LIM Kinase 1 Promotes Endothelial Barrier Disruption and Neutrophil Infiltration in Mouse Lungs

Matvey Gorovoy, Jingyan Han, Haiyun Pan, Emily Welch, Radu Neamtu, Zhengping Jia, Dan Predescu, Stephen Vogel, Richard D. Minshall, Richard D. Ye, Asrar B. Malik, Tatyana Voyno-Yasenetskaya

Rationale: Disruption of endothelial barrier function and neutrophil-mediated injury are two major mechanisms underlying the pathophysiology of sepsis-induced acute lung injury (ALI). Recently we reported that endotoxin induced activation of RhoA in mouse lungs that led to the disruption of endothelial barrier and lung edema formation; however, the molecular mechanism of this phenomenon remained unknown.

Objective: We reasoned that LIMK1, which participates in the regulation of endothelial cell contractility and is activated by RhoA/Rho kinase pathway, could mediate RhoA-dependent disruption of endothelial barrier function in mouse lungs during ALI. And if that is the case, then attenuation of endothelial cell contractility by downregulating LIMK1 may lead to the enhancement of endothelial barrier function, which could protect mice from endotoxin-induced ALI.

Methods and Results: Here we report that LIMK1 deficiency in mice significantly reduced mortality induced by endotoxin. Data showed that lung edema formation, lung microvascular permeability, and neutrophil infiltration into the lungs were suppressed in limk1−/− mice.

Conclusions: We identified that improvement of endothelial barrier function along with impaired neutrophil chemotaxis were the underlying mechanisms that reduced severity of ALI in limk1−/− mice, pointing to a new therapeutic target for diseases associated with acute inflammation of the lungs. (Circ Res. 2009;105:549-556.)

Key Words: LIMK1 ■ RhoA ■ endothelial barrier function ■ inflammation ■ acute lung injury ■ neutrophil infiltration ■ actin cytoskeleton

Sepsis is the most common cause of acute lung injury (ALI), a syndrome that results from acute pulmonary edema and inflammation. The pathogenesis of ALI includes disruption of endothelial barrier that leads to lung edema and activation of neutrophils by cytokines, which results in neutrophil infiltration and tissue damage.

RhoA regulates both endothelial barrier function and neutrophil function. In endothelial cells, RhoA promotes actin polymerization and actomyosin-based cell contractility, which leads to the disruption of endothelial barrier function. In neutrophils, RhoA regulates cell migration through signaling that leads to myosin light chain phosphorylation and actin polymerization.

We reported recently that endotoxin (LPS) induced activation of RhoA in mouse lungs in vivo, which resulted in the disruption of the endothelial barrier and lung edema formation. RhoA/Rho kinase (ROCK) signaling cascade was involved in the disruption of the endothelial barrier function in mouse lungs because LPS and peptide agonist corresponding to the tethered ligand sequence of human PAR-1 (SFLLRN) (PAR-1)-induced increases in vascular permeability were reversible by pharmacological inhibition of ROCK.

We hypothesized that LIMK1, which downstream of ROCK mediates endothelial cells contractility, regulates RhoA-dependent disruption of endothelial barrier function in mouse lungs during ALI. We also hypothesized that attenuation of endothelial cell contractility by downregulation of LIMK1 may lead to the enhancement of endothelial barrier function, which ultimately could protect mice during endotoxin-induced acute lung injury. In addition, as actin polymerization is required for the efficient neutrophils chemotaxis, we reasoned that lack of LIMK1 would impair neutrophil migration, leading to decreased diapedesis.

Here we demonstrated that LPS induced activation of RhoA and LIMK1 in the mouse lungs in vivo. To study the role of LIMK1 in ALI, we used limk1−/− mice. Importantly, the severity of ALI was decreased in limk1−/− mice, as was...
observed by increased survival rate, decreased edema formation, and polymorphonuclear leukocytes (PMN) infiltration in the lungs. We found that LIMK1 regulated RhoA-mediated LPS-induced increase in microvascular permeability. Our data showed that downregulation of LIMK1 resulted in the improvement of the endothelial barrier function both in vivo and in cell culture models. We demonstrated that, mechanistically, LIMK1 mediated endothelial barrier disruption through phosphorylation of its downstream target, cofilin. Finally, we determined that \( limk1^{-/-} \) PMN exhibited decreased chemotactic ability attributable to reduced actin polymerization.

Taking into account that genetic deletion of LIMK1 in mice resulted only in minor systemic effects,\(^{11} \) we propose that organ-specific (targeted) downregulation or inhibition of kinase activity of LIMK1 could be an effective strategy to (1) improve endothelial barrier function, leading to less permeable blood vessels, and to (2) decrease neutrophil infiltration in lungs that might be beneficial during acute lung injury.

### Methods

Detailed description of materials, animal procedures, lung preparation and Kfc measurements, tissue collection and preparation for biochemical assays, electron microscopy studies, immunohistochemistry, ECIS studies, PMN isolation and superoxide production, degranulation, and chemotaxis assays are provided in the Data Supplement (available online at http://circres.ahajournals.org).

### Animals

All animal procedures were carried out following the Public Health Service Policy on Humane Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of University of Illinois at Chicago.

### Statistical Analysis

For statistical analysis, paired 2-tailed Student \( t \) test was used to compare data between 2 groups. ANOVA test was used to compare several groups. Log-rank test was used to assess differences in mortalities. Values are expressed as mean±SEM. \( P<0.05 \) was considered statistically significant.

