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Caveolin-1 Regulates NF-κB Activation and Lung Inflammatory Response to Sepsis Induced by Lipopolysaccharide

Sean Garrean, Xiao-Pei Gao, Victor Brovkovych, Jun Shimizu, You-Yang Zhao, Stephen M. Vogel, and Asrar B. Malik

Caveolin-1, the principal structural and signaling protein of caveolae, is implicated in NO-mediated cell signaling events, but its precise role in inflammation is not well understood. Using caveolin-1-knockout (Cav-1−/−) mice, we addressed the role of caveolin-1 in the lung inflammatory response to sepsis induced by i.p. injection of LPS. LPS-challenged wild-type (WT) lungs exhibited significant increases in neutrophil sequestration (~16-fold), lung microvascular permeability $K_{fe}$ (~5.7-fold), and edema formation (~1.6-fold). Compared with WT, Cav-1−/− lungs showed marked attenuation of LPS-induced neutrophil sequestration (~11-fold increase) and inhibition of microvascular barrier breakdown and edema formation. Prevention of lung injury in Cav-1−/− mice was associated with decreased mortality in response to LPS challenge. To address the basis of the reduced inflammation and injury in Cav-1−/− lungs, we examined the role of NO because its plasma concentration is known to be increased in Cav-1−/− mice. Cav-1−/− mouse lungs demonstrated a significant increase in endothelial NO synthase (eNOS)-derived NO production relative to WT, which is consistent with the role of caveolin-1 as a negative regulator of eNOS activity. Cav-1−/− lungs concurrently showed suppression of NF-κB activity and decreased transcription of inducible NO synthase and ICAM-1. Coadministration of LPS with the NO synthase inhibitor nitro-L-arginine in Cav-1−/− mice prevented the suppression of NF-κB activity and restored lung polymorphonuclear leukocyte sequestration in response to LPS challenge. Thus, caveolin-1, through its ability to regulate eNOS-derived NO production, is a crucial determinant of NF-κB activation and the lung inflammatory response to LPS. The Journal of Immunology, 2006, 177: 4853–4860.

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$^4$ Abbreviations used in this paper: eNOS, endothelial NO synthase; iNOS, inducible NO synthase; PMN, polymorphonuclear leukocyte; l-NA, nitro-l-arginine; WT, wild type; MLVEC, mouse lung vascular endothelial cell; MPO, myeloperoxidase; siRNA, small interfering RNA.

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inhibitor mixture were purchased from Sigma-Aldrich; 1400W was from Calbiochem; Micro BCA Protein Assay and SuperSignal West Pico Chemiluminescent Substrate kits were from Pierce; NuPAGE MOPS SDS running buffer and TRIZol were from Invitrogen Life Technologies; and 5% nonfat milk and nitrocellulose membranes were from Bio-Rad. iNOS, eNOS, ICAM-1, IκBα, and GAPDH Abs for Western blot analysis were obtained from Santa Cruz Biotechnology, and caveolin-1 Ab was purchased from BD Biosciences. RNeasy Mini kit and Quantitech SYBR Green RT-PCR kit, used for RNA purification and quantification, were purchased from Qiagen.

**Animals**

Caveolin-1-knockout (Cav-1−/−) and B6129F2 control mice were obtained from The Jackson Laboratory and housed in the University of Illinois Animal Care Facility, in accordance with institutional and National Institutes of Health guidelines. Cav-1−/− mice were derived from B6129F2 mice by gene deletion and back-crossed into this background strain to obtain the homozygous Cav-1−/− genotype. All experiments were performed under anesthesia using either inhaled halothane or an i.p.-injected mixture of ketamine (60 mg/kg), xylazine (2.5 mg/kg), and acepromazine (2.5 mg/kg) in saline. Male mice (8 –10 wk) were matched according to weight, which ranged from 20 to 25 g.

**LPS challenge**

Lung injury was induced by i.p. injection of LPS (10 mg/kg, body weight). Lungs of B6129F2 and Cav-1−/− mice were then collected at various time points after LPS administration and used for determinations of NO production, NF-κB activity, expression of Cav-1, ICAM-1, eNOS, iNOS, and IκBα, formation of lung edema, and lung PMN sequestration.

