THE ROLE OF LSP1 IN CHEMOKINE-INDUCED NEUTROPHIL RECRUITMENT

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By

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ABSTRACT

Leukocyte-specific protein 1 (LSP1) is an intracellular, F-actin- and Ca^{2+}-binding, phosphoprotein expressed in leukocytes and endothelial cells (ECs). It is important in leukocyte motility and recruitment during inflammation. The role of LSP1 and its signaling mechanisms in chemokine-induced neutrophil transendothelial recruitment are not well understood. In this study, the role of neutrophil LSP1 (N-LSP1) and endothelial LSP1 (E-LSP1) in neutrophil-endothelial cell interactions in the post-capillary venules was investigated by using intravital microscopy and time-lapse video photography. This was determined in response to two very similar CXC chemokines keratinocyte-derived chemokine (KC, CXCL1) and macrophage inflammatory protein-2 (MIP-2, CXCL2). To induce neutrophil recruitment, one mm^3 piece of agarose gel containing MIP-2 or KC was carefully placed on 350 μm from the observed post-capillary venule of the mouse cremaster muscle. Neutrophil intraluminal crawling, transmigration, and chemotaxis in the muscle were analyzed by the use of LSP1-deficient (*Lsp1*$^{-/-}$) mice, wild-type (WT) 129/SvJ mice and their four types of chimeric mice (*Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$, WT→*Lsp1*$^{-/-}$, *Lsp1*$^{-/-}$→WT and WT→WT)*. We observed that *Lsp1*$^{-/-}$ mice exhibited similar responses to MIP-2 or KC-induced neutrophil recruitment compared to the WT mice. Emigration in neutrophil recruitment was significantly inhibited in *Lsp1*$^{-/-}$ mice. Neutrophils in *Lsp1*$^{-/-}$ mice, compared to those in the WT mice, displayed longer crawling time, slower crawling velocities and had lower chemotaxis index during their intraluminal crawling in venules in response to MIP-2. The transmigration time of *Lsp1*$^{-/-}$ neutrophils was longer than that of WT neutrophils, although LSP1 did not appear to play

a role during neutrophil detachment from endothelium in response to MIP-2. Substantial defects were observed in *Lsp1*$^{-/-}$ neutrophils during their chemotaxis in muscle tissue in response to MIP-2. For dissecting the role of LSP1 in neutrophils and ECs, we repeated these experiments using chimeric mice. We found that neutrophil emigration was significantly inhibited in WT→*Lsp1*$^{-/-}$ mice as well as in *Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$ mice but not in *Lsp1*$^{-/-}$→WT mice or WT→WT mice. N-LSP1 promoted intraluminal crawling and transmigration while E-LSP1 played more important roles in MIP-2-induced neutrophil transmigration and chemotaxis in tissue. In addition, p38 mitogen-activated protein kinase (MAPK) inhibitor SKF86002 abolished neutrophil recruitment and chemotaxis in tissue of WT mice. These inhibitory effects were partially achieved through LSP1 in MIP-2-induced neutrophil recruitment.

Taken together, these results suggest that both N-LSP1 and E-LSP1 are important in MIP-2-induced neutrophil intraluminal crawling and neutrophil transendothelial migration. These results also suggest that E-LSP1 may have a more important role in neutrophil transendothelial migration and in neutrophil chemotaxis both in the lumen and in extravascular tissues as well. Additionally, they demonstrate that MIP-2-induced neutrophil recruitment and chemotaxis in tissue is dependent on p38 MAPK activation.

*, Four types of LSP1 chimeric mice were generated; *Lsp1*$^{-/-}$ mice reconstituted with *Lsp1*$^{-/-}$ neutrophils and WT neutrophils were indicated as *Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$ and WT→*Lsp1*$^{-/-}$, respectively, and WT mice reconstituted with *Lsp1*$^{-/-}$ and WT neutrophils were indicated as *Lsp1*$^{-/-}$→WT and WT→WT, respectively.

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DEDICATION

The thesis is dedicated to my family.

