LSP1 is an endothelial gatekeeper of leukocyte transendothelial migration

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Leukocyte-specific protein 1 (LSP1), an F-actin binding protein and a major downstream substrate of p38 mitogen-activated protein kinase as well as protein kinase C, has been reported to be important in leukocyte chemotaxis. Although its distribution has been thought to be restricted to leukocytes, herein we report that LSP1 is expressed in endothelium and is essential to permit neutrophil emigration. Using intravital microscopy to directly visualize leukocyte rolling, adhesion, and emigration in postcapillary venules in LSP1-deficient (*Lsp1*$^{-/-}$) mice, we found that LSP1 deficiency inhibits neutrophil extravasation in response to various cytokines (tumor necrosis factor-α and interleukin-1β) and to neutrophil chemokine keratinocyte-derived chemokine in vivo. LSP1 deficiency did not affect leukocyte rolling or adhesion. Generation of *Lsp1*$^{-/-}$ chimeric mice using bone marrow transplantation revealed that in mice with *Lsp1*$^{-/-}$ endothelial cells and wild-type leukocytes, neutrophil transendothelial migration out of postcapillary venules is markedly restricted. In contrast, *Lsp1*$^{-/-}$ neutrophils in wild-type mice were able to extravasate normally. Consistent with altered endothelial function was a reduction in vascular permeability to histamine in *Lsp1*$^{-/-}$ animals. Western blot analysis and immunofluorescence microscopy examination confirmed the presence of LSP1 in wild-type but not in *Lsp1*$^{-/-}$ mouse microvascular endothelial cells. Cultured human endothelial cells also stained positive for LSP1. Our results suggest that LSP1 expressed in endothelium regulates neutrophil transendothelial migration.

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Abbreviations used: HUVEC, human umbilical vein endothelial cell; KC, keratinocyte-derived chemokine; LSP1, leukocyte-specific protein 1; *Lsp1*$^{-/-}$, LSP1-deficient; MAPK, mitogen-activated protein kinase; MAPKAP, MAPK-activated protein; PKC, protein kinase C.

Lymphocyte-specific gene 1, found in both mouse and humans, was initially thought to be restricted to B cells, functional T cells, and thymocytes (1, 2). However, more recently, it has been documented in monocytes, macrophages, and neutrophils and is now referred to as leukocyte-specific protein 1 (LSP1; references 3–5). LSP1 is an intracellular Ca^{2+} and F-actin binding protein (6–9). In its carboxyl-terminal region, the molecule contains a high affinity F-actin binding site which allows LSP1 to accumulate within the microfilament rich cortical cytoskeleton. LSP1 has been shown to be a major substrate of the mitogen-activated protein kinase (MAPK)-activated protein (MAPKAP) kinase-2 in the p38 MAPK pathway (10). MAPKAP kinase-2 and p38 MAPK were reported to be essential for neutrophil motility and chemotaxis (11–13), suggesting that LSP1 might be important in chemotaxis. However, it should be noted that MAPKAP kinase-2 phosphorylates numerous other molecules, including heat shock protein 25/27 (14), and so the importance of LSP1 after p38 MAPK activation remains unclear. In addition, LSP1 has also been shown to be a substrate for protein kinase C (PKC; 15, 16), which is another molecule implicated in numerous neutrophil functions (including adhesion and chemotaxis; reference 17), which raises the possibility that LSP1 may have multiple roles in neutrophil recruitment.

Although recent in vitro studies using LSP1-deficient (*Lsp1*$^{-/-}$) cells suggest that LSP1 does contribute to the process of chemotaxis, as yet unexplained opposing results have been observed. Jongstra-Bilen and colleagues generated *Lsp1*$^{-/-}$ mice and observed increased chemotactic responses in *Lsp1*$^{-/-}$ neutrophils in vitro (18, 19). In direct contrast, Hannigan et al. reported, in an in vitro study, reduced chemotactic responses of *Lsp1*$^{-/-}$ neutrophils, which may be associated with discontinuous primary actin-rich cortexes and large abnormal membrane protrusions (20). Although both

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groups used keratinocyte-derived chemokine (KC) as a chemoattractant, important differences in experimental conditions between these in vitro experiments included different substrates and different neutrophil populations (peritoneal elicited or bone marrow neutrophils vs. peripheral blood neutrophils). Clearly, a systematic examination of neutrophil function in vivo in the presence and absence of LSP1 is warranted.

In vivo, neutrophil recruitment is a very complex event that requires that neutrophils first tether to the endothelium, and upon activation via chemokines, firmly adhere. This appears to lead to cross-talk between the adherent neutrophil and the endothelium, whereby endothelial cells retract, allowing neutrophils to migrate across the endothelium (21, 22) before chemotaxing toward the source of the injury and/or infection. Recently, we reported that inhibition of p38 MAPK activity dramatically limited neutrophil transmigration across the endothelium and subsequent neutrophil chemotaxis through the interstitium (13). However, whether the p38 MAPK inhibitors were affecting the endothelium and/or the neutrophils was unclear. This is not trivial as both p38 MAPK and MAPKAP kinase-2 have been shown to play a role in endothelial cytoskeletal rearrangements and in increased endothelial permeability associated with hypoxic or oxidative stress (23–25), as well as in TNFα or VEGF stimulation (26–28). However, no one to date has assessed the possibility that LSP1 is found in endothelium.

During neutrophil recruitment, the endothelium is thought to actively retract to allow neutrophils to transmigrate (21, 22, 29, 30). Because LSP1 is a major substrate for the p38 MAPK–MAPKAP kinase-2 signaling pathway and MAPK appears to be important in both neutrophils and endothelium, we tested the hypothesis that LSP1 is an important protein in neutrophil extravasation in vivo as a result of endothelial LSP1. Indeed, our data do not support a critical role for neutrophil LSP1 in extravasation in vivo; however, our results reveal that endothelium does have LSP1 and it plays an essential role in transendothelial migration in chimeric mice where LSP1 was selectively expressed in the endothelium.

RESULTS

LSP1 does not affect leukocyte rolling and adhesion, but is important for leukocyte emigration in response to TNFα or IL-1β

Table I summarizes the hemodynamics of the microvasculature of *Lsp1*$^{-/-}$ and WT mice 4 h after intrascrotal injection of TNFα. The diameters of the chosen cremasteric venules were similar between WT and *Lsp1*$^{-/-}$ mice. There was no apparent difference in shear rate, red blood cell velocity (Table I), or calculated blood flow in these postcapillary venules (not depicted). Similarly, during the induction of inflammation, there was a similar decrease in blood flow in postcapillary venules in both WT and *Lsp1*$^{-/-}$ mice. Therefore, changes in leukocyte behavior described herein cannot be attributed to differences in hemodynamic parameters. There was a small but significant increase in circulating white blood cells in *Lsp1*$^{-/-}$ mice.

