltration, and if so, whether blocking P-Rex1 expression in endothelial cell prevents PMN transmigration. We found that, in the absence of P-Rex1, PMN transmigration was significantly reduced. Much fewer PMNs and macrophages were present in BAL fluid of P-Rex1 knockout mice compared with WT mice after instillation of murine recombinant TNF-\(\alpha\) into the airways. Silencing endothelial P-Rex1 expression resulted in significantly less PMN transmigration compared with sc-siRNA–transfected endothelium. This phenomenon was also confirmed ex vivo by PMN sequestration studies where radiolabeled WT or P-Rex1 knockout PMNs were perfused into WT and P-Rex1 knockout mice, respectively, and vice versa. It seems that the crosstalk between PMN and endothelial cell is key to PMN transendothelial migration.\(^2\) Our Western blot data showed that there is significantly less ICAM-1 expression in the absence of P-Rex1. As depicted in the model, P-Rex1 seems to play a role in the regulation of TNF-\(\alpha\)-induced ICAM-1 expression, which involves nuclear factor-\(\kappa\)B activation. The mechanism underlying P-Rex1 regulation of nuclear factor-\(\kappa\)B activation is yet to be delineated. An increase in ICAM-1 expression may in turn affect PMN transendothelial migration.

**A Potential Target for Therapeutic Intervention**

Our findings strongly implicate P-Rex1 in regulating TNF-\(\alpha\)-induced vascular permeability and lung edema. This function is mediated through the activation of Rac and generation of ROS, thus promoting endothelial barrier disruption and transendothelial PMN migration. These results demonstrate that downregulation of P-Rex1 may affect multiple proinflammatory pathways, and P-Rex1 may be a new therapeutic target in controlling lung vascular injury and PMN-mediated lung inflammation.

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**Disclosures**

None.

**References**


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Supplemental Material

For “A critical role for P-Rex1 in endothelial junction disruption and vascular hyper-permeability”

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Supplemental Methods

Animals: The generation of P-Rex1 knockout mice and its characterization has been reported previously 1. P-Rex1 heterozygous pairs were used for breeding. The resulting litter was genotyped. For all animal experiments, P-Rex1−/− mice (CD1/S129 background), and their P-Rex1+/+ littermate controls weighing 25-35 grams were used. Mice were maintained in pathogen-free conditions. All animal procedures were carried out using protocols approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago.

Materials: Human lung microvascular endothelial cells (HLMVEC), endothelial basal medium (EBM-2), and endothelial growth medium bullet kit (EGM-2MV) were purchased from Lonza (Walkersville, MD). Rac1 inhibitor (NSC23766), PI3K inhibitor (LY294002) and the Gβγ modulator II (Gallein) were purchased from EMD4biosciences (Gibbstown, NJ). P-Rex1 antibody was a kind gift from Dr. Marcus Thelen (The institute for Research in Biomedicine, Bellinzona, Switzerland). Rac1 Antibody was purchased from Millipore (Billerica, MA). Anti-phospho-Src (Y416) and anti-GEF-H1 were purchased from Cell Signaling Technologies (Danvers, MA). VE-cadherin antibody, c-Src antibody, ICAM-1 antibody, PY-20 antibody and protein A/G plus agarose were purchased from Santa Cruz biotechnology (Santa Cruz, CA). Alexafluor 488 conjugated secondary antibody, Fura-2AM and Hoechst stain were purchased from Invitrogen (Carlsbad, CA). Human and murine recombinant TNF-α were purchased from Peprotech (Rocky Hill, NJ). Halt Protease inhibitor cocktail was purchased from Thermo Scientific (Hudson, NH). TACS blue label apoptosis kit was purchased from Trevigen (Gaithersburg, MD). Dihydrorhodamine 123 and LPS were purchased from Sigma (St. Louis, MO). Glass bottom dishes were purchased from Mattek Corporation (Ashland, MA).

Endothelial Cell Culture: HLMVECs were grown in EBM2 medium supplemented with 2.5% FBS and EGM2-MV growth factors in 0.1% gelatin-coated flasks. ECs had typical cobblestone morphology and were grown to contact-inhibited monolayers in a 37°C incubator with 5% CO2 and 95% atmospheric air. For all experiments, HLMVECs were used between passages 3-5.

