Modulation of Mac-1 (CD11b/CD18)-Mediated Adhesion by the Leukocyte-Specific Protein 1 Is Key to Its Role in Neutrophil Polarization and Chemotaxis

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Modulation of Mac-1 (CD11b/CD18)-Mediated Adhesion by the Leukocyte-Specific Protein 1 Is Key to Its Role in Neutrophil Polarization and Chemotaxis

Chunjie Wang,†‡‡ Hisayoshi Hayashi,§§ Rene Harrison,§§ Basil Chiu,‡‡ Jason R. Chan,†¶ Hanne L. Ostergaard,‡‡ Robert D. Innis,§¶ Jan Jongstra,* Myron I. Cybulsky,†¶ and Jenny Jongstra-Bilen‡‡

Leukocyte-specific protein 1 (LSP1) is an intracellular filamentous-actin binding protein which modulates cell motility. The cellular process in which LSP1 functions to regulate motility is not yet identified. In this study, we show that LSP1 negatively regulates fMLP-induced polarization and chemotaxis of neutrophils through its function on adhesion via specific integrins. Using LSP1-deficient (Lsp1−/−) mice, we show increased neutrophil migration into mouse knee joints during zymosan-induced acute inflammation, an inflammatory model in which the number of resident synovioocytes are not affected by LSP1-deficiency. In vitro chemotaxis experiments performed by time-lapse videomicroscopy showed that purified Lsp1−/− bone-marrow neutrophils exhibit an increased migration rate toward a gradient of fMLP as compared with wild-type neutrophils. This difference was observed when cells migrated on fibrinogen, but not fibronectin, suggesting a role for LSP1 in modulating neutrophil adhesion by specific integrins. LSP1 is also a negative regulator of fMLP-induced adhesion to fibrinogen or ICAM-1, but not to ICAM-2, VCAM-1, or fibronectin. These results suggest that LSP1 regulates the function of Mac-1 (CD11b/CD18), which binds only to fibrinogen and ICAM-1 among the substrates we tested. fMLP-induced filamentous actin polarization is also increased in the absence of LSP1 when cells were layered on fibrinogen, but not on fibronectin. Our findings suggest that the increased neutrophil recruitment in Lsp1−/− mice during acute inflammation derives from the negative regulatory role of LSP1 on neutrophil adhesion, polarization, and migration via specific integrins, such as Mac-1, which mediate neutrophil responses to chemotactic stimuli. The Journal of Immunology, 2002, 169: 415–423.

Neutrophils are among the first leukocytes to reach an inflammatory site and initiate host-defense against pathogens. Activated neutrophils are able to fight infection effectively, together with monocytes and resident tissue macrophages, by phagocytosis of microorganisms, or by secreting several potent inflammatory compounds such as radical oxygen intermediates, proteases, or peroxidases. The importance of neutrophil function in innate immunity is best evidenced by several human neutrophil dysfunctions whereby defects in neutrophil migration or anti-inflammatory activity is the cause for severe susceptibility to bacterial infections and defective wound healing (1). In contrast, during chronic inflammatory diseases such as rheumatoïd arthritis or inflammatory bowel disease, uncontrolled accumulations of neutrophils yield aberrantly elevated levels of inflammatory compounds which become major contributors to tissue damage (2, 3). Thus, regulation of neutrophil recruitment into inflammatory sites and their clearance are critical processes assuring effective host defense without tissue injury.

The emigration of circulating neutrophils from blood to inflamed tissue is a complex process dependent upon the coordination of many cellular functions that culminate in directed cell movement (4–6). Adhesion receptors are key to several of these processes. Transendothelial migration is initiated by neutrophil rolling mediated by the selectin family of adhesion receptors. Rolling neutrophils arrest on endothelium and undergo firm adhesion, characterized by spreading and the formation of focal adhesion-like structures mediated by integrin receptors. Integrins are heterodimeric transmembrane proteins consisting of noncovalently bound α- and β-chains (7). In neutrophils, the β1 integrins comprise a common β-chain (CD29) and distinct α-chains (CD49) (8, 9). The β2 family of integrins share their β-chain (CD18) with distinct α-chains (CD11). The importance of the β2 integrins in adhesion and transmigration is evident by the analysis of patients with leukocyte adhesion deficiency type I syndrome which results from mutations in CD18 that lead to severe deficiency of the CD11/CD18 integrins and profound reduction in neutrophil recruitment during inflammation (10). The β2 integrins are critical for firm adhesion of rolling neutrophils on vascular surfaces and transendothelial migration (11, 12). LFA-1 (αLβ2, CD11a/CD18) and Mac-1 (αMβ2, CD11b/CD18) are the most abundant β2 integrins on neutrophils. Although these integrins share ICAM-1 as a ligand, the regulation of their surface expression and their function...
during neutrophil migration appear distinct. Neutrophil activation results in significant up-regulation of surface Mac-1 levels, but not LFA-1 (13). Although the importance of both integrin receptors in firm adhesion of neutrophils to ICAM-1 or to endothelial surfaces was demonstrated, the role of Mac-1 in recruitment of neutrophils into inflammatory sites is not completely understood. Although a number of studies showed inhibition of neutrophil migration by Mac-1 blocking Abs, LFA-1 appeared to play a more important role than Mac-1 in recruitment to inflammatory sites (13–15). Interestingly, increased accumulation of neutrophils was found at inflammatory sites in Mac-1-deficient mice which correlated with their increased survival (16). Thus, distinct or additional roles for Mac-1 have been demonstrated as compared with LFA-1 in inflammatory response.