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LIST OF ABBREVIATION

EC: endothelial cell

ECM: extracellular matrix

E-LSP1: endothelial LSP1

ERK: extracellular signal-regulated protein kinase

ERM: ezrin, radixin, moesin

ESAM: endothelial-specific adhesion molecules

F-actin: filamentous actin

fMLP: formyl-methionyl-leucyl-phenylalanine

GAGs: glycosaminoglycans

GPCRs: G-protein-coupled receptors

HSPGs: heparin sulfate proteoglycans

ICAM: intercellular cell adhesion molecule

JAM: junctional adhesion molecule

KC: keratinocyte-derived chemokine

LFA-1: lymphocyte function-associated antigen 1

IL: interleukin

LSP1: leukocyte-specific protein 1

Lsp1$^{-/-}$: LSP1-deficient (knockout)

Mac-1: macrophage antigen 1

MAPK: mitogen-activated protein kinase

MK2: MAPK-activated protein kinase-2

MIP-2: macrophage inflammatory protein-2

MLCK: myosin light chain kinase

NAD: neutrophil actin dysfunction

N-LSP1: neutrophil LSP1

P-: phosphorylated

PAK: p21-activated kinase

PECAM-1: platelet-endothelial cell adhesion molecule-1

PI3K: phosphatidylinoside 3-kinase

PIP3: phosphatidylinositol (3,4,5)-trisphosphate

PKB/C: protein kinase B/C

PMNs: polymorphonuclear leukocytes

RHO: RAS homologue

TEM: transendothelial migration

TNFα: tumor necrosis factor-α

VCAM-1: vascular cell adhesion molecule-1

VE-cadherin: vascular endothelial cadherin

WASP: Wiskott–Aldrich syndrome protein

WT: wild-type

1. Introduction

1.1. Neutrophil Recruitment during Inflammatory Response *in vivo*

Neutrophils, a subtype of leukocytes, are generally referred to polymorphonuclear leukocytes (PMNs). The endothelium is a thin and smooth layer of endothelial cells (ECs) which form the interface between circulating blood and the extravascular tissue. During acute inflammation, neutrophils in blood stream interact with vascular ECs and migrate towards the site of inflammation. This process is called neutrophil recruitment, which is the movement of circulating neutrophils from the blood stream to the inflamed site, and is a complex but crucial process for the development and outcome of inflammation. The current view of this process depicts a series of sequential steps that include neutrophils tethering and rolling, adhesion, intraluminal crawling on the luminal walls of the post-capillary venules, transendothelial migration (TEM) across the venules and migration in extravascular tissues to the inflamed site [1]. The process also involves many molecules such as selectins, integrins and the coordination of their cellular functions to induce directed cell movements. Fig. 1.1 presents a brief and schematic view of neutrophil recruitment process.

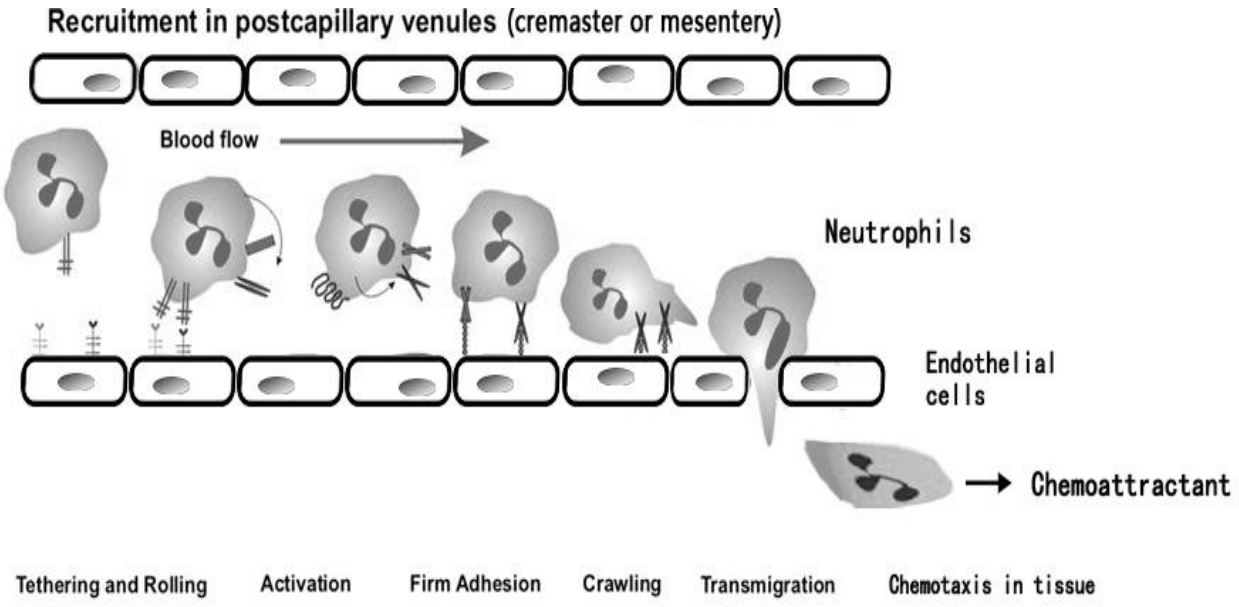


Fig. 1.1 The process of neutrophil recruitment during the interactions between neutrophils and ECs at post-capillary venules in cremasteric or mesenteric microvasculature. (Modified from Reference 1)

