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Intercellular Adhesion Molecule-1–Dependent Neutrophil Adhesion to Endothelial Cells Induces Caveolae-Mediated Pulmonary Vascular Hyperpermeability

Guochang Hu, Stephen M. Vogel, David E. Schwartz, Asrar B. Malik, and Richard D. Minshall

From the Departments of Pharmacology (G.H., S.M.V., A.B.M., R.D.M.) and Anesthesiology (D.E.S., R.D.M.) and Center for Lung and Vascular Biology (G.H., S.M.V., A.B.M., R.D.M.), University of Illinois College of Medicine, Chicago

Abstract

We investigated the role of caveolae in the mechanism of increased pulmonary vascular permeability and edema formation induced by the activation of polymorphonuclear neutrophils (PMNs). We observed that the increase in lung vascular permeability induced by the activation of PMNs required caveolin-1, the caveolae scaffold protein. The permeability increase induced by PMN activation was blocked in caveolin-1 knockout mice and by suppressing caveolin-1 expression in rats. The response was also dependent on *Src* phosphorylation of caveolin-1 known to activate caveolae-mediated endocytosis in endothelial cells. To address the role of PMN interaction with endothelial cells, we used an intercellular adhesion molecule (ICAM)-1 blocking monoclonal antibody. Preventing the ICAM-1–mediated PMN binding to endothelial cells abrogated *Src* phosphorylation of caveolin-1, as well as the increase in endothelial permeability. Direct ICAM-1 activation by crosslinking recapitulated these responses, suggesting that ICAM-1 activates caveolin-1 signaling responsible for caveolae-mediated endothelial hyperpermeability. Our results provide support for the novel concept that a large component of pulmonary vascular hyperpermeability induced by activation of PMNs adherent to the vessel wall is dependent on signaling via caveolin-1 and increased caveolae-mediated transcytosis. Thus, it is important to consider the role of the transendothelial vesicular permeability pathway that contributes to edema formation in developing therapeutic interventions against PMN-mediated inflammatory diseases such as acute lung injury.

Keywords

caveolin-1; *Src*; endothelial permeability; albumin transport; lung edema

Increased lung vascular albumin permeability leads to the accumulation of protein-rich interstitial and alveolar fluid, the characteristic feature of acute lung injury.^{1,2} Activation of pulmonary neutrophils (PMNs) sequestered in pulmonary microvessels is an important factor in the pathogenesis of increased lung vascular permeability and tissue injury.^{3–5} However, the mechanisms of increased vascular permeability induced by PMNs are not completely understood. Studies have shown that activation of PMNs results in the release of mediators (eg, oxidants and proteases) that increase vascular permeability by disrupting interendothelial junctions,^{1–9} which normally form a restrictive barrier excluding plasma proteins of the size

Correspondence to Richard D. Minshall, Department of Pharmacology (m/c 868), University of Illinois College of Medicine, 835 S Wolcott Ave, Chicago, IL 60612. E-mail rminsh@uic.edu.

Disclosures

None.

of albumin and greater.^{10,11} Other studies in lungs of sepsis-induced ARDS patients showed extensive fluid accumulation in interstitial and alveolar compartments, but in most cases, a well-preserved microvascular endothelial junctional barrier.¹² An unexamined mechanism of endothelial hyperpermeability is the possibility of increased transport of albumin occurring via the transcytosis pathway involving the trafficking of caveolae across the endothelial barrier.

¹ It is possible that in absence of gross interendothelial junctional alterations after PMN activation, increased caveolae-mediated transport of albumin, and concentration of albumin in the lung interstitium, provides the necessary transendothelial pressure gradient to maintain a high net transcapillary fluid filtration rate. Although studies have suggested that transcytosis is a mechanism of transendothelial albumin permeability,^{13–16} its pathophysiological significance in PMN activation-mediated inflammatory disease has not been examined.

The continuous microvascular endothelium, the type present in lung microvessels,¹ establishes a semipermeable barrier dependent on the assembly of adherens and tight junctions that anneal neighboring cells and restrict the passage of plasma proteins.^{1,17} Albumin, the most abundant plasma protein, traverses the endothelial barrier by a vesicular pathway.^{10,11} Albumin concentration of the interstitium is the primary determinant of interstitial oncotic pressure.¹ We and others have described a role of caveolin-1 (Cav-1), the primary structural component of caveolae and a scaffolding protein, in regulating endothelial transcytosis.^{10,14–16} Cav-1 controls the formation and release of caveolae from the plasma membrane, which shuttle macromolecules across the endothelial barrier.^{10,11,13–16} The uptake of albumin via endocytosis and its transcytosis to tissue is the result of fission and trafficking of caveolae.^{13–16} Studies in Cav-1-null mice (*Cav-1*^{-/-}) showed the absence of caveolae and defective transcellular transport of albumin,^{18,19} which could be rescued by expression of Cav-1.²⁰ Although mechanisms of Cav-1-regulated fission and trafficking of caveolae are still unclear, phosphorylation of Cav-1 on tyrosine 14 by *Src* kinase is a key “switch” initiating caveolar fission from the plasma membrane.^{21–27}

The role of *Src* activation on caveolae fission and trafficking and endothelial albumin transcytosis following PMN activation is not known. Activation of PMNs with the complement peptide C5a induced *Src* kinase activation and increased endothelial permeability.⁶ In addition, exposure of endothelial cells to H₂O₂ (a PMN-derived oxidant) increased *Src* activity in association with increased endothelial permeability.²⁸ Crosslinking of endothelial cell surface ICAM-1 increased *Src* kinase activity,^{29–32} raising the possibility that PMN adhesion to endothelial cells via the CD18/ICAM-1 interaction engages the endothelial cell caveolar transport machinery. Whereas previous studies indicate that activated PMNs induce an increase in albumin permeability, the role of caveolae in the mechanism of increased vascular albumin permeability has not been addressed. Our results suggest that endothelial albumin hyperpermeability induced by transcytosis of albumin contributes significantly to the development of pulmonary edema induced by the activation of PMNs. Thus, strategies aimed at blocking vascular hyperpermeability via transcytosis may prove to be useful in preventing pulmonary edema seen in acute lung injury.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement.

Materials

Adult Sprague–Dawley rats (250 to 300 g) were purchased from Charles River Laboratories (Wilmington, Mass). Cav-1-null mice and corresponding wild-type (WT) cohorts, in the black Swiss genetic background, were obtained from The Jackson Laboratory (Bar Harbor, Me) and Taconic (Hudson, NY). All animal experiments were performed after approval from the University of Illinois Animal Care and Use Committee. Rat lung microvascular endothelial

cells (RLMVECs) were obtained from Vec Technologies (Rensselaer, NY). Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (St Louis, Mo).

Cell culture, uptake and transendothelial transport of ^{125}I -albumin tracer, fluorescent albumin uptake, transendothelial electric resistance (TER), dominant negative (dn) *Src* construct transfections, and Western blotting were performed as described.^{21,23,24,33,34}

Isolation of Neutrophils

PMNs were isolated from rat or mouse whole blood using the hetastarch exchange transfusion and sedimentation technique.³⁵

Cav-1 Small Interfering RNA Transfection in Endothelial Cell and Rat Lung

Small interfering (si)RNA duplex oligonucleotides were purchased from Dharmacon (Lafayette, Colo). The sequences of Cav-1 siRNA and negative control duplex were 5'-UCUGUGAUCCACUCUUUGAUU-3' and 5'-UAAGGCUAUGAAGAGAUAC-3', respectively. Preliminary experiments showed effective siRNA-mediated knockdown of Cav-1 in RLMVEC by transfecting cells with 10 nmol/L siRNA at 50% to 70% confluence using the protocol provided by the manufacturer. All experiments were performed 48 hours after transfection. In vivo cationic liposome-siRNA complexes were made as described.³⁶ The liposome-siRNA complex was prepared by addition of 0.25 mg/kg siRNA into 500 μL of liposome suspensions. Successful transfection of Cav-1 siRNA was confirmed by Cav-1 Western blotting of lung homogenates.

