ICAM-1-mediated leukocyte adhesion is critical for the activation of endothelial LSP1

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Submitted 10 September 2012; accepted in final form 19 February 2013

Hossain M, Qadri SM, Su Y, Liu L. ICAM-1-mediated leukocyte adhesion is critical for the activation of endothelial LSP1. Am J Physiol Cell Physiol 304: C895–C904, 2013. First published February 27, 2013; doi:10.1152/ajpcell.00297.2012.—Leukocyte-endothelial interaction triggers signaling events in endothelial cells prior to transendothelial migration of leukocytes. Leukocyte-specific protein 1 (LSP1), expressed in endothelial cells, plays a pivotal role in regulating subsequent recruitment steps following leukocyte adhesion. In neutrophils, LSP1 is activated by phosphorylation of its serine residues by molecules downstream of p38 MAPK and PKC. Whether leukocyte adhesion to endothelial cells is required for endothelial LSP1 activation remains elusive. In addition, discrepancies in the functions of endothelial and leukocyte LSP1 in leukocyte adhesion prevail. We demonstrate that adhesion of wild-type (Lsp1+/+) neutrophils to LSP1-deficient (Lsp1−/−) endothelial cells was significantly reduced compared with adhesion to Lsp1+/+ endothelial cells. Immunoblotting revealed increased phosphorylated endothelial LSP1 in the presence of adherent Lsp1−/− neutrophils [stimulated by macrophage inflammatory protein-2 (CCL2), TNF-α, or thapsigargin], but not cytokine or chemokine alone. Pharmacological inhibition of p38 MAPK by SB-203580 (10 μM) significantly blunted the phosphorylation of endothelial LSP1. Functionally blocking endothelial ICAM-1 or neutrophil β2-integrins diminished neutrophil adhesion and phosphorylation of endothelial LSP1. The engagement of endothelial ICAM-1 cross-linking, which mimics leukocyte adhesion, resulted in phosphorylation of endothelial LSP1. In neutrophil-depleted Lsp1−/− mice, administration of ICAM-1 cross-linking antibody resulted in increased phosphorylation of LSP1 and p38 MAPK in TNF-α-stimulated cremaster muscle. In conclusion, endothelial LSP1 participates in leukocyte adhesion in vitro, and leukocyte adhesion through ICAM-1 fosters the activation of endothelial LSP1, an effect at least partially mediated by the activation of p38 MAPK. Endothelial LSP1, in contrast to neutrophil LSP1, is not phosphorylated by cytokine or chemokine stimulation alone.

LSP1; neutrophils; endothelial cells; p38 MAPK; adhesion; ICAM-1

A HALLMARK OF ACUTE INFLAMMATION is the recruitment of leukocytes from the bloodstream to the inflammation site. Leukocyte recruitment into inflamed tissue consists of molecularly defined steps, such as tethering, rolling, adhesion, intravascular crawling, and paracellular or transcellular transmigration across the endothelial lining (30, 41, 55, 56). Proinflammatory cytokines upregulate the expression of endothelial adhesion molecules. The β2-integrins expressed on leukocytes interact with endothelial adhesion molecules of the immunoglobulin superfamily, including ICAM-1 (CD54), which mediates firm arrest of leukocytes on endothelial cells prior to their transmigration (55). The leukocyte-endothelial cell interactions elicit a variety of signaling events that are critical for the passage of neutrophils to the extravascular tissue and the changes in microvascular permeability (22, 37, 40, 47, 48).

During inflammation, leukocyte arrest on the endothelium is mediated by the β2-integrins leukocyte function-associated antigen-1 and Mac-1 (30, 41, 56, 61). Stimulation of leukocytes by chemokines results in the activation of a number of intracellular signaling pathways and integrins, thereby enhancing the binding of leukocyte integrins to endothelial ligands such as ICAM-1 (2, 28). In endothelial cells, ICAM-1 signaling during leukocyte-endothelial interactions is mediated by receptor multimerization (15) and modulates the actin cytoskeleton by interactions with ERM proteins (ezrin, radixin, moesin), β-tubulin, caveolin-1, cortactins, and filamins A and B (6, 19, 27, 36, 59). ICAM-1 stimulation further activates mobilization of intracellular Ca2+ (18) and Rho GTPases (46). Moreover, activation of Rho GTPases by ICAM-1 modulates gene expression by stimulation of NF-κB (11). ICAM-1 engagement also triggers tyrosine phosphorylation of cytoskeleton-associated proteins, such as focal adhesion kinase, paxillin, and p130Cas (17), and phosphorylation of junctional proteins, such as vascular endothelial cadherin, which fosters the subsequent endothelial transmigration of leukocytes (3, 4). Leukocyte adhesion-triggered ICAM-1 signaling regulates transcellular and paracellular leukocyte transmigration (58) and microvascular permeability increases (44).