1.1.1. Neutrophil tethering, rolling and adhesion

The multistep process of neutrophil recruitment is initiated by neutrophil tethering and rolling on the endothelial lining of the post-capillary venules. The rolling of neutrophils is mediated by the selectin family adhesion molecules which include P-selectin, E-selectin and L-selectin, and, in some cases by α4-integrin, as well as their ligands [1]. P-selectins are expressed on the luminal surface of activated ECs and activated platelets. E-selectins are exclusively expressed on activated ECs such as those activated by cytokines, for example, interleukin (IL)-1 or tumor necrosis factor-α (TNF-α), whereas L-selectins are expressed only on leukocytes. In addition, it has been reported that the β_2 integrin lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18, $\alpha_L\beta_2$ integrin) is also involved in rolling step [2]. This step localizes neutrophils to the endothelial surface to sense chemokines and other chemoattractants on the venular surface.

The following step is neutrophil adhesion. In response to chemokines on the luminal surface of the endothelium, the rolling neutrophils are activated and arrested on the endothelium by the modulation of integrins such as α4-integrin and β_2 integrin and the binding to their ligands. During firm adhesion, neutrophils spread, flatten and form the focal adhesion-like structures. These structures consist of high affinity integrins which interact with their ligands such as intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1) [1]. It has been reported that the β_2 integrin LFA-1 ($\alpha_L\beta_2$) plays a dominant role during the neutrophil adhesion step [2, 3]. In addition, shear flow is another important factor to induce adhesion.

Under sheer flow, adhesion can be enhanced even if the activity of β_2 integrin is low in the presence of chemokines [4].

1.1.2. Neutrophil intraluminal crawling

Following adhesion is an important, recently identified step called intraluminal crawling. Intraluminal crawling means that many adhering neutrophils move from the initial adherent sites to the junctional extravasation sites on the endothelium in response to exogenous or endogenous chemokines or other chemoattractants *in vivo* [3]. In fact, this process appears to direct the cell to reach optimal emigration sites at endothelial junctions. In this step, neutrophils interact with ECs to accomplish cell morphological changes and motility. Reports showed that adhesion is mainly regulated by LFA-1 whereas intraluminal crawling is mediated by the β_2 integrin macrophage antigen 1 (Mac-1, CD11b/CD18, $\alpha_M\beta_2$ integrin) [3]. In the presence of chemoattractant in the extravascular environment, a chemoattractant gradient exists in the lumen and the crawling cells undergo chemotaxis in the lumen towards chemoattractant [5]. This neutrophil-endothelial cell interaction effectively promotes subsequent transendothelial migration. When the intraluminal crawling is disabled, neutrophil transmigration will be delayed and the transmigration route will be changed to mainly transcellular pathway instead of the predominant paracellular pathway [3]. In order to migrate, cells extend flat, filamentous (F)-actin-rich sheet-like structures (called lamellipodia) and thin, needle-like projections (called filopodia) from their leading edge and

attach to the extracellular substrate, while contracting their trailing edge to detach from the rear [6]. As a result, F-actin is polymerized and the cell moves the bulk of the cytoplasm and the nucleus forward [7-10]. In addition, F-actin filaments are bundled in filopodia and crosslinked in lamellipodia [6], and the podosome-like protrusions invaginate the endothelium to promote cell migration [11].

1.1.3. Neutrophil transendothelial migration

Transendothelial migration (TEM) is an intricate and very dynamic neutrophil-endothelial cell interaction process. Emigrating neutrophils encounter three distinct barriers: ECs, the endothelial-cell basement membrane, and pericytes. There are two transmigration routes, paracellular and transcellular pathways. The paracellular pathway means neutrophils transmigrate through endothelial junctions whereas the transcellular pathway means neutrophils transmigrate through the cell body of the endothelium. Neutrophils can switch from one to the other relying on the environmental conditions. For example, paracellular TEM can be shifted to transcellular TEM when certain adhesion molecules including Mac-1 or ICAM-1 are inhibited [12]. However, paracellular transmigration is the predominant route taken by neutrophils, since more than 90% of the transmigratory events are observed to take place in close proximity to endothelial cell junctions [3]. Cell motility is initiated by signaling transduction and involves lots of molecules both on neutrophils and endothelium such as LFA-1 and Mac-1 integrins and

endothelial cell-selective adhesion molecule (ESAM) as well as their ligands [13]. The process of TEM is very complex. Neutrophil TEM by paracellular or transcellular route is illustrated in Fig.1.2 below.

