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Endothelial LSP1 Modulates Extravascular Neutrophil Chemotaxis by Regulating Nonhematopoietic Vascular PECAM-1 Expression

Mokarram Hossain,*† Syed M. Qadri,*† Najia Xu,* Yang Su,* Francisco S. Cayabyab,† Bryan Heit,‡ and Lixin Liu*

During inflammation, leukocyte–endothelial cell interactions generate molecular signals that regulate cell functions. The Ca\(^{2+}\)- and F-actin–binding leukocyte-specific protein 1 (LSP1) expressed in leukocytes and nonhematopoietic endothelial cells is pivotal in regulating microvascular permeability and leukocyte recruitment. However, cell-specific function of LSP1 during leukocyte recruitment remains elusive. Using intravital microscopy of cremasteric microvasculature of chimeric LSP1-deficient mice, we show that not neutrophil but endothelial LSP1 regulates neutrophil transendothelial migration and extravascular directionality without affecting the speed of neutrophil migration in tissue in response to CXCL2 chemokine gradient. The expression of PECAM-1–sensitive \(\alpha_\beta_4\) integrins on the surface of transmigrated neutrophils was blunted in mice deficient in endothelial LSP1. Functional blocking studies in vivo and in vitro elucidated that \(\alpha_\beta_4\) integrins orchestrated extravascular directionality but not the speed of neutrophil migration. In LSP1-deficient mice, PECAM-1 expression was reduced in endothelial cells, but not in neutrophils. Similarly, LSP1-targeted small interfering RNA silencing in murine endothelial cells mitigated mRNA and protein expression of PECAM-1, but not ICAM-1 or VCAM-1. Overexpression of LSP1 in endothelial cells upregulated PECAM-1 expression. Furthermore, the expression of transcription factor GATA-2 that regulates endothelial PECAM-1 expression was blunted in LSP1-deficient or LSP1-silenced endothelial cells. The present study unravels endothelial LSP1 as a novel cell-specific regulator of integrin \(\alpha_\beta_4\)-dependent neutrophil extravascular chemotactic function in vivo, effective through GATA-2–dependent transcriptional regulation of endothelial PECAM-1 expression.

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Abbreviations used in this article: KC, keratinocyte-derived chemokine; KO, knock-out; LSP1, leukocyte-specific protein 1; PKC, protein kinase C; siRNA, small interfering RNA; WT, wild-type.

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bacterial formyl peptide fMLF in vitro (22). Discordantly, however, LSP1-deficient neutrophils exhibited impaired chemotaxis speed and directionality, but enhanced superoxide production, in response to keratinocyte-derived chemokine (KC/CXCL1) gradient in vitro (23). By intravital microscopy of mouse cremaster microvasculature in vivo, decreased transendothelial migration of neutrophils was demonstrated in LSP1-deficient mice in response to IL-1β or TNF-α, effects attributed to the supportive role of endothelial-expressed LSP1 in neutrophil extravasation (6). More recently, endothelial LSP1 was shown to regulate microvascular permeability by participating in endothelial dome formation during neutrophil transmigration (8).

The complexity of cell-specific LSP1 signaling has, thus, pro- mulgated discrepancies in the putative role of LSP1 in vivo. Whether LSP1 modulates other steps of neutrophil recruitment such as intraluminal crawling and posttransendothelial extravasation chemotaxis is not known. Adhesion and transendothelial migration of neutrophils are mainly mediated by β2 integrins (24). However, α4β1 (VLA-4) integrins can also mediate neutrophil recruitment independent of β2 (24–26). On the contrary, neutrophil extravascular chemotaxis was shown to be governed mainly by neutrophil β1 integrins such as α5β1, αvβ3, and αvβ5 (26–29).

Upregulation of these neutrophil β1 integrins is dependent on signaling events associated with engagement of β2 integrin during transendothelial migration (30) but may also be associated with homophilic interactions of both neutrophil PECAM-1 and endothelial PECAM-1 (31). The modulatory role of neutrophil LSP1 on the expression and function of adhesion molecules, however, is incompletely understood. Neutrophil LSP1 was previously shown to regulate the function, but not the expression, of the β2 integrin Mac-1 by modulating its translocation to the uropod (22). Similarly, the role of endothelial LSP1 in the expression and functions of adhesion molecules remain elusive.

In the current study, we explored the cell-specific molecular mechanisms by which LSP1 regulates different steps of CXC chemokine CXCL2-induced neutrophil recruitment in chimeric LSP1-deficient mice by using intravital microscopy and time-lapsed video analysis. By analyzing the expression of adhesion molecules on neutrophils and endothelial cells, we further investigated the cell-specific mechanisms involved in extravascular chemotaxis of transmigrated neutrophils regulated by LSP1 in endothelial cells.