**NO production measurement**

Basal and eNOS-derived NO production were determined by assaying nitrite accumulation using the Griess reagent. Briefly, lungs from LPS-un-treated mice were cut into 1-mm thick slices and incubated with t-arginine in DMEM for 1 h. Baseline NO levels were assessed as nitrite accumulation using the Griess reagent. Constitutive eNOS activity was then measured in the following manner. Lungs were isolated and immediately frozen at various time points after LPS injection. The samples were preincubated in t-arginine-free DMEM for 1 h and were subsequently incubated in a bath containing DMEM, 1 mM t-arginine, 2 μM iNOS inhibitor (1400W), and 1 μM calcium ionophore (A23187). NO production by eNOS was measured as nitrite accumulation in the medium using the Griess reagent.

**iNOS activity**

iNOS activity was determined using a three-electrode system as described previously (28). Briefly, lungs were cut into 1-mm thick slices and placed in HBSS at 37°C. NO production was assessed in this medium using a microsensor electrode (NOP-1, Quanteon; University of Colorado Health Sciences Center) coupled to a FAS1 femtostat and IBM-compatible computer. NO recording was generated by carefully applying the microsensor to the surface of the lung slice using a micromanipulator. Determination of iNOS-derived NO release was then made by preincubating the lung slices for 1 h in a t-arginine-free medium containing the eNOS inhibitor t-NA (2 μM), t-arginine (1 mM), and calcium ionophore A23187 (1 μM) were added to the medium and incubated for an additional 20 min. NO production was measured as area under the curve during the 20-min period.

**Nuclear protein isolation**

Lung samples were homogenized in lysis buffer containing 100 mM Tris (pH 7.5), 5 mM EGTA, 1 mM Na3VO4, 5 mM NaCl, 5 mM NaF, 5 mM EDTA, and protease inhibitor mixture. The homogenate was then centrifuged, and the supernatant was collected. Protein concentration was determined in an aliquot of the supernatant. GAPDH mRNA was measured as described previously (25). Briefly, the femur, tibia, and humerus were surgically removed, and bone marrow was extracted by flushing 0.1% BSA through the cut ends of the bones. The PMN-rich solution was added to a 96-well plate to which SYBR Green RT-PCR Master Mix components and appropriate primers were then added. The reaction mix was placed in a preprogrammed ABI PRISM real-time cycle (Applied Biosystems), which detected fluorescence produced by reaction of RNA with its respective primer. ICAM-1 mRNA levels were measured for each sample using GAPDH mRNA as a reference.

**Quantitative PCR**

Lung samples were homogenized in lysis buffer containing 100 mM Tris (pH 7.5), 5 mM EGTA, 1 mM Na3VO4, 5 mM NaCl, 5 mM NaF, 5 mM EDTA, and protease inhibitor mixture. The homogenate was then centrifuged, and the supernatant was collected. Protein concentration was determined in an aliquot of the supernatant. Equal amounts of protein from the homogenates were electrophoresed on gradient gels (4 –12%) and subsequently transferred to 0.22-μm nitrocellulose membranes (Osmonics). The membranes were blocked with 5% nonfat milk and probed with the following Abs: caveolin-1, iNOS, eNOS, ICAM-1, IκBα, and GAPDH.

**Isolation of murine PMN**

PMNs were isolated from murine bone marrow using Percoll density gradients as described previously (35). Briefly, the femur, tibia, and humerus were surgically removed, and bone marrow was extracted by flushing 0.1% BSA through the cut ends of the bones. The PMN-rich solution was added to a 96-well plate to which SYBR Green RT-PCR Master Mix components and appropriate primers were then added. The reaction mix was placed in a preprogrammed ABI PRISM real-time cycle (Applied Biosystems), which detected fluorescence produced by reaction of RNA with its respective primer. ICAM-1 mRNA levels were measured for each sample using GAPDH mRNA as a reference.