---

**Figure 1.** Genetic deletion of LIMK1 decreases endotoxin-induced mortality and lung edema. A, Genomic DNA was extracted from mice tails and analyzed by PCR for the presence of LIMK1 (330-bp product) and neomycin (370-bp product) DNAs in genomic DNA extracts. Lungs were extracted, homogenized and lysed (B) or neutrophils were isolated from the bone marrow and lysed (C), and total lysates were analyzed by Western blotting with anti-LIMK1, anti-LIMK2, and antiactin antibodies. D, LPS 23 mg/kg was given i.p. for 3 hours. Lungs were extracted, homogenized, lysed, and total LIMK1 was immunoprecipitated using anti-LIMK1 antibody and protein A/G agarose. Immunoprecipitates were analyzed by Western blotting with anti-LIMK1 and antiphospho LIMK1/LIMK2 antibody. Three independent experiments yielded similar results. n=3 lungs per experiment. E, Survival after LPS 23 mg/kg given i.p. to \( limk1^{-/-} \) and \( limk1^{-/-} \) mice \( (n=21 \), data were pooled from 2 experiments; differences in mortalities were assessed by log-rank test \( P=0.0468 \). F, Lung edema formation at 12 hours after 23 mg/kg LPS. Lungs were removed, weighed (wet weight), and placed in an oven at 70°C. Their weight was determined every morning until 2 readings were identical (dry weight). The wet/dry ratio was determined by dividing the wet weight to the dry weight. Data were pooled from 2 independent experiments. The average was calculated from \( n=8 \) for \( limk1^{-/-} \) mice, and \( n=5 \) for \( limk1^{-/-} \) mice. \( P=0.0393 \).

### Results

**Genetic Deletion of LIMK1 Decreases Severity of ALI in Mice**

LIMK1 and LIMK2 proteins are expressed in lungs and in neutrophils both in mice and humans\(^{12,13} \) (Figure 1A through 1C). Genetic deletion of LIMK1 did not result in the upregulation of LIMK2 expression (Figure 1B and 1C).

As a model of ALI, where lung is injured indirectly, and which targets primarily the capillary endothelium,\(^{14} \) we chose intraperitoneal administration of LPS. Initially we determined whether LIMK1 was activated in this model of ALI. LPS, at 23 mg/kg (\( LD_{50} \) for \( limk1^{-/-} \) mice; data not shown), induced activation and phosphorylation of LIMK1 in \( limk1^{-/-} \) mice (Figure 1D). To address the question whether genetic deletion of LIMK1 may attenuate the severity of ALI, LPS was given to the \( limk1^{-/+} \) and \( limk1^{-/-} \) mice, and the survival rate was assessed over period of 7 days. \( limk1^{-/-} \) mice displayed an increased survival rate as compared to \( limk1^{-/-} \) littermates (from 23.8% \( limk1^{-/-} \) to 52.38% \( limk1^{-/-} \); \( P=0.0468 \); Figure 1E). Pulmonary edema, which serves as a hallmark of this disease,\(^{15} \) is one of the factors contributing to the mortality of patients with ALI.\(^{1} \) LPS challenge resulted in the formation of pulmonary edema\(^{16} \) that was significantly re-
Downregulation of LIMK1 Enhances Endothelial Barrier Function In Vivo and in Cell Culture Models

Disruption of the endothelial barrier function is a major contributor to the lung edema development. To compare the endothelial barrier function in \( \text{limk}^{+/+} \) and \( \text{limk}^{-/-} \) mice, we determined the pulmonary microvessel filtration coefficient (Kfc), a quantitative measure of vascular permeability, using isolated-perfused lung model. Genetic deletion of LIMK1 resulted in a significant decrease of Kfc in a basal and LPS-stimulated conditions. No differences were observed between \( \text{limk}^{-/-} \) mice under basal conditions (Figure 2A). LPS-induced Kfc was also significantly smaller in \( \text{limk}^{+/+} \) mice (Figure 2A). Observed decrease in vascular permeability may serve as an explanation for the decreased lung edema formation in \( \text{limk}^{-/-} \) mice.

RhoA family GTPases regulate endothelial barrier function via modulation of the actin cytoskeleton dynamics. Activation of RhoA destabilizes, whereas activation of Rac1 and Cdc42 signaling pathways enhance the barrier function.

We assessed the possibility of compensatory upregulation/activation of RhoA proteins in \( \text{limk}^{-/-} \) mice, by evaluating the total and GTP-bound amounts of RhoA, Rac1 and Cdc42 in the lungs in the basal and LPS-stimulated conditions. No differences were observed between \( \text{limk}^{+/+} \) and \( \text{limk}^{-/-} \) genotypes in either condition (supplemental Figure 1).

Importantly, although LPS still induced activation of RhoA in the lungs of \( \text{limk}^{-/-} \) mice, the disruption of endothelial barrier function was significantly decreased in this genotype, therefore placing LIMK1 as a crucial regulator of endothelial permeability downstream of RhoA.

The mechanism of how LPS induces activation of RhoA is not understood and probably includes autocrine signaling from several receptors. For that reason, as a model to study the mechanism of LIMK1-mediated attenuation of RhoA-dependent disruption of endothelial barrier function in mouse lungs, we used a PAR-1 peptide, which has been shown to disrupt the endothelial barrier function both in vivo and in vitro almost exclusively through activation of RhoA signaling cascade. Although PAR-1 peptide induced the increase in Kfc in either genotype, in PAR-1–challenged \( \text{limk}^{-/-} \) mice, Kfc was significantly smaller and comparable to the Kfc of \( \text{limk}^{+/+} \) mice in the basal conditions (Figure 2A).