The tight adhesion of neutrophils to extracellular matrix proteins is accompanied by polarization toward a chemotactic gradient that mediates migration to the inflamed site. Polarization is achieved by the formations of lamellipodia (flat sheet-like structures) and filopodia (thin needle-like projections) at the leading edge and uropod at the rear of the cell (17). Cycles of attachment to the extracellular substrate at the front and detachment from the rear are mediated by temporal and spatial changes in the localization and strength of adhesive forces through integrin receptors (18, 19). Lamellipodia and filopodia are devoid of cytoplasm but contain mainly microfilaments that are formed by local filamentous actin (F-actin) polymerization. F-actin filaments are bundled in filopodia and cross-linked in lamellipodia (20). Thus, proteins which promote F-actin polymerization, or bundle or cross-link actin filaments, are key to provide the rigidity required for these structures to extend, and modulations in their expression have major impact on motile properties of cells (21–25).

The mouse leukocyte-specific protein 1 (LSP1; formerly lymphoocyte-specific protein 1 but renamed to reflect its expression in macrophages and neutrophils) (26, 27) is a 52-kDa intracellular F-actin binding protein which accumulates on the cortical cytoskeleton (28–30). LSP1 mRNA and protein is expressed in mature and immature B and T cells, macrophages, and neutrophils (31–33). Human and mouse LSP1 share the same expression pattern (26, 34–37) and the amino acid identity between the two species is 85% for the basic C-terminal half containing multiple F-actin binding domains and 53% for the acidic N-terminal half. LSP1 has two putative EF-hand motifs near the N terminus and rLSP1 binds Ca2+. LSP1 is a substrate for mitogen-activated protein kinase activated protein kinase 2 (37) and for protein kinase C (38).

Adult Lsp1−/− mice generated in our laboratory show no significant differences from wild-type (wt) mice in the development of the lymphoid and myeloid lineage (27). They display a 3-fold increase in resident peritoneal macrophages but normal proportions of B and T cells within the lymphoid population. Thioglycollate-induced influx of monocytes and neutrophils into the peritoneum is increased in Lsp1−/− mice. In vitro transmigration assays using elicited neutrophils demonstrated increased chemoattractant-induced motility of Lsp1−/− neutrophils. These results suggest that LSP1 is a negative regulator of neutrophil motility in vivo and in vitro. Studies by Howard and colleagues (39, 40) support this conclusion. LSP1 is over-expressed and a 89-kDa protein is under-expressed in patients with neutrophil actin dysfunction (NAD) disorder 47/89. Neutrophils from these patients and transfectants with high expression of LSP1 display formations of large F-actin bundles branching into hair-like cell surface projections and impaired motility (41, 42).

In this paper, we evaluated the in vivo role for LSP1 in regulating neutrophil influx to acute inflammation in the joint and investigated the requirement for LSP1 in various processes which culminate in fMLP-induced neutrophil motility with a long-term goal of elucidating the mechanism(s) by which LSP1 functions as a negative regulator of neutrophil migration during inflammation.

Materials and Methods

Mice

129/SvJ mice (wt and Lsp1−/−) were bred and housed in microisolators in the Toronto Western Research Institute Animal facility (Toronto, Ontario, Canada). Eight- to 12-wk-old female or male mice were used. All protocols were approved by the Animal Care Committee of the University Health Network.

Reagents and Abs

Zymosan, fMLP, PMA, fibrinogen, fibronectin (human plasma), poly-L-lysine, BSA (IgG-free, low endotoxin), and HBSS with or without Ca2+/Mg2+ were purchased from Sigma-Aldrich (St. Louis, MO); Immuno 2Hb microtier plates, used in adhesion assays, were obtained from Dynex Technologies (Chantilly, VA). Mouse ICAM-1 was purified from NS-1 B lymphoma cell membranes by affinity chromatography (43). Human chimeric VCAM-1/Fc and ICAM-2/Fc were purchased from R&D Systems (Minneapolis, MN). FITC-conjugated fMLP, Calcein-AM, and Alexa Fluor 488 Phalloidin were obtained from Molecular Probes (Eugene, OR).

For flow cytometry, mAbs against Mac-1 (FITC-anti-CD11b, M1/70, Serotec, Oxford, U.K.), Gr-1 (PE-conjugated), corresponding isotype controls (Cedarlane Laboratories, Hornby, Ontario, Canada), or LFA-1 (FD441.8; a kind gift from Dr. F. Takei, Terry Fox Laboratory, Vancouver, British Columbia, Canada), α4 or α5 (PS/2 or BMA-5, respectively; a kind gift from Dr. B. Chan, University of London, Ontario, Canada) in conjunction with biotinylated anti-rat Abs and streptavidin-PE (Cedarlane Laboratories) were used. F(ab)2 of goat anti-rabbit IgG (Fc-specific) and anti-FITC Abs were obtained from Caltag Laboratories (Burlingame, CA), unconjugated blocking Abs against mouse Mac-1 (M1/70), LFA-1 (M17/5.2), and CD18 (2E6) were purified from corresponding hybridoma cultures (American Type Culture Collection, Manassas, VA), isotype control rat IgG2b (low endotoxin) was obtained from BD PharMingen (San Diego, CA).