**Isolation of mouse lung vascular endothelial cells**

Mouse lung vascular endothelial cells (MLVEC) were isolated from WT and Cav-1−/− mice as follows. Briefly, lungs were extracted from 3– to 5-day-old mouse pups and minced in 1× HBSS. Collagenase A (10 ml) was then added to the minced lung tissue, and the mixture was incubated at 37°C in a shaking water bath for 60 min. Next, the minced samples were centrifuged at high speed for 10 min at room temperature. The resulting pellet was resuspended in 10 ml of 1× HBSS and filtered through a 60-μm nylon mesh. The cell suspension was centrifuged at high speed for 10 min at 4°C. The pellet was resuspended in 10 ml of suspension buffer (1× PBS, 0.5 g/100 ml BSA, 2 mM EDTA, and 25 mM d-glucose) containing 10 μg of anti-mouse PECAM-1 Ab. The cell suspension and primary Ab were
then incubated on ice for 1 h, during which time the secondary Ab was prepared. Dynabeads M-450 (100 µl) (sheep anti-rat IgG) were added to 5 ml of 1× HBSS and then attached to a magnetic column. The secondary Ab was washed twice with 5 ml of 1× HBSS. Next, the purified secondary Ab was added to the cell suspension and incubated on ice for 40 min. The cell suspension was placed in a separate container and affixed to the magnetic column. The supernatant was then removed, and the cells were resuspended in 1× HBSS. Trypsin (2 ml) was then added to the suspension, and the mixture was incubated for 2–3 min at room temperature. After attaching the suspension to a magnetic column, the trypsin-released cells were removed as supernatant, placed in a growth medium containing EBM-2 with 2% FBS, and grown to confluence on 96-well gelatin-coated plates.

**Determination of PMN adhesion to endothelium**

PMN adhesion to cultured MLVECs was measured as described previously (36). Briefly, bone marrow PMNs were loaded with calcein-AM (Molecular Probes) at 2 µg/ml for 30 min at room temperature and then added to MLVECs. The cells were then incubated with LPS (1 µg/ml) for 30 or 90 min. Following LPS treatment, the MLVECs were washed with PBS, and fluorescence was measured in duplicate with a spectrofluorometer (Photon Technology International) at excitation and emission wavelengths of 485 and 535 nm, respectively.

**Lung tissue myeloperoxidase (MPO) activity**

Lung tissue MPO activity was measured as described previously (28). Briefly, lungs were homogenized in 1 ml of PBS (pH 6) with 5% hexadecyltrimethylammonium bromide and 5 mM EDTA. The homogenates were sonicated, centrifuged at 40,000 × g for 20 min, and run through two freeze-thaw cycles. The samples were homogenized and centrifuged a second time. The supernatant was then collected and mixed 1/30 (v/v) with assay buffer (0.2 mg/ml o-dianisidine hydrochloride and 0.0005% H₂O₂). Absorbance change was measured at 460 nm for 3 min, and MPO activity was calculated as the change in absorbance over time. PMN sequestration was quantified as MPO activity normalized by dry lung weight.

**Determination of lung microvascular permeability and edema formation**

Microvascular liquid permeability and edema formation were quantified by measuring capillary filtration coefficient (Kₘ) and lung wet-to-dry weight ratios, respectively. As previously described (37), mice were anesthetized with an i.p. injection of ketamine (60 mg/kg), xylazine (2.5 mg/kg), and acepromazine (2.5 mg/kg) under an approved institutional animal protocol. The trachea was cannulated with a stainless steel tube for constant positive pressure ventilation (120 breaths/min). Heparin (50 U) was administered via the right internal jugular vein to prevent blood clotting. A thoracotomy was performed to expose the heart and lungs. An incision was made in the right ventricle for insertion of a pulmonary arterial catheter (polyethylene). The cannula was secured with suture. The lungs were perfused via a peristaltic pump with modified Krebs-Henseleit solution, supplemented with 5 g/100 ml of BSA (fraction V, 99% pure and endotoxin free; Sigma-Aldrich). The heart and lungs were rapidly excised and transferred en bloc to a perfusion device. Lung wet weight was monitored with an electronic beam balance and vascular pressures with transducers. Capillary pressure was measured by the double occlusion method (38). The isolated lungs were ventilated (120 breaths/min) and perfused at constant flow (2 ml/min), temperature (37°C), and venous pressure (+2 cm H₂O).

For Kₘ determinations, all lungs underwent a 20-min equilibration perfusion to establish an isogravimetric preparation. Outflow pressure was then rapidly elevated by + 6 cm H₂O for 30 min. The change in lung wet weight reflected both an initial rise in vascular volume followed by a slower phase of net fluid extravasation. A double exponential curve was fitted to the data, and Kₘ, in units of ml/min/cm H₂O/dry lung gram was calculated as the initial slope of the slow component of weight change divided by the change in capillary pressure and lung dry weight. Wet-to-dry weight ratio was measured in all preparations after determination of Kₘ. To do this, lungs were isolated, blotted, and weighed (lung wet weight). They were then placed in a 70°C incubator for 48 h, after which dry lung weights were determined.