Table I. Hemodynamic parameters in WT and *Lsp1*$^{-/-}$ mice 4 h after intrascrotal injection of TNFα (0.5 μg, $n = 3$ in each group)

Group	Venular diameter	V_{RBC}	Wall shear rate	WBC number ($\times 10^6$ cells)
	μm	*mm/s*	s^{-1}	
WT	31 ± 2.9	2.1 ± 0.3	331.8 ± 27.4	5.6 ± 0.2
Lsp1$^{-/-}$	30 ± 2.9	2.0 ± 0.5	320.5 ± 65.5	8.1 ± 0.4[a]

[a]$P < 0.05$ as compared with WBC number in WT mice.
WBC, white blood cell.

We treated WT and *Lsp1*$^{-/-}$ mice intrascrotally with 0.5 μg TNFα and measured leukocyte rolling flux, rolling velocity, and the number of adherent and emigrated leukocytes in cremasteric venules 3.5, 4, and 4.5 h after cytokine injection. Fig. 1 A demonstrates that ~40–60 cells rolled per minute in control preparations of both WT and *Lsp1*$^{-/-}$ mice. Exposure of the cremaster muscle microcirculation to TNFα induced very similar rolling flux in both WT and *Lsp1*$^{-/-}$ mice. The rolling velocity of leukocytes was ~80 μm/s under control conditions in both sets of mice and a very profound 80–90% decrease in rolling velocity was noted in both WT and *Lsp1*$^{-/-}$ mice after TNFα administration (Fig. 1 B). Fig. 1 C demonstrates a large increase in leukocyte adhesion in postcapillary venules after TNFα treatment, a response that was again close to identical in WT and *Lsp1*$^{-/-}$ mice. However, a very significant difference in leukocyte transendothelial migration was noted in WT and *Lsp1*$^{-/-}$ mice in response to TNFα (Fig. 1 D). Although ~40 cells emigrated out of vessels per field of view in WT

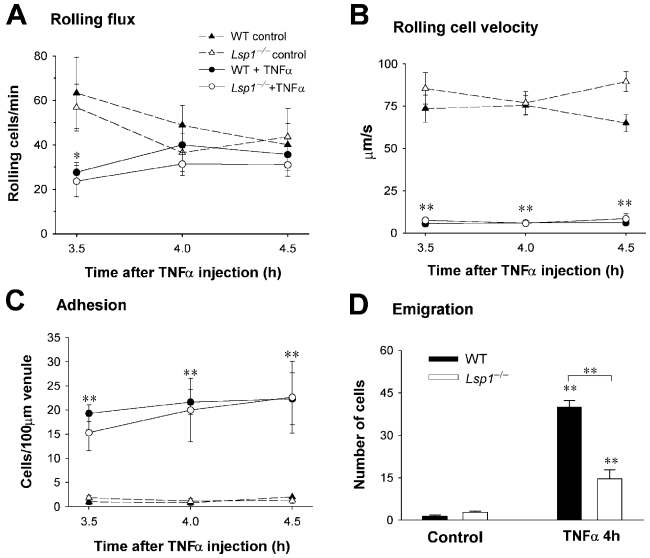

Figure 1. The flux of rolling leukocytes (A), rolling cell velocity (B), adherent (C), and emigrated (D) leukocytes in cremasteric venules of TNFα–treated and untreated WT and *Lsp1*$^{-/-}$ mice. Leukocyte recruitment was induced by intrascrotal injection of TNFα (0.5 μg in 200 μl saline) and the recruitment parameters determined in cremasteric venules from WT (WT control: $n = 4$; WT + TNFα: $n = 3$) and *Lsp1*$^{-/-}$ mice (*Lsp1*$^{-/-}$ control: $n = 6$; *Lsp1*$^{-/-}$ + TNFα: $n = 3$). *, $P < 0.05$ and **, $P < 0.01$, as compared with each untreated control group.

mice, only 15 cells emigrated in *Lsp1*$^{-/-}$ mice ($P < 0.01$). In an additional group of WT mice, one fifth the concentration of TNFα was used. This caused fewer cells to adhere than in *Lsp1*$^{-/-}$ mice treated with the higher concentration of TNFα, yet the emigration was still higher in WT mice than that in *Lsp1*$^{-/-}$ mice (unpublished data).

Previous papers have suggested that the mechanisms underlying leukocyte emigration can be quite different for TNFα versus, for example, IL-1β (31, 32). To determine whether the impaired emigration was limited to TNFα, we injected mice intrascrotally with an optimal dose (12.5 ng) of IL-1β (31), and measured leukocyte rolling flux, rolling velocity, and the number of adherent and emigrated leukocytes in the tissues 3.5, 4, and 4.5 h after injection of cytokine. After IL-1β local administration, leukocyte rolling flux was increased in both WT and *Lsp1*$^{-/-}$ mice at least twofold greater than untreated control mice (Fig. 2 A). Similar decrease in rolling velocity (Fig. 2 B) and increase in adhesion (Fig. 2 C) to IL-1β was noted in WT and *Lsp1*$^{-/-}$ mice. Fig. 2 D demonstrates a profound 75% inhibition in leukocyte transendothelial migration in *Lsp1*$^{-/-}$ mice ($P < 0.05$). Clearly, LSP1 plays a role in leukocyte emigration in response to proinflammatory cytokines.

The impairment in *Lsp1*$^{-/-}$ mice could be due to an impairment in cytokine signaling and subsequent synthesis of chemokines or it could be an impairment in the emigration process per se. Previous work has demonstrated that essentially all of the emigrated cells at 4-h TNFα or IL-1β stimulation are neutrophils (31, 33). Therefore, we examined responses of WT and *Lsp1*$^{-/-}$ neutrophils after activation of their chemokine receptors to the neutrophil chemoattractant KC in vivo.

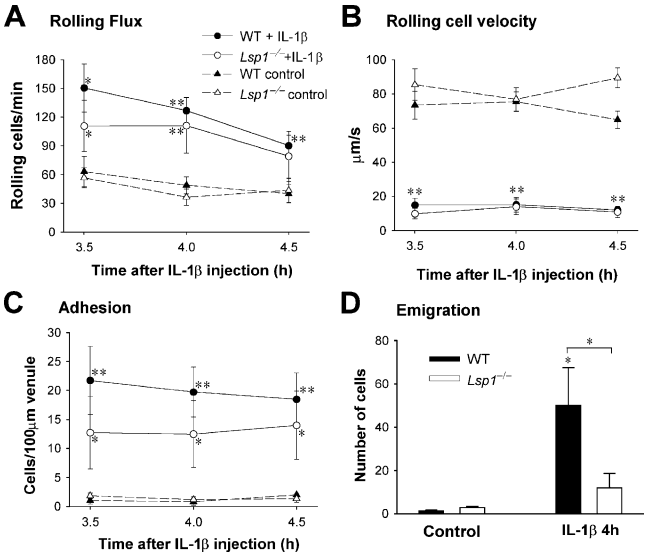

Figure 2. The flux of rolling leukocytes (A), rolling cell velocity (B), adherent (C), and emigrated (D) leukocytes in cremasteric venules of IL-1β–treated and untreated WT and *Lsp1*$^{-/-}$ mice. Leukocyte recruitment was induced by intrascrotal injection of IL-1β (12.5 ng in 200 μl saline) and the recruitment parameters determined in cremasteric venules from WT (WT control: $n = 4$; WT + IL-1β: $n = 4$) and *Lsp1*$^{-/-}$ mice (*Lsp1*$^{-/-}$ control: $n = 6$; *Lsp1*$^{-/-}$ + IL-1β: $n = 4$). *, $P < 0.05$ and **, $P < 0.01$, as compared with each untreated control group.