Cell transfection: Transfection was performed by nucleofection per manufacturer’s recommendations (Lonza Walkersville, MD). Briefly, cells were harvested by trypsinization and cell density was determined. Approximately 0.5 X 10⁶ cells were used per transfection. The cell pellet was resuspended in a mixture of 100 μl of nucleofector solution and scrambled siRNA (5’-AGTACGTCTACGATACGGTT-3’) or P-Rex1 siRNA (5’-GCAACGACTTCAA GCTGGTGGAGAA-3’) was used at a concentration of 100 nmol/L. For GEF-H1 knockdown, we have used GEF-H1-specific siRNA pool containing 4 oligos 2. Oligo 6 (5’-GAAUUAAGAU GGAGUUGCAUU-3’), oligo 7 (5’-GUCGGAACUGGUGUAAU-3’), oligo 8 (5’-GAAGGUAGCAGCGUCUGUU-3’) and oligo 9 (5’-CCACCGAAACUGGCAUUCUU-3’). The suspended mixture was transferred into a cuvette (provided in kit) and was placed in cuvette holder of the nucleofection device. An appropriate program for human lung microvascular endothelial cells was selected on the nucleofector. After completion of the
nucleofection, 500 µl of culture medium (EGM2-MV) was added to the nucleofected cell suspension. The cell suspension was immediately transferred to a 10 cm dish (pre-coated with 0.1% gelatin) with culture medium. The transfection medium was replaced with complete medium after 5 h. Experiments were performed 48 h after transfection.

**RNA isolation and PCR:** RNA was isolated from HLMVECs, HUVECs and HPAECs per manufacturer’s instructions. Reverse transcription of isolated RNA was performed and the resulting cDNA was used for PCR reaction. For the PCR reaction, 1µl of cDNA, 0.5 µl of 10 µmol/L P-Rex1 forward primer (5’-CGGCGTGGTGTAGGATGATG-3’), 0.5 µl of 10 µmol/L P-Rex1 reverse primer (5’-TCTCCAGCTTGGTGCACAG-3’), 0.5 µl of 10 µmol/L GAPDH forward primer (5’-GGTCTCCTCTGTGACTTCAACA-3’), 0.5 µl of 10 µmol/L GAPDH reverse primer (5’AGCCAAATTGCTGTACTACA-3’), 12.5 µl of SYBR Green mix (2X), 0.5 µl of Taq polymerase were used and the reaction was made up to 25 µl with DEPC-treated water. The reaction was run for 35 cycles at 94°C for 2 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, 72°C for 7 min, and stored at 4°C. The PCR products were analyzed on a 1.2% agarose gel. To detect GEF-H1 in HLMVECs, we used 0.5 µl of 10 µmol/L GEF-H1 forward primer (5’-ACACGCTTCCTAGCCAGCTATT-3’) and 0.5 µl of 10 µmol/L GEF-H1 reverse primer (5’-AATTGCTGGAAAGCTTGTCTCGG-3’). Negative controls, consisting of reaction mixtures containing all components except target RNA, were included with each of the reverse transcription PCR runs. To over rule the contamination with genomic DNA in PCR products, representative PCR mixtures for the target gene were run in the absence of superscript III RT enzyme that resulted no PCR products. In addition, the primers used crossed over the exons also overruling genomic DNA contamination.

**Rac Pull down assay:** Rac activation in cells was determined by GST-PAK1-PBD probe as described previously 3 with minor modifications. HLMVECs were used for the assay and stimulated with TNF-α. At the end of stimulation, cells were washed and lysed with cell lysis buffer (6 mmol/L Na2HPO4, 4 mmol/L NaH2PO4, 1% Nonidet P-40, 150 mmol/L NaCl, 30 mmol/L MgCl2) supplemented with 1 mmol/L phenylmethlysulfonyl fluoride (PMSF), protease inhibitor cocktail I, 0.1 mmol/L Na3VO4, and 50 mmol/L NaF. After centrifugation of the cell lysate, 50 µl of the supernatant was aliquoted for detection of total Rac. The remaining sample was incubated with 30 µl of glutathione sepharose 4B beads (that were previously fused with purified PBD protein expressed in *Escherichia coli*) for 1 h at 4°C. Beads were pelleted and washed with Rac wash buffer followed by suspension in the loading buffer. Samples were analyzed on a 12% SDS-PAGE gel. The membrane was probed with Rac1 antibody for detection of GTP-Rac and total Rac.