Zymosan-induced acute inflammation in mouse knee joints

Intra-articular injection of 180 μg zymosan was performed along the suprapatellar ligament into the joint cavity of anesthetized mice (44). The contralateral knee was injected similarly with an equal volume of nonpyrogenic saline. Both knee injections were completed by injection of the dye primaquine chromoglycine (43). Human chimeric VCAM-1/Fc and ICAM-2/Fc were purchased from R&D Systems (Minneapolis, MN). FITC-conjugated fMLP, Calcein-AM, and Alexa Fluor 488 Phalloidin were obtained from Molecular Probes (Eugene, OR).

Isolation of bone marrow (BM) neutrophils

Mouse BM cells were flushed from the femur and tibia of wt and Lsp1−/− male mice with Ca2+/Mg2+-free HBSS. Cells were washed and resuspended in 4% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) and neutrophils were purified from Percoll density gradients (45). RBC were lysed with E-lyse solution (Cardinal Associates, Santa Fe, NM). Cells were washed with ice-cold Ca2+/Mg2+-free HBSS (for Calcein-AM labeling) or HBSS with Ca2+/Mg2+ and 0.1% BSA (HB, for all other studies). Flow cytometry revealed high expression of the murine granulocytic marker Gr-1 typically on 75–80% of the isolated cells.

Chemotaxis and chemokinesis assays

Chemokinesis was performed by adding IMLP directly to BM neutrophils and chemotaxis was performed by using a point source of IMLP derived from guest on August 31, 2017 http://www.jimmunol.org/ Downloaded from

<http://www.jimmunol.org/>
from a micropipette. Micropipettes with tips 30–100 μm in diameter were filled with 10−5 M fMLP in 1% low-melt agarose (FMC Bioproducts, Rockland, ME). Neutrophils (0.4 × 106 in HB) were plated on fibrinogen- or fibrin-coated coverslips and were incubated for 10 min in a 37°C heated chamber to allow for adhesion. Nonadherent cells were removed by several washes with HB. Time-lapse video microscopy was performed by capturing differential interference contrast images every 15 s under a Leica DMRB microscope using MetaFluor software (Universal Imaging, West Chester, PA). Image acquisition was initiated 1 min before the placement of the micropipette or addition of fMLP into the chamber to obtain resting neutrophil images. The average speed was calculated from the total migration distance determined by MetaMorph software (Universal Imaging) and the elapsed time. In chemokinesis experiments the response criterion was cell movement at least over a distance corresponding to one cell body.

Coating with extracellular matrix proteins
Fibrinogen (1 mg/ml), fibronectin (20 μg/ml), or ICAM-1 (1 μg/ml) in PBS were added to microtiter wells or to acid-washed coverslips and incubated for 2 h at 37°C. Unbound proteins were removed, plates coated with ICAM-1 were blocked with 2% BSA (IgG free, low endotoxin; Sigma-Aldrich) in PBS for 30 min at room temperature (RT). For coating with VCAM-1/Fc and ICAM-2/Fc, goat anti-human IgG (Fc-specific F(ab′)2), 100 μg/ml were added to each well for 60 min at RT, followed by blocking with 2% BSA before the addition of 20 μg/ml VCAM-1/Fc or ICAM-2/Fc overnight at 4°C. All coating steps were followed by three washes with PBS. Poly-L-lysine was coated (10 μg/ml) as described by the supplier.

For adhesion assays under shear flow, 20 μl goat anti-rabbit IgG (Fc-specific F(ab′)2), 50 μg/ml and fibrinogen (100 μg/ml) were adsorbed onto the center of a 35-mm dish for 60 min in a humidified atmosphere at RT; 10 μl rabbit anti-FITC Abs (20 μg/ml) were subsequently added for 60 min. After blocking with 0.5% BSA overnight at 4°C, 10 μl fMLP-FITC (20 μg/ml) were added at RT.

Neutrophil adhesion assays
For static adhesion assays, Calcein-AM-labeled (27) BM neutrophils in HB (107/100 μl) were plated into precoated microtiter wells (in triplicate) and incubated at 37°C with 7% CO2 for 60 min following the addition of 100 μl agonists. Plates were centrifuged upside-down as described (45) and a CytoFluor 2300 fluorometric plate reader (Millipore, Nepean, Ontario, Canada) was used to calculate the percentage of adhered cells. A standard curve was constructed with increasing amounts of labeled cells added into a separate plate.

For assays under shear flow, BM neutrophils (107/ml) settled for 2 min in a parallel flow chamber as previously described (46). Shear stress was applied at 4 dynes/cm² for 30 s. Cells were observed under an inverted phase contrast microscope, videotaped, and analyzed (46).

fMLP-induced Mac-1 up-regulation and F-actin polymerization
For Mac-1 up-regulation, BM neutrophils in HB (0.5 × 106 cells) were prewarmed for 4 min at 37°C and stimulated with different concentrations of fMLP for 10 min, and stained with FITC-conjugated anti-Mac-1, PE-conjugated anti-Gr-1, or isotype controls (47). Mac-1⁺ live cells were analyzed within the gated Gr-1⁺/+ neutrophils by flow cytometry using FACS-Calibur and CellQuest Software (BD Biosciences, San Jose, CA).

For F-actin assays, BM neutrophils in HB (2.5 × 106/ml) were prewarmed at 25°C and incubated with or without 10−5 M fMLP for different times, fixed with 3.7% paraformaldehyde in PBS for 20 min at RT, washed, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After washing, cells were stained with Alexa Fluor 488 phalloidin for 30 min and analyzed by flow cytometry as above.