An essential regulatory protein that plays a pivotal role in leukocyte recruitment and microvascular permeability changes is leukocyte-specific protein 1 (LSP1) (24, 32, 40). In leukocytes, LSP1 is an intracellular Ca2+ - and F-actin-binding protein that interacts with the cytoskeleton (24). It serves as a substrate for PKC and p38 MAPK (10, 23). Previous studies suggest the negative regulatory roles of neutrophil LSP1 on adhesion, polarization, and migration (49). In endothelial cells, however, LSP1 is primarily localized in the nucleus, with a smaller amount colocalized with the cytoskeleton (32). Endothelial LSP1 has been shown to regulate transendothelial migration of leukocytes (32, 40). Interestingly, LSP1-deficient (Lsp1−/−) mice showed a compromised formation of endothelial transmigratory domelike structures, resulting in a disproportionate increase in vascular permeability, despite impaired transendothelial migration of leukocytes (29, 40). In leukocytes, LSP1 has been shown to be activated by chemoattractants (57). However, the mechanisms of endothelial LSP1 activation during leukocyte recruitment remain completely unknown.

The present study explores whether endothelial LSP1 is involved in leukocyte adhesion to endothelial cells, whether endothelial LSP1 is activated after stimulation with soluble, receptor-mediated chemotactic mediators or cytokines, and whether neutrophil adhesion activates endothelial LSP1.

MATERIALS AND METHODS

Animals. Lsp1−/− mice on the 129/SvJ background were generated by homologous recombination by Jongstra-Bilen and colleagues (25); both mouse strains were then transferred to the University of Saskatchewan. Lsp1−/− mice were crossed with Lsp1+/+ mice to generate heterozygotes and homozygotes (F2). The Lsp1−/− and
**Lsp1**-/- homozygotes were confirmed by genotyping. Mice of these two genotypes were bred to obtain age-matched and sex-matched controls. Five- to 7-day-old and 8- to 16-wk-old mice were used in the experiments. The study was carried out with the approval of animal protocols from the University Committee on Animal Care and Supply at the University of Saskatchewan and following the standards of the Canadian Association of Animal Care. All surgeries were performed under ketamine-xylazine anesthesia, as previously described (32), and all efforts were made to minimize animal suffering.

**Isolation of murine neutrophils.** Femurs and tibias from mice were dissected, and the marrow was flushed with ice-cold Ca²⁺-/Mg²⁺-free PBS solution. Neutrophils were isolated using three-step Percoll (GE Healthcare, Uppsala, Sweden) gradient (72%, 64%, and 52%) centrifugation at 1,060 g for 30 min, as described previously (31). This procedure yielded 80–90% morphologically mature neutrophils.

**Harvest of primary murine endothelial cells.** Primary murine endothelial cells were isolated from the lungs or hearts of **Lsp1**-/- or **Lsp1**+/+ mice (aged 5–7 days) according to a previously described protocol (7, 32). Briefly, endothelial cells were immunomagnetically isolated utilizing anti-ICAM-2 (CD102) antibody (clone 3C4, BD Pharmingen, Quebec City, QC, Canada), seeded in 35-mm laminin-coated petri dishes, and cultured in microvascular endothelial cell culture medium (EBM-2) supplemented with the EGM-2-MV BulletKit (Lonza, Mississauga, ON, Canada). After the cells reached confluency, they were passaged on laminin-coated 24-well plates for in vitro adhesion assay or 35-mm cell culture dishes (1 × 10⁶ cells) for detection of protein expression.

**Culture of endothelial cell line cells.** Murine microvascular endothelial cells (SVEC4-10EE2 cell line, American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% FBS and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) in a 5% CO₂ incubator at 37°C. Cells of confluent monolayers were passaged on 24-well plates for in vitro adhesion assay or 100-mm cell culture dishes (8 × 10⁶ cells) for detection of protein expression.

**In vitro adhesion assay.** Neutrophil adhesion to endothelial cells was quantified as previously described with minor modifications (21). Briefly, freshly isolated bone marrow neutrophils were stimulated for 5 min with macrophage inflammatory protein-2 (CXCL2), 100 nM; R & D Systems, Burlington, ON, Canada), KC (CXCL1, 100 nM; Peprotech, Rocky Hill, NJ), or thapsigargin (100 nM; Sigma, St. Louis, MO). The neutrophils were allowed to adhere at 37°C for 30 min to confluent monolayers of primary endothelial cells or mouse endothelial cell line cells in 24-well plates. Where indicated, unstimulated neutrophils were allowed to adhere to TNF-α-stimulated (20 ng/ml for 4 h; R & D Systems) endothelial cells. Unlike MIP-2- or KC-stimulated neutrophils, thapsigargin-treated neutrophils were subsequently washed twice in ice-cold PBS before they were allowed to adhere to endothelial cells. The ratios of neutrophils to endothelial cells were 0:1, 0:5:1, 1:1, 2:1, and 5:1. Where indicated, the p38 MAPK inhibitor SB-203580 (10 µM; EMD Millipore, Billerica, MA) or a PKC inhibitor [staurosporine (100 nM; Tocris, Bristol, UK)] was added during the assay. Unbound cells were removed by two washes with prewarmed (37°C) PBS, and the adherent neutrophils were stained using a three-step staining set (Richard-Allan Scientific, Kalamazoo, MI), counted microscopically in triplicates, and averaged from five different fields of view (325 µm²). To assess the role of endothelial LSP1, neutrophils from **Lsp1**-/- mice were layered on primary endothelial cells from **Lsp1**+/+ or **Lsp1**-/- mice. To study phosphorylation of LSP1 in endothelial cells, only **Lsp1**-/- neutrophils were used in all experiments to rule out the influence of neutrophil LSP1.