LSP1 is essential for neutrophil emigration in response to the chemokine KC

We measured leukocyte rolling, adhesion, and transendothelial migration upon slow release of the chemokine KC from an agarose gel positioned 350 μm from the observed cremasteric postcapillary venule. Rolling was not affected by KC (Fig. 3 A), whereas neutrophils began adhering quite rapidly after the KC-containing gel was placed on the cremaster preparation (Fig. 3 B). However, there was no significant difference in the rolling flux and adhesion response between WT and *Lsp1*$^{-/-}$ mice. Fig. 3 C summarizes the number of emigrated neutrophils per field of view 60 min after local KC-containing gel addition. In WT animals, ~25 neutrophils could be seen outside the venule of study and all of the cells were migrating toward the KC-containing gel (unpublished data). In contrast, in the *Lsp1*$^{-/-}$ mice, the transendothelial migration was even more impaired in response to the chemokine KC at 60 min than it was in response to cytokines. Less than four cells were seen to migrate across the endothelium.

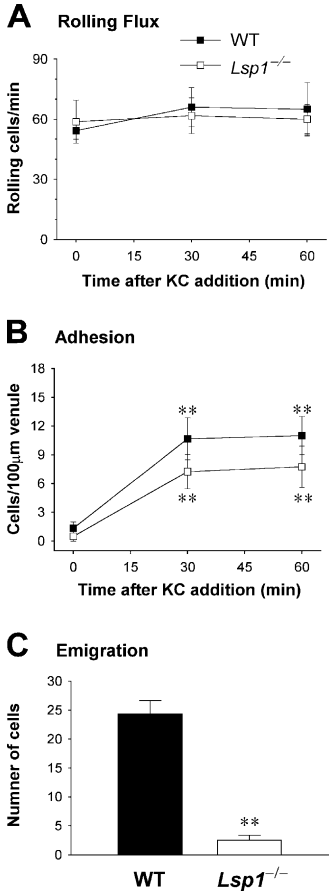

Figure 3. The flux of rolling leukocytes (A), adherent leukocytes (B), and emigrated leukocytes (C) induced by KC in agarose gel placed 350 μm from the observed cremasteric venule of WT ($n = 3$) and *Lsp1*$^{-/-}$ ($n = 4$) mice. **, $P < 0.01$ as compared with time 0 (B) or with the WT control (C).

LSP1 is expressed in mouse and human endothelial cells

Because transendothelial migration is an active process of both leukocytes and endothelium, we isolated leukocytes from the peritoneal cavity in adult mice and primary endothelial cells from the whole lung of 5–7-d-old WT and *Lsp1*$^{-/-}$ mice. There was insufficient cremaster muscle tissue to harvest sufficient numbers of endothelial cells. The large majority of endothelium isolated from the lung is microvascular in origin. RT-PCR revealed that both WT leukocytes and WT endothelium, but not *Lsp1*$^{-/-}$ endothelium, had mRNA for LSP1 (Fig. 4 A). By Western blotting and using the original polyclonal anti-LSP1 serum, we observed that WT but not *Lsp1*$^{-/-}$ mouse primary lung endothelial cells expressed LSP1 protein (52-kD band) and both WT and *Lsp1*$^{-/-}$ endothelial cells also showed an additional ~78 kD band (unpublished data). To obtain more specific antibodies against mouse LSP1, we partially purified the polyclonal anti-LSP1 serum, and by affinity absorption, we made anti-NH_2-terminal LSP1 and anti–COOH-terminal LSP1. Fig. 4 shows that both anti–NH_2-terminal LSP1 (Fig. 4 B) and anti–COOH-terminal LSP1 (Fig. 4 C) stained the 52-kD LSP1 in WT but not in *Lsp1*$^{-/-}$ endothelial cells. The molecular mass of the mouse endothelial LSP1 was identical in size to the leukocyte LSP1 (~52 kD). This 52-kD band was not observed in endothelial extracts from *Lsp1*$^{-/-}$ mice (Fig. 4, B and C). Moreover, the band was lost in WT endothelial cells when the LPS1 antibody was preabsorbed against both GST–LSP1 fusion proteins described in Materials and Methods (unpublished data).

There was an additional ~78-kD band cross-reacting only with anti–NH_2-terminal LSP1 antibody expressed in endothelial cells from both WT and *Lsp1*$^{-/-}$ mice, but not in mouse leukocytes (Fig. 4 B). With two partially purified antibodies against LSP1, we observed fluorescence staining of WT and *Lsp1*$^{-/-}$ endothelial cells. Anti–NH_2-terminal LSP1 antibody stained much brighter on both WT and *Lsp1*$^{-/-}$ endothelial cells than the anti–COOH-terminal LSP1 staining, and the endothelial cells showed strong cytoskeletal staining with anti–NH_2-terminal LSP1 antibody (Fig. 5, top). Because this pattern was also seen in *Lsp1*$^{-/-}$ mice, this cross-reactivity is likely with another cytoskeletal-associated protein. Anti–COOH-terminal LSP1 antibody specifically stained WT mouse endothelial cells but not *Lsp1*$^{-/-}$ endothelial cells (Fig. 5, bottom). Interestingly, the distribution of LSP1 in resting WT endothelial cells appeared in the nuclei and very diffusely throughout the cytoplasm. Because LSP1 was stained weakly in the cytoplasm of WT endothelial cells, it was difficult to determine whether LSP1 associated with the endothelial cytoskeleton (Fig. 5, bottom). In *Lsp1*$^{-/-}$ endothelial cells, the ~78-kD protein was still present, but the overall staining was diminished consistent with the lack of LSP1 in *Lsp1*$^{-/-}$ endothelium.