**Mortality study**

Cav-1⁻/⁻ and WT mice (15 mice/group) were injected with a lethal LPS dose (125 mg/kg, i.p.). Percentage of surviving mice was noted at 6, 8, 10, and 12 h after LPS administration. l-NA (100 mg/kg, i.p.) was given 1 h before LPS in one group of Cav-1⁻/⁻ mice to assess the effect of NO inhibition on LPS-induced mortality in the absence of caveolin-1 protein. Percent survival was again documented at 6, 8, 10, and 12 h following LPS challenge.

**Statistical analysis**

Data are represented as mean ± SEM. Statistical comparisons were made using Dunnet’s t test and the log rank test (for survival curve comparisons). Data were considered statistically different at p < 0.05.

**Results**

*Increased eNOS-derived NO production and impaired iNOS-derived NO production in lungs of Cav-1⁻/⁻ mice after LPS challenge*

We found that in the absence of a calcium ionophore, NO concentration (measured as nitrite metabolite) was significantly greater in Cav-1⁻/⁻ lungs (5.1 ± 0.6 µM) than WT (0.27 ± 0.08 µM). To distinguish the enzymatic source of NO, we measured NO production in the presence of inhibitors of eNOS (l-NA) or iNOS (1400W). We incubated lung slices with calcium ionophore A23187 plus 1400W to determine eNOS-derived NO levels. In both control and LPS-treated Cav-1⁻/⁻ lung slices, eNOS-derived NO concentrations were significantly greater than in WT (Fig. 1A). Interestingly, the addition of calcium ionophore A23187 (plus the iNOS blocker) produced a rise in NO concentration in the control WT lungs (3.0 µM) but not in Cav-1⁻/⁻ lung slices (data not shown), which is consistent with our observation of constitutive eNOS activation in Cav-1⁻/⁻ lungs.

We next measured NO production by iNOS using the eNOS inhibitor l-NA. Lungs from LPS-untreated WT and Cav-1⁻/⁻ lungs produced the same quantity of NO under basal conditions in the presence of eNOS inhibitor (Fig. 1B). Challenge of WT mice with LPS had no effect on iNOS-derived NO production until 4 h after injection (Fig. 1B). At this time, NO production rose steeply; however, this increase was small in Cav-1⁻/⁻ lungs relative to WT (Fig. 1B). Thus, while eNOS-derived NO production was augmented in Cav-1⁻/⁻ mice, iNOS-generated NO exhibited the opposite trend.

*Reduced iNOS expression in lungs of Cav-1⁻/⁻ mice after LPS challenge*

We next addressed the basis of the reduced LPS-induced NO production in Cav-1⁻/⁻ lungs; i.e., whether the difference was due to changes in enzyme expression or activation of eNOS and iNOS. eNOS protein levels were similar in WT and Cav-1⁻/⁻ mice and remained constant at all time points following LPS administration (Fig. 1C), indicating that increase in eNOS activity rather than level of expression was responsible for the augmented eNOS-derived NO production observed in Cav-1⁻/⁻ lungs.

By contrast, iNOS expression in lungs increased significantly after LPS administration in WT lungs (Fig. 1C). The time course of LPS-induced iNOS expression paralleled the rise in iNOS-derived NO production observed after LPS. Furthermore, as with NO production, iNOS expression was markedly reduced in Cav-1⁻/⁻ lungs relative to WT (Fig. 1C), suggesting that the decrease in iNOS-derived NO production observed in Cav-1⁻/⁻ mice is principally due to diminished expression.