To exclude the possibility of endothelial LSP1 activation by soluble mediators released from activated neutrophils or endothelial cells during neutrophil adhesion, endothelial cells were additionally treated with conditioned medium. Freshly isolated bone marrow neutrophils were stimulated with MIP-2 (100 nM for 10 min, 37°C) and then allowed to adhere to endothelial cells at 37°C for 30 min before centrifugation and collection of the supernatant (conditioned medium). Another confluent monolayer of endothelial cells was treated with this conditioned medium for 30 min at 37°C before endothelial phosphorylated LSP1 was detected.

**Functional blocking of β₂-integrins and ICAM-1.** For determination of the role of β₂-integrins in **Lsp1**-/- neutrophil adhesion and endothelial LSP1 phosphorylation, freshly isolated and unstimulated **Lsp1**-/- bone marrow neutrophils were preincubated with CD18 blocking antibodies (10 µg/ml; clone M18/2, BD Pharmingen) or the isotype control (10 µg/ml rat IgG2a κ; clone R35-95, BD Pharmingen) for 20 min at 37°C, as described elsewhere (38), prior to incubation with MIP-2 (10 min, 37°C). Next, the neutrophils were allowed to adhere to murine heart or lung endothelial cells from **Lsp1**-/- mice or mouse SVEC4-10EE2 endothelial cells, respectively.

To study the role of ICAM-1, following stimulation with TNF-α (20 ng/ml, 4 h), murine SVEC4-10EE2 endothelial cells were treated with ICAM-1 blocking antibody (10 µg/ml; clone YN1/7.4, ebioscience, San Diego, CA) or isotype control (10 µg/ml rat IgG2b κ; ebioscience) for 1 h, as described elsewhere (60). Then, freshly isolated and unstimulated **Lsp1**-/- bone marrow neutrophils were layered on the endothelial cells. After incubation for 30 min, nonadherent neutrophils were removed by two washes with prewarmed PBS. Endothelial cells were lysed for detection of phosphorylated LSP1 or fixed and stained for counting of the number of adherent neutrophils.

**ICAM-1 cross-linking.** ICAM-1 cross-linking was used to mimic leukocyte adhesion. To study the effect of ICAM-1 cross-linking in vivo, circulating neutrophils were depleted in **Lsp1**-/- mice by intraperitoneal administration of 200 µg of anti-mouse Ly-6G (Gr-1) antibodies (1 mg/ml; clone RB6-8C5, ebioscience) 24 h prior to treatment with cross-linking antibodies (62), thus ruling out the detection of neutrophil LSP1. For ligation of ICAM-1, rat anti-mouse ICAM-1 antibody (100 µg/mouse; clone YN1/7.4, ebioscience) and the respective isotype control antibody (100 µg/mouse rat IgG2b κ; ebioscience) were administered as described previously (43). Saline was administered to control mice. ICAM-1 expression was upregulated by an intrascrotal injection of TNF-α (300 ng) 20 h after administration of Gr-1 antibody. After 4 h of TNF-α treatment, ICAM-1 ligation antibody or isotype control was infused via the jugular vein followed by the secondary anti-rat cross-linking IgG2b (1:100 dilution; clone RB2-7C3, ebioscience). After 30 min of ICAM-1 cross-linking, the cremaster muscle from the TNF-α-treated scrotum was carefully excised and snap-frozen in liquid nitrogen for the detection of total and phosphorylated LSP1 and p38 MAPK by immunoblotting.

ICAM-1 engagement in vitro was performed as previously described (50) by incubation of TNF-α-pretreated (20 ng/ml, 4 h) endothelial cells with ICAM-1 ligation antibody, the isotype control antibody, and the secondary cross-linking antibody for 30 min. Endothelial cells were then prepared for immunoblotting to determine the abundance of phosphorylated LSP1 and total LSP1.

**Immunoblotting.** Cells were lysed in a lysis buffer (pH 8.0) containing 150 mM NaCl, 50 mM Tris, 1% NP-40, and protease and phosphatase inhibitor cocktails (Fisher Scientific, Toronto, ON, Canada). The lysate was centrifuged at 10,000 g at −80°C. The same protocol was followed to prepare homogenates from mouse cremaster muscle.