To determine whether human endothelial cells express LSP1, we double stained human umbilical vein endothelial cells (HUVECs) with anti-LSP1 and anti–VE-cadherin or phalloidin (F-actin). Fig. 6 demonstrates that, similar to mouse endothelial cells, the VE-cadherin–expressing human endothelial cells also express LSP1. Although the majority of LSP1 staining was found in the nucleus, LSP1 staining in the cytoplasm was weak but detectable (Fig. 6 A). Isotype control IgG and secondary Ab alone did not show any significant fluorescence in the nucleus or the cytoplasm (not depicted and Fig. 6 D, respectively). To better detect the LSP1 staining pattern that was relatively weak in the cytoplasm compared with the nuclear staining, we enhanced the fluorescence signals of the dual-labeled LSP1 and phalloidin im-

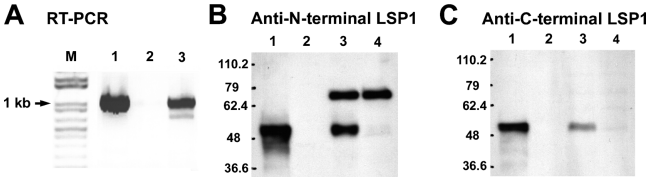

Figure 4. LSP1 expression in mouse primary lung endothelial cells. (A) RT-PCR analysis of LSP1 mRNA was performed using total RNA extracted from mouse leukocytes and primary lung endothelial cells. (lane M) DNA molecular weight markers (Invitrogen) and (lanes 1–3) RT-PCR products from WT leukocyte RNA extracts (lane 1), *Lsp1*$^{-/-}$ endothelial RNA extracts (lane 2), and WT endothelial RNA extracts (lane 3). (B and C) Immunoblotting was performed with anti–NH_2-terminal mouse LSP1 (B) and anti–COOH-terminal mouse LSP1 (C) antibodies and with cell extracts as described in Materials and Methods. Cell lysates from $4-8 \times 10^4$ leukocytes were loaded in lanes 1 and 2, and 2×10^4 endothelial cells in lanes 3 and 4. Equal protein extracts were loaded in B and C. The protein concentrations in anti–NH_2-terminal LSP1 and anti–COOH-terminal LSP1 solutions used for both blottings were 20 μg/ml. (lane 1) Protein extracts of leukocytes from WT mice; (lane 2) protein extracts of leukocytes from *Lsp1*$^{-/-}$ mice; (lane 3) protein extracts of lung endothelial cells from WT mice; and (lane 4) protein extracts of lung endothelial cells from *Lsp1*$^{-/-}$ mice. The numbers to the left are molecular sizes (BenchMark prestained protein ladder; Invitrogen) in kilodaltons. Similar results were observed in three experiments with three batches of endothelial cell isolation.

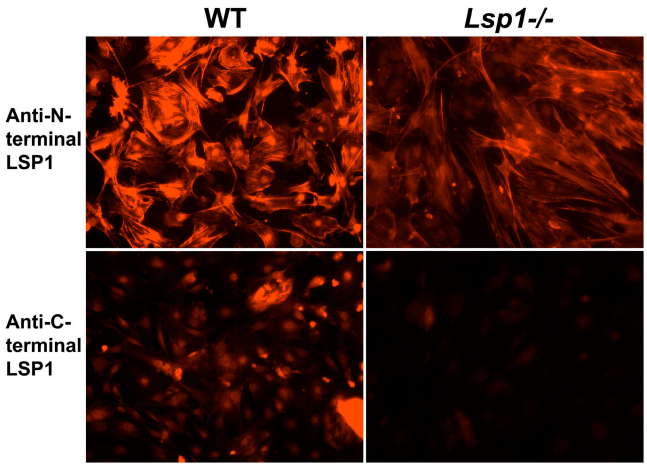

Figure 5. Immunofluorescence staining pattern of cultured mouse lung endothelial cells with anti–NH_2-terminal LSP1 and anti–COOH-terminal LSP1. WT (left) and *Lsp1*$^{-/-}$ (right) mouse lung endothelial cells of passage 1 at subconfluence on glass coverslips were stained with anti–NH_2-terminal LSP1 (top) or anti–COOH-terminal LSP1 (bottom), respectively, as described in Materials and Methods. Magnification, 200.

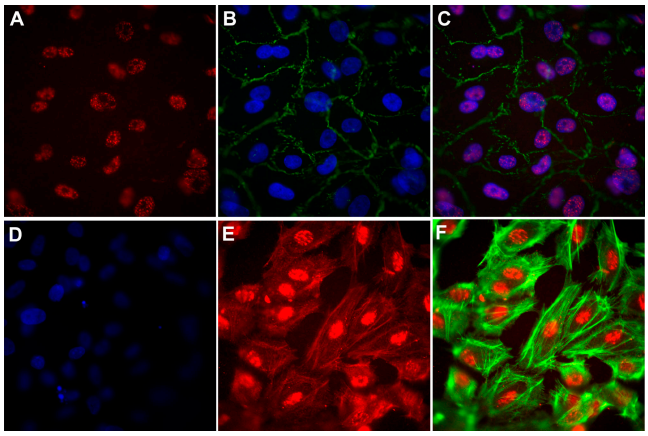

Figure 6. Immunofluorescence localization of LSP1 in cultured human endothelial cells. HUVECs cultured on coverslips were double stained for LSP1 (red; A, C, E, and F) and VE-cadherin (green; B and C). (B–D) Cells were also counterstained for DAPI (blue). (C) The overlaid image of A and B. (D) Secondary Ab alone (Texas red) with DAPI staining. (E) The enhanced image (by increasing the contrast) of LSP1 staining pattern. (F) The enhanced image of LSP1 (from E) overlaid with phalloidin (green). Magnification, 400.

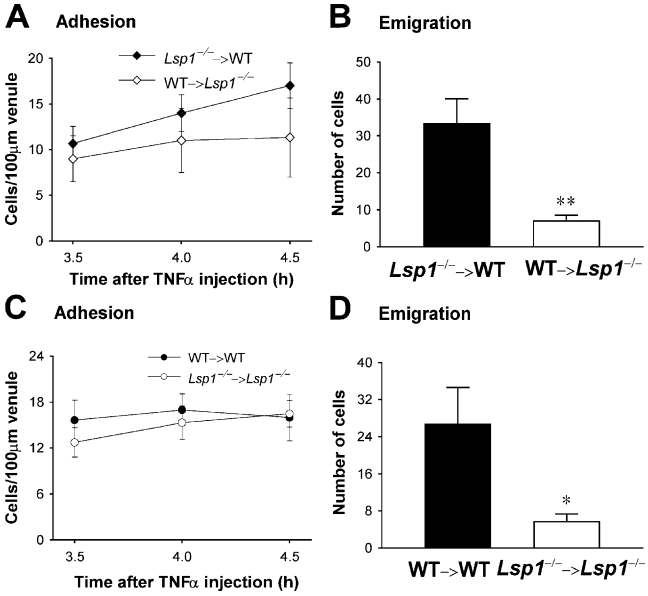

Figure 7. The number of adherent (A and C) and emigrated (B and D) leukocytes in a cremasteric venule of TNFα-treated chimeric mice. WT and *Lsp1*$^{-/-}$ mice were reconstituted with *Lsp1*$^{-/-}$ and WT leukocytes, and indicated as *Lsp1*$^{-/-}$→WT and WT→*Lsp1*$^{-/-}$, respectively (A and B, $n = 3$). WT and *Lsp1*$^{-/-}$ mice were also reconstituted with WT and *Lsp1*$^{-/-}$ leukocytes, and indicated as WT→WT and *Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$, respectively (C and D, $n = 3\sim4$). Leukocyte recruitment was induced by intrascrotal injection of TNFα (0.5 μg in 200 μl saline) and the recruitment parameters determined in cremasteric venules from these chimeric mice. **, $P < 0.01$ as compared with the group of *Lsp1*$^{-/-}$→WT mice at 4 h. *, $P < 0.05$ as compared with the group of WT→WT mice at 4 h.

ages (via increasing the contrast; Fig. 6, E and F). The cytoplasmic LSP1 appeared to be distributed throughout the cytoplasm, with some of the cytoplasmic LSP1 overlapping with F-actin (Fig. 6, E and F). Although F-actin formed clear finger-like projections characteristic of cytoskeleton (Fig. 6 F), a significant amount of LSP1 was diffuse and not always associated with these cytoskeletal structures (Fig. 6 E). These results suggest the following: (a) the majority of endothelial LSP1 is nuclear, (b) LSP1 distributes throughout the endothelial cytoplasm, and (c) some endothelial cytoplasmic LSP1 remains associated with F-actin or at least localizes extremely proximate to the endothelial cytoskeleton.