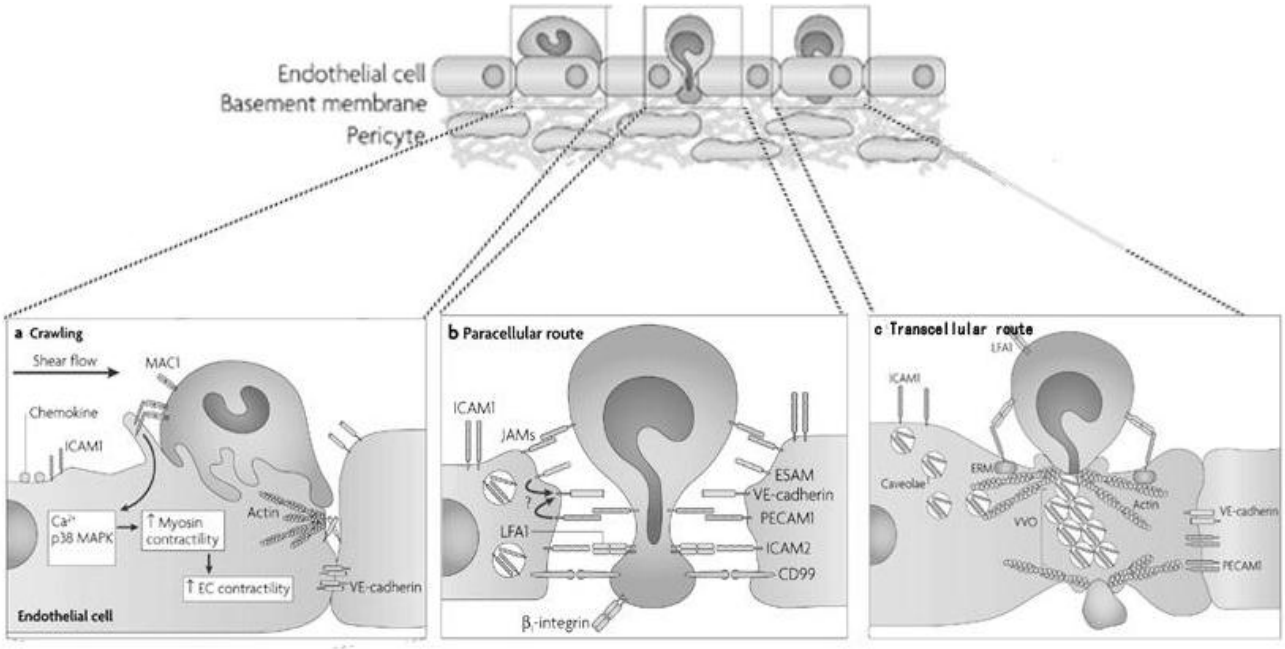


Fig. 1.2 The mechanisms of neutrophil transendothelial migration. **(a)** Under shear flow, crawling neutrophils with podosome-like protrusions invaginate into the endothelium. Endothelial-cell junctions are activated by the binding of integrins and their ligands (e.g. , ICAM-1 and Mac-1). Once activated, the increased EC intracellular Ca^{2+} leads to the activation of p38 mitogen-activated protein kinase (MAPK), which finally induces EC contractions and inter-endothelial contacts opening to promote neutrophil paracellular or transcellular migration [14, 15]. **(b)** Paracellular migration involves a lot of endothelial-cell junctional molecules, such as junctional adhesion molecule A (JAM-A), platelet/endothelial-cell adhesion molecule 1 (PECAM-1), ICAM-1/2, CD99. JAM-A, PECAM-1 and ESAM, which regulate the release of endothelial-expressed vascular endothelial cadherin (VE-cadherin) by activating different signaling pathways, induce endothelial cell contraction, permeability changes and facilitate

neutrophil transmigration [14, 16, 17]. **(c)** Transcellular migration occurs in areas of endothelial-cell thinning, so that the neutrophils only cross small distances [18]. Ligation of ICAM-1 leads to the transportation of ICAM-1 with caveolin-1 to the basal plasma membrane which results in the formation of vesiculo-vacuolar organelles (VVOs) that establish an intracellular channel channels in endothelium to promote the neutrophil transmigration [4, 19]. (Modified from Reference 13)