For the detection of total and phosphorylated LSP1, immunoblotting was performed in the nonreduced and nondenatured state, as described previously (8). The samples were run in native MiniPROTEAN TGX precast gels (Bio-Rad, Mississauga, ON, Canada) by electrophoresis. Proteins were transferred onto an Immobilon-FL membrane (Millipore, Billerica, MA) followed by dual blotting of total and phosphorylated LSP1. Total LSP1 was detected using rabbit
anti-LSP1 (1:500 dilution; a generous gift from Dr. J. Jongstra, University of Toronto) and goat anti-rabbit IgG tagged with Alexa Fluor 647 (1:5,000 dilution; Invitrogen, Burlington, ON, Canada) as the secondary antibody. Phosphorylated LSP1 was detected using mouse monoclonal antibody against phosphorylated LSP1 (1:500 dilution; clone AT-1E6, Cylex, Nagano, Japan) and goat anti-mouse IgG tagged with Alexa Fluor 488 (1:5,000 dilution; Invitrogen) as the secondary antibody. Membranes were developed with Versa Doc 5000 using appropriate filters. Band densities were quantified using ImageJ. As proteins in their native condition do not run according to their molecular weight, the identity of the protein was confirmed using mass spectrometry in manually excised bands from Coomassie blue-stained gel.

Total and phosphorylated p38 MAPK was detected in reduced and denatured conditions, as previously described (29). Protein samples as prepared and mentioned above were solubilized in equal volume of Laemmli sample buffer at 95°C and resolved by 10% SDS-PAGE. For immunoblotting, proteins were transferred onto a nitrocellulose membrane and blocked with 5% BSA in Tris-buffered saline-Tween 20 at room temperature for 1 h. Then the membrane was incubated with affinity-purified rabbit anti-phosphorylated p38 MAPK antibody (1:1,000 dilution; Cell Signaling Technology, Danvers, MA) at 4°C overnight. After incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, antibody binding was detected with enhanced chemiluminescence detection reagent (GE Healthcare, Baie d’Urfe, QC, Canada). Total p38 MAPK (rabbit anti-p38 antibody, 1:1,000 dilution; Cell Signaling Technology) and β-actin (mouse anti-β-actin antibody, 1:1,000 dilution; Santa Cruz Biotechnology) were detected after stripping with a buffer (pH 6.8) containing 0.5 M Tris-HCl, 2% SDS, and 0.7% 2-β-mercaptoethanol. Densitometric quantification of the detected bands was performed using Gene Snap software (Syngene, Frederick, MD).

Mass spectrometry. The excised bands with the protein of interest were processed using a Micromass MassPrep Station according to a previously reported protocol (8). Briefly, the gel fragments were destained, reduced, alkylated, digested with trypsin, and then excised overnight at room temperature. The resulting tryptic digest was analyzed by mass spectrometry. For electrospray, quadruple time-of-flight (Q-TOF) analysis, 1 μl of the solution was used. Liquid chromatography-mass spectrometry (LC/MS) was performed on a CapLC high-performance liquid chromatography unit (Waters) coupled with a Q-TOF-2 mass spectrometer (Micromass). A mass deviation of 0.2 was tolerated, and one missed cleavage site was allowed. Resulting values from mass spectrometry (MS/MS) analysis were used to search against the SwissProt database with Rodentia specified. Resulting values from mass spectrometry (MS/MS) analysis were used to search against the SwissProt database with Rodentia specified. We used the Mascot (www.matrixscience.com) search engine to search the protein database.

Statistical analysis. Values are arithmetic means ± SD; n denotes the number of different mice and different batches of neutrophils and endothelial cells studied in each group. Statistical analysis was carried out using Student’s t-test or one-way ANOVA with Tukey’s post hoc comparison test. P < 0.05 was considered statistically significant.

RESULTS

Because there have been discrepancies regarding the role of endothelial LSP1 in leukocyte adhesion in different model systems, we investigated whether endothelial LSP1 participates in neutrophil adhesion. Using an in vitro assay, we measured the adhesion of Lsp1+/+ neutrophils to Lsp1+/+ and Lsp1−/− endothelial cells. As depicted in Fig. 1, incubation of chemokine-stimulated (KC or MIP-2, 100 nM) neutrophils with endothelial cells for 30 min significantly enhanced the number of adherent neutrophils on Lsp1+/+ and Lsp1−/− endothelial cells compared with neutrophil adhesion in the absence of chemokine stimulation. The number of adherent neutrophils, however, was slightly, but significantly, lower on Lsp1−/− endothelial cells than Lsp1+/+ endothelial cells, suggesting that endothelial LSP1 plays a role in neutrophil adhesion (Fig. 1).