Lung Preparation

Methods of isolation and perfusion of lung preparations were performed as described.³⁷ Permeability-surface area (PS) product, an index of vascular permeability to albumin, was measured as described.³⁸ The animals were anesthetized with pentobarbital sodium (40 mg/kg IP). The lungs were perfused with Krebs solution at a constant flow and venous pressure.

Statistic Analysis

Data were expressed as means \pm SEM. One-way ANOVA and the Student-Newman-Keuls test were used to compare the different groups; $P < 0.05$ was considered significant.

Results

Caveolae-Dependent Increase in Transendothelial ^{125}I -Albumin Transport Following PMN Activation

Activation of PMNs with *N*-formyl-methionyl-leucyl-phenyl-alanine (fMLP) resulted in a 75% increase in transendothelial transport of ^{125}I -albumin (Figure 1A). Neither addition of unstimulated PMNs nor fMLP alone altered ^{125}I -albumin permeability (Figure 1A). The increase in albumin permeability was dependent on the number of PMNs added (Figure 1B). To address whether intact caveolae were required for the response, endothelial caveolae were disrupted by pretreatment with cholesterol-binding agent methyl- β -cyclodextrin.^{33,39} Cyclodextrin prevented the PMN activation-dependent increase in ^{125}I -albumin flux (Figure 1C). To determine whether increased transendothelial albumin flux was the result of opening of interendothelial junctions, we measured changes in TER. Activation of PMNs with fMLP did not alter TER (Figure 1D), whereas in a positive-control experiment, TER decreased by 60% from baseline ($5.95 \pm 0.58 \Omega \cdot \text{cm}^2$) for up to 60 minutes on activation of PMNs with the phorbol ester 4-phorbol myristate 13-acetate (PMA), which is known to severely injure the endothelial barrier (Figure 1D).

PMN Activation With fMLP Induces Albumin Endocytosis in Endothelial Cells

We next addressed the possibility that PMN activation stimulated caveolae-mediated endocytosis of albumin in endothelial cells, the initial step in albumin transport via transcytosis. ^{1,13–16} As shown in Figure 2A and 2B, fMLP activation of PMNs increased ¹²⁵I-albumin endocytosis by endothelial cells within minutes. The response was dependent on the number of PMNs added to endothelial cell monolayers. Neither PMNs nor fMLP alone affected the uptake of ¹²⁵I-albumin (data not shown). The increase in albumin endocytosis was abolished by pretreatment with cyclodextrin (Figure 2C). Fluorescence quantified as pixel intensity per cell using confocal microscopy showed that fMLP stimulation of 10⁶, 5×10⁶, or 10×10⁶ PMNs induced significant increases in Alexa 488 albumin uptake (Figure 2D), paralleling the responses observed with ¹²⁵I-albumin tracer.

Cav-1 Is Required for PMN Activation–Induced Endocytosis and Transendothelial Flux of Albumin

fMLP activation of PMNs increased the phosphorylation of Cav-1 in endothelial cells (Figure 3A and Figure IA in the online data supplement), whereas PMNs or fMLP alone had no effect (data not shown). Endothelial cells transduced with Cav-1 siRNA showed 90% decrease in caveolin-1 protein after 48 hours (Figure 3A and supplemental Figure IA). These cells were used to address the role of Cav-1 in mediating endothelial transcytosis. As shown in Figure 3B and 3C, fMLP activation of PMNs increased ¹²⁵I-albumin endocytosis and flux 2-fold in endothelial cells transduced with scrambled siRNA, whereas Cav-1 knockdown abolished this effect. To address further the role of Cav-1 in the PMN activation–induced increase in albumin permeability, we compared the effects of activated PMNs on endothelial endocytosis and transendothelial transport of ¹²⁵I-albumin in WT and Y14F-Cav-1 mutant (nonphosphorylatable Cav-1)–expressing RLMVECs (Figure 3D and supplemental Figure IB).²⁶ PMN activation-induced increases in endocytosis and transcytosis of ¹²⁵I-albumin were abolished in Y14F-Cav-1 mutant–expressing cells (Figure 3E and 3F).

Requirement for *Src* Phosphorylation of Cav-1 in Signaling Endocytosis and Transendothelial Albumin Transport Induced by PMN Activation

fMLP activation of PMNs significantly increased *Src* activity in endothelial cells and pretreatment with the *Src* inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo [3,4,d] pyrimidine prevented this response (Figure 4A and supplemental Figure IC). Activation of increasing numbers of PMNs with fMLP also increased Cav-1 phosphorylation (Figure 4A and supplemental Figure IC), ¹²⁵I-albumin endocytosis (Figure 4B), and transendothelial ¹²⁵I-albumin flux; in all cases, these responses were inhibited by PP2. Neither PMNs alone nor fMLP alone elicited these effects (data not shown).

To address further the role of *Src* phosphorylation of Cav-1 in signaling endocytosis and transendothelial albumin transport induced by PMN activation, we transfected endothelial cells with the pFB retroviral vector encoding dn-*Src*. Total *Src* protein in dn-*Src*–transfected cells was significantly greater than endogenous *Src* protein (Figure 4D and supplemental Figure ID); ie, the exogenously introduced dn-*Src* kinase represented most of the total *Src* protein. dn-*Src* expression blocked basal Cav-1 phosphorylation, indicating that inhibiting the activity of endogenous *Src* kinase prevented Cav-1 phosphorylation (Figure 4D and supplemental Figure ID). Expression of dn-*Src* also prevented ¹²⁵I-albumin endocytosis (Figure 4E) and transendothelial ¹²⁵I-albumin flux stimulated by activated PMNs (Figure 4F).

PMN Activation With fMLP Increases Vascular Albumin Permeability and Tissue Water Content via Cav-1-Dependent Pathway in Rat Lungs

Administration of 2×10^7 PMNs or 5×10^7 PMNs followed by $1 \mu\text{mol/L}$ fMLP increased ^{125}I -albumin PS product by 74% and 140%, respectively, whereas PMN (2×10^7 or 5×10^7 cells) or fMLP alone ($1 \mu\text{mol/L}$) did not alter ^{125}I -albumin PS (Figure 5A). To address whether the caveolae-mediated transcytosis pathway was involved, we pretreated lungs with the cholesterol-binding agent filipin to ablate caveolae⁴⁰ before injection of the ^{125}I -albumin tracer. As shown in Figure 5B, filipin blocked the stimulatory effect of fMLP-activated PMNs (2×10^7 cells) on ^{125}I -albumin PS product. However, transport of ^3H -mannitol, a low-molecular-weight (182-Da) tracer that traverses the endothelial barrier via the paracellular (junctional) pathway,³³ was not affected by fMLP activation of PMNs. Also, *Src* inhibitor PP2 prevented the increase in ^{125}I -albumin PS product but did not alter the ^3H -mannitol PS product (Figure 5B).