To rule out other possible compensatory effects that might be associated with gene deletion in mice, we addressed the question of whether downregulation of LIMK1 would decrease permeability in cultured endothelial cells. Endogenous LIMK1 was downregulated in human umbilical vein endothelial cells (HUVECs) using siRNA (Figure 2B). Endothelial barrier function was evaluated using electric cell-substrate impedance sensor (ECIS). Downregulation of LIMK1 in HUVECs resulted in the enhancement of endothelial barrier function (Figure 2C).

We previously showed that overexpression of wild type LIMK1 in HUVECs that possesses kinase activity resulted in the production of actin stress fibers and therefore in increased contractility of endothelial cells. To determine if kinase activity of LIMK1 plays role in the disruption endothelial barrier, we overexpressed the wild type (wt) or kinase-dead (kd) mutants of LIMK1 in HUVECs. kdLIMK1 had no effect, whereas overexpression of wtLIMK1 significantly decreased endothelial permeability (Figure 2D). The known target of LIMK1 is cofilin, an actin-depolymerizing factor. To elucidate the mechanism of how LIMK1 regulates endothelial barrier disruption, we coexpressed nonphosphorylatable mutant of cofilin S3A-cofilin with wtLIMK1. Importantly, coexpression of S3A-cofilin resulted in the complete recovery of phenotype induced by wtLIMK1 (Figure 2E), suggesting that LIMK1 exerts its effect on endothelial barrier disruption through phosphorylation of cofilin.

\[ \text{wtLIMK1} + \text{S3A-cofilin} \] The data shown was collected from 3 independent experiments performed in duplicates. Error bars represent SEM (P<0.05).
LIMK1 Destabilizes Interendothelial Junctions

Barrier breakdown that is observed during inflammation, is regulated, in part, by the integrity of intercellular junctions. To analyze ultrastructural changes of the endothelial barrier in limk1−/− mice, we used quantitative transmission electron microscopy. Tight junctions, formed between adjacent endothelial cells in the capillary segment of the lungs, were detected as characteristic central dark element (fused outer layers) separated symmetrically by two light zones (central, lipid layers of unit) from two dark lines which are the cytoplasmic layers of unit membrane of each endothelial cell.22

We did not observe differences in the structure of endothelium in the lungs of nonstimulated wild-type and limk1−/− mice (Figure 3A and 3B). PAR1 peptide induced opening of interendothelial junctions in both genotypes, however quantitative morphometric analysis of the interendothelial junctions revealed that the number of open junctions was significantly smaller in limk1−/− mice (Figure 3C).

To address the question about the role of LIMK1 kinase activity in morphology of endothelial junctions, we overexpressed kdLIMK1 or wtLIMK1 in HUVECs. Continuous junctional immunostaining was displayed by staining with anti–VE-cadherin antibody in HUVECs transfected with the kdLIMK1 (Figure 3D). On the contrary, overexpression of wtLIMK1 resulted in appearance of intercellular gaps (Figure 3D). Importantly, destabilization of endothelial junctions, induced by wtLIMK1, was eliminated by coexpression of S3A-cofilin together with wtLIMK1 (Figure 3D). Similar results were obtained by staining with anti-Zonula Occludence-1 (ZO-1) antibody (data not shown).

To summarize, these data indicated that downregulation of LIMK1 enhanced endothelial barrier function both in vivo and in cell culture models. Lesser number of the open interendothelial junctions, as observed after PAR1 stimulation, could be the underlying cause of enhanced endothelial barrier function in limk1−/− mice. The decrease in vascular permeability, attributable to the improvement of endothelial barrier function, could have contributed to the attenuation of edema formation in limk1−/− mice. Mechanistically, LIMK1 exerted its effect on the disruption of endothelial barrier through phosphorylation of actin-depolymerizing factor, cofilin.

Reduced Diapedesis of limk1−/− PMN in the Lungs During ALI

It is well established that infiltration of neutrophils in lungs, followed by tissue damage and lung edema, plays a crucial role in the pathogenesis of ALI. Increased production of reactive oxygen species and proteolytic enzymes by neutrophils are known mechanisms underlying the development of tissue damage in ALI.

We tested whether production of reactive oxygen species or degranulation were decreased in limk1−/− mice. Data showed that fMLP or phorbol myristate acetate–induced O2− production in neutrophils as well as the total amount of superoxide produced did not differ between limk1−/− and limk1+/− mice (supplemental Figure IIA). Neutrophil degranulation, a measure of proteolytic enzymes release, induced by fMLP or C5a (an anaphylatoxin, which is produced upon compliment activation in sepsis, was similar in limk1−/− and limk1+/− PMN (supplemental Figure IIB).

Next, we evaluated the extent of PMN extravasation into the lung tissues using hemotaxilin/eosin and leukocyte-specific Leder staining. In both limk1+/+ and limk1−/− mice,
lung sections showed infiltration of neutrophils at 6 hours after LPS challenge. Notably, the number of neutrophils in the lungs of limk1−/− mice was significantly smaller (Figure 4A through 4C).

Decreased number of neutrophils in lungs of limk1−/− mice was not attributable to a decrease of the total amount of circulating neutrophils, because hemograms obtained before and after LPS injection showed no difference between limk1+/+ and limk1−/− genotypes (supplemental Table I).

ALI causes increased secretion of cytokines that promote neutrophil infiltration in the lungs.3 We determined whether decreased cytokine production accounted for decreased neutrophil infiltration in limk1−/− mice. Data showed that IL-6, MIP-1α, TNFα, and RANTES production in response to LPS did not differ between limk1+/+ and limk1−/− mice (supplemental Figure III).