Next, we explored whether neutrophil adhesion to endothelial cells stimulates phosphorylation of endothelial LSP1. As illustrated in Fig. 2A, stimulation of neutrophils with MIP-2 or thapsigargin or of endothelial cells with TNF-α significantly enhanced the number of adherent neutrophils. Phosphorylated LSP1 was quantified in endothelial cells in the presence and absence of adherent Lsp1+/− neutrophils. Neutrophils stimulated with MIP-2 or pretreated with thapsigargin and allowed to adhere to endothelial cells significantly increased phosphorylated LSP1 in endothelial cells compared with endothelial cells treated with MIP-2 alone or with untreated neutrophils (Fig. 2, B and C). Similarly, the addition of untreated Lsp1−/− neutrophils to TNF-α-stimulated endothelial cells significantly augmented endothelial LSP1 phosphorylation. In contrast, TNF-α stimulation did not enhance phosphorylation of endothelial LSP1 in the absence of neutrophils. These data clearly indicate that adhesion of neutrophils to endothelial cells is critical for LSP1 phosphorylation in endothelial cells which is not affected by cytokine or chemokine stimulation alone (Fig. 2, B and C). During adhesion, neutrophils may secrete a variety of soluble mediators and substances that might lead to activation of endothelial LSP1. To exclude this possibility, we tested whether the conditioned medium harvested after neutrophil adhesion to endothelial cells triggered the activation of endothelial LSP1. As shown in Fig. 2, D and E, not the presence of conditioned medium and MIP-2 but, rather, the adherence of Lsp1−/− neutrophils in the presence of MIP-2 elicited phosphorylation of endothelial LSP1.

Mass spectroscopic analysis of the protein bands confirmed the identity of mouse endothelial LSP1 detected by immunoblotting (Mouse/ions score: 881; queries matched: 42; sequence coverage: 52%; p/mol wt: 4.7 397.6). Ion score is $-10\log(P)$, where $P$ is the probability that the match is a random event. Individual ion score $>32$ indicates identity or extensive homology ($P < 0.05$).
In neutrophils, activation of LSP1 is effectively accomplished by p38 MAPK-mediated signaling events downstream of receptor-mediated activation signals from chemoattractants (23, 26, 57). We provided direct and specific experimental verification that phosphorylation of endothelial LSP1 is blunted by pharmacological inhibition of p38 MAPK. It is documented that inhibition of p38 MAPK signaling downregulates the expression of B2-integrins (45) and decreases leucocyte adhesion to endothelial cells (12, 16, 39, 42). In light of these results, LSP1 phosphorylation in endothelial cells could be diminished as a result of decreased neutrophil adhesion to endothelial cells. To address this issue, we analyzed neutrophil adhesion using different ratios of added Lsp1<sup>−/−</sup> neutrophils to endothelial cells, which affected the number of adherent cells. As the first step, we show that neutrophil adhesion and abundance of phosphorylated endothelial LSP1 were significantly enhanced with increased neutrophil-to-endothelial cell ratio (Fig. 3, A–C). As illustrated in Fig. 3D, we exploited the effects of increased neutrophil-to-endothelial cell ratio to demonstrate that the number of adherent neutrophils was significantly higher at 2:1 than 1:1 neutrophil-to-endothelial cell ratio, and addition of SB-203580 (10 μM) significantly decreased the number of adherent neutrophils. However, the number of adherent neutrophils in the group with a 1:1 neutrophil-to-endothelial cell ratio and without SB-203580 did not significantly differ from the neutrophil adhesion number in the group with a 2:1 neutrophil-to-endothelial cell ratio (Fig. 3D). Accordingly, analysis of phosphorylated LSP1 in these treatment groups showed that pharmacological inhibition of p38 MAPK by SB-203580 indeed blunted the phosphorylation of endothelial LSP1, despite similar numbers of adherent neutrophils (Fig. 3, D–F). This indicates that phosphorylation of endothelial LSP1 is downstream of p38 MAPK signaling in endothelial cells.

In neutrophils, LSP1 was previously described as a substrate for PKC (57). A further series of experiments were performed to elucidate the role of PKC in the modulation of endothelial LSP1 phosphorylation triggered by neutrophil adhesion. Treatment of endothelial cells with the PKC inhibitors staurosporine (100 nM), sotrastaurin (5 μM), or Gö-6983 (1 μM) tended to
decrease neutrophil adhesion triggered by MIP-2, an effect not reaching statistical significance. The number of adherent neutrophils (counted in a 325-μm² area) was 38.3 ± 7.3, 40.2 ± 6.5, and 41.2 ± 5.9 in the presence of staurosporine, sotrastaurin, and Gö-6983, respectively, compared with 49.2 ± 6.5, and 41.2 ± 5.9 in the control group without PKC inhibition (n = 3 each, P > 0.05, ANOVA). Similarly, enhanced expression of phosphorylated endothelial LSP1 triggered by MIP-2-activated neutrophils did not significantly differ upon treatment with staurosporine, sotrastaurin, or Gö-6983. The relative LSP1 phosphorylation was 65.0 ± 7.0 in the absence of PKC inhibitors and 47.3 ± 10.2, 49.6 ± 9.9, and 49.8 ± 8.5 in the presence of staurosporine, sotrastaurin, and Gö-6983 (arbitrary units, n = 3 each, P > 0.05, ANOVA), respectively. Clearly, p38 MAPK plays a more prominent role than PKC in endothelial LSP1 activation.