Because measurement of ^{125}I -albumin PS product provides an assessment of the transvascular flux of albumin, we also addressed whether increase in PS value resulted in alterations in the final wet-to-dry (W/D) lung weight ratio, a measure of pulmonary edema formation (Figure 5C). Activation of 2×10^7 PMNs in lung microvessels with fMLP ($1 \mu\text{mol/L}$) did not alter W/D lung weight ratio (Figure 5C) in the presence of 74% increase in ^{125}I -albumin PS product (Figure 5A), whereas activation of 5×10^7 PMNs significantly increased lung W/D ratio (Figure 5C) concomitant with a 140% increase in ^{125}I -albumin PS product (Figure 5A). Neither PMNs nor fMLP alone affected ^{125}I -albumin PS product or W/D ratio values. Thus, 2×10^7 PMNs resulted in an increase in ^{125}I -albumin PS product but without pulmonary edema formation, whereas 5×10^7 PMNs resulted in a 2-fold greater increase in ^{125}I -albumin PS product and edema formation.

We next assessed the effects of siRNA depletion of Cav-1 in rat lung microvessels on vascular permeability. As shown in Figure 5D and supplemental Figure IIA, Cav-1 siRNA significantly decreased total rat lung Cav-1 expression compared to scrambled siRNA-treated lungs. In lungs treated with Cav-1 siRNA, we observed a significant decrease in basal, as well as PMN-stimulated, ^{125}I -albumin PS relative to scrambled siRNA-treated rat lungs (Figure 5E), consistent with the filipin study shown above (Figure 5B).

We also carried out studies in lungs from mice with targeted deletion of Cav-1 (*Cav-1*^{-/-}). Figure 5F compares the effect of fMLP-activated WT mouse PMNs on ^{125}I -albumin PS product in WT and *Cav-1*^{-/-} mouse lungs. Infusion of PMNs (2×10^6 cells) followed by fMLP ($1 \mu\text{mol/L}$) induced 1.6-fold increase in pulmonary vascular ^{125}I -albumin PS in WT mouse lungs. In *Cav-1*^{-/-} mouse lungs, we observed a decrease in basal ^{125}I -albumin PS, as evidenced by the role of caveolae in transporting albumin across the endothelial barrier. In contrast to WT, activation of PMNs did not result in increased ^{125}I -albumin PS in *Cav-1*^{-/-} mouse lungs.

Requirement for ICAM-1 Signaling Increased Pulmonary Vascular Albumin Permeability Induced by Activation of PMN

Confocal imaging showed clustering of endothelial cell surface ICAM-1 within 30 minutes following the addition of fMLP to PMN-endothelial cell cocultures (Figure 6A). Neither PMNs nor fMLP alone altered ICAM-1 surface distribution (data not shown). fMLP activation of PMNs also induced *Src* activation and *Src*-dependent phosphorylation of Cav-1 in RLMVEC monolayers, and these effects were blocked by anti-ICAM-1 monoclonal antibody (mAb) (Figure 6B and supplemental Figure IIB). Compared with control IgG group, treatment of endothelial cells with anti-ICAM-1 blocking mAb also prevented the increased endocytosis of fluorescent-albumin and ^{125}I -albumin tracer, as well as increased transendothelial ^{125}I -albumin flux (Figure 6C through 6E) induced by fMLP activation of PMNs. Neither anti-

ICAM-1 mAb nor control IgG alone affected ICAM-1 surface distribution and endocytosis of fluorescent-albumin (data not shown).

In the rat lung preparation, we observed that administration of anti-ICAM-1 blocking mAb (1A29), but not the isotype-matched control mAb, significantly reduced PMN activation-dependent increase in pulmonary vascular ^{125}I -albumin PS product (Figure 6F). Using the ICAM-1 crosslinking method in endothelial cells,^{29,41} we observed activation of *Src* and Cav-1 phosphorylation (Figure 7A and supplemental Figure IIC). Neither *Src* activation nor Cav-1 phosphorylation was seen following treatment with anti-ICAM-1 mAb, control mouse IgG plus antimouse IgG (Fab')₂ (secondary Ab), or secondary Ab IgG (Fab')₂ alone (Figure 7A and supplemental Figure IIC). Confocal imaging showed ICAM-1 clustering on the endothelial cell surface and increased Alexa 488-albumin endocytosis (Figure 7B). ICAM-1 crosslinking also increased ^{125}I -albumin endocytosis in endothelial cells (Figure 7C) and transendothelial albumin permeability (Figure 7D).

Discussion

The present study demonstrates a novel role of caveolae-mediated transcytosis of albumin in microvascular endothelial cells in the mechanism of PMN activation-induced increase in lung vascular permeability. Our results show that ICAM-1 signaling activated by the binding of PMNs to the endothelial cell surface promotes *Src* phosphorylation of Cav-1, a requirement for activation of the caveolar transport machinery.^{21–27} The increase in albumin permeability by means of caveolae was also shown to be an important mechanism of pulmonary edema formation. These results suggest that caveolae-mediated transport of albumin following PMN activation is an important determinant of the formation of pulmonary edema.

In our studies, we induced PMN adhesion to endothelial cells and activated PMNs with fMLP, a secretagogue specific to PMNs (versus endothelial cells).⁴² We observed that activation of PMNs increased endothelial permeability to albumin dependent on *Src* phosphorylation of Cav-1. This finding is consistent with the evidence that *Src* signaling is required for the engagement of the caveolae-mediated transcytosis machinery.^{21–27} Because endocytosis is the required initial step in transcytosis, we used several approaches to address the effects of activation of PMNs on albumin endocytosis. Using ^{125}I -albumin and Alexa 488-albumin tracers, we observed that PMN activation with fMLP induced albumin endocytosis. The cholesterol-depleting agent cyclodextrin or siRNA-induced depletion of Cav-1 prevented the endocytosis of albumin, indicative of a caveolae-dependent mechanism. Using a number of PMNs sufficient to activate caveolae-mediated albumin transcytosis in endothelial monolayers, we did not observe a decrease in TER, a measure of loss of endothelial junctional integrity. Finally, data showed the inability of fMLP-activated PMNs (at a PMN:endothelial cell ratio of 10:1) to disrupt interendothelial junctions consistent with previous findings,⁴³ whereas PMN activation with PMA, a potent PMN stimulating agent, resulted in decreased TER, a characteristic of severe endothelial junctional injury. Thus, activation of transcytosis under these conditions was not directly coupled to a disruption of endothelial junctional integrity.

The present studies were also carried out in rat and mouse lungs to buttress the endothelial monolayer studies and to address in vivo significance of PMN activation-induced increase in albumin transcytosis in endothelial cells. These results also showed that the PMN activation-induced increase in lung vascular permeability was dependent on caveolae-mediated transcytosis of albumin. Pulmonary edema developed only in isolated rat lungs in which 5×10^7 PMNs were added to the perfusate. Interestingly, a lower number of PMNs (2×10^7 PMNs) added to the perfusate and then activated with fMLP increased albumin permeability but produced no pulmonary edema, whereas a higher number of PMNs (5×10^7 cells) induced a greater increase in albumin permeability, as well as pulmonary edema. These results in lungs,

which are consistent with the effects of addition of different PMN numbers to endothelial monolayers, suggest that PMNs activated with fMLP increase lung microvessel permeability via a caveolae-mediated pathway in a PMN number-dependent manner. The more severe edema observed with the higher PMN number may be the result of a greater increase in vascular permeability to albumin because of increases in both junctional permeability and transcytosis.

Our finding of reduced basal pulmonary microvascular albumin permeability in *Cav-1*^{-/-} mice is consistent with other studies in these mice.¹⁸ Using siRNA to decrease Cav-1 expression in mouse lungs, we have shown an increase in the number of open interendothelial junctions and reduction in caveolae-mediated albumin transcytosis.⁴⁴ The present studies are different because we have defined for the first time the important role for activation of caveolae-mediated transport induced by activated PMNs in pulmonary edema formation. Although a constitutive level of transcytosis via caveolae may be important in maintaining tissue fluid balance,^{1,10,13} the present studies reveal the potentially crucial pathogenic role of caveolae trafficking in the mechanism of edema formation.