Endothelial LSP1 regulates leukocyte transendothelial migration

The surprising discovery of LSP1 in endothelium led us to ask whether the protein had any function in the dramatic reduction in leukocyte transendothelial migration in *Lsp1*$^{-/-}$ mice. We made chimeric mice that lacked LSP1 only in the leukocytes. We also made chimeric mice where the *Lsp1*$^{-/-}$ mice received a BM transplant from WT mice. These mice lack LSP1 in endothelium. Upon TNFα local administration, both types of chimeric mice demonstrated similar responsiveness in leukocyte rolling flux (not depicted), rolling velocity (not depicted), and adhesion (Fig. 7 A) in cremasteric venules. Surprisingly, chimeric mice that lacked LSP1 only in their leukocytes emigrated as effectively across the vasculature as WT mice in response to TNFα injection, suggesting that the impaired transendothelial emigration was unrelated to leukocyte-derived LSP1 (Fig. 7 B). However, WT leukocytes reconstituted in *Lsp1*$^{-/-}$ mice (i.e., lacking LSP1 in endothelium) had difficulties in migrating through the *Lsp1*$^{-/-}$ venules and into the tissue ($P < 0.01$, as compared with the reversed chimeric mice). Fig. 7 (C and D) demonstrates that WT mice receiving WT BM behaved just like the WT mice in Fig. 1 and *Lsp1*$^{-/-}$ mice receiving *Lsp1*$^{-/-}$ BM behaved like *Lsp1*$^{-/-}$ mice in Fig. 1.

In a second series of experiments, we tested responses to KC in the chimeric mice. Both sets of chimeric mice demonstrated similar responsiveness in the leukocyte rolling flux (not depicted), rolling velocity (not depicted), and adhesion (Fig. 8 A) upon placement of the KC-containing gel onto the muscle microvasculature. Again, chimeric mice that lacked LSP1 only in their leukocytes emigrated as effectively across the vasculature as WT mice (Fig. 8 B) in response to KC administration. In contrast, WT leukocytes reconstituted in *Lsp1*$^{-/-}$ mice (i.e., lacking LSP1 in endothelium) did not display significant transendothelial migration (Fig. 8 B).

LSP1 is important in histamine-stimulated permeability increases in postcapillary venules

This is the first demonstration of LSP1 in endothelium and, more importantly, the first demonstration of a functional role for endothelial LSP1 in regulating leukocyte emigration. Although the mechanism by which *Lsp1*$^{-/-}$ endothelium restricts leukocyte recruitment is unclear, it is clear that LSP1 is an F-actin binding protein and involved in cytoskeletal changes in leukocytes (7, 8, 19). A very likely possibility is that the *Lsp1*$^{-/-}$ endothelium did not actively retract to permit leukocyte transendothelial migration. Therefore, we

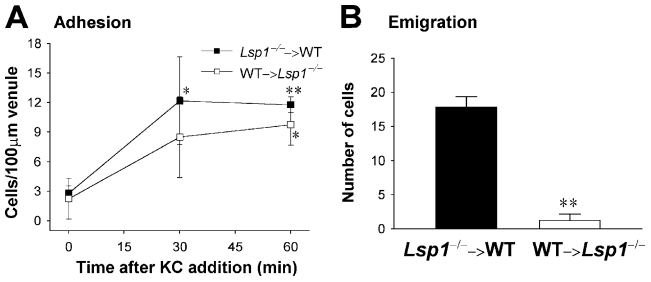

Figure 8. The number of adherent (A) and emigrated (B) leukocytes induced by KC in an agarose gel placed 350 μm from the observed cremasteric venule of chimeric mice. WT and *Lsp1*$^{-/-}$ mice were reconstituted with *Lsp1*$^{-/-}$ leukocytes and WT leukocytes, and indicated as *Lsp1*$^{-/-}$→WT ($n = 5$) and WT→*Lsp1*$^{-/-}$ ($n = 4$), respectively. *, $P < 0.05$ and **, $P < 0.01$, as compared with time 0 in A, or with the data of the WT mice reconstituted with *Lsp1*$^{-/-}$ leukocytes in B.

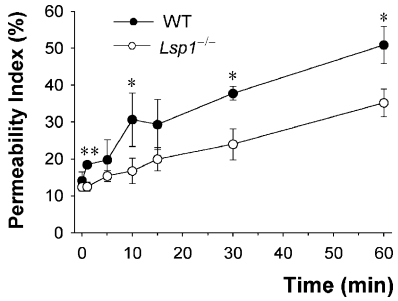

Figure 9. Microvascular permeability changes in cremasteric venules of WT ($n = 4$) and *Lsp1*$^{-/-}$ mice ($n = 7$) upon histamine superfusion. Measurements were taken before (time 0) and after 0.1 mM histamine superfusion of the cremaster muscle preparation. *, $P < 0.05$ and **, $P < 0.01$, as compared with the *Lsp1*$^{-/-}$ mice at the same time points.

measured permeability responses in the postcapillary venules in the control WT and *Lsp1*$^{-/-}$ mice. We chose histamine, a stimulus that did not induce neutrophil emigration per se, to avoid complications associated with neutrophils emigrating in WT but not in *Lsp1*$^{-/-}$ mice. In each case, more FITC-albumin leaked into the interstitium in WT than in *Lsp1*$^{-/-}$ mice. Fig. 9 shows that at 1, 10, 30, and 60 min after 0.1 mM histamine superfusion, the permeability index in venules of WT mice was significantly higher than in venules of *Lsp1*$^{-/-}$ mice ($P < 0.05$). Thus, the results indicated that LSP1 has a functional role in regulating the stimulated permeability changes in postcapillary venules.

LSP1 deficiency does not decrease all forms of leukocyte recruitment

When we administered IL-1β to the peritoneal cavities of WT and *Lsp1*$^{-/-}$ mice, there was very significant neutrophil recruitment in both strains of mice. In fact, the total leukocytes recovered from the peritoneal lavage were slightly higher in *Lsp1*$^{-/-}$ mice than in WT mice (at 4 h, $11.1 \times 10^6 \pm 1.2 \times 10^6$ [$n = 5$] cells in *Lsp1*$^{-/-}$ mice vs. $8.0 \times 10^6 \pm 0.7 \times 10^6$ [$n = 5$] cells in WT mice; $P < 0.05$), consistent with previously published results (18).