Next, we investigated whether altered chemotactic activity in response to C5a and IL-8 (a chemokine that mediates the influx of neutrophils in the lungs during ALI) contributed to decreased neutrophil infiltration in the lungs of limk1−/− mice. Interestingly, directed chemotaxis of limk1−/− PMNs was decreased by ~20% in response to C5a (Figure 4D); and by ~40% in response to IL-8 (Figure 4E). That led us to conclusion that reduced number of infiltrated PMN in the lungs of limk1−/− mice could be attributed to defects in limk1−/− PMN chemotaxis.

Polymerization of actin is one of the early events during neutrophils activation and is required for the efficient chemotaxis.30,31 Considering a significant role of LIMK1 in actin polymerization, we analyzed C5a-induced changes in F-actin content in neutrophils from limk1+/+ and limk1−/− mice. C5a-induced actin polymerization in limk1−/− PMNs was significantly decreased and less sustained than in limk1+/+ PMNs (Figure 4F), thus providing an explanation for the defects in limk1−/− PMN chemotaxis.

To summarize, genetic deletion of LIMK1 reduced the severity of ALI as was indicated by increased survival rate, decreased lung edema formation, and decreased PMN infiltration in the lungs. We propose that enhancement of the endothelial barrier function in lungs of limk1−/− mice, through attenuation of RhoA-dependent signaling, along with a decrease of limk1−/− PMN chemotaxis, attributable to defects in actin polymerization, were the underlying mechanisms that reduced the severity of ALI.

Discussion

In this study we demonstrated that genetic deletion of LIMK1 resulted in increased survival rate and decreased lung edema formation during ALI. For the first time, we provided the evidence that LIMK1 mediated RhoA-dependent endothelial barrier disruption during inflammation, induced by LPS or PAR-1 peptide. We suggested that improvement of the endothelial barrier function through attenuation of LIMK1-mediated contractility; and decrease in PMN chemotaxis attributable to defects in actin polymerization were the
underlying mechanisms that led to the reduction of the inflammatory responses in limk1−/− mice during ALI. Downregulation of LIMK1 in vivo and in cell culture models resulted in the improvement of endothelial barrier function. Mechanistically, LIMK1 mediated disruption of endothelial barrier through phosphorylation of actin-depolymerizing factor, cofolin.

The pathogenesis of ALI includes disruption of endothelial barrier that leads to protein-rich edema,2 which ultimately contributes to significant mortality of the patients affected with ALI.1,23–26 Two actin cytoskeleton-dependent events are linked in a positive feed-forward cycle and are primarily responsible for the development of inflammatory response and lung edema during ALI: impairment of the endothelial barrier function and neutrophil-dependent tissue damage.23–26

Endothelial Barrier Function: Actin Cytoskeleton and LIMK1

Endothelial barrier function is controlled by the actomyosin cytoskeleton via regulation of the stability of intercellular junctions and tensile force within the monolayer.34–36 Here, for the first time, we showed that downregulation of LIMK1 resulted in decreased microvascular permeability in mouse lungs and improved endothelial barrier function both in vivo and in cell culture models. The lower basal permeability induced by downregulation of LIMK1 could be explained by reduced homeostatic tensile force in endothelial cells attributable to reduced actin polymerization. Our current data also showed that LIMK1 regulated disruption of endothelial barrier function primarily through phosphorylation of actin depolymerizing factor, cofolin (Figure 2E).

RhoA-ROCK-LIMK1 Signaling Axis Regulates Endothelial Barrier Disruption During Inflammation

It has been speculated that RhoA-mediated increase in actomyosin contractility plays a significant role in the disruption of endothelial barrier during inflammation.37,38 Our current data together with the previous data from our laboratory7 and others8 provided the evidence that RhoA, through RhoA-ROCK-LIMK1 pathway, induced disruption of endothelial barrier during inflammation in mouse lungs in vivo. We showed here that LPS induced activation of both RhoA and LIMK1 in mouse lungs (Figure 1D and supplemental Figure I). Notably, genetic deletion of LIMK1 significantly decreased LPS- and PAR-1-induced RhoA-mediated effects on the disruption of endothelial barrier function, without affecting the activation cycle of small GTPases per se (supplemental Figure I). The mechanism of LPS-induced RhoA activation in endothelial cells in vivo still remains to be determined. It may include autocrine signaling through G protein–coupled receptors facilitated by anaphylatoxins, which are products of complement activation,37,38 or by chemokines, because it has been shown that chemokines exert their effects through at least nineteen G protein–coupled receptors.39,40

The Role of Other Organs and Cell Types in the Pathogenesis of ALI in limk1−/− Mice

Endothelial cells and neutrophils are two cell types primarily involved in the development of inflammatory response in lungs during ALI, therefore it is plausible to conclude that decreased microvascular permeability and PMN infiltration in limk1−/− mice played the major role in increased survival rate during ALI. However, there is still a possibility that defects in other cell types also contributed to the protection against mortality, because LIMK1 is ubiquitously expressed in all tissues.13 On the other hand, although we observed that LPS-induced lung edema formation was significantly suppressed (Figure 1G), about half of limk1−/− mice were mortal by LPS treatment. This phenomenon can be also explained by the fact that the development of LPS-induced sepsis is manifested by multiple organ failure that includes kidney, lung, liver, etc.41 Therefore, defects in multiple organs contribute to overall mortality, and suppression of lung edema formation may not be enough to completely abolish LPS-induced mortality.