It has been documented that neutrophil recruitment in the lung can be integrin- and ICAM-1-independent (13, 14). To test whether these organ-specific mechanisms affect phosphorylation of endothelial LSP1, a series of experiments were performed using blocking antibodies against β2-integrins to analyze neutrophil adhesion and subsequent LSP1 phosphorylation in endothelial cells derived from different organs. In primary lung endothelial cells, MIP-2-triggered neutrophil adhesion and subsequent endothelial LSP1 phosphorylation were significantly blunted by the β2-integrin blocking antibodies (Fig. 4, A–C). Similarly, primary heart endothelial cells and murine SVEC4-10EE2 endothelial cells showed inhibition of neutrophil adhesion (Fig. 4, D and G) and phosphorylation of endothelial LSP1 (Figs. 4, E, F, H, and I). As depicted in Fig. 4, blocking neutrophil β2-integrins resulted in 45%, 65%, and 63% decreased neutrophil adhesion to primary lung endothelial cells, primary heart endothelial cells, and murine SVEC4-10EE2 endothelial cells, respectively. Similarly, blocking neutrophil β2-integrins resulted in 69%, 73%, and 85% reduction of phosphorylated LSP1 in primary lung endothelial cells.
Fig. 4. Blocking neutrophil $\beta_2$-integrins/CD18 or endothelial ICAM-1 inhibits neutrophil adhesion and phosphorylation of endothelial LSP1. A, D, and G: number of adherent $Lsp1^{+/+}$ neutrophils (counted in a 325-$\mu$m$^2$ area) stimulated with 100 nM MIP-2 in the absence (control) and presence of $\beta_2$-integrin blocking antibodies or respective isotype control (isotype) on primary lung (A) and heart (D) endothelial cells from $Lsp1^{+/+}$ mice and murine SVEC4-10EE2 endothelial cells (G). Values are means ± SD ($n = 3$). Significantly different from control and isotype: **$P < 0.01$ and ***$P < 0.001$ (by ANOVA). B, E, and H: original dual immunoblots (representative of 3 similar experiments) demonstrating expression of phosphorylated LSP1 (Alexa Fluor 488, green), total LSP1 (Alexa Fluor 647, red), and overlap of phosphorylated and total LSP1 (yellow) in primary lung (B) and heart (E) endothelial cells from $Lsp1^{+/+}$ mice and murine SVEC4-10EE2 endothelial cells (H) after 30 min of incubation in the presence of neutrophils stimulated with MIP-2 (100 nM) and in the presence or absence of anti-$\beta_2$-integrin blocking antibodies or the respective isotype control antibodies. C, F, and I: densitometric analysis of abundance of phosphorylated LSP1 relative to total LSP1 in primary lung (C) and heart (F) endothelial cells from $Lsp1^{+/+}$ mice and murine SVEC4-10EE2 endothelial cells (I) after 30 min of incubation in the presence of neutrophils stimulated with MIP-2 (100 nM) and in the absence (control) or presence of anti-$\beta_2$-integrin blocking antibodies or the respective isotype control antibodies. Values are means ± SD ($n = 3$). ***Significantly different ($P < 0.001$) from control and isotype (by ANOVA). J: number of adherent $Lsp1^{+/+}$ neutrophils (counted in a 325-$\mu$m$^2$ area) in the absence (control) and presence of anti-ICAM-1 blocking antibodies or the respective isotype control on TNF-α-stimulated (20 ng/ml, 4 h) murine SVEC4-10EE2 endothelial cells. Values are means ± SD ($n = 3$). ***Significantly different ($P < 0.001$) from control and isotype (by ANOVA). K: original dual immunoblots (representative of 3 similar experiments) demonstrating expression of phosphorylated LSP1 (Alexa Fluor 488, green), total LSP1 (Alexa Fluor 647, red), and overlap of phosphorylated LSP1 and total LSP1 (yellow) determined in TNF-α-stimulated (20 ng/ml, 4 h) murine SVEC4-10EE2 endothelial cells after 30 min in the presence of adherent neutrophils and the absence (control) or presence of anti-ICAM-1 blocking antibodies or the respective isotype control antibodies. L: densitometric analysis of abundance of phosphorylated LSP1 relative to total LSP1 in TNF-α-stimulated (20 ng/ml, 4 h) murine SVEC4-10EE2 endothelial cells after 30 min in the presence of adherent neutrophils and in the absence (control) or presence of anti-ICAM-1 blocking antibodies or the respective isotype control antibodies. Values are means ± SD ($n = 3$). ***Significantly different ($P < 0.001$) from control and isotype (by ANOVA).
primary heart endothelial cells, and murine SVEC4-10EE2 endothelial cells, respectively.

To further substantiate our findings, we assessed the effects of blocking endothelial ICAM-1 on neutrophil adhesion and activation of endothelial LSP1 in vitro. Treatment with anti-ICAM-1 blocking antibodies significantly reduced neutrophil adhesion (Fig. 4J) and resulted in significant inhibition of LSP1 phosphorylation in TNF-α-treated endothelial cells (Fig. 4, K and L). Treatment with isotype control antibodies, however, did not significantly modify neutrophil adhesion to endothelial cells (Fig. 4J) and the phosphorylation of endothelial LSP1 (Fig. 4, K and L).