**Determination of ROS formation:** HLMVECs were transfected with scrambled or P-Rex1 siRNA and plated on glass bottom dishes (Mattek corporation, Ashland, MA). ECs were washed with HBSS and incubated in 20 µmol/L of Dihydrorhodamine 123 4 in HBSS with 0.5% FBS for 30 min. A cell-permeant, DNA binding, Hoechst stain (2.5 µmol/L) was added to the cells for 10 min. At the end of incubation, the buffer with both the stains was aspirated and the cells were incubated with fresh phenol red free HBSS buffer with 0.5% FBS. The dish was then mounted on the stage of an LSM 510 laser scanning meta confocal microscope heated to 37°C. Dihydrorhodamine 123 undergoes excitation at 488 nm. A series of images were captured before and after stimulation with TNF-α. The intensity of fluorescence was quantified at regions of interest by Zeiss LSM 510 Meta confocal software.
**Immunofluorescence Microscopy:** HLMVECs grown on 0.1% gelatin coated coverslips, were treated as indicated, washed with cold PBS, fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized for 4 min in permeabilization-fixation medium (0.25% Triton X-100 in 4% paraformaldehyde). Cells were then washed in TBST followed by incubation for 30 min in the blocking buffer (1% bovine serum albumin in TBST). Primary antibodies prepared in blocking buffer were used at 1:200 dilutions for 1 h at room temperature. After thorough washing with TBST thrice, Alexa Fluor 488 secondary antibody prepared in blocking buffer was used at 1:200 dilution for 1 h at room temperature taking care to avoid exposure to light. After three more washes in TBST, mounting of the coverslips was done on glass slides with Prolong Gold Antifade mounting medium (Invitrogen, Carlsbad, CA) and images were acquired with Zeiss LSM 510 Meta confocal microscope.

**Preparation of Cell Lysates, Immunoprecipitation and Western Blotting:** HLMVECs were serum starved for 2 h in EBM2. After stimulation with TNF-α, cells were washed with ice cold PBS scraped in 1 ml of lysis buffer (50 mmol/L Tris-HCl, pH 7.4; NaCl; Nonidet P-40; 0.25% sodium deoxycholate; 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonylfluoride; 1 mmol/L sodium orthovanadate; 1 mmol/L sodium fluoride) containing protease inhibitor cocktail, sonicated on ice for 30 sec and centrifuged at 5000 X g for 5 min in a microcentrifuge at 4°C. The supernatant was used to determine protein concentration using a Bio-Rad protein assay kit. Equal volume of supernatants were adjusted to 1 mg of protein /ml, dissolved in 4X SDS sample buffer and denatured by boiling for 5 min and samples were separated on SDS-PAGE gels and analyzed by western blotting. For immunoprecipitation, cell lysates (1 mg of protein) were incubated in appropriate antibodies (1 µg/ml) overnight at 4°C, followed by incubation with A/G plus agarose beads (50 µl) for 1 h at 4°C and centrifuged in a microcentrifuge at 5000 X g. The pellets obtained were washed in lysis buffer twice followed by denaturation by boiling in 2X SDS sample buffer for 5 min. Samples were run on 10% SDS-PAGE gels transferred onto 0.2 µm nitrocellulose membrane, blocked in 5% milk in TBST buffer (10 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl and 0.1% Tween-20) and incubated with appropriate primary and secondary antibodies per manufacturer’s specifications. The proteins were detected by ECL kit, scanned and analyzed by Image J software (NIH).

**Cell Fractionation:** Cell fractionation is performed as described with minor modifications. Endothelial cells plated to confluency were stimulated with TNF-α for 0, 1 and 2 min. Ice cold PBS was added to stop the stimulation. Cells were pelleted in 500 µl of HES I buffer (0.25 mol/L sucrose, 20 mmol/L Tris pH 7.6, 1 mmol/L EDTA, protease inhibitor cocktail) . The pellet was sonicated for 10 sec followed by three freeze thaw cycles in liquid nitrogen and at 37°C. Samples were then centrifuged at 600 x g to remove unlysed cells. The total cell lysate obtained was layered on 500 µl of HES II buffer (1.12 mol/L sucrose, 20 mmol/L Tris pH 7.6, 1 mmol/L EDTA) and centrifuged at 16,000 x g for 60 min. The supernatant obtained was designated as cytosolic fraction. The solution at the interphase was collected from sucrose gradient, and transferred to new eppendorf tubes, suspended in HES I buffer and centrifuged at 16,000 x g for 60 min. The resulting pellet is plasma membrane fraction.

