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. 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 Ca2+ 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 cortices and large abnormal membrane protrusions (20). Although both
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 1 summarizes the hemodynamics of the microvascularity 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 1), 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.

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

Table 1. Hemodynamic parameters in WT and Lsp1−/− mice 4 h after intrascrotal injection of TNFα (0.5 μg, n = 3 in each group)

| Group   | Venules diameter (μm) | VbSc (μm/s) | µm/s | Wall shear rate (s⁻¹) | WBC number (× 10⁶ cells) |
|---------|-----------------------|-------------|------|-----------------------|---------------------------|
| 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             |                            |

*p < 0.05 as compared with WBC number in WT mice.

WBC, white blood cell.

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 chemotactic KC in vivo.

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 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.

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. 4A). 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-NH2-terminal LSP1 and anti–COOH-terminal LSP1. Fig. 4 shows that both anti–NH2-terminal LSP1 (Fig. 4B) and anti–COOH-terminal LSP1 (Fig. 4C) 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 with both GST–LSP1 fusion proteins described in Materials and Methods (unpublished data).

There was an additional ~78-kD band cross-reacting only with anti–NH2-terminal LSP1 antibody expressed in endothelial cells from both WT and Lsp1−/− mice, but not in mouse leukocytes (Fig. 4B). With two partially purified antibodies against LSP1, we observed fluorescence staining of WT and Lsp1−/− endothelial cells. Anti–NH2-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–NH2-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, staining in the cytoplasm was weak but detectable (Fig. 6A). Isotype control IgG and secondary Ab alone did not show any significant fluorescence in the nucleus or the cytoplasm (not depicted and Fig. 6D, 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 images.
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 distributed 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 resect to permit leukocyte transendothelial migration. Therefore, we
mice reconstituted with Lsp1<sup>+/−</sup> leukocytes and WT leukocytes, and indicated as Lsp1<sup>+/−</sup>→WT (n = 5) and WT→Lsp1<sup>+/−</sup> (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<sup>+/−</sup> leukocytes in B.

measured permeability responses in the postcapillary venules in the control WT and Lsp1<sup>+/−</sup> 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<sup>+/−</sup> mice. In each case, more FITC-albumin leaked into the interstitium in WT than in Lsp1<sup>+/−</sup> 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<sup>+/−</sup> 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<sup>+/−</sup> 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<sup>+/−</sup> mice than in WT mice (at 4 h, 11.1 × 10<sup>6</sup> ± 1.2 × 10<sup>6</sup> [n = 5] cells in Lsp1<sup>+/−</sup> mice vs. 8.0 × 10<sup>6</sup> ± 0.7 × 10<sup>6</sup> [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<sup>+/−</sup> mice when compared with WT littermates. Not all leukocyte functions were inhibited in a manner as much as rolling and adhesion within the vasculature (selectin- and integrin-dependent events, respectively) were not altered in Lsp1<sup>+/−</sup> 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<sup>+/−</sup> 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<sup>+/−</sup> mice. Second, chimeric mice were generated that had WT leukocytes and Lsp1<sup>+/−</sup> endothelium to delineate the importance of endothelial LSP1. The results clearly demonstrated that WT neutrophils had a profound inability to transmigrate across Lsp1<sup>+/−</sup> endothelium. In contrast, Lsp1<sup>+/−</sup> neutrophils migrated across WT endothelium with no impairment. In addition, we report that Lsp1<sup>+/−</sup> 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-
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 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).

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 thioglycollate; 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 isola- tion of mouse endothelial cells (5–7 d old). TIE2-GFP mice were also pur- chased 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 sus- pendings (8 × 10⁶ 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, 137Cs 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.

**Intervital 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 intervital microscope (Axioskop; Carl Zeiss Microimaging, Inc.) with a ×25 objective lens (Wetzlar, L25/0.35; E. Leitz Inc.), and a ×10 eyepiece was used to examine the cremaster microcirculation. A video camera (S100 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 length of venule. A leukocyte was considered to be adherent if it remained calculated from the time required for a leukocyte to roll along a 100-μm length of 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 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 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 length of venule.

**Microvascular permeability measurement.** The degree of vascular albumin leakage from cremasteric venules of Lsp1+/− and control 129/SvJ mice was quantitated as described previously (31). 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-enhanced 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 lumina of the venule and in the adjacent parenchymal 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) (31).

**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/coverglass) 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 kDa 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–NH2-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 analyses. Whole cell lysates were prepared from these cells using Laemmli buffer with 10% β-mercaptoethanol, 10 μg/ml leupeptin, and 10 μg/ml aprotonin. 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–NH2-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 mM NaCl, 5 mM KCl, 1.1 mM Na2HPO4, 0.4 mM KH2PO4, 5.5 mM glucose, 4.15 mM PIPES dihydrochloride, 2 mM EGTA, and 4.15 mM PIPES 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 of primary antibodies. All rinsing, incubation, and dilution 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.

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