To verify that the phosphorylation of endothelial LSP1 induced by Lsp1−/− neutrophils was indeed triggered by the binding through endothelial adhesion molecules, we used ICAM-1 antibody cross-linking, which engages ICAM-1 on endothelial cells, to determine the effect of ICAM-1-mediated adhesion in triggering of endothelial LSP1 phosphorylation. After stimulation of endothelial cells with TNF-α in vitro, cross-linking ICAM-1 significantly enhanced the phosphorylation of LSP1 in endothelial cells compared with that in the presence of the control isotype immunoglobulins on TNF-α-treated endothelial cells or the same cross-linking on unstimulated endothelial cells (Fig. 5). After ICAM-1 ligation and then cross-linking on unstimulated endothelial cells, we observed a subtle, but significant, increase in the abundance of phosphorylated endothelial LSP1 underlying baseline levels of ICAM-1 expression and its functions in LSP1 activation upon cross-linking on unstimulated endothelial cells (Fig. 5).

As an additional approach, we determined the effect of ICAM-1 engagement on endothelial LSP1 phosphorylation in vivo. Administration of ICAM-1 cross-linking antibodies to Lsp1−/− mice following depletion of circulating neutrophils significantly enhanced the abundance of phosphorylated LSP1 in cremaster muscle. Administration of isotype control immunoglobulins, however, did not modify the level of phosphorylated LSP1 (Fig. 6, A and B). These results indicate that ICAM-1-mediated adhesion is important for the phosphorylation of endothelial LSP1. Consistent with our findings that pharmacological inhibition of p38 MAPK attenuates phosphorylation of endothelial LSP1 (Fig. 3), we observed robust phosphorylation of p38 MAPK in cremaster muscle upon ICAM-1 cross-linking, but not upon administration of the isotype immunoglobulins (Fig. 6, C and D), thus highlighting that phosphorylation of LSP1 is the signaling event downstream of ICAM-1-mediated p38 MAPK activation in endothelial cells.

DISCUSSION

The present study discloses that the adhesion of neutrophils to endothelial cells through ICAM-1 engagement, but not the stimulation with chemokine or cytokine alone, triggers phosphorylation of endothelial LSP1. Our data provide mechanistic evidence that the ICAM-1-mediated adhesion mechanism during leukocyte-endothelial interactions is essential for the activation of endothelial LSP1.

It is well appreciated that p38 MAPK plays an important role in leukocyte recruitment. Pharmacological p38 MAPK inhibition was shown to impair neutrophil transendothelial migration and chemotaxis (9). However, whether the p38 MAPK inhibitors were affecting the endothelium and/or the neutrophils remained obscure. The p38 MAPK downstream molecule LSP1 was shown to play a more important role in endothelial cells than in leukocytes during transendothelial migration (32). The specific roles of endothelial and neutrophil LSP1 in leukocyte adhesion are not completely understood. Endothelial and neutrophil LSP1 deficiency indeed attenuated neutrophil adhesion after stimulation with KC or MIP-2, suggesting that endothelial LSP1 is involved in neutrophil adhesion.

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Fig. 5. Phosphorylation of endothelial LSP1 by ICAM-1 engagement in vitro. A: original dual immunoblots (representative of 3 similar experiments) demonstrating expression of phosphorylated LSP1 (Alexa Fluor 488, green), total LSP1 (Alexa Fluor 647, red), and overlap of phosphorylated LSP1 and total LSP1 (yellow) determined in endothelial cells after stimulation with TNF-α (20 ng/ml) in the presence of cross-linking antibodies following addition of anti-ICAM-1 antibodies (ICAM-1) or isotype controls (isotype). B: densitometric analysis of abundance of phosphorylated LSP1 relative to total LSP1 of endothelial cells determined in endothelial cells without stimulation with TNF-α and in the presence of cross-linking antibodies and after stimulation with TNF-α (20 ng/ml) in the absence (control) or presence of cross-linking antibodies following addition of anti-ICAM-1 antibodies (ICAM-1) or the isotype control. Values are means ± SD (n = 3). Significantly different from control and isotype: *P < 0.05 and ***P < 0.001 (by ANOVA). ###Significantly different (P < 0.001) from cross-linking antibodies + TNF-α stimulation (by ANOVA).
It has been reported that LSP1 regulates neutrophil chemotaxis during inflammation (25). Lsp1−/− neutrophils showed impaired locomotive machinery of migration speed and chemotaxis directionality (49). The negative regulatory role of LSP1 in neutrophils is mediated by the β2-integrin Mac-1 (49), and the function of LSP1 may be regulated through the phosphorylation of LSP1 by MAPK-activated protein kinase 2, a specific downstream target of p38 MAPK (23). Moreover, it was shown that phosphorylated LSP1 was colocalized with F-actin at the leading edge of the polarized neutrophils (57). In addition, it was also documented that the neutrophil chemotaxis induced by the bacterial homoserine lactone AHL-12 was paralleled by the phosphorylation of LSP1 and its colocalization with F-actin (26). Along those lines, phosphorylation was shown to be essential for the activation of LSP1 in other cells. In dendritic cells, the viral protein gp120 induced migration and phosphorylation of LSP1, effects that were significantly blunted by pharmacological inhibition of p38 MAPK (5). In T cells, p50/LSP1 was shown to be phosphorylated by PKC in response to mitogen activation, suggesting a p38 MAPK-independent signaling event for LSP1 activation (34, 35). Accumulating evidence suggests that, in leukocytes, LSP1 is activated by phosphorylation of its Ser residues. In murine leukocytes, Ser243 is the only phosphorylation site recognized on LSP1 by MAPK-activated protein kinase 2, whereas PKC was shown to phosphorylate LSP1 at other Ser/Thr residues (57). To the best of our knowledge, phosphorylation of endothelial LSP1 has not been reported.