**Collection of bronchoalveolar fluid:** WT and P-Rex1−/− mice were instilled with PBS or 0.5 µg of murine recombinant TNF-α intratracheally. After 24 h, mice were anesthetized with an i.p injection of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) and bronchoalveolar fluid was collected by flushing the lungs with 1 ml of cold PBS. The BAL fluid was centrifuged, red cells lysed with ACK, and resuspended in 1 ml of PBS. The total number of cells was counted using a hemocytometer. A 500 µl aliquot was taken from each sample for cytoospin at 700 rpm for 5 min. Cells were dried for an hour and stained using Hema-3. Neutrophils and macrophages were
counted in twenty different fields by random selection. Based on the percentage of neutrophils and macrophages in each of the high power field, the total number of neutrophils and macrophages was calculated.

**Evans blue albumin dye extravasation:** The assay was carried out as described. Mice were anesthetized using ketamine (100 mg/kg) and xylazine (2.5 mg/kg). In deep state anesthesia, Evans blue dye albumin (EBA) (20 mg/kg) was injected into the tail vein 30 min before the termination of 6 h of PBS/LPS/TNF-α treatment to assess vascular leakage. An abdominal incision was made to expose the thoracic cavity. An incision was given on abdominal aorta to drain the blood and the lungs were perfused free of blood (perfusion pressure of 5 mm Hg) with phosphate-buffered saline (PBS) containing 5 mmol/L ethylenediaminetetraacetic acid. Lungs were excised out of thoracic cavity homogenized in PBS (1 ml per 100 mg of lung tissue), followed by addition of 2 volumes of formamide for 18 h at 60°C. At the end of incubation, the homogenate was centrifuged at 10,000 rpm for 30 min and the supernatant was used to determine optical density spectrophotometrically at 620 nm. A standard curve was plotted and EBA concentration in each sample was calculated as micrograms of Evans blue dye per gram of lung tissue.

**Determination of lung wet-to-dry weight ratio:** TNF-α (0.5 µg) was instilled intra-tracheally. After 6 h, mice were anesthetized with an i.p injection of ketamine (100 mg/kg) and xylazine (2.5 mg/kg). In deep state anesthesia, thoracotomy was performed and lungs were exposed. Lungs were excised and immediately weighed to get the wet weight. Lungs were dried in an oven at 60°C for 3 days and dry weight is recorded. Lung wet weight to dry weight ratio was calculated.

**Transendothelial Electrical Resistance:** *Electric Cell-Substrate Impedance Sensing (ECIS)*. Endothelial monolayer permeability was determined by measuring changes in electrical resistance using an electric cell-substrate impedance sensor (ECIS, Applied BioPhysics, Inc.) as previously published. Briefly, HLMVECs (Approximately 80,000 cells) were seeded onto ECIS 10E cultureware (0.8 cm²/well) pre-coated with 0.1% gelatin. The electrical impedance across the monolayer was measured at 1V, 4000 Hz with current flowing through 10 small gold electrodes per well plus one large counter-electrode, using the culture medium as source of electrolytes. Impedance was monitored by the lock-in amplifier, stored, and then used to calculate resistance and capacitance by the manufacturer’s software. Data is presented as a plot of normalized resistance vs. time. Absolute resistance values recorded were used to quantify the change in resistance from basal.

**Leukocyte transendothelial migration:** HLMVECs transfected with either sc-siRNA or P-Rex1 siRNA were plated on 3 µm gelatin coated porous inserts (Corning Incorporated, Corning, NY) and left overnight. Confluent monolayers were washed in HBSS followed by incubation with TNF-α for 2 h. Freshly isolated PMNs (0.1 ml of 2 X10⁶ cells/ml PMN suspension) were added to the endothelial monolayer and were allowed to transmigrate for 4 h. Transmigrated PMNs were collected in the RPMI buffer alone or in RPMI medium consisting of 100 nmol/L formyl peptide (fMIVIL) in the lower chamber. A small aliquot of the buffer was taken and cells were counted on a hemocytometer.