Fluorescence microscopy
BM neutrophils (0.4 × 106 cells) in HB were preincubated on fibrinogen- or fibronectin-coated coverslips for 10 min before incubation with or without 10−5 M fMLP for 30 min at 37°C with 5% CO2. After washing briefly with prewarmed HB, the adhered cells were fixed with 4% paraformaldehyde solution (Canemco, Quebec, Canada) for 30 min at RT. Cells were stained for F-actin as described above. Coverslips were mounted with DAKO fluorescent mounting medium (Carpinteria, CA). Slides were examined with a Zeiss LSM 510 scanning laser confocal mounted on a Zeiss Axiovert 100 microscope with a 100× oil immersion objective. Alexa Fluor 488 phalloidin-stained neutrophils were visualized with a fluorescein filter set (515 nm dichroic mirror, 530 nm long-pass barrier filter) using Argon laser (488 nm) illumination. Digital images were prepared using Adobe Photoshop 6.0 software (Adobe Systems, Mountain View, CA). Two hundred cells were counted under a Leica DMRB immunofluorescence microscope to determine the proportion of cells with head-to-tail morphology. The overall F-actin levels in adhered cells was determined from merged stacked confocal images of cells, acquired under non-saturating laser intensities, using NIH Image 1.52 software. Cell outlines were manually traced on images and pixel intensities in the Alexa Fluor 488 phalloidin-stained areas were expressed as relative fluorescent intensity (RFI).

Statistical Analysis
All data are expressed as mean values ± 1 SEM. Unpaired Student’s t tests or Welch’s t tests were performed using InStat software (GraphPad Software, San Diego).

Results
Increased influx of Lsp1−/− neutrophils into zymosan-injected knee joints
We previously reported increased accumulation of Lsp1−/− neutrophils as compared with wt during thioglycollate-induced peritonitis. Lsp1−/− mice exhibit increased resident macrophage levels in the peritoneum but not in the lung, indicating that LSP1 deficiency does not affect all resident macrophage populations. Because, in our former study, we did not eliminate the possibility that the increased level of resident macrophages contribute to the increased influx of neutrophils to the peritoneum, we chose to induce acute inflammation in another tissue where resident cell levels would be unaffected by LSP1 deficiency.

The thin cell layer of resident synoviocytes along the synovial membrane are comprised of macrophages and fibroblasts (48, 49). To verify whether the unstimulated synovial lining displays a similar number of resident cells in the knee joints from wt vs Lsp1−/− mice, we counted 200–500 total cells/mouse synovial lining and determined the average cell number per unit length. We found no differences between five mice from each genotype (not shown), confirming that the resident cells of unstimulated knee joints are equivalent. To determine the accumulation rate of Lsp1−/− vs wt neutrophils in this system, we injected zymosan into the knee joints. Within 24 h, zymosan induced a typical acute inflammation characterized by swelling of the joints and the influx of inflammatory cells (50, 51). Swelling which is a manifestation of edema due to increased vascular permeability was not affected by LSP1 deficiency (Table I). Neutrophils, identified by their typical multilobed morphology by H&E staining, were the predominant cells accumulating in the joint at this early time point of inflammation. Fig. 1 illustrates an example for each genotype analyzed and Table I summarizes the average scores for inflammation. Fig. 1, A and B, shows control joints injected with saline, which exhibit similar morphology in both genotypes. Zymosan-induced accumulation of neutrophils was significantly increased in the synovial cavity of Lsp1−/− mice (Fig. 1, D and F) as compared with wt (Fig. 1, C and Table I. Histological evaluation of acute inflammation in zymosan-injected mouse knees

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Joint Inflammationa</th>
<th>Cavity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lsp1−/−</td>
<td>1.17 ± 0.03 (8)</td>
<td>1.50 ± 0.20 (14)</td>
</tr>
<tr>
<td>Lsp1−/−</td>
<td>1.19 ± 0.03 (7)</td>
<td>1.92 ± 0.14 (13)</td>
</tr>
</tbody>
</table>

a Values are mean ± 1 SEM. Numbers in parentheses correspond to the number of mice analyzed.

b Swelling of the joints was measured after removal of the surrounding muscles and was expressed as a ratio of zymosan over saline-injected knee joint (L,R, left to right).

c The extent of inflammation in the femorotibial joints was scored as the exudation of neutrophils into the joint cavity and infiltration into the subsynovium using a linear scale 0–4 according to cell number and area occupied: 0 = normal histology, 4 = extensive coverage with densely packed neutrophils.

p < 0.02.
E) (Table I). Although increased levels of infiltration into the subsynovium were also found in Lsp1−/− mice, the differences were not statistically significant. These results demonstrate that LSP1 negatively regulates neutrophil recruitment into the joint during acute inflammation independently from changes in resident cell numbers.

Increased chemotaxis of Lsp1−/− neutrophils on fibrinogen

We previously demonstrated that in the absence of LSP1, there is increased in vitro transmigration of thioglycollate-elicited neutrophils in response to chemoattractants, fMLP, or KC. These findings raised the question of whether the increased chemotaxis of Lsp1−/− neutrophils depends on their activation state since we used elicited neutrophils. Furthermore, the transmigration assays do not distinguish between increased speed per cell or an increased number of cells responding. To better elucidate the role of LSP1 in neutrophil motility, we performed time-lapse in vitro fMLP-induced chemokinesis and chemotaxis assays on fibrinogen or fibronectin using BM neutrophils purified from Lsp1−/− and wt mice. Chemokinesis assays, in which a uniform concentration of fMLP (10−8 or 10−5 M) induced unidirectional movement, revealed similar number of cells responding (75–80 of 100 cells/genotype, not shown) on either substrate. Chemotaxis assays were used to determine the average migration rate toward a gradient of fMLP per cell. Table II shows that in the absence of LSP1, neutrophils gained a significant increase in their average speed during chemotaxis on fibrinogen-coated coverslips. Interestingly, the average speed of wt and Lsp1−/− neutrophils was similar on fibronectin-coated coverslips. These data raised the question of whether the negative regulatory role of LSP1 in neutrophil motility is related to its role in adhesion and whether such a role would be specific for a particular class of integrins.