There are several pathways available for the transport of plasma protein the size of albumin and greater across the continuous endothelium: caveolae, clathrin-coated vesicles, interendothelial junctions, and micropinocytosis involving caveolin- and clathrin-independent mechanisms.¹ Caveolae-mediated transcytosis is thought to be the primary mechanism of basal albumin permeability across continuous endothelial cell barrier.¹ Transcytosis was reduced by disruption of caveolae and inhibition of Src-dependent Cav-1 phosphorylation.^{21–25} Our findings suggest that binding of activated PMNs increases vascular permeability through the augmentation of this mechanism. Thus, caveolae trafficking represents the primary albumin transport pathway under basal conditions in continuous endothelial cells, and, as we have shown, it also contributes to the mechanism of increased albumin permeability after PMN activation. Caveolae are not detectable in Cav-1-null mice.^{19,20} However, a few vesicles of the same size or slightly greater than caveolae were detected^{19,20} but whose molecular identity has not been characterized. These structures were noted in the original Cav-1 mouse knockout studies by Drab et al¹⁹ and Razani et al²⁰ but do not appear to compensate sufficiently for the loss of caveolae.

Transcytosis of albumin in endothelial cells requires the binding of albumin to the albumin binding protein gp60 on the cell surface,^{23,33} interaction of gp60 with Cav-1,²³ and Src activation.²² We have shown that Src phosphorylates Cav-1, gp60, and dynamin-2 to initiate caveolae fission from the plasma membrane.^{21,22,24} In the present study, we showed that PMN activation also induced Src phosphorylation of Cav-1, whereas expression dn-Src prevented the phosphorylation, and, importantly, it inhibited endocytosis and transcytosis of albumin. Thus, Src phosphorylation of Cav-1 is a key determinant of PMN activation-mediated increase in albumin transcytosis in endothelial cells.

To address how Src kinase may be activated by PMNs, we focused on the role of ICAM-1 localized on the endothelial cell plasma membrane. Previous studies have shown that ICAM-1 binding to CD18 on PMNs mediates “outside-in” signaling that can activate Src.³⁰ We observed here that activation of PMNs with fMLP caused endothelial cell surface clustering of ICAM-1 and that anti-ICAM-1 mAb blocked Src activation and Cav-1 phosphorylation, consistent with a role of ICAM-1-induced signaling. We also observed that anti-ICAM-1 blocking mAb prevented the PMN activation-induced increase in ¹²⁵I-albumin endocytosis and transcytosis in endothelial cells. To address whether ICAM-1 may be directly responsible for signaling, we crosslinked cell surface ICAM-1^{29,41} to induce clustering and observed activation of Src and Cav-1 phosphorylation. Moreover, ICAM-1 crosslinking increased albumin endocytosis and transendothelial albumin permeability, thus mimicking the effects of PMN activation of endothelial cells. Recent studies have shown a role for engagement of

ICAM-1 by leukocytes in mediating *Src* activation and tyrosine phosphorylation of VE-cadherin as a requirement for transendothelial migration of PMNs.⁴³ Thus, our results are consistent with the emerging concept that ICAM-1 engagement transmits signals into endothelial cells that promote PMN migration⁴⁵ and, as shown in the present study, the activation of caveolae-mediated transcytosis of albumin contributing to pulmonary edema formation.

In summary, fMLP activation of PMNs increased caveolae-mediated transendothelial albumin permeability and induced pulmonary edema formation in rats and mice. The response was initiated by ICAM-1–dependent *Src* activation and *Src* phosphorylation of Cav-1 following the binding of PMN to endothelial cells, which resulted in caveolae-mediated transcytosis of albumin. Therefore, pulmonary vascular transcytosis of albumin is a potentially important mechanism contributing to pulmonary edema formation and needs to be considered as a crucial factor in the pathogenesis of acute lung injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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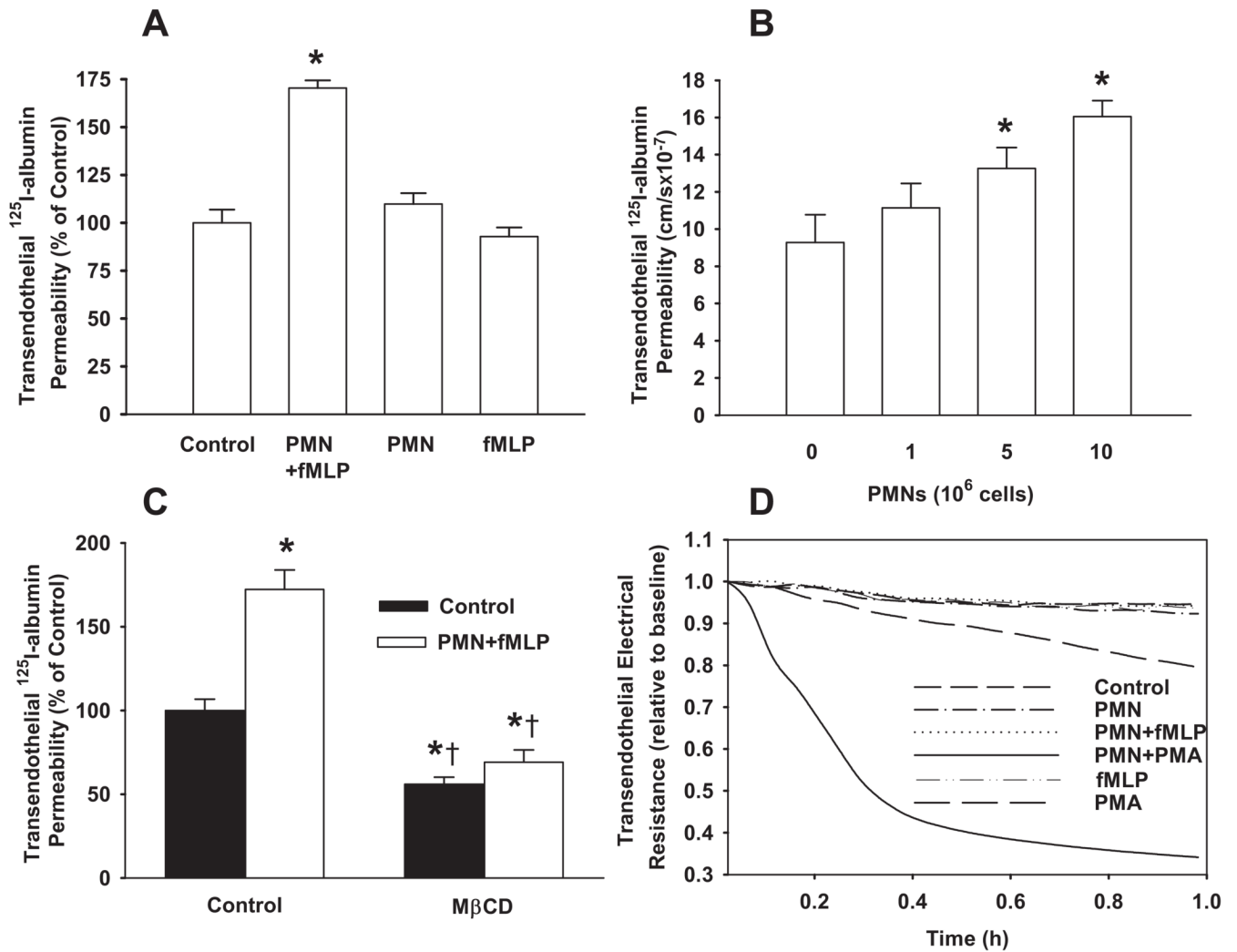
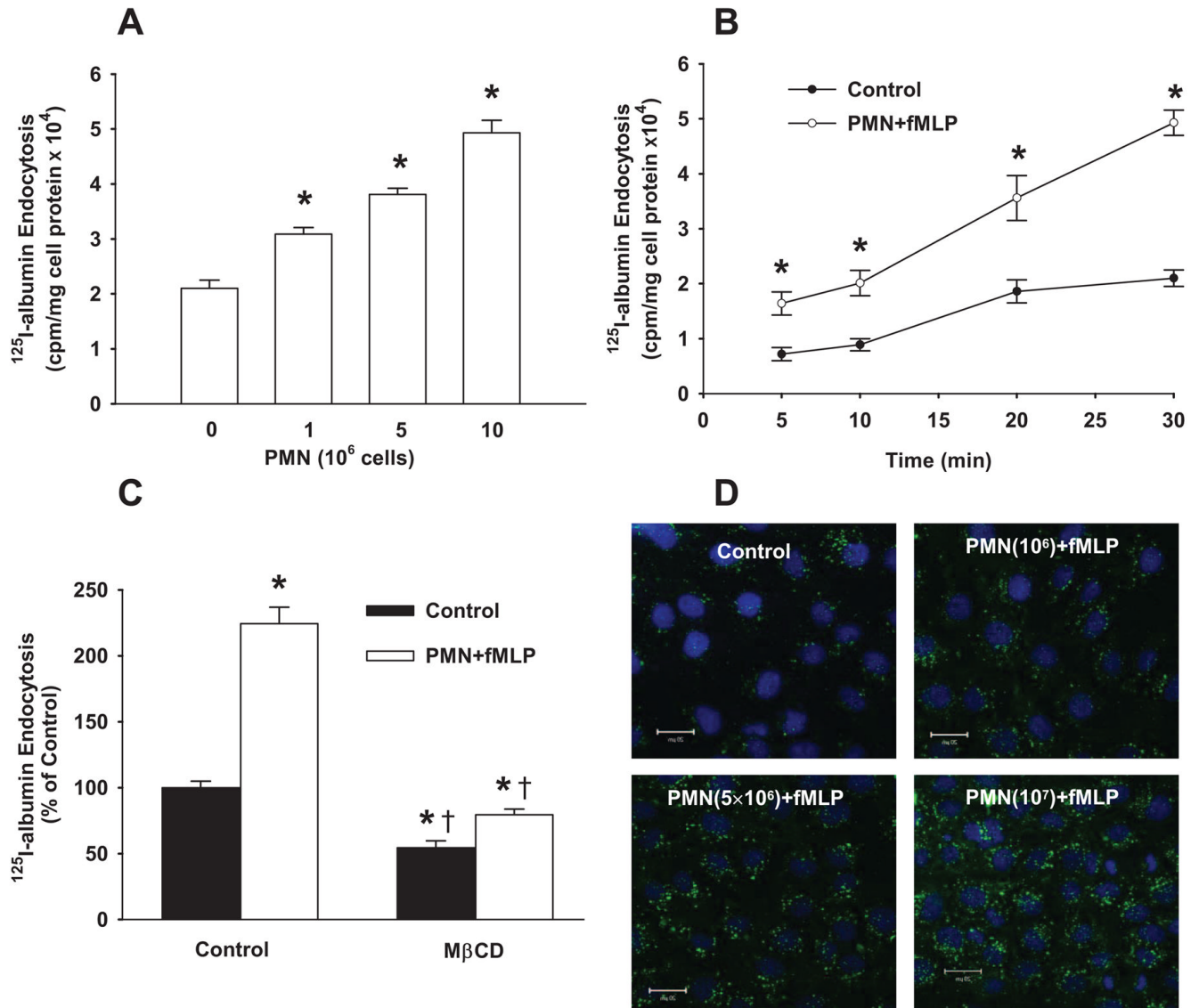