**Materials and Methods**

**Mice**

Five- to seven-day-old and 8–16-wk-old LSP1-deficient (KO) mice, on the 129/SvJ background, generated as described previously (20) and transferred from the University of Toronto, and the control wild-type (WT) 129/SvJ mice were used in this study with the approval of animal protocols from University Committee on Animal Care and Supply at the University of Saskatchewan. All surgeries were performed under ketamine-xylazine anesthesia, as described (7), and all efforts were made to minimize animal suffering. Bone marrow chimeric mice were generated according to a previously described protocol (6) and housed in specific pathogen-free facilities for 6–8 wk to allow full hemonal reconstitution before use in experiments. Bone marrows from WT mice transplanted into WT and KO mice are designated as WT→WT and WT→KO mice, and bone marrows from KO mice transplanted into WT and KO mice are designated as KO→WT and KO→KO mice, respectively. Chimeric mice generated according to this protocol were confirmed to have ∼99% of leukocytes from donor mice (32). The neutrophil responses determined in WT→WT and KO→KO mice in this study were found to be indistin- guishable from those in WT and KO mice, respectively (N. Xu and L. Liu, unpublished observations and Ref. 6).

**Intravital microscopy**

The mouse cremaster muscle preparation was used to study dynamic leukocyte-endothelial interactions in microvasculature as described pre- viously (6, 7, 11, 33). For induction of neutrophil recruitment, a piece of agarose gel (∼1 mm3) containing the optimal concentration of murine chemokine CXCL2 (0.5 µmol/l; R&D Systems, Minneapolis, MN) was placed and held on the surface of the cremaster muscle in a preselected area 350 µm from and parallel to the observed postcapillary venule. Leukocyte rolling velocity (µm/s), leukocyte rolling flux (cells/min), and the number of adherent cells(100-µm venule) and emigrated neutrophils (cells/235 × 200 µm2 field) were determined in the cremaster postcapillary venule (25–40 µm diameter) using video-playback analysis (6, 7, 11, 33). By using ImageJ software (National Institutes of Health) and time- lapse video analysis, neutrophil intraluminal crawling, transmigration time, and extravascular chemotaxis were quantified as described previously (11, 33, 34). Chemotaxis index was calculated by dividing the chemotaxis distance (distance in the direction of chemotactic gradient) by the total migration distance in tissue. At least 40 cells were tracked and analyzed for each group. Where indicated, functional blocking Abs against α4 (3 mg/kg; clone NKI-GoH3; Serotec, Oxford, U.K.) and the respective isotype control (3 mg/kg; rat IgG2a; eBioscience, San Diego, CA) were administered i.v. 30 min prior to the placement of CXCL2-containing gel.

**Isolation of neutrophils and FACS analysis**

Neutrophils from murine femurs and tibias were isolated using three-step Percoll (GE Healthcare, Baie d’Urfé, QC, Canada) gradient (72, 64, and 52%) centrifugation at 1060 g for 30 min as described (19).

Acute mouse peritonitis was induced to obtain emigrated neutrophils 4 h after an i.p. injection of CXCL2 (0.5 µg in saline). Neutrophils were then lavaged and harvested from the peritoneum (with ≥95% purity), and intracellular expression was determined as described previously (11) using the following FITC-conjugated fluorescent Abs and the respective isotype controls (purchased from eBioscience, San Diego, CA) against α4 (CD49f, 1:250 dilution; clone HMa2), α5 (CD49d, 1:200 dilution; clone R1-2), α2 (CD49c, 1:200 dilution; clone HMa5-1), α6 (CD49f, 1:100 dilution; clone GoH3), and β2 (CD29, 1:100 dilution; clone HMb1-1). For FACS analysis, neutrophils were gated on a linearly plotted forward scatter–side scatter dot plot.

PECAM-1 expression in murine peripheral blood neutrophils was determined following a previously reported protocol (35) with minor modi- fication. Mouse blood was collected by cardiac puncture in a heparinized syringe. A blood sample (100 µl) was incubated with FITC-conjugated anti-mouse CD31 mbAb (1:100 dilution; clone 390; eBioscience) for 30 min at room temperature. The erythrocytes were lysed using a lysis buffer (Beckman Coulter, Mississauga, ON, Canada). The cells were then washed and analyzed by flow cytometry.