**Isolation of bone marrow-derived mouse neutrophils:** Mouse neutrophils were isolated from Femurs and tibias as described. Femurs and tibias were collected from mice following their sacrifice. Bones collected were flushed using HBSS prep (Ca²⁺/Mg²⁺-free Hank's balanced salt solution (HBSS) containing 0.5% BSA). Cells flushed were resuspended using 18 gauge needle and passed through the 70 µm strainer. Cells collected were centrifuged for 10 min at 1500 rpm.
A gradient is prepared by adding 3 ml of Nycoprep and underlaying 3 ml of 72% Percoll. The pellet obtained was resuspended in 2 ml of HBSS prep and layered on top of Nycoprep and centrifuged at 2400 rpm for 30 min. Neutrophils were collected from the interface of Nycoprep and Percoll. After a couple of washes in HBSS prep, cells were lysed in ACK buffer (155 mmol/L NH₄Cl, 10 mmol/L KHCO₃ and 111 µmol/L Disodium EDTA) to get rid of contaminating RBC’s followed by a wash with HBSS prep. The resulting pellet was resuspended in RPMI medium for further use.

**Calcium ratiometric assay:** HLMVECs were plated to confluency in glass bottom dishes (Mattek Corporation, Ashland, MA). ECs were given no pre-treatment or pre-treated with Gβγ modulator II. ECs were loaded with Fura-2/AM (2 µmol/L) for 20 min at 37°C followed by 3 washes. The dishes were mounted on the stage of calcium ratio imaging fluorescence microscope (Zeiss Axiovert 200) fitted with AxiocamHS camera. A baseline calcium measurement was recorded for selected cells. Under stable basal conditions, ECs were stimulated with Thrombin and observed for calcium release at the excitation wavelength of 340 nm and 380 nm. The emission wavelength was 510 nm. Calcium levels were calculated as ratio of fluorescence at 340/380 using Axiovision software (Zeiss).

**Determination of Apoptosis:** HLMVECs (scrambled and P-Rex1 siRNA transfected) were plated to confluency on gelatin-coated coverslips kept in 12 well dishes. ECs were stimulated with TNF-α for 10 h, washed and fixed in 3.7 % buffered formalin. Fixed cells were processed per manufacturer’s protocol (TACS blue label in situ apoptosis detection kit (Trevigen, Gaithersburg, MD). Fixed samples were immersed in PBS for 10 min followed by incubation of the coverslips with 50 µl of Cytonin for 20 min. After 2 washes, samples were drenched in Quenching solution for 5 min. After 1 wash in PBS, samples were immersed in 1X TDT labeling buffer for 5 min. Samples were covered with 50 µl of Labeling reaction mix and incubated at 37°C for 30 min. The samples were immersed in 1X TdT stop buffer for 5 min followed by 2 washes in PBS. Samples were covered with 50 µl of Antibody solution and incubated for 30 min at 37°C followed by 3 washes in 1X PBS-Tween, 2 min each. Samples were covered with 50 µl of Strep-HRP solution, incubated for 10 min at room temperature followed by 2 washes in PBS and once in deionized water. Samples were covered with 50 µl of TACS Blue Label for 3 min. Samples were washed in deionized water twice followed by counterstaining with nuclear fast red. Samples were washed by dipping 10 times in deionized water, air dried and dehydrated by dipping 10 times in 100% ethanol, clarified by dipping 10 times in O-xylene. Coverslips were mounted onto the slides using mounting medium. TACS nuclease treated samples served as positive controls. The slides were viewed under microscope (40X magnification).