Figure 1.

Activation of PMNs with fMLP increases caveolae-mediated transendothelial ^{125}I -albumin permeability in RLMVEC monolayers. A, Effect of PMNs in the absence and presence of fMLP on transendothelial ^{125}I -albumin permeability. B, Activation of PMNs with fMLP increased transendothelial ^{125}I -albumin permeability in a PMN number-dependent manner. C, Effect of pretreatment of RLMVECs with M β CD on ^{125}I -albumin permeability. The baseline permeability value for control group (without PMNs and fMLP) is 9.4 ± 1.1 cm/sec. D, Effect of activation of PMNs (10^7 cells/mL) with fMLP ($1.0 \mu\text{mol/L}$) or PMA ($0.1 \mu\text{mol/L}$) on TER. Results are typical of 3 experiments (A through C). $n=4$ to 6 for each group. * $P < 0.05$ compared with control group (without PMNs and fMLP), † $P < 0.05$ compared with respective groups.

**Figure 2.**

Activation of PMNs with fMLP increases caveolae-mediated endocytosis of albumin in endothelial cells. A, Activation of PMNs with fMLP increased ^{125}I -albumin endocytosis in a PMN number-dependent manner. B, Time course of effects of fMLP activation of PMNs (10^7 cells/mL) on ^{125}I -albumin endocytosis. C, Effect of pretreatment of RLMVECs with M β CD on PMN-induced (10^7 cells/mL) ^{125}I -albumin endocytosis. Results are representative of 3 experiments. Scale bars=10 μm . n=4 to 6 for each group (A and C) and time point (B). * P <0.05 compared with control group (without PMNs and fMLP), $\dagger P$ <0.05 compared with respective groups. D, Confocal images showing effect of fMLP activation of PMNs induced a PMN number-dependent increase in uptake of Alexa 488-labeled albumin (green). The nucleus (blue) was stained with DAPI. Results are representative of 3 experiments.