PMN Diapedesis During ALI

Neutrophil migration into the lung tissue plays a crucial role in the pathogenesis of ALI.23–26 Activation of PMN results in the responses such as migration, degranulation, and phagocytosis, which are mediated by actin cytoskeleton.42 We studied the role of LIMK1 during LPS-induced acute lung injury in 2 classes of cellular responses of PMN: (1) migratory (chemotaxis and transmigration) and (2) secretory (generation of oxygen radicals and secretion of lysosomal enzymes).

The transendothelial migration (diapedesis) is governed by coordinated action of both endothelial cells and neutrophils. In endothelial cells Rho protein–dependent43 remodeling of the F-actin cytoskeleton and actin-associated interendothelial adherens junctions has been observed as the leukocyte completes diapedesis step.44,45 In neutrophils sequential activation of RhoA family small GTPases by microenvironmental signals leads to the actin cytoskeleton, which results in cell orientation and extension of extrapary filopodia in the gap between endothelial cells.30

Here we show that genetic deletion of LIMK1 decreased PMN infiltration in mouse lungs in vivo and produced defects in PMN chemotaxis in vitro (Figure 4A through 4E). Observed reduction in limk1−/− PMN chemotaxis can be explained by perturbances in the dynamics of actin polymerization (Figure 4F).

It is reasonable to attribute decreased limk1−/− PMN infiltration in lungs to defects in PMN chemotaxis. However, it is also possible that impaired actin cytoskeleton dynamics in endothelial cells contributed to reduced diapedesis, because it was shown that disruption of actin cytoskeleton in endothelial cells resulted in reduced transendothelial migration of monocytes.46

We suggest that impaired actin cytoskeleton dynamics, which resulted in the enhancement of endothelial barrier, together with decreased PMN chemotaxis contributed to the decreased PMN diapedesis. The relative degree to which improvement of the endothelial barrier or decreased PMN chemotaxis contributed to the reduced PMN infiltration and lung injury remains to be determined.
PMN Superoxide Production and Degranulation

PMN cytoskeletal dynamics plays a crucial role in superoxide production and degranulation.\textsuperscript{30} It was shown that inhibition of actin polymerization in activated neutrophils resulted in augmentation of superoxide anion production and degranulation induced by different agents.\textsuperscript{37–40} Interestingly, we found that stimulus-induced superoxide production and degranulation in \textit{limk1}\textsuperscript{−/−} PMN did not differ from \textit{limk1}\textsuperscript{+/+} cells, although actin polymerization was impaired. Possible explanation underlying this phenomenon might be in the difference of the extent to which actin cytoskeleton dynamics was perturbed, because there are multiple ways to regulate actin cytoskeleton dynamics and LIMK1 affects only one of those.

To summarize, we demonstrated that the severity of acute lung injury was decreased in \textit{limk1}\textsuperscript{−/−} mice, as was observed by increased survival rate, decreased edema formation, and PMN infiltration in the lungs. Based on our findings, we propose that organ-specific downregulation or inhibition of LIMK1 activity could be a potential strategy to reduce vascular leakage during many life-threatening diseases including ALI.

Sources of Funding

This work was supported by National Institutes of Health grants GM56159 and P01 HL60678.

Disclosures

None.

References


LIM Kinase 1 Promotes Endothelial Barrier Disruption and Neutrophil Infiltration in Mouse Lungs
Matvey Gorovoy, Jingyan Han, Haiyun Pan, Emily Welch, Radu Neamu, Zhengping Jia, Dan Predescu, Stephen Vogel, Richard D. Minshall, Richard D. Ye, Asrar B. Malik and Tatyana Voyno-Yasenetskaya

Circ Res. 2009;105:549-556; originally published online August 13, 2009;
doi: 10.1161/CIRCRESAHA.109.195883

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/105/6/549

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/08/14/CIRCRESAHA.109.195883.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supplement Material

Materials and Methods

Materials:

Lipopolysaccharides (LPS) from Escherichia coli 0111:B4, C5a and IL-8 were from Sigma; PAR-1 peptide Ac-TFLLRNPN-DK-NH2 was from Biosource; S3A mutant of cofolin was a gift from Dr. Bernard; ketamine, xilazine, acepromazine were from Abbott Laboratories; anti-RhoA, anti-ZO-1, and anti-VE-cadherin antibodies were from Abcam; anti-Cdc42, anti-Rac1 antibodies were from BD Transduction Laboratories; anti-LIMK1 and anti-phospho-LIMK1/LIMK2 antibodies were from Cell Signaling Technology; anti-mouse Alexa Fluor 594 was from Molecular Probes.

siRNA against human LIMK1 was described previously. Double-stranded siRNA targeted against human LIMK1: CCU GGA GGG AAG AAC GUA UUU, and mismatch siRNA CCU GAA AGA AAA AAC GUA UUU (where 4 nucleotides were mutated G/A) were from Dharmacon (Chicago, IL). The specificity of the LIMK1 siRNA was verified or the siRNA study was validated by using 1) mismatch controls, where mutation of only several nucleotides completely abolished the silencing effect; and 2) several siRNAs targeted against different regions on LIMK1 mRNA, which showed similar silencing effects.

Animals

All animal procedures were carried out following the Public Health Service Policy on Humane Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of University of Illinois at Chicago. Mice were kept under standard conditions in the Biological Resources Laboratory facilities of the University of Illinois at
Chicago. The LIMK1 knockout mice generation and characteristics are described elsewhere ⁴. limk1+/- pairs were used for breeding. The genotype of the mice was identified on DNA extracted from tail biopsy according to the manufacturer’s protocol Wizard Genomic DNA purification kit Promega (Madison, WI) by polymerase chain reaction assay. Sex-matched limk1+/+ littermates were used as controls for limk1-/- mice.