Our data directly show that adhesion-triggered phosphorylation of endothelial LSP1 in vitro was significantly attenuated by the p38 MAPK inhibitor SB-203580, a finding that is consistent with previous reports on the role of p38 MAPK activation in the phosphorylation of neutrophil LSP1 (23, 26, 57). In the present study, however, we did not observe an effect of pharmacological inhibition of PKC on phosphorylation of endothelial LSP1, suggesting that p38 MAPK, but not PKC, is the dominant upstream molecule in the activation of endothelial LSP1.

ICAM-1 has been identified as a critical signaling molecule connecting leukocyte adhesion with downstream events in endothelial cells. Phosphorylation of endothelial LSP1 upon ligation and cross-linking of ICAM-1 in vitro or in vivo substantiates our findings that leukocyte adhesion is essential for the activation of endothelial LSP1. ICAM-1 cross-linking was previously shown to activate endothelial p38 MAPK signaling (51), which is partially mediated by xanthine oxidase (52). ICAM-1 engagement by cross-linking antibodies or human rhinovirus binding was also shown to induce phosphorylation of p38 MAPK in a Syk tyrosine kinase-dependent manner (54). Interestingly, leukocyte adhesion further enhanced the phosphorylation of endothelial p38 MAPK elicited by TNF-α, pointing to a synergism between leukocyte adhesion and cytokine stimulation (53). Our observation that basal abundance of phosphorylated p38 MAPK in TNF-α-stimulated cremaster muscle tissue was increased after ICAM-1 cross-linking is consistent with previous reports. Moreover, various other studies have shown that ICAM-1 engagement may also induce PKC activation (1, 18, 44). It is intriguing to speculate that endothelial LSP1 phosphorylation following leukocyte adhesion contributes to the physiological functions of microvascular permeability and transendothelial migration of leukocytes, thus explaining the impaired inflammatory phenotypes in Lsp1−/− mice (29, 32, 40).

Thapsigargin is a pharmacological tool that bypasses cell membrane receptor-mediated signaling mechanisms, triggers intracellular Ca2+ responses, and upregulates the expression and functions of β2-integrins, such as Mac-1, on leukocytes (20, 33). In our study, thapsigargin-pretreated neutrophils adhered to endothelial cells, with subsequent phosphorylation of endothelial LSP1 similar to the effect of neutrophil adhesion induced by the chemokine MIP-2 or the cytokine TNF-α. This suggests that the participation of cytokines or chemokines...
themsevles is not mandatory for the phosphorylation and activation of endothelial LSP1. This phenomenon is further corroborated by the phosphorylation of endothelial LSP1 in response to ICAM-I cross-linking in vitro and in vivo.

In conclusion, activation of endothelial LSP1 is triggered by leukocyte adhesion to endothelial cells, but not by stimulation with chemokine or cytokine alone, and the engagement of ICAM-1 induces phosphorylation of LSP1, an effect downstream of the p38 MAPK signaling cascade in endothelial cells.

ACKNOWLEDGMENTS
The authors thank Dr. Greg Sawicki for valuable suggestions and use of imaging facilities and Jolanta Sawicka and Dr. Virgilio Cadete for expert technical help.

GRANTS
This study was supported by a research grant from the Canadian Institutes of Health Research. L. Liu is a Canadian Institutes of Health Research New Investigator, and S. M. Qadri is a recipient of a fellowship from the Saskatchewan Health Research Foundation.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
M.H., S.M.Q., and L.L. are responsible for conception and design of the experiments; M.H., S.M.Q., and L.L. analyzed the data; M.H., S.M.Q., and L.L. interpreted the results of the experiments; M.H. and Y.S. performed the experiments; M.H., S.M.Q., and L.L. analyzed the data; M.H., S.M.Q., and L.L. interpreted the results of the experiments; M.H. and Y.S. prepared the figures; M.H., S.M.Q., and L.L. interpreted the results of the research; M.H., S.M.Q., and L.L. edited and revised the manuscript; M.H., S.M.Q., Y.S., and L.L. approved the final version of the manuscript.

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