DISCUSSION

In this paper, we have demonstrated that LSP1 appears to be an essential intracellular molecule involved in the crucial event of transendothelial migration that permits leukocytes to be recruited to sites of inflammation. Neutrophil transendothelial migration across postcapillary venules was clearly impaired in vivo in *Lsp1*$^{-/-}$ mice when compared with WT littermates. Not all leukocyte functions were inhibited inasmuch as rolling and adhesion within the vasculature (selectin- and integrin-dependent events, respectively) were not altered in *Lsp1*$^{-/-}$ mice. The impaired recruitment appeared to occur regardless of whether an exogenous chemokine was introduced or whether endogenous chemokines were produced by the local administration of TNFα or IL-1β. Until now, LSP1 has been considered to be leukocyte specific; however, we report herein that this F-actin binding protein is also located in microvascular endothelium and functions to permit neutrophil transendothelial migration, suggesting for the first time both a new cellular source of LSP1 and a new critical functional role for LSP1 in endothelium.

Although previous findings of altered neutrophil recruitment in *Lsp1*$^{-/-}$ mice were considered to exclusively reflect an alteration in neutrophil function per se (18–20), in this paper, we provide evidence that endothelial LSP1 also contributes to the neutrophil recruitment. First, we demonstrated that LSP1 was located in endothelium. The protein was the same size as leukocyte LSP1 and was absent in *Lsp1*$^{-/-}$ mice. Second, chimeric mice were generated that had WT leukocytes and *Lsp1*$^{-/-}$ endothelium to delineate the importance of endothelial LSP1. The results clearly demonstrated that WT neutrophils had a profound inability to transmigrate across *Lsp1*$^{-/-}$ endothelium. In contrast, *Lsp1*$^{-/-}$ neutrophils migrated across WT endothelium with no impairment. In addition, we report that *Lsp1*$^{-/-}$ endothelium was less responsive to histamine, a molecule known to activate the endothelial cytoskeleton and induce endothelial retraction.

There is a growing body of evidence that endothelial cells actively contribute to leukocyte transendothelial migration, not only by presenting adhesion molecules including PECAM-1, CD99, and JAM-1, but also by actively retracting and forming gaps upon leukocyte adhesion to the endothelial cells and providing leukocytes a passage of lesser resistance (21, 22, 29, 34, 35). Endothelial cell to cell adherens junctions contain VE-cadherin, which links to different intracellular proteins including β-catenin, γ-catenin (plakoglobin), and p120; the former two connect to actin cytoskeleton through the binding to α-catenin (36, 37). The retraction process has been shown in vitro and in vivo to involve cytoskeletal rearrangement within the endothelium, leading to the disengagement of catenins and VE-cadherins and to the increase in endothelial permeability (38, 39); this retraction can be triggered by both inflammatory mediators, such as histamine, as well as by the direct process of leukocyte adhesion (21, 22, 29, 34–37, 40). Disruption of endothelial microfilaments significantly reduced leukocyte transmigration (41, 42). Although the signaling events leading to the retraction of the endothelium upstream of the cytoskele-

ton are not entirely clear, the prevailing view at this stage is that leukocytes engage surface molecules on the endothelium that activate intracellular signaling events, including increased intracellular Ca^{2+} and activation of PKC and/or myosin light chain kinase, leading to active retraction of endothelium (21, 29, 43, 44). Because LSP1 has actin-binding sites (8, 9), has been shown to play a role in adhesion-dependent polarization (19), and is associated with the cytoplasmic face of the plasma membrane (6), we propose that disruption of LSP1 perturbs the ability of endothelium to retract and, thus, decreases the histamine-induced permeability response of the endothelium and neutrophil extravasation observed in this paper. However, whether LSP1 simply functions upstream of the cytoskeletal changes or physically binds cytoskeleton remains unclear from our immunofluorescence data. It should be noted that overexpression of LSP1 in endothelium (via transfection) revealed significant cytoskeletal association (unpublished data).

It is well appreciated that, in leukocytes, LSP1 is one of several key substrates of MAPKAP kinase-2 and the latter is the direct target of p38 MAPK (10, 45). Several in vitro studies have revealed that both p38 MAPK and MAPKAP kinase-2 are important intracellular signaling molecules in the induction of chemotaxis (11–13). Recently, we have reported that inhibition of p38 MAPK in vivo resulted in both an impairment in transendothelial migration and neutrophil chemotaxis in response to the chemokine KC (13). In contrast, inhibition of p38 MAPK in vitro in human endothelium did not impair recruitment in response to the cytokine TNFα (46). In this paper, lack of LSP1 caused impaired emigration in response to both TNFα and KC. This clearly suggests that the LSP1 deficiency extends well beyond the role of substrate for p38 MAPK. Indeed, it has been reported that LSP1 is also a major substrate for PKC (15, 16), a molecule known to regulate endothelial retraction. An alternative explanation is that the p38 MAPK inhibitors were less effective than LSP1 deficiency. The p38 MAPK inhibitors used previously in this regard are known to specifically inhibit the p38α and p38β isoform, but not the p38γ and p38δ isoforms. Because endothelium can express p38α, β, γ, and δ (45), if LSP1 is downstream of each of these p38 isoforms, then this may explain the additional inhibition seen in *Lsp1*$^{-/-}$ mice versus p38 inhibitors.

The nonlethal phenotype of *Lsp1*$^{-/-}$ mice permitted manipulations that resolved the importance of this molecule in different cell types. Indeed, if the LSP1 pathway is critical within neutrophils for transendothelial migration, then, removal of LSP1 only in leukocytes by generating LSP1 chimeric mice should have still resulted in an inability of *Lsp1*$^{-/-}$ neutrophils to emigrate out of WT vasculature. Surprisingly, BM transplantation of *Lsp1*$^{-/-}$ leukocytes into WT mice restored normal transendothelial migration, suggesting that the lack of neutrophil LSP1 was not responsible for the impairment of transendothelial migration in *Lsp1*$^{-/-}$ mice. Alternative explanations included the possibilities that the irradiation process in some nonspecific manner altered the transendothelial migration phenotype and that the irradiated mice now had an enhanced responsiveness to chemokines. However, our data from the reversed BM chimeras, in which WT neutrophils failed to emigrate out of *Lsp1*$^{-/-}$ vasculature, refute these alternative explanations and support the view that neutrophil transendothelial migration is dependent on a nonleukocyte source of LSP1.