LSP1 is a negative regulator of β2 integrin-mediated adhesion

Neutrophils require an optimal level of adhesion via β1 and β2 integrins on activated endothelial surfaces to mediate spreading and chemotaxis (19, 52). The β1 or β2 family of integrins on neutrophils mediate binding to extracellular matrix proteins or their counterreceptors such as fibronectin (α5β1, α6β1), VCAM-1 (α4β1, α9β1) or fibrinogen (αMβ2, αXβ2), ICAM-1 (αLβ2, αMβ2) or ICAM-2 (αLβ2).

We performed in vitro adhesion assays with BM neutrophils using various substrates which serve as ligands for β2 (Fig. 2) or β1 (Fig. 3) integrins. Fig. 2, A, B, and D, illustrates fMLP and PMA-induced tight adhesion to ICAM-1, fibrinogen, or ICAM-2, respectively, which are mediated by β2 integrins. Adhesion of unstimulated wt and Lsp1−/− neutrophils is similar with each substrate tested. Upon fMLP stimulation, we observed a significant

![Image](http://www.jimmunol.org/)

### Table II. Chemotaxis of BM neutrophilsa

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Substratum</th>
<th>Speed (micrometers per minute)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lsp1+/+</td>
<td>Fibrinogen</td>
<td>5.16 ± 0.72</td>
<td>13</td>
</tr>
<tr>
<td>Lsp1−/−</td>
<td>Fibrinogen</td>
<td>7.02 ± 0.51*</td>
<td>27</td>
</tr>
<tr>
<td>Lsp1+/+</td>
<td>Fibronectin</td>
<td>8.58 ± 0.61</td>
<td>16</td>
</tr>
<tr>
<td>Lsp1−/−</td>
<td>Fibronectin</td>
<td>8.58 ± 0.54</td>
<td>21</td>
</tr>
</tbody>
</table>

* A point source of fMLP was used for chemotaxis as described in Materials and Methods. Values are mean ± 1 SEM; n, the number of cells tracked. * p < 0.05.
increase in tight adhesion of wt neutrophils to fibrinogen, but not to ICAM-1, while fMLP-stimulated Lsp1−/− neutrophils adhered more tightly (1.5- to 2-fold) to both extracellular proteins (Fig. 2, A and B). We obtained a similar increase in fMLP-induced adhesion of Lsp1−/− neutrophils when cells were layered on wells coated with five times less fibrinogen (0.2 mg/ml): wt 8.90 ± 0.83%; Lsp1−/− 12.56 ± 1.05% (n = 5, p < 0.03), indicating that saturating amounts of fibrinogen have been used throughout this study. Because under physiological conditions neutrophils sense and respond to stimulus under shear flow, we repeated the fMLP-induced adhesion experiments to fibrinogen under conditions where neutrophils were subjected to a shear force of 4 dyn/cm² (Fig. 2C). Under these conditions, 58.2 ± 1.6% of wt neutrophils and 86.9 ± 1.4% of Lsp1−/− neutrophils remained adhered to fibrinogen. A shear force of 10 dyn/cm² yielded similar results (not shown). We have thus confirmed by two different assays that LSP1 deficiency results in enhanced fMLP-induced adhesion to fibrinogen. These results, together with significantly increased adhesion to ICAM-1, suggest strongly that LSP1 negatively regulates β2 integrin-mediated adhesion. Although fibrinogen is a ligand for Mac-1, the increased binding of Lsp1−/− neutrophils to ICAM-1 may be the result of binding to both Mac-1 and LFA-1. To verify the capacity of neutrophil adhesion to LFA-1 in the presence or absence of LSP1, we conducted adhesion assays to the specific LFA-1 ligand ICAM-2. Although, fMLP did not induce adhesion to ICAM-2 in wt or in LSP1-deficient neutrophils (Fig. 2D), PMA induced similar adhesion to ICAM-2 in neutrophils from both genotypes showing that murine neutrophils are capable of adhering to this extracellular protein. Similar levels of PMA-induced adhesion were also observed with ICAM-1 (Fig. 2A) while adhesion of Lsp1−/− neutrophils to fibrinogen was significantly increased as compared with wt in the presence of PMA. The lack of difference in PMA-induced adhesion to ICAM-1 of wt and Lsp1−/− neutrophils may be due to their similar binding via LFA-1 as was shown for ICAM-2 (Fig. 2D).

To investigate a role for LSP1 in β1 integrin-mediated firm adhesion, we performed adhesion assays with extracellular matrix proteins VCAM-1 or fibronectin. Fig. 3, A and B, shows that unstimulated neutrophils of both genotypes bind similarly at background levels to VCAM-1 or fibronectin. Interestingly, the increase in adhesion by fMLP or PMA to these substrates is indistinguishable between wt and Lsp1−/− neutrophils. Neutrophil adhesion was similar between the two genotypes when lower (5 µg/ml) or higher (100 µg/ml) concentrations of fibronectin were used to coat the wells. The fMLP-induced adhesion on 100 µg/ml fibronectin was: wt 19.93% ± 1.60; Lsp1−/− 18.67% ± 2.86 (n = 3), indicating that the 20 µg/ml fibronectin used throughout this paper is saturating.