**Neutrophil sequestration assay:** WT and P-Rex1−/− mice were anesthetized using ketamine (100 mg/kg) and xylazine (2.5 mg/kg). Tracheal cannulation was done and mice were kept on a ventilator. In deep state anesthesia, surgical procedure was performed and lungs were excised en bloc with heart and trachea under ventilation. Lungs were perfused (37°C, 2 ml/min, RPMI) to remove blood cells. Lung preparations underwent equilibration for 20 min followed by perfusion of these lungs with TNF-α, 2000 U/ml. Both WT and P-Rex1−/− lungs received an infusion of 1 million PMNs (purified from bone marrow of WT mice of the same genetic background and also from P-Rex1−/− mice) pre-labeled with 111Indium oxine via the side-arm of pulmonary-artery cannula. i.e, WT lungs independently received WT PMNs and P-Rex1−/− PMNs. P-Rex1−/− lungs also received WT PMNs and P-Rex1−/− PMNs independently. Lung preparations were rinsed for 30 min to remove non-adherent PMNs from the pulmonary vasculature. Lung tissue and sample of the infusate were counted for gamma radioactivity, and data from duplicate experiments was expressed as ratio of tissue cpm to total infused cpm.
**Lung histology:** WT and P-Rex1\(^{+/–}\) mice were intratracheally instilled with murine TNF-α (0.5 ug / mouse). After 24 h, mice were anesthetized using ketamine (100 mg/kg) and xylazine (2.5 mg/kg). In deep state anesthesia, an abdominal incision was made and lungs were exsanguinated followed by excision from abdominal cavity. Multiple lung tissue specimen were collected from both WT and P-Rex1\(^{+/–}\) mice for histological analysis. Lung sections were stained with haematoxylin and eosin.

**Capillary filtration (Kf,c):** WT and P-Rex1\(^{+/–}\) mice were administered with 5mg/kg body weight of LPS intraperitoneally. After 6 h, mice were anesthetized using ketamine (100 mg/kg) and xylazine (2.5 mg/kg) and heparinized to drain blood. Mice were kept on a ventilator and surgical procedure was performed in deep state anesthesia. Mice were exsanguinated and RPMI buffer (37°C, 2 ml/min, RPMI) was used to perfuse through the lungs. Lungs were excised en bloc along with the heart and trachea on ventilation free from thoracic cavity. Excised lungs were mounted on a weight transducer to record the changes in weight of the lung. After acquiring a stable baseline for 20 min, a step-up increase in pressure was applied to observe the change in weight of the lung as recorded electronically. An edematous lung shows a gain in weight that is calculated based on the slope recorded. Capillary filtration coefficient (Kf, c) is calculated based on the dry weight of the lung and the magnitude of the change in pressure.

**Collection of blood:** WT and P-Rex1\(^{+/–}\) mice were intraperitoneally injected with PBS or LPS (5 mg/kg body weight, 6 h). After 6 h, mice were anesthetized with an i.p injection of ketamine (100 mg/kg) and xylazine (2.5 mg/kg). Under deep stage anesthesia blood was collected from caudal vena cava and used for CBC (Complete Blood Count) analysis.

**Rho pull-down assay:** Rho pull-down assay was performed (as per manufacturer's instructions) using a kit from Thermoscientific. Human lung microvascular endothelial cells that were plated in 6 well dishes were lysed in lysis buffer provided. Cells were scraped, briefly incubated on ice for 5 min and centrifuged at 16,000 X g at 4°C for 15 min. A small sample of cell lysate was saved for BCA protein assay. A 50 % Glutathione resin beads slurry (100ul) was placed in spin cup with collection tube followed by centrifugation for 10-30 s at 6000 X g. The flow through was discarded. GST-Rhotekin-RBD (400 ug) was added to spin cup containing glutathione resin. Immediately, 700 ul of cell lysate was transferred to the spin cup, sealed and vortexed. The reaction mixture was incubated at 4ºC for 1 h with gentle rocking. The spin cup was centrifuged at 6000 X g for 10-30 sec. The spin cup was transferred to a new collection tube. Resin was washed with 400ul of lysis buffer and the tubes were inverted thrice followed by centrifugation at 6000 X g for 10 -30 sec. Washing step was repeated two more times. Spin cup was transferred to a new collection tube and 50 ul of 2X reducing sample buffer was added to the resin. Samples were vortexed and incubated at room temperature for 2 min. Samples were centrifuged at 6000 X g for 2 min. The eluted samples were heated for 5 min. Samples were electrophoresed on 12 % acrylamide gel.