**Lung preparation**

According to an approved protocol of the University of Illinois at Chicago Animal Care Committee, limk1-/- mice or their wild-type littermates mice weighing between 25 and 35 grams were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg), xylazine (2.5 mg/kg) and acepromazine (2.5 mg/kg). The detailed protocol is described elsewhere ⁵. Briefly, a tracheal cannula was inserted for positive-pressure ventilation and 100U heparin injected into the internal jugular vein for anticoagulation. The diaphragm was exposed through a midline abdominal incision, and excised from the rib cage. A sternotomy was performed and the thoracic cavity was exposed. A polyethylene cannula (PE-90, Becton Dickinson Sparks, MD) was inserted in the pulmonary artery through a small incision in the right ventricle. The cannula was secured using a suture around the pulmonary artery and aorta. The left atrium was cut free for drainage of the venous effluent. The lungs were perfused with RPM I 1640 solution at a constant flow rate (2mL/min), temperature (37°C) and pH (7.4), using a peristaltic pump (Gilson Minipuls 3). Positive-pressure ventilation (rate, 120 breaths/min; peak inspiratory pressure, 8-10 cm H₂O; peak end-expiratory pressure, 2 cm H₂O) was maintained by means of a dual mode ventilator (Kent Scientific, Litchfield, CT).
Capillary filtration coefficient determination was performed as described. The lungs and heart were rapidly removed en bloc from the thoracic cavity and suspended from a lever arm connected to a force displacement transducer (FT03, Grass Telefactor, W Warwick, RI). Pulmonary arterial pressure was recorded during the experiment using a Gould pressure transducer (CP122 AC/DC strain gage Amplifier, Gould Instruments, Dayton, OH). Both transducers were connected to amplifiers (Grass Instrument Co, W. Warwick, RI) and the recordings were displayed on a computer monitor with the aid of an analog to digital converter (DAS 1800ST board; Keithly Metabyte, Solon, OH) and software for acquisition of data (Notebook Pro for Windows, Labtech, Andover, MA).

After mounting, the weight was zeroed and the lungs were given a 20-minute equilibration period. The lungs that were not isogravimetric at the end of this period were discarded. The pressure was increased abruptly by 8-10 cm H$_2$O for 5 minutes every 20 minutes, which gave a characteristic weight recording: an initial fast phase (almost vertical) followed by a slow phase that represented the net fluid extravasation. The venous pressure was assumed to be zero. For every mouse 4 capillary filtration coefficient determinations were made and the average was taken into consideration. At the end of experiment the lungs were cut free of any other tissue and placed in a oven for at least 48 hours until a constant weight was reached, representing the dry weight. Data analysis was carried out off-line, using a LOTUS 1-2-3 macro© developed in this laboratory.
**Drug infusion**

The infusions were made at a constant rate of 0.2 ml/min using a side-port tube passing through a peristaltic pump and connected to the main line. The flow rate in the main line was decreased to 1.8 ml/min when infusion of the drugs was started. In experiments testing the effect of PAR-1 agonist peptide on the Kfc infusion was begun 5 minutes before the 1st Kfc measurement. In every case the perfusion of drug was stopped when the Kfc measurement was begun. PAR-1 agonist peptide was used at the same concentration (6 µM) in all the experiments.

**Tissue preparation for biochemical assays**

The blood was removed by a 10-minute perfusion with RPMI 1640 at RT and thereafter lungs were quickly cut free of any other tissue and snap-frozen in liquid nitrogen.

**Gravimetric assessment of pulmonary water content**

LPS at 23 mg/kg was injected into the peritoneal of conscious mice. Animals were anesthetized with ketamine/xylazine after 12 hours post-LPS and lungs were removed, immediately weighed to measure wet weight. Dry weight was determined after heating the lung at 80°C for 36 hours. Pulmonary water content index was calculated by dividing the lung wet weight over the lung dry weight as described previously.

**Survival studies**

Wild type (n=21) and LIMK1 knockout mice (n=21) animals were injected with 23 mg/kg LPS intraperitoneally. Mice were returned to their cages and allowed access to food and water ad libitum. Animals were observed at regular intervals for occurrence of
mortality over the subsequent 7 days post-LPS. Moribund animals (defined as bradycardia to a heart rate less than 40; severe lethargy; and irresponsive to painful stimulations) were sacrificed with a lethal dose of ketamine/xylazine as defined by the University of Illinois at Chicago Animal Care Committee.

Electron microscopy studies

After removal of blood from the vascular space with a 10-minute HBSS perfusion, lungs were fixed in situ (20 min at RT) by injecting through a pulmonary artery cannula a mixture of 3% formaldehyde + 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. Excised specimens were further fixed in the same mixture (1h at RT) then post-fixed in 2% OsO₄ in acetate veronal buffer, pH 6.8 (1h on ice), stained in the dark (1h at RT) with Kellenberg uranyl acetate, dehydrated through graded ethanol and then embedded in Epon 812. Tissue blocks were cured (72 h at 90º C), cut on a Leica microtome, and 60 nm sections were counterstained with uranyl acetate and lead citrate and examined and photographed in a JEOL 1220 transmission electron microscope.

Immunocytochemistry

HUVECs grown to confluency on coverslips coated with gelatin were serum-starved for 6 hours before each experiment. Cells were washed with Hank’s balanced salt solution (HBSS) and fixed with 3.3% paraformaldehyde for 30 min. Cells were permeabilized for 5 min with 0.1% Triton X-100/ PBS and washed extensively with HBSS. After blocking with 1% BSA/0.2% fish skin gelatin in HBSS for 1 h at room temperature (RT), cells were incubated with primary antibody in blocking solution for 1 h at RT followed by incubation with secondary antibodies. Slides were mounted using ProLong Antifade Kit (Molecular Probes). Microscopy was performed using Zeiss LSM
510 confocal microscope equipped with 63x water-immersion objective with appropriate filter sets.