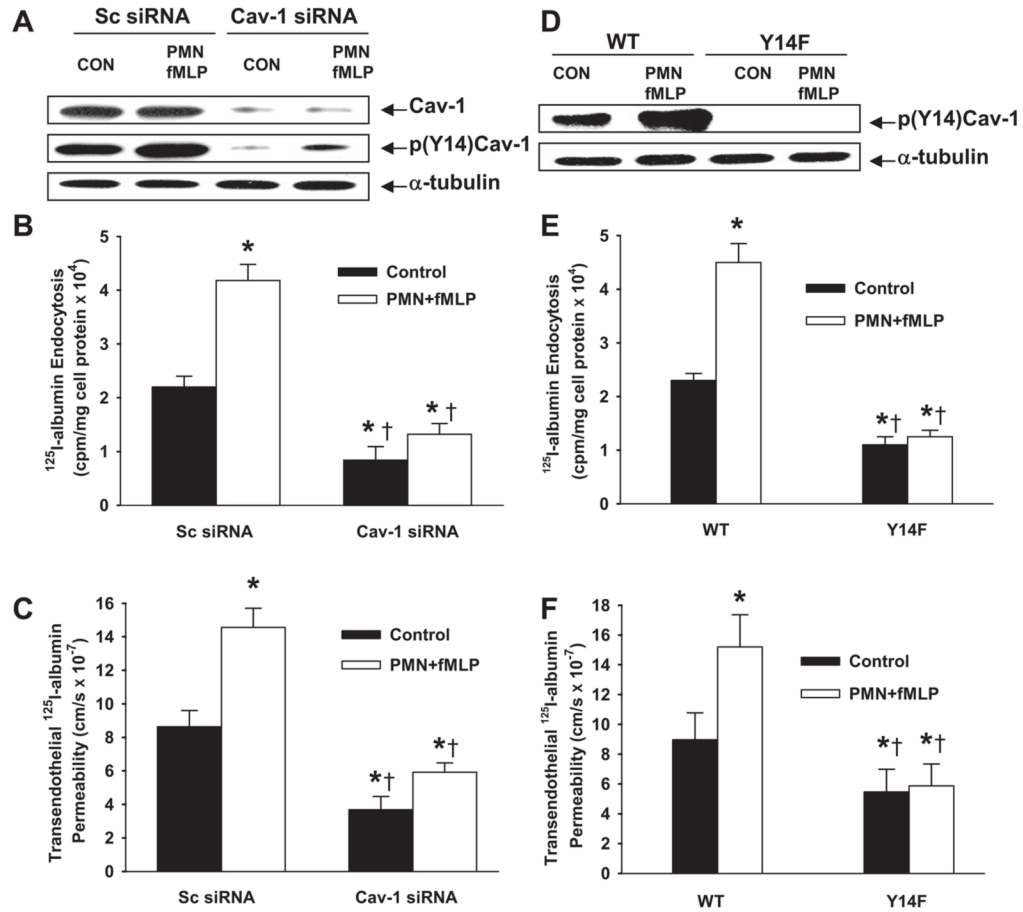


Figure 3.

Activation of PMNs with fMLP increases ¹²⁵I-albumin endocytosis and transendothelial permeability through Cav-1 phosphorylation. RLMVECs were coincubated with PMNs in the presence or absence of fMLP (1.0 μmol/L) for 30 minutes at 37°C. A through C, Effects of activation of PMNs with fMLP on Cav-1 expression and phosphorylation (A and supplemental Figure IA) and ¹²⁵I-albumin endocytosis (B) and transendothelial albumin permeability (C) in cells transfected with Cav-1 siRNA or scrambled (Sc) siRNA. D through F, Effects of activation of PMNs with fMLP on Cav-1 phosphorylation (D and supplemental Figure IB) and ¹²⁵I-albumin endocytosis (E) and transendothelial albumin permeability (F) in cells stably expressing phosphorylation-defective Y14F-Cav-1 mutant. Exogenous Cav-1 is myc-tagged (D). Results are typical of 3 experiments (A and D). n=4 to 6 for each group (B, C, E, and F). *P<0.05 compared with control group, †P<0.05 compared with respective groups.

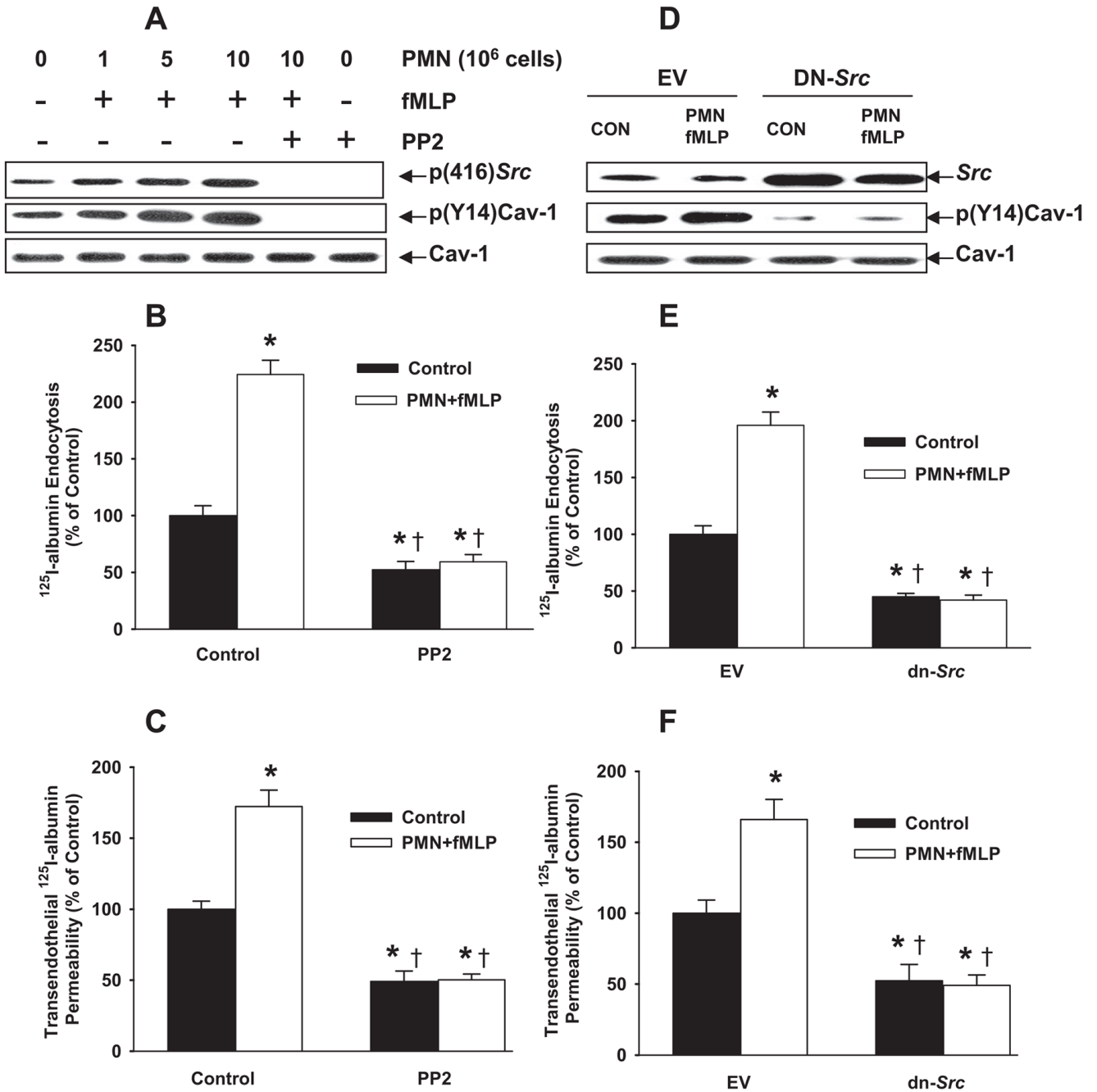
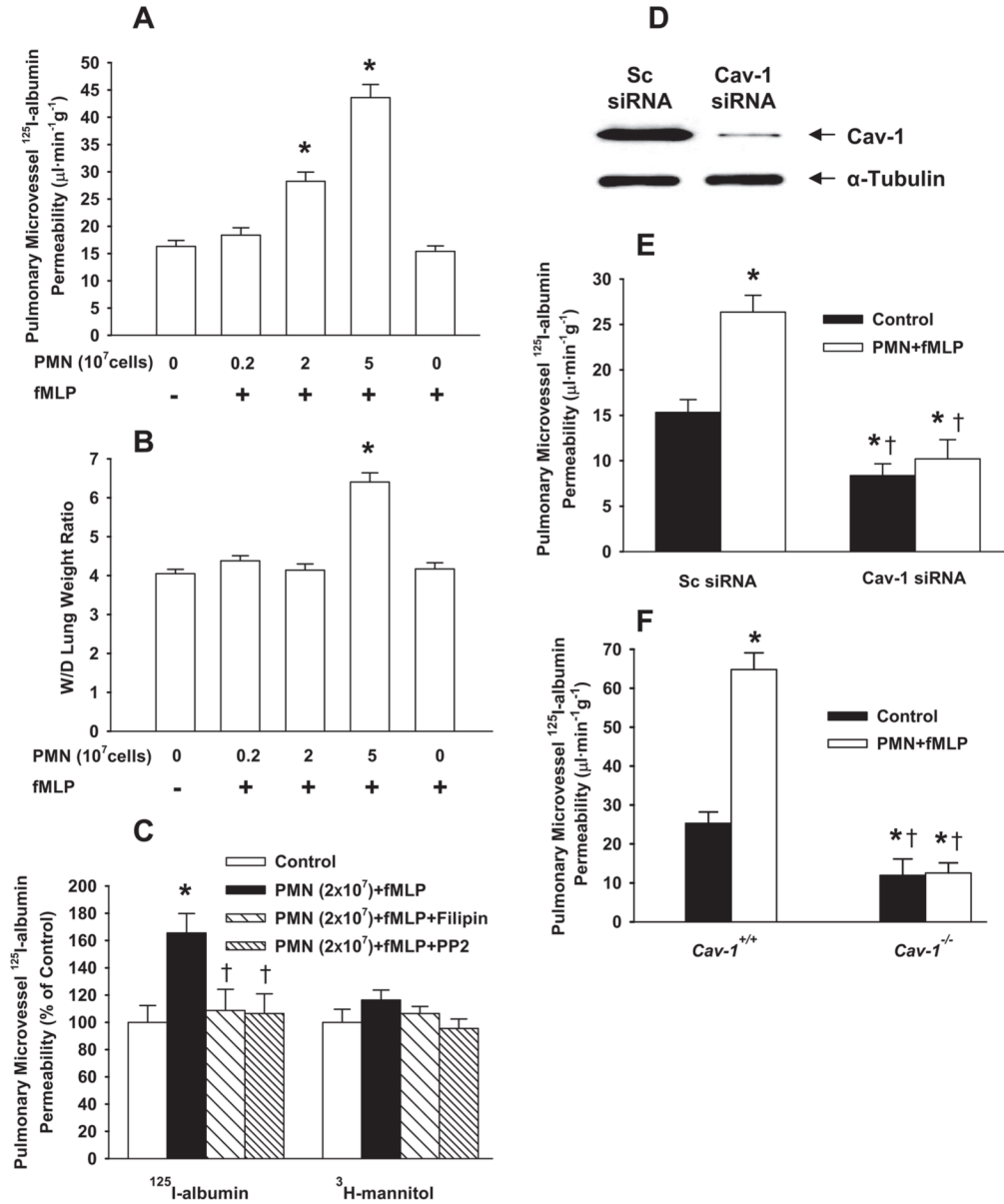