**Harvest of primary endothelial cells**

Primary murine endothelial cells were isolated from the lungs and hearts of 5–7-d-old WT and KO mice according to a previously described protocol (19). Briefly, endothelial cells were immunomagnetically isolated using anti–ICAM-2 (CD102) Ab (clone 3C4; BD Pharmingen, Quebec City, QC, Canada), cultured, and passed once on laminin-coated 24-well plates after reaching confluence. The purity of isolated endothelial cells was determined and at the time of experiment was determined by immunocyto-chemistry and image analysis as described (36). CD31 (PECAM-1) and nuclei of the cells were labeled using rabbit anti–PECAM-1 (PA5-24411; Thermo Fisher Scientific, Mississauga, ON, Canada) and Alexa Fluor 488– conjugated goat anti-rabbit IgG (Life Technologies, Burlington, ON, Canada) Abs and stained with Hoechst (Life Technologies), respectively. Purity at the time of experiment was ≥90% (n = 6; for each sample, a minimum of 500 cells was analyzed).

**In vitro chemotaxis assay**

Time-lapsed video microscopy was used to determine speed of migration and directionality of chemotaxis in response to CXCL2 chemokine gradient using a previously described method with slight modifications (37, 38). Transmigrated neutrophils were harvested by peritoneal lavage 4 h after an i.p. injection of 1% oyster glycogen (Sigma-Aldrich, Oakville and Mississauga, ON, Canada). Neutrophils were then resuspended at 3 × 106/ml in DMEM cell culture medium containing 10% FCS and 15 mmol HEPES, and 6 µl cell suspension containing functional blocking Abs against α4 (10 µg/ml) or the respective isotype control (10 µg/ml) were seeded into the narrow channel of an uncoated ibiTreat Chemotaxis2D µ-Slide (ibidi, Martinsried, Germany). The slide was then incubated in a humidified incubator at 37˚C and 5% CO2 to allow neutrophils to adhere, and nonadherent neutrophils were removed by flushing the channel. The two opposing reservoirs adjacent to the channel were filled with cell-free medium, and 18 µl 1 µmol/l CXCL2 was introduced into one reservoir. Neutrophil migration was recorded for...
60 min at 37°C using an ibidi heating and incubation chamber on an inverted microscope (Applied Precision) equipped with a digital color video camera. The DMEM cell culture medium was supplemented with 15 mmol/l HEPES to maintain stable pH of the medium during the chemotaxis assay. Time-lapsed video analysis of chemotaxis was performed using ImageJ software (National Institutes of Health), and at least 60 cells were tracked and analyzed for each group.

**In vitro neutrophil transendothelial migration assay**

Murine microvascular SVEC4-10EE2 endothelial cell line cells (American Type Culture Collection, Manassas, VA) were cultured onto tissue culture-treated polycarbonate transwell inserts (3-μm pore size; Corning, Corning, NY) until 85% confluence. After endothelial cells were treated with or without GATA-2-specific or nontargeting silencing RNA for 24 h, neutrophil transendothelial migration was examined as previously described (39, 40) with minor modification. In brief, freshly isolated murine neutrophils were suspended at 5 × 10⁶ cells/ml in DMEM medium supplemented with 5% FBS. Neutrophils in suspension were added to the top inserts and chemotactic gradient. Time-lapsed video analysis of neutrophil transmigration was performed at 37°C in 5% CO₂. Then, 60 μl of 0.5 mol/l EDTA was added to the bottom chamber, and the plate was incubated for 10–15 min at 4°C. Inserts were removed from the well, and the total number of transmigrated cells in each bottom well was counted using a hemocytometer.

**Gene silencing and overexpression**

Murine microvascular SVEC4-10EE2 endothelial cells were cultured as described (19). Targeted gene silencing was achieved by a 48-h transfection of the cells with small interfering RNA (siRNA) specifically targeting LSP1 (Santa Cruz Biotechnology, Dallas, TX) or GATA-2 (Santa Cruz Biotechnology) and with siRNA transfection medium and reagent (Santa Cruz Biotechnology) according to the manufacturer’s protocol (18). The control cells were transfected with negative control scrambled siRNA (Santa Cruz Biotechnology) having no homology to any known RNA sequence.

For overexpression of LSP1, mouse pCMV-SPORT6-LSP1 plasmids (Thermo Fisher Scientific), amplified using Escherichia coli DH5α and purified using the Plasmid Midi Kit (Qiagen), were transfected in endothelial cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol.