Our attempts to confirm the identity of the β2 integrin for which LSP1 is a negative regulator of adhesion by use of blocking Abs against murine integrin receptors have not been successful. We have used anti-CD11b (M1/70), anti-LFA-1 (M17/5.2, FD441.8), and anti-CD18 (2E6) Abs which we purified from hybridoma supernatants to block adhesion (13). However, these and the control IgG2b Abs (low endotoxin) stimulated, rather than blocked, adhesion of BM neutrophils from both genotypes even when F(ab′)2 were used. As a test for the involvement of integrins in the increased adhesion of LSP1-deficient neutrophils, we repeated the adhesion assays in the absence of Ca2+ and Mg2+ which are required for integrin function (7, 53, 54). Table III illustrates that unstimulated, fMLP-, or PMA-induced neutrophils of either genotype were unable to bind to ICAM-1 in the absence of the divalent ions and consequently, the increase in binding of Lsp1−/− neutrophils to ICAM-1 as compared with their wt counterparts was abolished. Similarly, PMA-induced adhesion to ICAM-2 was also abolished in the absence of Ca2+ and Mg2+ (not shown). In contrast, adhesion of unstimulated or stimulated neutrophils of either genotype to poly-L-lysine, which does not involve integrins, was not significantly affected by the absence of Ca2+ and Mg2+. It is notable that LSP1 deficiency did not affect adhesion to poly-L-lysine under any of the conditions tested.
Table III. \( \text{Ca}^{2+}/\text{Mg}^{2+} \)-dependent adhesion to ICAM-1

<table>
<thead>
<tr>
<th>ICAM-1</th>
<th>% Adhesion with ( \text{Ca}^{2+}, \text{Mg}^{2+} )</th>
<th>PMA</th>
<th>% Adhesion ( \text{Ca}^{2+}, \text{Mg}^{2+} )-Free</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>5.1 ± 1.1 (7)</td>
<td>6.7 ± 0.5 (7)</td>
<td>20.7 ± 1.1 (7)</td>
</tr>
<tr>
<td>KO</td>
<td>5.1 ± 1.0 (7)</td>
<td>15.5 ± 2.5 (7)*</td>
<td>22.7 ± 2.1 (7)</td>
</tr>
<tr>
<td>Plysine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>27.4 ± 4.3 (3)</td>
<td>16.8 ± 3.7 (3)</td>
<td>13.6 ± 2.5 (3)</td>
</tr>
<tr>
<td>KO</td>
<td>25.4 ± 5.6 (3)</td>
<td>19.6 ± 2.0 (3)</td>
<td>17.7 ± 2.2 (3)</td>
</tr>
</tbody>
</table>

* Tight adhesion of BM neutrophils to ICAM-1 and to poly-L-lysine (Plysine) in response to \( 10^{-6} \text{M fMLP}, 25 \text{ng/ml PMA} \), or the carrier 0.1% DMSO (cnt) was performed in the presence or absence of \( \text{Ca}^{2+}/\text{Mg}^{2+} \) for 60 min. For adhesion in the absence of \( \text{Ca}^{2+}/\text{Mg}^{2+} \), 0.1 mM EDTA was added to \( \text{Ca}^{2+}/\text{Mg}^{2+} \)-free HBSS. Values are mean ± 1 SEM. Numbers in parentheses correspond to the number of experiments.

†, \( p < 0.01 \) when compared to wt.

†, \( p < 0.02 \) when compared to the corresponding values in the presence of \( \text{Ca}^{2+}/\text{Mg}^{2+} \).

Taken together, these results demonstrate that LSP1 is not a ubiquitous negative regulator of adhesion but it is involved in integrin-mediated adhesion. In addition, our results also demonstrate that the requirement for LSP1 depends on the class of integrin being activated. In this study, we have identified \( \beta_2 \) integrins, and among them most likely Mac-1, rather than LFA-1, as one of the integrins being regulated by LSP1.

**LSP1 is not required for fMLP-induced Mac-1 up-regulation and F-actin polymerization**

Neutrophils up-regulate their surface Mac-1 levels within minutes of agonist stimulation (55, 56). We asked whether the negative regulatory role of LSP1 in Mac-1-mediated adhesion could be at the level of Mac-1 expression in fMLP-stimulated neutrophils. Fig. 4A shows a robust increase in surface Mac-1 levels (3- to 4-fold) 10 min after fMLP stimulation which was similar for wt and Lsp1\(^{-/-}\) neutrophils. This demonstrates that LSP1 is not involved in the up-regulation of Mac-1 surface expression in stimulated neutrophils. Surface expression of other integrins, LFA-1, \( \alpha_4\beta_1 \) or \( \alpha_5\beta_1 \), which do not significantly change after fMLP stimulation, were similar in neutrophils from both genotypes (not shown).