Figure 4. Activation of PMNs with fMLP stimulates ¹²⁵I-albumin endocytosis and transendothelial permeability through *Src* signaling. RLMVECs were coincubated with PMNs in the presence or absence of fMLP (1.0 μmol/L) for 30 minutes at 37°C. A, Effects of fMLP activation of PMNs and PP2 (15 μmol/L) pretreatment on Cav-1 protein expression and phosphorylation of *Src* and Cav-1. B, C, and supplemental Figure IC, Effects of PP2 pretreatment on PMN activation-induced change in ¹²⁵I-albumin endocytosis (B) and transendothelial albumin permeability (C). D through F, Effects of activation of PMNs with fMLP on *Src* protein expression and Cav-1 phosphorylation (D and supplemental Figure ID) and ¹²⁵I-albumin endocytosis (E) and transendothelial albumin permeability (F) in RLMVECs infected with dn-

Src pFB adenovirus or empty vector (EV). Results are typical of 3 experiments (A and D). n=4 to 6 for each group (B, C, E, and F). The baseline permeability values for control group (without PMNs and fMLP) are 9.2 ± 1.4 cm/sec (C) and 9.1 ± 1.3 cm/sec (F), respectively. * $P < 0.05$ compared with control group, † $P < 0.05$ compared with respective groups.

**Figure 5.**

Activation of PMNs with fMLP induces caveolae-mediated albumin hyperpermeability in rat and mouse lung vessels. After lung perfusion stabilization of 20 minutes, PMNs and fMLP were separately infused in the pulmonary circulation for 30 minutes. A and B, Effect of fMLP-activated rat PMNs (2×10^6 , 2×10^7 , or 5×10^7 cells) on ^{125}I -albumin PS product (A) and W/D lung weight ratio (B). C, Effects of filipin and PP2 ($1\ \mu\text{g}/\text{kg}$) on PMN (2×10^7 cells) activation-induced changes in ^{125}I -albumin and ^3H -mannitol PS products. D and E, Rats were injected with liposomes containing scrambled (Sc) or Cav-1 siRNA in the tail vein. After 48 hours, Western blot analyses of Cav-1 expression (D and supplemental Figure IIA) and pulmonary vascular ^{125}I -albumin PS (E) were assessed. F, Effects of activation of mouse PMNs (2×10^6 cells) on pulmonary vascular ^{125}I -albumin PS product in *Cav-1*^{+/+} and *Cav-1*^{-/-} mouse lungs. * $P<0.05$ compared with control groups (without PMNs and fMLP) (A through C and F) or with scrambled siRNA control (E). † $P<0.05$ compared with PMN (2×10^7 cells)+fMLP group (C) or with respective groups (E and F).