**Transfection and culturing of HUVECs:**

Human umbilical vein endothelial cells (HUVECs) were obtained at first passage from Cambrex (Walkersville, MD, culture line CC-2519) and were utilized at passages 6-10. Cells were cultured in EBM-2 medium (Cambrex) supplemented with 10% (v/v) fetal bovine serum (Cellgro) and EGM-2 SingleQuots (Cambrex) and maintained at 37°C in humidified atmosphere of 5% CO₂-95% air. Transient transfections of HUVECs with cDNAs were performed with SuperFect Transfection reagent (Qiagen) according to the manufacturer’s protocol. Transfection of HUVECs with siRNAs was performed either with DharmaFect1 (Dharmacon) (for TER measurements and Western blotting) or using Amaxa Nucleofector (Lonza) (for Western blotting). TER experiments were performed from 12h to 48h after transfection (Figure 2C). Western blotting was performed 48h after transfection (Figure 2B).

**Measurements of trans-endothelial electrical resistance**

The total electrical resistance was measured dynamically across the HUVECs monolayer using an electrical cell-substrate impedance sensing system (ECIS) (Applied Biophysics, Troy) described previously. Briefly, HUVECs were plated in a well containing a small gold electrode and transfected as described above. Culture medium was used as the electrolyte. The small electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier. A 1-V, 4,000-Hz AC signal was supplied through a resistance of 1 megohm to approximate a constant current of 1 μA. The voltage change between the small electrode and the larger counter electrode was
continuously monitored, stored, and processed on a computer. The data are presented as the change in the resistive (in-phase) portion of the impedance normalized to its initial value at time zero.

Quantification of PMN infiltration into the lungs

After removal of blood from the vascular space with a 10-minute HBSS perfusion, lungs were fixed in situ (20 min at RT) by injecting through a pulmonary artery cannula 3% formaldehyde solution; and thereafter were kept in 3% formaldehyde solution overnight at 4°C. Hemotaxilin/eosin and Leder (naphtol AS-D chloroacetate esterase) staining were performed according to the manufacturer’s protocol (Sigma-Aldrich). Thereafter, lung sections were analyzed by light microscopy (magnification 40X). Infiltrated neutrophils were counted in a blinded manner to the mice genotype and experimental conditions. Data represent mean count from five different sections (four different fields were analyzed per each section) from each of 3 mice lung preparations per condition.

Chemotactic assay

Bone marrow derived neutrophils were used, chemotaxis was quantified using a Transwell system (polycarbonate filters with pore size 5µm). Cells were placed in the top well in a total volume of 100 µl, and 600 µl of chemoattractant or medium (RPMI 1640 with 1% FBS) was added to the lower chamber. After 40-min incubation at 37°C, cells in the bottom chamber were collected and the number of migrated PMN was determined.

Superoxide production
Mouse bone marrow neutrophils were resuspended in BSA buffer (0.5% BSA in Hank’s balanced salt solution with Ca$^{2+}$ and Mg$^{2+}$, and 10 mM Hepes) at 5x10^6 cells/ml. Superoxide anion released from neutrophils were measured as described elsewhere. Briefly, isoluminol was added to the cell suspension to a final concentration of 50 µM and horseradish peroxidase to a final concentration of 40 U/ml. Cells (200 µl/well) were then seeded into 96-well flat bottom culture dish (E&K Scientific, Campbell, CA). Chemiluminescence was measured at least every minute using a Wallac Multilabel Counter plate reader (Perkin Elmer Life Sciences, Boston, MA) starting from 10 min before and continuing to 30 min after ligand stimulation. Unstimulated controls were recorded simultaneously.

**Degranulation assay**

Mouse bone marrow neutrophils were resuspended in BSA buffer (0.5% BSA in Hank’s balanced salt solution with Ca$^{2+}$ and Mg$^{2+}$, and 10 mM Hepes) at 6.25x10^6 cells/ml and placed on ice. Cytochalasin B (10 µM, Sigma) was then added, cells were aliquotted (5x10^5 cells/tube), then returned to ice for 15 min. After 15 min, cells were transferred to 37°C and incubated for another 15 min. Agonist was prepared at 2x concentration and warmed to 37°C, then added to the cells to a final concentration of 1x. After 10 min, the reaction was terminated by transferring samples to ice. Cells were pelleted at 1500 rpm for 5 min at 4°C, and the supernatants were transferred to new tubes. Cell pellet was resuspended in 4x supernatant volume (640 µl) of 0.1% Triton X-100 in HBSS. Substrate (4-Methylumbelliferyl β-D-glucuronide hydrate, Sigma) was prepared fresh at a concentration of 2.5 mM in 0.1M Sodium acetate (pH 4.0) with 0.1% Triton X-100. Cell supernatant (40 µl) was then combined with 40 µl substrate in a black
96 well plate (E and K Scientific), and then incubated for 15 min at 37°C. At the end of the incubation, 170 µl stop solution (50 mM glycine and 5 mM EDTA) was added to terminate the reaction. Fluorescence was measured on the Flex Station (Molecular Devices) with excitation wavelength at 365 nm and emission wavelength at 460 nm.

**Actin polymerization**

Bone marrow-derived neutrophils were stimulated for indicated amount of time with 20 nM C5a at room temperature and fixed in 3% formaldehyde in HBSS for 30 min. F-actin was stained by addition of 0.2 µM FITC-phalloidin/100µg/ml lysophosphatidicholine in HBSS for 30 min. Fluorescence intensity was determined by flow cytometry.