To verify the genotype of the Cav-1⁻/⁻ mice, Western blots were performed. Cav-1⁻/⁻ mice showed complete absence of caveolin-1 protein, which did not change after LPS challenge (Fig. 1D). Caveolin-1 expression in WT mouse lungs also did not increase as a result of LPS challenge (Fig. 1D).
Caveolin-1 deletion impairs IkBα degradation and NF-κB activation through a NO-dependent mechanism

Administration of LPS resulted in increased NF-κB activity, with peak activation occurring at 1 h in WT and Cav-1−/− mice (Fig. 2, A and D). The levels of NF-κB activity after LPS administration were significantly reduced in Cav-1−/− mice relative to WT (Fig. 2, A and D). Administration of the eNOS inhibitor L-NA (100 mg/kg, i.p.) 1 h before LPS injection, restored NF-κB activity in Cav-1−/− lungs to WT level at 1 h after LPS treatment (Fig. 2, B and D).

IkBα degradation mirrored NF-κB activation (Fig. 2C). In both WT and Cav-1−/− lungs, IkBα protein levels were lowest at 1 h after LPS challenge, corresponding to the peak of NF-κB activation. Furthermore, IkBα expression levels were greater in Cav-1−/− lungs than WT at 1 and 3 h post-LPS. Administration of L-NA (100 mg/kg, i.p.) reduced IkBα in Cav-1−/− lungs to near WT levels.

Reduced ICAM-1 expression in Cav-1−/− mice

We assessed NF-κB-dependent ICAM-1 expression using both quantitative RT-PCR and Western blotting. LPS treatment resulted in increased ICAM-1 mRNA levels in WT and Cav-1−/− lungs (Fig. 3, A and B). Peak expression paralleled the NF-κB activity. When compared with their WT counterparts, however, Cav-1−/− lungs exhibited a marked reduction in LPS-induced ICAM-1 expression (Fig. 3, A and B). We observed the same pattern with ICAM-1 protein expression. Western blots showed up-regulation of ICAM-1 protein after LPS treatment, but this response was markedly attenuated in Cav-1−/− mice relative to WT (Fig. 3B).
Caveolin-1 deletion reduces PMN adhesion to endothelial cells and PMN sequestration in lungs after LPS challenge

PMN binding was assessed in vitro by adhesion of WT PMN to cultured MLVEC. Adhesion of WT PMN increased significantly after exposure of endothelial cells to LPS; however, the response was markedly reduced in MLVEC cultured from Cav-1

-/- mice relative to WT (Fig. 4A). This reduction in PMN binding paralleled the diminished ICAM-1 expression seen with Western blotting. Lung PMN sequestration was studied in vivo with the MPO assay. Like PMN adhesion, MPO activity was up-regulated by LPS administration, but the response was reduced significantly in Cav-1

-/- lungs relative to WT (Fig. 4B). Administration of L-NA 1 h before LPS injection prevented the reduction in MPO activity seen in Cav-1

-/- mice at 4 h post-LPS (Fig. 4B). This effect was not seen 1 h after LPS injection, suggesting the time dependence of the NO effect in down-regulating the expression of ICAM-1 and thereby PMN uptake.

Deletion of caveolin-1 gene prevents LPS-induced increase in lung microvascular permeability and edema formation

We assessed pulmonary microvascular liquid permeability by measuring the capillary filtration coefficient (Kf,c) (Fig. 5A). Under basal conditions (i.e., no LPS pretreatment), Kf,c in Cav-1

-/- lungs was not significantly elevated above WT levels. LPS induced a significant increase in Kf,c only in WT lungs, thus indicating that Cav-1

-/- lungs were protected from LPS-induced lung injury. To assess alterations in lung fluid balance in response to LPS challenge, we also measured the wet-to-dry lung weight ratio in the perfused lung preparations (Fig. 5B). We observed no difference in the wet-to-dry ratio of Cav-1

-/- and WT lung preparations under basal conditions. The wet-to-dry weight ratio was increased significantly in WT mice after LPS challenge but remained the same in Cav-1

-/- lungs.

Reduced mortality in Cav-1

-/- mice following LPS challenge

To demonstrate the possible protective effect of the Cav-1

-/- genotype, we performed mortality studies with LPS using a dose chosen to be lethal to WT control mice. Therefore, we increased the dosage of LPS to a level (i.e., 125 mg/kg, i.p.) well above its LD50. The administration of this high LPS dose was lethal to 87% of WT mice within 12 h. By contrast, Cav-1

-/- mice receiving the same LPS dose clearly had resistance to its lethal effects: only 40% of the mice so treated died at 12 h (Fig. 6). To determine whether elevated eNOS-derived NO was responsible for this protection, we administered L-NA 1 h before LPS in Cav-1

-/- mice. We observed 100% mortality at 12 h in Cav-1

-/- mice given the eNOS inhibitor.