**Immunoblotting**

Endothelial cells or neutrophils were lysed using RIPA buffer or Nuclear Extraction Kit (Cayman Chemical, Ann Arbor, MI). Proteins were solubilized in equal volume of Laemmli buffer at 95°C, resolved by 10% SDS-PAGE, and thereafter transferred to a nitrocellulose membrane and immunoblotted as described previously (19). Briefly, the nitrocellulose membrane was blocked with 5% BSA at room temperature for 1 h and then incubated with rabbit anti–PECAM-1 (1:1000 dilution; Thermo Fisher Scientific), rat anti–ICAM-1 (1:1000 dilution; eBioscience), rat anti–VCAM-1 (1:1000 dilution; Abcam, Toronto, ON, Canada), rabbit anti–GATA-2 (1:1000 dilution; Santa Cruz Biotechnology), anti-LSP1 rabbit serum (1:1000 dilution; a generous gift from Dr. J. Jongstra, University of Toronto), or mouse anti–β-actin (1:2500 dilution; Santa Cruz Biotechnology) Ab. After incubation with HRP-conjugated respective secondary antibodies (Ab) (1:5000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature, Ab binding was detected with ECL detection reagent (GE Healthcare). ImageJ software (v1.47; National Institutes of Health; http://rsweb.nih.gov/ij) was used for densitometric quantification of the detected bands. Intensity values for the proteins were normalized to β-actin.

**RT-PCR**

RT-PCR was performed to determine PECAM-1, ICAM-1, and β-actin mRNA expression as described previously (41). Briefly, RNA was isolated from the cells using RNA isolation kit (Qiagen) and reverse-transcribed using reverse transcription kit (Qiagen). RT-PCR was performed using SYBR Green PCR kit (Qiagen) in an iCycler iQ apparatus (Bio-Rad, Hercules, CA) with primers targeting PECAM-1 (QT01052044; Qiagen), ICAM-1 (QT00155078; Qiagen), LSP1 (QT01046227; Qiagen), and β-actin (QT00005242; Qiagen). All PCRs were performed in triplicate and run for 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 40 s.

**Confocal imaging**

Whole-mount staining of cremaster muscle was performed to examine vascular PECAM-1 expression as described previously (8). Briefly, 3 h after an intrascrotal injection of TNF-α (0.1 μg; R&D Systems), the cremaster muscle was excised and fixed using 4% paraformaldehyde (10 min prior to and 1 h after excision of the muscle) and subsequently washed with PBS. The muscle was then permeabilized by 0.5% Triton X-100, blocked with 20% normal goat serum, and then incubated overnight at 4°C with FITC-conjugated rat anti-mouse PECAM-1 Ab (1:250; ebioscience). The samples were then washed with 0.1% Tween-20, fixed on glass slides with fluorescent mounting medium (Sigma-Aldrich), and visualized with a laser scanning confocal microscope (LSM700; Zeiss).

**Statistics**

Data are shown as arithmetic mean ± SEM. Statistical analysis was made using Student t test or ANOVA with Tukey post hoc comparison test. n denotes the number of different mice, different batches of endothelial cells, or neutrophils studied in each group. p values < 0.05 were considered statistically significant.

**Results**

To study the cell-specific functions of neutrophil LSP1 and endothelial LSP1 in CXC chemokine CXCL2-induced neutrophil recruitment in vivo, we used intravital microscopy to analyze different steps of recruitment in the cremaster microvasculature of chimeric LSP1-deficient mice. First, we examined the intravascular dynamics of neutrophil recruitment in the presence of a CXCL2 chemokine gradient. After placing the CXCL2-containing gel, 350 μm from the venule, we observed that leukocyte rolling velocity and rolling flux were not significantly altered in all four types of chimeric mice (Fig. 1A, 1B), Chimeric mice deficient in endothelial LSP1 (KO→KO and WT→KO mice) tended to have lower neutrophil adhesion as compared with WT→WT and KO→WT mice (Fig. 1C). The number of transmigrated neutrophils was significantly lower in KO→KO and WT→KO mice as compared with WT→WT and KO→WT mice, suggesting that not neutrophil LSP1 but endothelial LSP1 is important in neutrophil transendothelial migration in response to CXCL2 (Fig. 1D). As most adherent neutrophils crawl to optimal sites in the vascular lumen for transmigration (34), we analyzed whether cell-specific LSP1 influences intraluminal crawling velocity. Fig. 1E shows that the velocity of intraluminal crawling of neutrophils was not significantly different in all four types of chimeric mice, indicating that neither endothelial LSP1 nor neutrophil LSP1 participates in intraluminal crawling in response to CXCL2 chemo tactic gradient. Time-lapsed video analysis of neutrophil transmigration time revealed that comparing to WT→WT and KO→WT mice, transmigrating neutrophils in KO→KO and WT→KO mice took a longer time from the start of neutrophil transmigration to the first appearance of the whole neutrophil outside the venule in the tissue, underlining that endothelial LSP1 deficiency impairs endothelial cell function in promoting neutrophil transmigration (Fig. 1F).