We had previously shown that rLSP1 does not modify the kinetics of F-actin polymerization in vitro (29). We investigated whether in BM neutrophils LSP1 plays a role in fMLP-induced F-actin polymerization. F-actin levels are similar in unstimulated neutrophils from both genotypes (Fig. 4B). Neutrophils stimulated with \( 10^{-6} \text{M fMLP} \) displayed a maximal F-actin polymerization at 30 s which declined and reached a plateau level after 1 min. LSP1 deficiency did not substantially change this kinetics except at 2 min where a small (14.3%) but statistically significant decrease in F-actin content in Lsp1\(^{-/-}\) neutrophils was observed. No differences in F-actin content were observed with \( 10^{-5} \text{M fMLP} \) in any of the time points tested (not shown). Taken together, these results demonstrate a lack of LSP1 involvement in F-actin polymerization in fMLP-induced neutrophils.

**FIGURE 4.** LSP1 is not required for fMLP-induced Mac-1 up-regulation or F-actin polymerization. A, wt (●) and Lsp1\(^{-/-}\) (■) neutrophils were incubated without (cnt) or with carrier (0.1% DMSO), or with indicated concentrations of fMLP for 10 min and surface Mac-1 levels were determined by flow cytometry in two experiments. B, wt (■) or Lsp1\(^{-/-}\) (●) neutrophils were incubated with \( 10^{-6} \text{M fMLP} \) at 25°C for indicated times and F-actin levels were measured by flow cytometry in three experiments.

**FIGURE 5.** LSP1 negatively regulates neutrophil polarization on fibrinogen. Confocal images of F-actin stained wt (A and B) and Lsp1\(^{-/-}\) (C and D) neutrophils, stimulated with \( 10^{-5} \text{M fMLP} \) for 30 min on fibrinogen-coated coverslips. A1 and C1 correspond to the optical sections at the most basal region of the cell adjacent to the coverslips. Each subsequent frame (2–5) represents a 0.4-μm increment from the level of the previous section. B and D are merged images from other fields of view. Insets are merged images from additional cells. White arrows indicate lamellipodia, open arrowheads indicate trailing filopodia. Note the more extensive F-actin stained polarizations in Lsp1\(^{-/-}\) neutrophils. (Bars = 10 μm).
LSP1-deficiency leads to increased polarization of neutrophils on fibrinogen

Because LSP1 exhibits F-actin bundling capacity (29, 41), it was logical to determine whether the specific negative regulatory role for LSP1 in fMLP-induced neutrophil adhesion to fibrinogen, but not to fibronectin, would be accompanied by similar differences in F-actin polarization on these substrates. Adhesion was induced by fMLP on fibrinogen- or fibronectin-coated coverslips and neutrophils were stained for F-actin. When analyzed by confocal microscopy, a small number of unstimulated neutrophils with typical unpolarized spherical appearance were found on coverslips coated with either substrate (see insets in Fig. 6, A1 and B1). With fMLP, both wt and Lsp1−/− neutrophils underwent polarization on either substrate (Figs. 5 and 6). On fibrinogen, these changes were much more pronounced in Lsp1−/− neutrophils than in wt, while they were similar between the two genotypes on fibronectin. As shown in Fig. 5, A1, A2, and B, wt neutrophils on fibrinogen displayed polarized F-actin patches at the anterior end (lamellipodia, white arrows) which were lost in regions further away from the substratum (Fig. 5A, 3–5). Polarized wt neutrophils displayed one or two trailing filopodia at the posterior end (Fig. 5A, open arrowheads; Fig. 5B, inset). Lsp1−/− neutrophils on fibrinogen (Fig. 5C) displayed larger areas of intense F-actin caps as compared with wt (white arrows). These caps were thicker, as they could be seen in optical sections away from the substratum (see Fig. 5C, 1–5). Some neutrophils from both genotypes had more than one lamellipodia (insets), but again the Lsp1−/− neutrophils displayed more polarized structures. We also found a significant number of Lsp1−/− neutrophils with bright actin-stained focal contacts at the

proximity of lamellipodia which often had more extensive trailing filopodia formations than in wt (Fig. 5D, arrowheads). Quantitatively, on fibrinogen, there was a 2-fold increase (2.06 ± 0.19, n = 5) in the proportion of Lsp1−/− neutrophils which exhibit highly polarized F-actin with head-to-tail morphology as compared with wt. On fibronectin, the extent of head-to-tail polarization of F-actin in wt neutrophils was similar to that of Lsp1−/− neutrophils (Fig. 6, A and B, and inset B). Lamellipodia could be observed throughout the different optical sections of both genotypes (Fig. 6, A or B, 1–6). The ratio of polarized wt vs Lsp1−/− neutrophils was 1.11 ± 0.16, n = 3.

RFI of F-actin is indistinguishable between fMLP-treated neutrophils from the two genotypes plated on fibrinogen (wt, 40.2 ± 3.5; Lsp1−/−, 38.7 ± 3.1) or fibronectin (wt, 30.7 ± 5.5; Lsp1−/−, 26.1 ± 6.7). Thus, consistent with the lack of LSP1 involvement in F-actin polymerization in fMLP-induced neutrophils in suspension (Fig. 4), these data show that LSP1-deficiency does not affect the overall F-actin levels stimulated by fMLP even when neutrophils exhibit increased adhesion and F-actin polarization on fibrinogen.