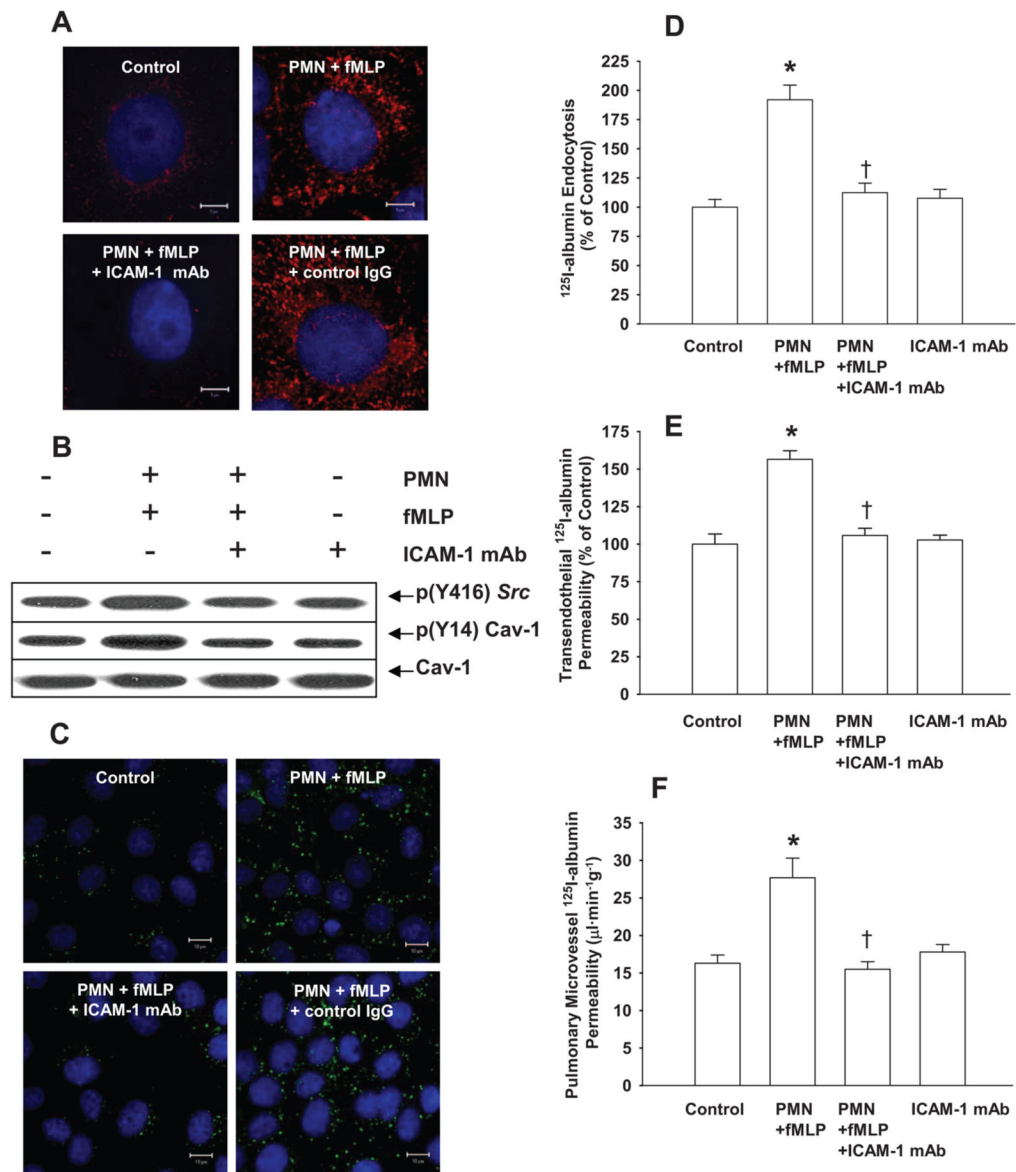
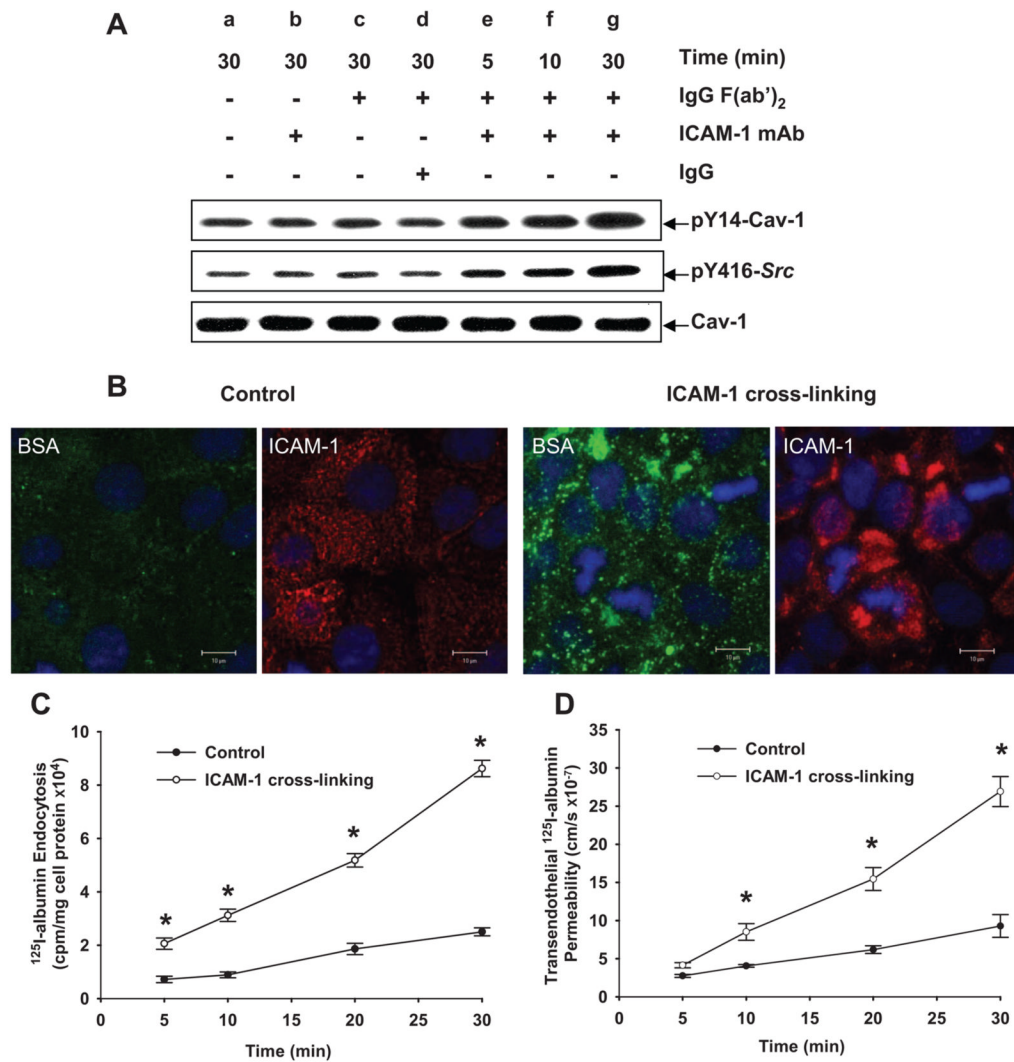


Figure 6. Role of ICAM-1 in mediating *Src* activation and Cav-1 phosphorylation in endothelial cells and its consequence in increasing caveolae-mediated endothelial hyperpermeability. **A**, Effects of fMLP activation of PMNs with and without anti-ICAM-1 mAb pretreatment on cell surface ICAM-1 distribution. Each image shows merged ICAM-1 immunostaining (red) and nucleus (blue). Scale bars=5 μ m. **B** and supplemental Figure IIB, Effects of fMLP activation of PMNs with and without anti-ICAM-1 Ab pretreatment on *Src* and Cav-1 phosphorylation. **C**, Effects of activation of PMNs with fMLP or combined with anti-ICAM-1 Ab pretreatment on Alexa 488-labeled albumin (green) endocytosis. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) to label nuclei (blue). Results are typical of 3 experiments. Scales bar=10 μ m. **D** and **E**, Effects of anti-ICAM-1 Ab on PMN activation-induced increase in ¹²⁵I-albumin endocytosis (**D**) and transcytosis (**E**). **F**, Effects of anti-ICAM-1 Ab on PMN activation-induced increase in pulmonary trans-vascular ¹²⁵I-albumin PS product in rats. The baseline permeability value for control group (without PMNs and fMLP) was 9.1 \pm 1.1 cm/sec (**E**) (n=4

to 6 for each group). * $P < 0.05$ compared with control group, † $P < 0.05$ compared with PMN +fMLP groups.

**Figure 7.**

ICAM-1 crosslinking induces *Src* activation and Cav-1 phosphorylation and activates albumin endocytosis and transcytosis. RLMVECs were incubated with 10 $\mu\text{g}/\text{mL}$ anti-ICAM-1 Ab at 37°C for 30 minutes and washed and incubated with 0.1 $\mu\text{g}/\text{mL}$ goat antimouse IgG F(ab')₂ at 37°C for 5, 10, and 30 minutes. A and supplemental Figure IIC, Effects of ICAM-1 crosslinking on *Src* activation and Cav-1 phosphorylation. B, Effects of ICAM-1 crosslinking on Alexa 488-labeled albumin uptake (green) and anti-ICAM-1 IgG+anti-mouse Alexa 568 immunostaining (red). Confocal images show ICAM-1 clustering and increased tracer albumin uptake following ICAM-1 crosslinking. Scales bar=10 μm . C and D, Time course of ICAM-1 crosslinking effect on ¹²⁵I-albumin endocytosis (C) and transcytosis (D). Results are typical of 3 experiments (A and B). n=4 to 6 for each time point (C and D). * $P<0.05$ compared with control group.