The impaired neutrophil recruitment due to LSP1 deficiency in our study in muscle appeared to be either stimulus or site specific as the work by Jongstra-Bilen and colleagues clearly revealed the opposite response (i.e., enhanced recruitment of leukocytes into the peritoneal cavity in response to thioglycolate; reference 18). We found that, whereas the recruitment of *Lsp1*$^{-/-}$ neutrophils was decreased in cremaster muscle upon IL-1β local administration, the recruitment of *Lsp1*$^{-/-}$ leukocytes into peritoneal cavity was not impaired (but increased) after IL-1β i.p. injection. Although *Lsp1*$^{-/-}$ neutrophils emigrated less into muscle in response to TNFα, we also found that there was no apparent difference in the recruitment of *Lsp1*$^{-/-}$ and WT leukocytes into peritoneal cavity upon TNFα i.p. administration (unpublished data). These results argue against stimulus specificity and support the notion that structural or physiological differences of the organ microvasculatures dictate organ-specific mechanisms of neutrophil recruitment (47).

This is the first documentation of a functional role for LSP1 in endothelium. Some LSP1 could be detected associated with the cytoskeleton; however, the amount was quite low. It may be that the protein only binds the cytoskeleton after leukocyte binding to the endothelium, or perhaps under flow conditions when the endothelium is under constant state of shear force. In addition, under stimulating conditions with a permeabilizing agent, perhaps the movement of LSP1 to cytoskeleton might be observed. Future studies will need to examine under what conditions and how LSP1 interacts with the actin cytoskeleton in endothelium. We will also need to determine whether the recently described function of LSP1 as a cytoskeletal ERK/MAP kinase pathway targeting protein (48) plays a role in its function as an endothelial gatekeeper of leukocyte transendothelial migration.

MATERIALS AND METHODS

Animals. 129/SvJ WT mice were purchased from The Jackson Laboratory. *Lsp1*$^{-/-}$ mice on the 129/SvJ background were generated by homologous recombination by Jongstra-Bilen and colleagues as described previously (18) and transferred to the University of Calgary Health Sciences Centre. Mice of these two genotypes were bred in the University Animal Centre to obtain age- and sex-matched controls. The mice between 8 and 16 wk of age were used in experiments except for the mice used for isolation of mouse endothelial cells (5–7 d old). TIE2-GFP mice were also purchased from The Jackson Laboratory. All animal protocols were approved by the Animal Care Committee of the University of Calgary and met the standards of the Canadian Association of Animal Care. All animals were kept in specific pathogen-free conditions.

Two types of BM chimeric mice were generated following the standard protocols in our laboratory (33). In brief, BM was isolated from 6–8-wk-old donor mice killed by spinal cord displacement. The BM cell suspensions (8×10^6 cells) from donor *Lsp1*$^{-/-}$ and 129/SvJ WT mice were injected into the tail vein of 129/SvJ WT and *Lsp1*$^{-/-}$ mice, respectively.

Before BM cell injection, recipients were irradiated with two doses of 5 Gy γ-ray (Gammacell, ^{137}Cs γ-irradiation source) with a 3-h interval between the two irradiations. These chimeric mice were housed in specific pathogen-free facilities for 6–8 wk to allow full humoral reconstitution before use in experiments. Initial experiments confirmed that ~99% of leukocytes were from donor mice (33). Two additional control groups of mice (WT into WT and *Lsp1*$^{-/-}$ into *Lsp1*$^{-/-}$) went through an identical protocol to ensure that the transplant procedure did not cause any untoward effects.

Intravital microscopy. Male mice were anesthetized with an i.p. injection of a mixture of 10 mg/kg xylazine (Animal Health; Bayer Inc.) and 200 mg/kg ketamine hydrochloride (Rogar/STB Inc.). For all protocols, the left jugular vein was cannulated to administer additional anesthetic or drugs when necessary. The mouse cremaster muscle preparation was used to study the behavior of leukocytes in the microcirculation and adjacent connective tissue as described previously (49). In brief, an incision was made in the scrotal skin to expose the left cremaster muscle, which was then carefully dissected free of the associated fascia. The cremaster muscle was cut longitudinally with a cautery. The testicle and the epididymis were separated from the underlying muscle and were moved into the abdominal cavity. The muscle was held flat on an optically clear viewing pedestal and was secured along the edges with 4–0 suture. The exposed tissue was superfused with 37°C warmed bicarbonate-buffered saline, pH 7.4. An intravital microscope (Axioskip; Carl Zeiss MicroImaging, Inc.) with a ×25 objective lens (Weltzlar L25/0.35; E. Leitz Inc.), and a ×10 eyepiece was used to examine the cremasteric microcirculation. A video camera (5100 HS; Panasonic) was used to project the images onto a monitor, and the images were recorded for playback analysis using a videocassette recorder.

Single unbranched cremasteric venules (25–40 μm in diameter) were selected, and to minimize variability, the same section of cremasteric venule was observed throughout the experiment. The number of rolling, adherent, and emigrated leukocytes was determined offline during video playback analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. The flux of rolling cells was measured as the number of rolling leukocytes passing by a given point in the venule per minute. Leukocyte rolling velocity was measured for the first 20 leukocytes entering the field of view at the time of recording and calculated from the time required for a leukocyte to roll along a 100-μm length of venule. A leukocyte was considered to be adherent if it remained stationary for at least 30 s, and total leukocyte adhesion was quantified as the number of adherent cells within a 100-μm length of venule in 5 min. Leukocyte emigration was defined as the number of cells in the extravascular space within a 200 × 300-μm area (0.06 mm^2), adjacent to the observed venule. More than 90% of these emigrated cells were neutrophils (13, 31). Only cells adjacent to and clearly outside the vessel under study were counted as emigrated.

Induction of leukocyte recruitment in cremaster muscle. To determine whether emigration was impaired in response to cytokines, recombinant mouse TNFα (0.5 or 0.1 μg; R&D Systems) or IL-1β (12.5 ng; BD Biosciences) in 200 μl of saline was injected intrascrotally into *Lsp1*$^{-/-}$, 129/SvJ WT, or chimeric mice. The leukocyte rolling flux, rolling velocity, adherence, and emigration were measured in the cremasteric venule at 3.5, 4, and 4.5 h after the injection.

To induce neutrophil recruitment independent of cytokines, an agarose gel containing KC (CXCL1; R&D Systems) was used (13, 50). The agarose gel was prepared by adding 10 ml of 2 × HBSS to a boiling concentrated agarose solution (4% in 10 ml of distilled water). A 100-μl aliquot of this solution was removed, and KC was added to this aliquot and mixed to achieve a final concentration of 0.5 μM. To enable visualization of the gel on the cremaster muscle, a small amount of India ink was added to each preparation. A 1-mm^3 piece of the mixture (KC-containing gel) was punched out using the tip of a Pasteur pipette. This piece of KC-containing gel was carefully placed on the surface of the cremaster in a preselected area 350 μm (two monitor screens wide) from a postcapillary venule. The gel was held in place using a 22 × 22-mm glass coverslip, and the tissue was superfused beneath the coverslip at a slow rate (0.35 ml/min) to create a chemotactic gradient that permitted emigration. The image was recorded for 90 min: 30 min for control without gel and 60 min with KC-containing gel. In control experiments, only the gel (without KC addition) was placed on the surface of the cremaster muscle.