Discussion

Although it has previously been reported that resident macrophages disappear from the inflamed site within the first hour of thioglycollate injection to the peritoneum, resident macrophages do participate in the production of proinflammatory agonists (such as TNF-α, GM-CSF, IL-8, and other chemokines) that activate neutrophils (57, 58). We reported previously that neutrophil influx into the peritoneum is increased in response to thioglycollate in Lsp1−/− mice which harbor increased levels of resident peritoneal macrophages. Therefore, it was important to study the role of LSP1 in neutrophil migration in vivo, independently from changes in resident cell levels to distinguish between intrinsic vs environmental factors influencing the migration of activated neutrophils. In this study, we found that in unstimulated knee joints the number of the synoviocytes was indistinguishable between the two genotypes while the accumulation in the synovial fluid of Lsp1−/− neutrophils was significantly increased in response to zymosan. These findings demonstrate that the increased neutrophil recruitment in Lsp1−/− mice is independent from the resident cell numbers and is likely due to the changes in the chemotactic characteristics of neutrophils.

Our in vitro studies using single cell analysis by time-lapse video microscopy of BM neutrophils further confirmed the notion that LSP1 deficiency affects the locomotive machinery of neutrophils because we showed that the average migration rate per cell toward a gradient of fMLP is greater in the absence of LSP1 and this does not require previous activation of neutrophils. Interestingly, we also found that this was dependent on the substrate on which the neutrophils migrate. Accelerated migration of LSP1-deficient neutrophils occurred on fibrinogen, but not fibronectin, raising the possibility that the role of LSP1 on neutrophil migration stems from a specific role for this protein in adhesion via certain integrins. In vitro adhesion assays using different extracellular substrate proteins indeed showed that fMLP-stimulated Lsp1−/− neutrophils exhibit increased adhesion to the β2 integrin ligands, fibrinogen (Mac-1), or to ICAM-1 (LFA-1 and Mac-1) but not to ICAM-2 (LFA-1). Adhesion to β2 integrin ligands VCAM-1 or fibronectin were similar. These results suggest strongly for the first time that the negative regulatory role of LSP1 in cell migration is dependent on its role in adhesion via specific integrins. Although adhesion to fibrinogen may not exclusively occur via Mac-1, the fact that increased adhesion of Lsp1−/− neutrophils occurs both to ICAM-1 and fibrinogen and not to ICAM-2, fibronectin, and

FIGURE 6. LSP1 does not modulate the polarization of neutrophils on fibrinogen. Confocal images of F-actin-stained wt (A) and Lsp1−/− (B) neutrophils, stimulated with 10−8 M fMLP for 30 min on fibronectin-coated coverslips. Panels 1–6 illustrate similar image acquisition as described in Fig. 5. Insets in A1 and B1 illustrate typical merged images of unstimulated neutrophils and the inset in B6 illustrates a merged image of another polarized Lsp1−/− neutrophil. Note a similar extent of F-actin polarization in neutrophils of both genotypes. (Bars = 10 μm).
VCAM-1 strongly suggests that at least one integrin modulated by LSP1 is Mac-1, while LFA-1 and \( \beta_1 \) integrins are not negatively regulated by this protein. The tight adhesion of wt neutrophils to fibronectin or VCAM-1 was stronger than to fibrinogen or ICAM-1 and similarly the extent of polarization and average migration rate of wt neutrophils were higher on fibronectin than fibrinogen. Thus, at least in the case of \( \beta_1 \) integrins, the strength of adhesion may compensate for the negative regulatory role of LSP1.

During chemotaxis, neutrophils undergo transient shape changes by sending forward lamellipodia in the direction of migration. These attach to the extracellular matrix and allow the cell body to move toward this new attachment. Cells also release attachments of their posterior ends (6, 59). In this study, we observed striking differences in fMLP-induced F-actin polarization between wt and LSP1-deficient neutrophils when they were induced to adhere on fibrinogen, while adhesion to fibronectin did not result in such differences. It is notable that in suspended neutrophils, in the absence of any attachment, fMLP-stimulated F-actin polarization, as measured by percent-polarized cells or the area occupied by F-actin caps was similar between wt and Lsp1−/− neutrophils (not shown). These findings support the notion that LSP1 is not involved directly in fMLP-induced polarization of F-actin but rather emphasize the function of LSP1 in regulating the gradient of adhesive strength which develops during chemotaxis.

Hamnigan et al. (60) recently reported that LSP1-deficient blood neutrophils exhibit impaired chemotaxis toward a gradient of the chemokine KC on glass coverslips, which they attributed to unidirectional protrusions produced in the absence of LSP1. Our data show the importance of the substrate used for understanding the role of LSP1 in chemotaxis. They also reported that LSP1 is not required for adhesion to fibrinogen in response to KC or PMA. Besides the differences in the experimental conditions, these authors used total bone marrow cells as opposed to purified neutrophils, which may have masked the increased adhesion of neutrophils.

A specific role of LSP1 in the functioning of Mac-1 rather than LFA-1 can be considered if LSP1 interacts with Mac-1 directly or through an adapter protein and provides a linkage to F-actin in unstimulated neutrophils. Given that LSP1 is an abundant protein involved directly in fMLP-induced polarization of F-actin but rather emphasizes the function of LSP1 in regulating the gradient of adhesive strength which develops during chemotaxis.

In conclusion, we showed that LSP1 negatively regulates neutrophil chemotaxis and polarization through its regulatory function on adhesion via specific integrins such as Mac-1. We suggest that these findings account for our in vivo observations that LSP1 modulates neutrophil accumulation during acute inflammation. Future studies will focus on the mechanisms by which LSP1 regulates Mac-1 function in adhesion. It also remains to be investigated whether this negative regulatory role of LSP1 affects other neutrophil functions involving Mac-1, i.e., phagocytosis and cytotoxicity (72–74).

References

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