Next, using time-lapsed video analysis, we examined neutrophil extravascular chemotaxis in response to CXCL2 chemo tactic gradient. To this end, the extravascular speed of neutrophil migration was significantly reduced in KO→KO and KO→WT mice as compared with WT→WT and WT→KO mice, suggesting that LSP1 in neutrophils, but not in endothelial cells, is decisive in regulating the speed of neutrophil extravascular migration (Fig. 2A, 2B). Furthermore, as illustrated in Fig. 2A and 2C, chemotaxis index of extravascular neutrophils was significantly lower in KO→KO and WT→KO mice as compared with that in WT→WT and KO→WT mice, suggesting that endothelial LSP1 is pivotal in regulating the extravascular directionalities of chemotaxing neutrophils.

In view of the surprising modulatory effects of endothelial LSP1 on the directionality of chemotaxing neutrophils in the tissue, we performed a further series of experiments to investigate whether integrin-dependent mechanisms participate in endothelial LSP1-regulated extravascular chemotaxis of emigrated neutrophils. Intraperitoneal injection of CXCL2 resulted in recruitment of neutrophils...
with the unstimulated bone marrow neutrophils, had 5-fold higher surface expression of $\beta_1$ integrins (N. Xu and L. Liu, unpublished observations and Ref. 30). Increased expression of $\alpha_6$ and $\beta_1$, but not $\alpha_2$, $\alpha_4$, or $\alpha_5$ integrins, on the transmigrated neutrophils was significantly lower in KO→KO and WT→KO mice as compared with WT→WT and KO→WT mice, suggesting that not neutrophil LSP1, but endothelial LSP1 participates in mediating the upregulated expression of $\alpha_6\beta_1$ integrins on transmigrated neutrophils (Fig. 3A). Because the expression of only $\alpha_6\beta_1$ integrins on transmigrated neutrophils was significantly altered in mice with endothelial LSP1 deficiency, we sought to address the question of whether $\alpha_6\beta_1$ integrins participate in regulating endothelial LSP1-sensitive extravascular directionality of chemotaxing neutrophils. We analyzed chemotaxis index and speed of migration of transmigrated neutrophils after functionally blocking $\alpha_6$ in cremasteric microvasculature of WT mice and corroborated those observations by determining these parameters using an in vitro μ-slide chemotaxis assay. As depicted in Fig. 3B and 3C, the i.v. administration of anti-$\alpha_6$ blocking Abs in WT mice or treatment of transmigrated WT neutrophils in the medium in vitro with anti-$\alpha_6$ blocking Abs did not significantly change the speed of migration, but significantly mitigated chemotaxis index as compared with the treatment with isotype control Abs, indicating that upregulated chemotaxis index on transmigrated neutrophils regulates the directionality of extravascular neutrophil chemotaxis both in vivo and in vitro.

The upregulation of $\alpha_6\beta_1$ integrins on transmigrated neutrophils is fostered by homophilic PECAM-1 interactions during neutrophil–endothelial cell interactions (31). We, therefore, hypothesized that decreased surface expression of $\alpha_6\beta_1$ on transmigrated neutrophils in endothelial-specific LSP1 deficiency could be a result of deranged PECAM-1 expression. Accordingly, additional in vivo and in vitro experiments were performed to explore whether endothelial or neutrophil LSP1 modulates PECAM-1 expression. Using fluorescence confocal imaging, we visualized the cremasteric microvasculature to examine vascular and neutrophil PECAM-1 expression in WT and LSP1 KO mice. As depicted in Fig. 4A, LSP1 KO mice showed a remarkable decrease in vascular PECAM-1 expression as compared with WT mice. Leukocyte PECAM-1–dependent fluorescence in cremasteric venules, however, similar in both WT and KO mice. These results indicate that endothelial LSP1 regulates endothelial PECAM-1 expression, whereas neutrophil LSP1 does not regulate PECAM-1 expression in neutrophils. Additionally, using flow cytometric determination of PECAM-1–dependent fluorescence

### FIGURE 1. Cell-specific regulation of various neutrophil recruitment steps by LSP1.

(A) Representative original tracings (four traces for each one of the four types of chimeric mice) of extravascular migrating neutrophils in cremasteric muscle tissue. (B) Means ± SEM (n = 6) of speed of neutrophil migration (μm/min) in extravascular cremasteric tissue of WT→WT, KO→WT, WT→KO, and KO→KO chimeric mice determined within 90 min after stimulation with CXCL2 chemokine gradient. *p < 0.05 from WT→WT, †p < 0.05 from WT→KO. (C) Means ± SEM (n = 6) of velocity of intraluminal crawling (μm/min) and transmigration time (min) determined within 90 min after stimulation with CXCL2 chemokine gradient. *p < 0.05 from WT→WT, †p < 0.05 from KO→WT.