Microvascular permeability measurement. The degree of vascular albumin leakage from cremasteric venules of *Lsp1*$^{-/-}$ and control 129/SvJ mice was quantified as described previously (51). In brief, 25 mg/kg FITC-labeled BSA (Sigma-Aldrich) was administered to the mice i.v. at the start of the experiment, and FITC-derived fluorescence (excitation wavelength, 450–490 nm; emission wavelength, 520 nm) was detected using a silicon-intensified charge-coupled device camera (model C-2400-80; Hamamatsu Photonics). Image analysis software (Optimas; Bioscan Inc.) was used to determine the intensity of FITC-albumin–derived fluorescence within the lumen of the venule and in the adjacent perivascular tissue. Background was defined as the fluorescence intensity before FITC-albumin administration. The exposed cremaster muscle was superfused with 0.1 mM histamine dihydrochloride (Sigma-Aldrich) in 37°C warmed bicarbonate-buffered saline. The index of vascular albumin leakage (permeability index) at different time points after histamine superfusion was determined according to the following ratio expressed as a percentage: (mean interstitial intensity − background)/(venular intensity − background) (51).

Harvesting endothelial cells and leukocytes. Acute mouse peritonitis was induced to obtain emigrated leukocytes from *Lsp1*$^{-/-}$ and control 129/SvJ WT mice. 3 h after an i.p. injection of 1% oyster glycogen (in 1 ml saline; Sigma-Aldrich), leukocytes were lavaged from the peritoneum and prepared for Western blotting (see the next paragraph). Mouse primary lung endothelial cells were isolated from 5–7-d-old *Lsp1*$^{-/-}$ and control 129/SvJ WT mice, and were cultured according to the protocols described previously (52). Using this protocol with TIE2-GFP mice and flow cytometry, we verified that ~93–98% of the isolated cells were GFP positive, confirming that the majority of the purified cells were of endothelial cell origin (53). Freshly isolated mouse endothelial cells were cultured in microvascular endothelial cell medium-2 (Clonetics EGM-2MV BulletKit; Cambrex Bio Science) in 35-mm Petri dishes precoated with 20 μg/ml mouse laminin (Upstate Biotechnology). After reaching confluence in 5–6 d, the cells were either used for Western blotting or trypsinized and subcultured on laminin-coated 22 × 22-mm glass coverslips (at 30,000 cells/coverslip) contained in 35-mm Petri dishes.

Western blot and RT-PCR analysis. The polyclonal anti-LSP1 serum was made in rabbits against mouse recombinant LSP1 protein (6). Although, in leukocytes, the anti-LSP1 serum detected a single band at the appropriate size for LSP1, a second band of ~78 kD was detected in endothelium. To remove this reactivity, the GST-LSP1 fusion proteins containing LSP1 residues 1–178 or 179–330 were constructed, subcloned, and expressed in *Escherichia coli* BL21 (DE3) cells as described previously (54). These two fusion proteins were allowed to conjugate agarose-glutathione beads and were packed into separate glass columns. The polyclonal anti-LSP1 serum was flowed at a rate of <0.15 ml/min (at 4°C) through the different columns containing at least 10-fold molar excess fusion proteins (residues 1–178 or 179–330). The flow-through fractions containing >1.5 mg/ml protein concentrations, which were the anti–COOH-terminal LSP1 and anti–NH_2-terminal LSP1, respectively, were collected, pooled separately, and used in Western blotting assays and immunofluorescence microscopy. As a control, the anti-LSP1 serum was also absorbed against both GST-LSP1 fusion proteins and used in Western blotting.

Freshly isolated mouse leukocytes and mouse primary lung endothelial cells were used for Western blot and RT-PCR analysis. Whole cell lysates were prepared from these cells using Laemmli buffer with 10% β-mercaptoethanol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The proteins were separated by electrophoresis in 10% SDS-polyacrylamide gels, transferred to

a PVDF Hybond-P transfer membrane (Amersham Biosciences), and blotted using a specific polyclonal rabbit anti–mouse LSP1 serum (at 1:2,000 dilution) as described previously (6) or the anti–COOH-terminal LSP1 and anti–NH_2-terminal LSP1 as described before. After washing, the membrane was incubated with a secondary, horseradish peroxidase–conjugated goat anti–rabbit IgG and treated with enhanced chemiluminescence reagents (ECL kit; Amersham Biosciences). The blotted bands were detected with high performance autoradiography films from Amersham Biosciences. RT-PCR was performed using total RNA (100 ng for each cell type) extracted from freshly isolated leukocytes, mouse primary lung endothelial cells, and LSP1 primer pair A1/A4 as described previously (4). The PCR products were electrophoresed by agarose gel, stained with ethidium bromide, and analyzed by high sensitivity Fluor-S Multimager MAX scanner (Bio-Rad Laboratories) upon dark subtraction.

Immunofluorescence microscopy. All rinsing, incubation, and dilution of antibodies was performed in basal buffer that contained 137 NaCl, 5 KCl, 1.1 Na_2HPO_4, 0.4 KH_2PO_4, 4 $NaHCO_3$, 5.5 glucose, 4.15 PIPES disodium salt, 2 EGTA, and 4.15 $MgCl_2$ in mM, pH 7.2, at room temperature. Mouse lung primary endothelial cells grown on glass coverslips for 24 h were fixed with 4% formalin, permeabilized with 0.1% Triton X-100, and incubated with 10 μg/ml glycine. Primary antibodies used were the anti–COOH-terminal LSP1 and anti–NH_2-terminal LSP1 as described before for 30 min where the protein concentrations were both 0.4 mg/ml in the two blotting solutions, or the original rabbit polyclonal anti-LSP1 serum (at 1:100 dilution). After rinsing three times with 0.1% Tween-20, the coverslips were incubated with Cy3-conjugated goat anti–rabbit IgG for 30 min. After rinsing with basal buffer, the coverslips were mounted in 90% glycerol. Observations were performed on an Olympus IX-70 fluorescence microscope (Olympus). Fluorescence images were captured using OpenLab software (version 3.1.5; Improvision Inc.).

To determine whether human endothelial cells express LSP1, we isolated and cultured HUVECs as described previously (46). Once the HUVECs were confluent, they were passaged onto fibronectin-coated glass coverslips. After culture for 24–48 h, these HUVECs were stained as outlined before with 2.5 μg/ml mouse anti–human LSP1 mAb (clone 16; BD Biosciences) and goat anti–mouse IgG conjugated with Texas Red (Molecular Probes). DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride; Molecular Probes) was used for nuclear staining. To confirm that endothelial cells and not a contaminating cell type expressed LSP1, we dual labeled the HUVECs with FITC-conjugated anti–human VE-cadherin (Bender Medsystems) in combination with LSP1 staining. To assess the association of LSP1 with the cytoskeleton, we labeled HUVECs with anti-LSP1 and phalloidin (Alexa Fluor conjugated; Molecular Probes).

Statistical analysis. The data are expressed as means ± SEM. A Student's *t* test was applied to compare the statistical difference within two groups, and analysis of variance was used for the comparison of the differences in more than two groups. A p-value of <0.05 was considered statistically significant.

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