**Supplement figure legends:**

**Online Figure I.** A, B, C) LPS at 23 mg/kg was given i.p., where indicated. Lungs were extracted 3 h upon LPS treatment. GTP-bound small GTPases pull-down assays were performed using whole lung homogenates from limk1+/+ and limk1−/− mice as described elsewhere. Precipitates and total lysates were analyzed by Western blotting using (A) anti-RhoA, (B) anti-Rac1, (C) anti-Cdc42 antibodies. Relative amount of GTP-bound GTPases over total amount of GTPases is presented on the bar graph (n=5 lung preparations per group).

**Online Table I.** Hemograms from limk1+/+ and limk1−/− mice before and after LPS treatment. Animals were injected i.p. with LPS (23 mg/kg) and peripheral blood was collected from the retro-orbital sinus 6 h pos-LPS. Cell counts were performed using an automated cell counter with veterinary parameters and reagents. WBC, white blood cells; NE, neutrophils; LY, lymphocytes; MO, monocytes; EO, eosinophils; BA, basophils;
RBC, red blood cells; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets. (n=3 mice per group).

**Online Figure II.** A) Superoxide production by PMNs of limk1+/+ and limk1−/− mice. PMNs were stimulated with either fMLP or PMA for 15 minutes. Superoxide production was determined as described in Methods (n=3 per group). B) Degranulation of PMNs isolated from limk1+/+ and limk1−/− mice. PMNs were stimulated with either fMLP or C5a for 30 minutes. Degranulation was determined as described in Methods (n=3 per group).

**Online Figure III.** Cytokine production in limk1+/+ and limk1−/− mice before and after LPS treatment. Animals were injected i.p. with LPS (23 mg/kg) and peripheral blood was collected 2 h later. Serum was isolated and cytokine production was measured according to the manufacturer’s protocol (BioPlex, BioRad). (n=3 mice per group)
REFERENCES


Online Figure II
<table>
<thead>
<tr>
<th>Variable</th>
<th>limk1+/+ Mean</th>
<th>limk1+/+ SD</th>
<th>limk1-/ - Mean</th>
<th>limk1-/ - SD</th>
<th>limk1+/+, LPS Mean</th>
<th>limk1+/+, LPS SD</th>
<th>limk1-/ -, LPS Mean</th>
<th>limk1-/ -, LPS SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (x10^9/L)</td>
<td>7.226667</td>
<td>1.120238</td>
<td>6.406667</td>
<td>1.540692</td>
<td>1.6</td>
<td>0.480833</td>
<td>2.05</td>
<td>0.381838</td>
</tr>
<tr>
<td>NE (x10^9/L)</td>
<td>1.543333</td>
<td>0.347035</td>
<td>1.42</td>
<td>0.38223</td>
<td>0.61</td>
<td>0.169706</td>
<td>0.56</td>
<td>0.325269</td>
</tr>
<tr>
<td>LY (x10^9/L)</td>
<td>5.323333</td>
<td>0.680759</td>
<td>4.65</td>
<td>1.162282</td>
<td>0.905</td>
<td>0.233345</td>
<td>1.35</td>
<td>0.014142</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>0.263333</td>
<td>0.037859</td>
<td>0.236667</td>
<td>0.110151</td>
<td>0.055</td>
<td>0.06364</td>
<td>0.06</td>
<td>0</td>
</tr>
<tr>
<td>EO (x10^9/L)</td>
<td>0.073333</td>
<td>0.092916</td>
<td>0.073333</td>
<td>0.049329</td>
<td>0.025</td>
<td>0.021213</td>
<td>0.06</td>
<td>0.056569</td>
</tr>
<tr>
<td>BA (x10^9/L)</td>
<td>0.023333</td>
<td>0.025166</td>
<td>0.023333</td>
<td>0.023094</td>
<td>0.005</td>
<td>0.007071</td>
<td>0.02</td>
<td>0.028284</td>
</tr>
<tr>
<td>RBC (M/µL)</td>
<td>8.92</td>
<td>0.600083</td>
<td>8.946667</td>
<td>0.855005</td>
<td>8.425</td>
<td>0.516188</td>
<td>9.9</td>
<td>2.390021</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>12.6667</td>
<td>0.929157</td>
<td>13.63333</td>
<td>1.582193</td>
<td>12.55</td>
<td>0.636396</td>
<td>14.2</td>
<td>2.404163</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>40</td>
<td>2.751363</td>
<td>42.93333</td>
<td>3.126233</td>
<td>43.8</td>
<td>3.252691</td>
<td>48.1</td>
<td>5.515433</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>44.83333</td>
<td>0.305505</td>
<td>48.06667</td>
<td>1.209683</td>
<td>52</td>
<td>0.707107</td>
<td>49.35</td>
<td>6.29325</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>14.23333</td>
<td>0.378594</td>
<td>15.2</td>
<td>0.458258</td>
<td>14.9</td>
<td>0.141421</td>
<td>14.45</td>
<td>1.06066</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>31.6667</td>
<td>0.665833</td>
<td>31.73333</td>
<td>1.436431</td>
<td>28.7</td>
<td>0.707107</td>
<td>29.45</td>
<td>1.626346</td>
</tr>
<tr>
<td>PLT (x10^9/L)</td>
<td>622.6667</td>
<td>231.7096</td>
<td>387</td>
<td>115.113</td>
<td>416</td>
<td>70.71068</td>
<td>339</td>
<td>226.2742</td>
</tr>
</tbody>
</table>
Online Figure III