**Statistical analysis:** ANOVA (one factor, two factor or three factor) was used for data analysis. Multiple means comparison was performed using Tukey’s post-hoc procedure. A family-wise type I error rate at 0.05 or less is considered statistically significant. Statistical analysis was done using the PROC ANOVA in SAS 9.2 (Cary, NC). Whereas, "ns" indicates that the comparisons made were not statistically significant at the family-wise type I error rate of 0.05. In addition, PROC t-test (two sample t-test) was used for analysis of data sets with only two groups. Comparisons that gave p-value less than 0.05 were considered significant.
Supplemental References:


Supplemental Figures

Online Figure I. Expression of P-Rex1 in endothelial cells.

(A) PCR detection of transcript of P-Rex1 in human lung microvascular endothelial cells (HLMVEC), human pulmonary artery endothelial cells (HPAEC), and human umbilical vein endothelial cells (HUVEC). The product with expected size of 226 bp was detected in all 3 endothelial cells. P-Rex1 cDNA was used as positive control, and GAPDH was used as loading control. (B) Protein expression of P-Rex1 in HLMVEC, HPAEC and HUVEC. Endogenous P-Rex1 (196 KDa) was detected using a P-Rex1 antibody. Macrophage cell lysate from WT and P-Rex1 knockout mice were used as positive and negative controls, respectively. β-actin was used as loading control. (C) siRNA-mediated knockdown of P-Rex1 expression in HLMVEC. EC were either untransfected (U) or transfected with scrambled (Sc) or P-Rex1 (P) siRNA. The level of P-Rex1 protein was detected with a monoclonal anti-P-Rex1 48 h after transfection. (D) Quantification by densitometry of the P-Rex1 protein expression after siRNA knockdown based on 3 independent experiments. Data shown are mean ± SEM. Asterisks (**): P < 0.01.
Online Figure II. Apoptotic index of HLMVEC stimulated with TNF-α.

(A) Scrambled (Sc) or P-Rex1 siRNA transfected HLMVECs were unstimulated (negative control) or stimulated with TNF-α (25ng/ml) and observed for DNA fragmentation as an indication of apoptosis. TACS nuclease treated cells were used as positive control. Nuclear fast red was used for counter staining. Apoptotic cells show intense TACS staining. (B) Number of apoptotic cells was counted in 20 random fields of each group based on 5 independent experiments. Data shown are mean ± SEM. Asterisks (**): P < 0.01, ns = not significant.
Online Figure III. Rac inhibitor (NSC-23766) ablates TNF-α-induced lung microvascular barrier permeability.

HLMVECs plated on electrodes in the wells of ECIS arrays were untreated or pre-treated with 50 μM Rac inhibitor for 1 h. After acquiring stable baseline for 20 min, EC were stimulated with 25ng/ml of TNF-α. (A) Representative graph showing real-time changes in barrier function among different groups indicated as normalized resistance. Arrow indicates time point of stimulation. (B) Quantification of data in terms of absolute resistance values in ohms at the 5h time point, subtracted from the basal resistance values. Data shown are mean ± SEM from 3 independent experiments. Asterisks (**): P < 0.01.
Online Figure IV. Dominant negative Rac significantly reduced TNF-α-induced lung microvascular permeability.

A) HLMVECs were untransfected or transfected with dominant negative Rac (T17NRac) to determine the role for Rac in TNF-α-induced endothelial permeability. Western blot showing expression of Rac when probed with pan Rac antibody. β-actin was used as loading control. B) TER to determine effect of dominant negative Rac in ECs upon stimulation with TNF-α depicted as normalized resistance. C) Quantification of TER studies showing significantly less endothelial barrier dysfunction in T17NRac transfected group compared to untransfected group upon stimulation with TNF-α. n=4 indicating absolute resistance values obtained from 4 independent experiments. Data shown are mean ± SEM. Asterisks (**): P < 0.01.
Online Figure V. GEF-H1 knockdown did not alter TNF-α-induced lung microvascular permeability.

A) Western blot showing knockdown of GEF-H1 after ECs were transfected with GEF-H1 siRNA. Knockdown of P-Rex1 in the ECs did not affect expression of GEF-H1. β-actin was used as loading control. B) Representative tracings showing response of GEF-H1 knockdown on TER in terms of normalized resistance after ECs were stimulated with TNF-α. C) Quantification of TER in terms of absolute resistance showing the change in resistance from basal after stimulation with TNF-α. Scrambled siRNA and GEF-H1 siRNA transfected groups are not significantly different after stimulation with TNF-α. n= 5 where n stands for absolute resistance values of each group acquired from 5 different set of experiments on different days. Data shown are mean ± SEM. ns = not significant.
Online Figure VI. TNF-α-induced activation of Rho was not compromised in P-Rex1 knockdown ECs.