Discussion

Our results demonstrate an important regulatory role of caveolin-1, the principal signaling and structural protein of caveolae, in the
Significant reduction in wet-to-dry ratio vs WT at 6 h.

NF-κB observed that caveolin-1 deficiency interfered with LPS-induced mechanism of lung inflammation and injury induced by LPS. We addressed the role of caveolin-1 in the regulation of the lung vascular permeability was assessed by measuring the capillary filtration coefficient ($K_f$) (see Materials and Methods). In the absence of LPS, lung microvascular fluid permeability was not significantly different between Cav-1$^{-/-}$ and WT lungs. However, 6 h after LPS, WT lung exhibited a marked increase in $K_f$, whereas Cav-1$^{-/-}$ lungs showed no significant change from baseline. *, $p < 0.05$ vs WT untreated. †, Significant reduction in $K_f$ vs WT at 6 h. B, LPS-induced pulmonary edema. Lung wet-to-dry weight ratio was used to quantify pulmonary edema. No significant differences were observed in the wet-to-dry ratios of WT and Cav-1$^{-/-}$ lung under basal conditions. However, at 6 h after LPS (10 mg/kg, i.p.), WT lungs had a significant increase in wet-to-dry ratio, whereas the ratio in Cav-1$^{-/-}$ lungs did not increase above baseline. *, $p < 0.05$ vs WT untreated. †, Significant reduction in wet-to-dry ratio vs WT at 6 h.

mechanism of lung inflammatory response to LPS-induced sepsis. Caveolae have been shown to facilitate transendothelial transport via transcytosis and migration of leukocytes across the vascular endothelial barrier (39, 40). It is unclear, however, whether caveolin-1 plays a role in the organization of inflammatory signaling events leading to lung injury. Thus, we used Cav-1$^{-/-}$ mice to address the role of caveolin-1 in the regulation of the lung vascular response to sepsis. As caveolin-1 is a crucial negative regulator of eNOS-derived NO production (13–19), we addressed the possibility that caveolin-1 regulates NO signaling and participates in the mechanism of lung inflammation and injury induced by LPS. We observed that caveolin-1 deficiency interfered with LPS-induced NF-κB activation and the consequent expression of NF-κB-regulated iNOS and ICAM-1 genes. These effects of caveolin-1 deletion were ascribed to enhanced eNOS activity and eNOS-derived NO production in the caveolin-1 null mice.

We showed in these studies that caveolin-1 negatively regulates eNOS activity in vivo consistent with previous findings (13–19). Lungs from Cav-1$^{-/-}$ mice exhibited greater eNOS-derived NO production than WT lungs. Since eNOS protein expression was the same in both WT and Cav-1$^{-/-}$ lungs, the increase in eNOS-derived NO production in Cav-1$^{-/-}$ lung was attributed to enhanced enzymatic activity.

Increased total and eNOS-derived NO was associated with suppression in NF-κB activation in Cav-1$^{-/-}$ lungs. We observed a significant reduction in LPS-induced IkBα degradation and NF-κB activation in Cav-1$^{-/-}$ lungs relative to WT. Since NO regulates NF-κB in a concentration-dependent manner, the attenuation in NF-κB activation is likely the result of high basal NO levels in Cav-1$^{-/-}$ lungs. Elevated NO during the early inflammatory response may serve a negative feedback function suppressing NF-κB activation. This was indeed the case as we observed that inhibition of NO by l-NA restored NF-κB activity in Cav-1$^{-/-}$ lungs to WT levels. The restoration of NF-κB activity occurred within 1 h of LPS exposure, suggesting that suppression of NF-κB activation in Cav-1$^{-/-}$ lungs was the result of increased eNOS activity and resultant NO production. This finding raises the possibility that increasing NO levels using NO donors during the crucial early period after sepsis may prevent NF-κB activation and expression of proinflammatory genes. Several mechanisms have been postulated to explain the ability of NO to down-regulate NF-κB activity (34). These include stabilization of IkBα (30), nitrosation of Cys (62) on p50 (41–44), and rapid interaction with reactive oxygen species (45–47). Our Western blot data, which showed increased LPS-induced IkBα protein levels in Cav-1$^{-/-}$ lungs, indicate that elevated NO may stabilize IkBα, thus preventing its degradation and nuclear translocation of NF-κB.