(A) Rolling velocity

(B) Rolling flux

(C) Adhesion

(D) Emigration

(E) Velocity of intraluminal crawling

(F) Transmigration time

### FIGURE 2. Cell-specific regulation of extravascular neutrophil migration by LSP1.

(A) Representative original tracings (four traces for each one of the four types of chimeric mice) of extravascular migrating neutrophils in cremasteric muscle tissue. (B) Means ± SEM (n = 6) of speed of neutrophil migration (μm/min) in extravascular cremasteric tissue of WT→WT, KO→WT, WT→KO, and KO→KO chimeric mice determined within 90 min after stimulation with CXCL2 chemokine gradient. *p < 0.05 from WT→WT, †p < 0.05 from WT→KO. (C) Means ± SEM (n = 6) of chemotaxis index (C.I.) of neutrophil chemotactic migration in extravascular cremasteric tissue of WT→WT, KO→WT, WT→KO, and KO→KO chimeric mice determined within 90 min after stimulation with CXCL2 chemokine gradient. *p < 0.05 from WT→WT, †p < 0.05 from KO→WT.
intensity, we revealed that the surface expression of PECAM-1 on peripheral blood neutrophils did not vary between WT and KO mice (mean fluorescence intensity, 224.8 ± 23.2 and 243.4 ± 49.7 arbitrary units after background subtraction [mean ± SEM]; n = 3, respectively) (Supplemental Fig. 1). To corroborate these findings and to determine whether LSP1 regulates other adhesion molecules, we analyzed PECAM-1 and ICAM-1 protein expression in primary endothelial cells from WT and KO mice. As shown in Fig. 4B, PECAM-1, but not ICAM-1, expression was significantly reduced in endothelial cells of LSP1 KO mice as compared with WT mice. To support these data, we determined PECAM-1 protein expression after siRNA-targeted silencing of LSP1 in murine microvascular SVEC4-10EE2 endothelial cells. As a result, siRNA-targeted silencing of endothelial LSP1 significantly reduced PECAM-1 (Fig. 4D), without any effect on ICAM-1 (Fig. 4D) and VCAM-1 (Supplemental Fig. 2) expression in these murine endothelial cells. Silencing LSP1 using gene targeted-siRNA significantly suppressed LSP1 mRNA (Fig. 4C) and protein expression (Fig. 4D), indicating the experimental efficiency of LSP1 silencing in these endothelial cells. Additional experiments explored whether overexpression of endothelial LSP1 modulates PECAM-1 expression. Fig. 4E shows that overexpression of LSP1 in SVEC4-10EE2 endothelial cells resulted in increased PECAM-1. To confirm the cell-specific regulation of PECAM-1 expression by LSP1, we detected PECAM-1 expression in mouse bone marrow neutrophils. As shown in Fig. 4F, neutrophil PECAM-1 expression was not significantly different in either genotype (KO or WT mice). These results and our fluorescence confocal imaging studies shown in Fig. 4A suggest that endothelial LSP1 regulates PECAM-1 expression in endothelial cells, whereas neutrophil LSP1 does not regulate PECAM-1 expression in neutrophils.

We performed an additional series of experiments to disclose the underlying molecular mechanisms of endothelial LSP1-sensitive PECAM-1 expression. As shown in Fig. 5A, targeted siRNA silencing of LSP1 in murine SVEC4-10EE2 endothelial cells significantly blunted PECAM-1, but not ICAM-1, mRNA levels, suggesting that LSP1 transcriptionally regulates PECAM-1 expression in endothelial cells. To elucidate the transcriptional regulation of PECAM-1 by LSP1, we explored the participation of the transcription factor GATA-2 that is expressed in microvascular endothelial cells (42). As shown in Fig. 5B, GATA-2 expression in LSP1-deficient murine primary endothelial cells was significantly lower as compared with that in WT endothelial cells. Furthermore, silencing LSP1 in SVEC4-10EE2 endothelial cells significantly mitigated GATA-2 expression (Fig. 5C), alluding to LSP1 sensitivity of endothelial GATA-2 expression. Overexpression of LSP1 in these endothelial cells significantly increased the expression of GATA-2 (Fig. 5D). To validate the role of LSP1-sensitive GATA-2 expression in selectively regulating endothelial PECAM-1 expression, we silenced GATA-2 and analyzed adhesion molecule expression. Silencing endothelial GATA-2 significantly attenuated GATA-2 and PECAM-1, but not ICAM-1, protein expression in endothelial cells, confirming the endothelial cell LSP1-specific regulation of PECAM-1 expression by GATA-2 (Fig. 5E). We also explored whether endothelial cell GATA-2 indeed plays a role in neutrophil transmigration. In vitro neutrophil transendothelial migration assay revealed that targeted GATA-2 silencing in SVEC4-10EE2 endothelial cells significantly reduced neutrophil transendothelial migration in response to CXCL2, confirming that endothelial cell GATA-2 is an important regulator of neutrophil transendothelial migration (Fig. 5F).