(A) HLMVECs transfected with either scrambled or P-Rex1 siRNA were unstimulated or stimulated with TNF-α (25 ng/ml) for 2 and 5 min. Rho pull-down assay was performed to detect activated Rho. Activation of Rho was seen at 2 and 5 min. knockdown of P-Rex1 did not affect activation of Rho. Total Rho was used as loading control. GTPγS and GDP were used as positive and negative controls (data not shown). (B) Quantification of western blot data shows that there is no significant difference in the activated Rho between scrambled and P-Rex1 siRNA transfected ECs. Data are mean ± SEM from 3 independent experiments. n=3. ns = not significant.
Online Figure VII. TNF-α-induced Rac activation is PI3K dependent.

(A) Western blots showing activated Rac proteins. HLMVECs were pre-incubated with 20 µM of LY-294002 or vehicle for 30 min as indicated. ECs were stimulated with 25 ng/ml of TNF-α in the continued presence of the inhibitor. Rac pull-down assay and antibody detection of activated Rac was as described in Figure 2. (B) Bar graph showing quantification of Western blot data by densitometry (mean ± SEM based on 3 independent experiments). Fold change is indicates ratio of active Rac to total Rac. Data are quantified relative to basal value of TNF-α group set to 1. Asterisk (*): p < 0.05; (**) p<0.01.
Online Figure VIII. Histological sections showing reduced infiltration in the lungs of P-Rex1 knockout mice.

WT and P-Rex1/− mice were intratracheally instilled with PBS or murine recombinant TNF-α (0.5 µg/mouse) for 24 h. Histological sections were prepared and subjected to Haematoxylin and Eosin (H&E) staining to determine cellular infiltration. TNF-α treated WT mice show severe cellular infiltration and thickening of interstitium. P-Rex1 knockout mice show markedly low cellular infiltration and interstitial thickening. Images are representative of 3 independent experiments. A total of 3 mice were used for each group.
Online Figure IX. TNF-α-induced ICAM-1 expression is compromised in P-Rex1 knockdown endothelial cells.

(A) Western blots showing TNF-α-induced ICAM-1 expression. HLMVECs transfected with sc-siRNA or P-Rex1 siRNA were stimulated with 25ng/ml TNF-α for 0, 2, 4 and 8 h. Cell lysates were sonicated, denatured by boiling in 2X SDS buffer and separated on a 10% SDS gel. β-actin was used as loading control. (B) Quantification of western blots by densitometry using Image J software, shown as mean ± SEM from 3 experiments. Fold change is calculated based on the ratio of density of ICAM-1 to β-actin. Asterisk (*): p < 0.05; (**) p<0.01.
Online Figure X. Role for P-Rex1 in neutrophil transmigration across endothelial monolayer.

Transmigration of WT or P-Rex1−/− neutrophils across monolayers of HLMVECs, transfected with either scrambled siRNA or P-Rex1 siRNA, and plated on gelatin-coated 3 µm pore filters. The endothelium was pre-stimulated with TNF-α (25ng/ml) for 2 h in the indicated groups. The formyl peptide (fMIVIL, 100 nM) was placed in the bottom well in indicated groups. Data shown are mean ± SEM from 3 independent experiments. Asterisk (*): p < 0.05; (**: p<0.01; ns = not significant.
Online Figure XI. WBC and platelet counts after LPS challenge.

WT mice were injected with PBS or 5 mg/kg body weight of LPS intraperitoneally for 6 h. Blood samples were collected for CBC (Complete Blood Counts) profiling. (A) WBC counts in PBS and LPS groups were expressed as counts per micro liter of blood. The normal range of WBC count in mouse blood is (3.2-12.7) x 10^3 / µL. Two sample t-Test. P-value<0.01 indicated by double asterisks (**). (B) Platelet counts in PBS and LPS treated groups were expressed as counts per micro liter of blood. The normal range of platelet count in mouse blood is (766-1657) x 10^3 / µL. Two sample t-Test. P-value<0.05 indicated by single asterisk (*).