To address the effects of caveolin-1-regulated NO signaling downstream of NF-κB, we measured the expression of two NF-κB-dependent inflammatory proteins: ICAM-1 and iNOS. Western blotting showed significant reductions in LPS-induced ICAM-1 and iNOS protein expression. The difference in iNOS protein expression in WT and Cav-1$^{-/-}$ lungs was not detected until 4 h after LPS challenge, a finding consistent with the hypothesis that caveolin-1 deficiency negatively regulates NF-κB-dependent de novo synthesis of inflammatory proteins. Interestingly, ICAM-1 protein expression, while it behaved similarly to iNOS in response to LPS stimulation in Cav-1$^{-/-}$ lungs, was also reduced during baseline relative to WT. This reduction in basal ICAM-1 expression in Cav-1$^{-/-}$ lungs may be the result of increased production of eNOS-derived NO during the baseline period. Previous studies have also shown that ICAM-1 dynamically interacts with caveolar membrane during leukocyte trafficking (39); thus, it is possible that in the absence of caveolae in Cav-1$^{-/-}$ mice, ICAM-1 is not appropriately localized to caveolae to promote leukocyte transmigration. This notion is consistent with the findings that both PMN adhesion to Cav-1$^{-/-}$ endothelial cells and lung PMN infiltration in Cav-1$^{-/-}$ lungs were markedly reduced after LPS challenge.

**FIGURE 5.** Lung microvascular permeability and edema formation in Cav-1$^{-/-}$ mice in response to LPS. Lungs were isolated and perfused (see Materials and Methods). A, LPS-induced increase in lung microvascular permeability was assessed by measuring the capillary filtration coefficient ($K_f$) (see Materials and Methods). In the absence of LPS, lung microvascular fluid permeability was not significantly different between Cav-1$^{-/-}$ and WT lungs. However, 6 h after LPS, WT lung exhibited a marked increase in $K_f$, whereas Cav-1$^{-/-}$ lungs showed no significant change from baseline. *, $p < 0.05$ vs WT untreated. †, Significant reduction in $K_f$ vs WT at 6 h. B, LPS-induced pulmonary edema. Lung wet-to-dry weight ratio was used to quantify pulmonary edema. No significant differences were observed in the wet-to-dry ratios of WT and Cav-1$^{-/-}$ lung under basal conditions. However, at 6 h after LPS (10 mg/kg, i.p.), WT lungs had a significant increase in wet-to-dry ratio, whereas the ratio in Cav-1$^{-/-}$ lungs did not increase above baseline. *, $p < 0.05$ vs WT untreated. †, Significant reduction in wet-to-dry ratio vs WT at 6 h.

**FIGURE 6.** Decreased mortality in Cav-1$^{-/-}$ mice compared with WT 12 h after administration of a lethal dose of LPS. Mice were injected with LPS (125 mg/kg, i.p.), and the percentage of surviving animals was observed at 6, 8, 10, and 12 h post-LPS. At 12 h, Cav-1$^{-/-}$ mice exhibited significantly decreased mortality (40%) relative to WT (87%). Administration of l-NA (100 mg/kg, i.p.) to Cav-1$^{-/-}$ mice 1 h before LPS produced 100% mortality. *, $p < 0.05$ vs LPS-treated WT control.
To ascertain the functional significance of reduced ICAM-1 expression in Cav-1−/− mice, we investigated PMN sequestration in lungs and PMN adhesion to endothelial cells obtained from these mice. As ICAM-1 is the primary adhesion molecule involved in firm PMN binding to the endothelium (48), we determined whether the reduced ICAM-1 expression seen in Cav-1−/− lungs would interfere with PMN adhesion and recruitment in lungs. We found that PMN adhered less to MLVECs cultured from Cav-1−/− mice than WT MLVECs. This was the case under both basal conditions and after LPS stimulation. Using the MPO assay, we observed diminished WT MLVECs. This was the case under both basal conditions and after LPS challenge relative to WT. Administration of L-NA restored the magnitude of PMN sequestration in Cav-1−/− lungs to the level seen in WT lungs at 4 hr after LPS. That WT L-NA showed the same results in Cav-1−/− lungs was the latter step in reversing the Cav-1−/− phenotype at 4 hr, but not at 1 hr after LPS, lends further support to the hypothesis that caveolin-1 deficiency impairs NF-κB-dependent ICAM-1 synthesis through elevated eNOS-derived NO production.