**Discussion**

The present observations disclose the hitherto unknown endothelial cell–specific role of LSP1 in regulating integrin-dependent neutrophil functions during neutrophil recruitment. By using intravital microscopy and time-lapsed video analysis of multiple steps of
neutrophil recruitment, our data demonstrate that endothelial, but not neutrophil, LSP1 is important in the transendothelial migration of neutrophils in response to chemokine CXCL2, confirming the role of endothelial LSP1 as a gatekeeper for neutrophil transendothelial migration (6). It is our novel and surprising finding that the nonhematopoietic, endothelial LSP1 further regulates the directionality, but not the speed, of extravascular migrating neutrophils in tissue after they leave the vasculature. This effect on the directionality of migrating neutrophils in tissue is, at least in part, due to endothelial, but not neutrophil, LSP1-dependent transcriptional regulation by GATA-2 of endothelial PECAM-1 expression, which dictates the expression of α6β1 integrins on the surface of transmigrating neutrophils.

In this study, we demonstrate that, in the absence of endothelial LSP1 alone, CXCL2-elicted rolling and adhesion of neutrophils is not substantially affected, whereas transendothelial migration of neutrophils is markedly reduced. A similar effect was previously reported upon stimulation of leukocyte recruitment by chemokine KC/CXCL1 and cytokines TNF-α and IL-1β (6). Transendothelial migration of neutrophils is preceded by Mac-1–dependent intraluminal crawling mediated by endothelial ICAM-1 (34). Despite a previous report showing that neutrophil LSP1 modulates Mac-1 functions (22), our results do not find alterations in neutrophil intraluminal crawling. However, our data of unaltered ICAM-1 expression in LSP1-deficient endothelial cells substantiate our observation that LSP1 signaling does not participate in intraluminal crawling of neutrophils.

Mechanistically, extravascular migration of neutrophils is effectively accomplished by high-affinity interactions of β1 integrins with proteins of the extracellular matrix (30). We observed that deficiency of neutrophil, but not endothelial, LSP1 resulted in decreased speed of migration in response to CXCL2. Consistent with our findings, LSP1-deficient neutrophils were previously shown to exhibit reduced speed of neutrophil migration in KC/CXCL1 gradient in vitro (23). Surprisingly, our results show that the directionality of chemotaxing neutrophils toward CXCL2 gradient was affected in mice with endothelial cell-specific deficiency of LSP1 in contrast to previous reports showing that neutrophil chemotaxis toward KC/CXCL1 gradient was neutrophil LSP1 dependent (23). It is documented that the inhibitory effect of α6 integrin blocking on neutrophil recruitment is chemotactant specific and involves reduced transendothelial migration, and the impact of α6 integrin blocking on extravascular chemotaxis was not determined (29, 30, 43). However, our in vitro and in vivo results demonstrate that functionally blocking α6 integrin did not affect the speed of neutrophil migration but impaired the directionality, an observation that has not been reported previously. Along this line, LSP1-deficient endothelial cells, therefore, render decreased α6β1 expression on the transmigrating neutrophils during neutrophil–endothelial cell interactions, resulting in impaired directionality of extravascular neutrophil migration in the tissue. In our in vivo study, the effect of α6 functional blocking on the directionality, not the speed of migration, suggests the possible involvement of interactions between α6 integrin and its ligand laminin and the subsequent signaling in dictating extravascular chemotactic directionality of emigrated neutrophils. However, the results of our in vitro α6 functional blocking studies in uncoated, but serum-containing chemotaxis μ-slide indicate that in addition to laminin binding, α6 integrin may also be probably involved in other signaling events such as integrin cross-talk or binding to serum proteins, which could determine the directionality of extravascular neutrophil chemotaxis. Extravascular neutrophil locomotion requires coordinated engagement and detachment of different integrins and their ligands. Apart from ligand binding, integrins are also
involved in a wide range of immune function–related signaling events (30). Because of the complex nature and insufficient knowledge of leukocyte tissue chemotaxis, more investigations are needed to uncover the molecular mechanisms of extravascular neutrophil chemotaxis.

Extravasated neutrophils are also known to secrete soluble mediators such as LTβ4, which is capable of attracting more neutrophils to the site of inflammation (44). The reduction in transmigration of LSP1-deficient neutrophils may possibly be attributed, in part, to the impaired secretion of such soluble mediators. However, whether transmigrated neutrophil-derived soluble chemoattractants are involved in regulating the directionality of neutrophil chemotaxis in tissue is unknown. Further research is warranted to investigate whether such inflammatory mediators are involved in extravascular chemotaxis of neutrophils.