We observed marked reductions in pulmonary edema formation and microvascular barrier permeability in Cav-1−/− mice consistent with the findings of reduced ICAM-1 synthesis and PMN recruitment in Cav-1−/− lungs. Decreased edema formation in Cav-1−/− lung is likely related to preservation of the intact pulmonary microvascular barrier in Cav-1−/− lungs. Thus, the reduction in NF-κB-mediated ICAM-1 expression observed in caveolin-1 deficient lungs results in impaired PMN adhesion and sequestration in lungs. This in turn protects Cav-1−/− lungs from PMN-mediated injury of the microvascular barrier and edema formation. The reduced PMN-mediated lung injury may be responsible for the greater survival seen at 12 h after LPS challenge in Cav-1−/− mice compared with WT. Interestingly, coadministration of NO inhibitor with LPS to Cav-1−/− mice resulted in significantly greater mortality at 12 h. This finding is consistent with published data showing that systemic administration of NO inhibitor to patients in septic shock worsens survival despite ameliorating hypotension (49–52).

It is informative to compare our result that increased eNOS-derived NO production is responsible for protection against LPS-induced lung injury in Cav-1−/− mice with those of Connolly et al. (53). Connolly et al. (53) showed that eNOS-derived NO plays a key role in facilitating iNOS expression in LPS-induced endotoxemia. They observed a temporal reduction in iNOS expression and activity (in lung, liver, heart, and aorta) in LPS-challenged Cav-1−/− mice compared with WT. However, our results are not discrepant with Connolly’s findings. Their study did not address the consequence of augmentation of eNOS activity. We showed that increased eNOS activity in Cav-1−/− lungs suppressed NF-κB activation and NO production levels. This is in contrast to our observations in Cav-1−/− lungs challenged by LPS, whereas Cav-1 null mice exhibited no significant change in permeability after LPS due to the reduction in PMN-mediated endothelial barrier damage.

Wang et al. (54) showed that siRNA-induced Cav-1 knockdown resulted in increased NF-κB activation in LPS-stimulated alveolar macrophages. Their study, however, did not assess the effects of Cav-1 knockdown on eNOS activity and NO levels in macrophages. This is an important consideration because the NO level is arguably related to the effectiveness of the knockdown and percentage of transduced cells. Thus, it is difficult to compare their results to ours. We showed that caveolin-1 deletion resulted in increased eNOS-derived NO in lungs and that this was responsible for the attenuation of LPS-induced NF-κB activity in Cav-1−/− lungs. In addition, there are several other important differences between the study of Wang et al. and ours. Wang et al. (54) studied NF-κB activation in macrophages in an in vitro system using siRNA to knockdown caveolin-1 expression. We measured NF-κB activity in Cav-1−/− lungs from Cav-1 knockout mice whereas Wang et al. (54) used a knockdown model. Caveolin-1 knockout mice are devoid of both caveolin-1 and caveolin-2, whereas caveolin-1 knockout animals expressed caveolin-2 (55); thus, these models should not be considered equivalent.

In summary, we have described a novel caveolin-1-mediated signaling pathway in a mouse model of LPS-induced lung injury. We showed that caveolin-1 through its ability to regulate eNOS-derived NO production inhibits NF-κB activation and expression of proinflammatory proteins, iNOS and ICAM-1. WT lungs with low eNOS activity achieved much higher iNOS expression levels than Cav-1−/− deficient lungs in response to LPS, indicating that eNOS can effectively control high-output (i.e., iNOS-dependent) NO production. We also demonstrated that caveolin-1 gene knockouts exert a protective effect in the lung inflammatory response by reducing PMN-mediated microvascular barrier injury and edema formation. These findings may be of value in the design of novel therapeutic strategies to block the lung inflammatory response to sepsis. Peptides that interfere with binding of caveolin-1 to eNOS may prevent NF-κB activation and the resultant expression of inflammatory proteins contributing to the mechanism of lung injury.

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Disclosures
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