Interestingly, PECAM–1-deficient neutrophils exhibit loss of directionality during migration in vitro (45). This mechanism, however, may not be operative in endothelial LSP1-sensitive directionality changes of migrating neutrophils, as our results show that PECAM–1 expression was reduced in LSP1-deficient endothelial cells, but not in LSP1-deficient neutrophils. PECAM–1-dependent transendothelial migration of neutrophils was previously shown to be mediated by α6β1 integrin, which is upregulated by homophilic interactions of neutrophil and endothelial cell PECAM–1 (31, 46). This cross-talk is supported by our present observation that implicates endothelial LSP1 sensitivity in regulating endothelial PECAM–1–modulated and α6β1–dependent extravascular chemotaxis of neutrophils. Ample evidence underscores the importance of PECAM–1 in orchestrating leukocyte recruitment and microvascular permeability (47, 48). It is, therefore, tempting to speculate that endothelial LSP1-sensitive PECAM–1 expression contributes to the recently reported impairment in microvascular permeability during neutrophil recruitment in LSP1-deficient mice (7, 8).

Very little is known about the signaling mechanisms that regulate PECAM–1 expression in endothelial cells. Endothelial PECAM–1 expression was previously shown to be regulated by inflammatory cytokines (49) and by activation of NO synthase (50), PKC, and phospholipase A2 (51). Transcriptional regulation of PECAM–1 was documented to involve modulation of its gene promoter activity by the p65 subunit of NF–κB (52). The zinc finger transcription factor GATA–2 was initially shown to be crucial in modulating gene expression in megakaryocytic and erythroid lineages (53). Mounting evidence suggests that GATA–2 plays an important role in nonhematopoietic endothelial cells (54, 55), where it is associated with PECAM–1 expression and participates in endothelial cell dedifferentiation (42, 56). PECAM–1 transcription was shown to be regulated by the putative TATA-less promoter region that contains relevant EGR–1 and GATA–2 cis-regulatory elements (55, 57, 58). In addition to regulating PECAM–1 expression, GATA–2 is decisive in endothelial-selective gene expression of endothelial NO synthase, endomucin, and VCAM–1 (42, 58–60). Recently, endothelial GATA–2 was shown to participate in regulating angiogenesis (61) and maintenance of vascular integrity (62). Although GATA–2 regulated the transcription of both PECAM–1 and VCAM–1 (M. Hossain and L. Liu, unpublished observations) in endothelial cells, we found that LSP1 selectively regulated GATA–2–mediated transcription of PECAM–1 but not ICAM–1 or VCAM–1 in these cells. Although both endothelial-specific PECAM–1 and GATA–2 were downregulated.
in LSP1 deficiency, PECAM-1 expression was not altered in LSP1-deficient neutrophils. In light of the strategic nuclear localization of endothelial LSP1 (6) in contrast to its cytosolic presence in neutrophils (23), it is intriguing to consider the role of endothelial cell specificity of GATA-2 function (42, 58) in fostering cell-specific transcriptional regulation of PECAM-1 expression. Interestingly, endothelial GATA-2 was shown to be activated by PKC (63, 64). By the same token, LSP1 serves as a major substrate for PKC (9). However, the possible signaling cross-talk among these molecules requires further investigation. Remarkably, we observed that the expression of ICAM-1, unlike PECAM-1, was not altered in LSP1-deficient endothelial cells. Our result that silencing endothelial expression of ICAM-1, unlike PECAM-1, was not altered in LSP1-deficient endothelial cells is in contrast to its cytosolic presence in neutrophils (23), it is intriguing to consider the role of endothelial cell-specific protein 1 is key to its role in neutrophil polarization and chemotaxis. J. Immunol. 169: 415–423.


**Figure S1. PECAM-1 expression in murine peripheral blood neutrophils**

Original histograms of PECAM-1-dependent fluorescence in peripheral blood neutrophils from WT (red lines) and KO (blue lines) mice. Neutrophils were stained with FITC-conjugated PECAM-1 (solid lines) or isotype control (dashed lines) antibodies.
**Figure S2: LSP1 silencing does not affect VCAM-1 expression in endothelial cells**

Representative original Western blot and means ± SEM (n=3) showing VCAM-1 protein expression (relative to β-actin) in TNF-α-treated (20 ng/ml; 4 h) SVEC4-10EE2 endothelial cells in the absence (Control) or in the presence of treatment with negative control scrambled siRNA or LSP1-targeted siRNA.