However, the endothelium is critically important in the transmigration step no matter which transmigration routes neutrophils use. During transendothelial migration, the endothelium forms docking structures (also known as transmigration cups) around adherent neutrophils which extend pseudopodia to facilitate neutrophil migration [11]. These structures strongly express integrins such as LFA-1 as well as their ligands (e.g. ICAM-1 and VCAM-1), and they allow very close contact between neutrophils and the endothelium [20, 21]. ICAM-1 and VCAM-1 connect to the actin-cytoskeleton through the adaptor proteins such as ERM (ezrin, radixin and moesin) [22]. The interactions of integrins with their ligands lead to the reorganization of the cytoskeleton by anchorage of adaptor proteins with α-actinin and vinculin [23]. In addition, an amazing observation is the dome structure that was observed *in vivo* (Fig. 1.3). Before neutrophils migrate out of the endothelium, the involving endothelial cell extends its cell body and envelopes the whole adherent neutrophils by the formation of dome structure without any disruptions of endothelium [11]. The dome–like encapsulations develop from the docking structures [11].

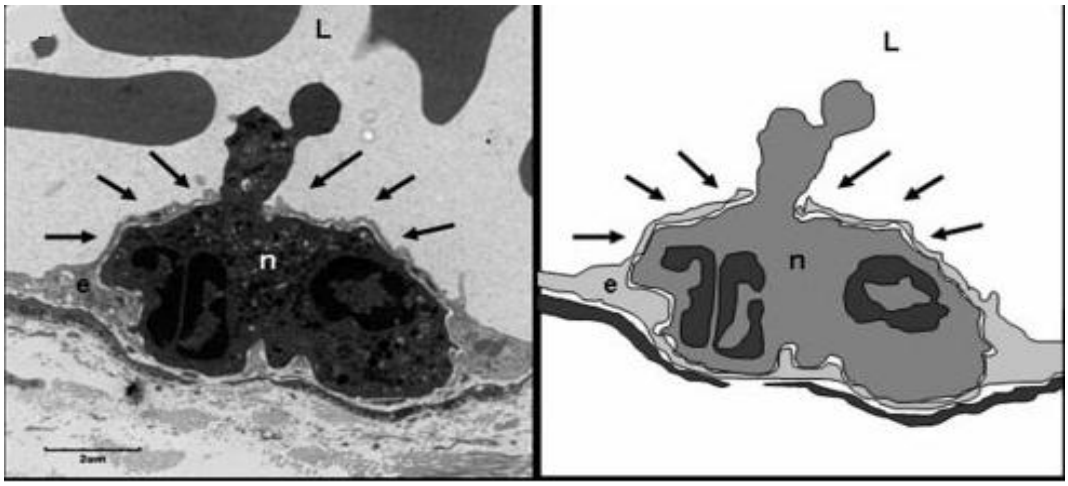


Fig.1.3 The novel "dome"-like structures (arrows) *in vivo*. This electron microscopy picture shows the formation of the “dome”-like structure surrounding a transmigrating neutrophil. Abbreviations: e, endothelial cell; n, neutrophil; L, lumen. (Modified from Reference 1)

1.1.4. Neutrophil chemotaxis in tissue

The ability of cells to sense external chemokines or other chemoattractants and to respond by directionally migrating towards them, is a fundamental process called chemotaxis [24]. Chemoattractants are derived from the endogenous host cells such as ECs, macrophages and neutrophils, or from exogenous bacteria components such as formyl-methionyl-leucyl-phenylalanine (fMLP). After migration across the endothelium barrier, neutrophils reach the extravascular tissues and undergo chemotaxis. Neutrophil chemotaxis is a dynamic process including directional sensing, signaling transduction, cell polarization, cell adhesion and motility [25]. It is initiated by neutrophil sensing a chemoattractant gradient in its extravasular environment via tyrosine kinase receptors and G protein-coupled receptors [26-28]. Chemotaxing neutrophils have an amazing ability to detect small changes in the concentration of a chemoattractant and can amplify their responses to very subtle chemoattractant gradients [25]. After sensing the chemoattractant gradient, neutrophils undergo polarization by the activation of spatially localized cellular signals. The polarized cell has well-defined leading lamellipodia or filopodia which are filled with F-actin and extend towards the chemoattractant, and a trailing ‘‘tail’’, called a uropod, which is filled with actin filament and myosin II complexes to control uropod movement and promote the tail contraction (Fig. 1.4). By the regulation of dynamic signaling interactions between the front and the back of chemotaxing cells, neutrophils translocate their bodies forward and detach from the adhesion sites [27].

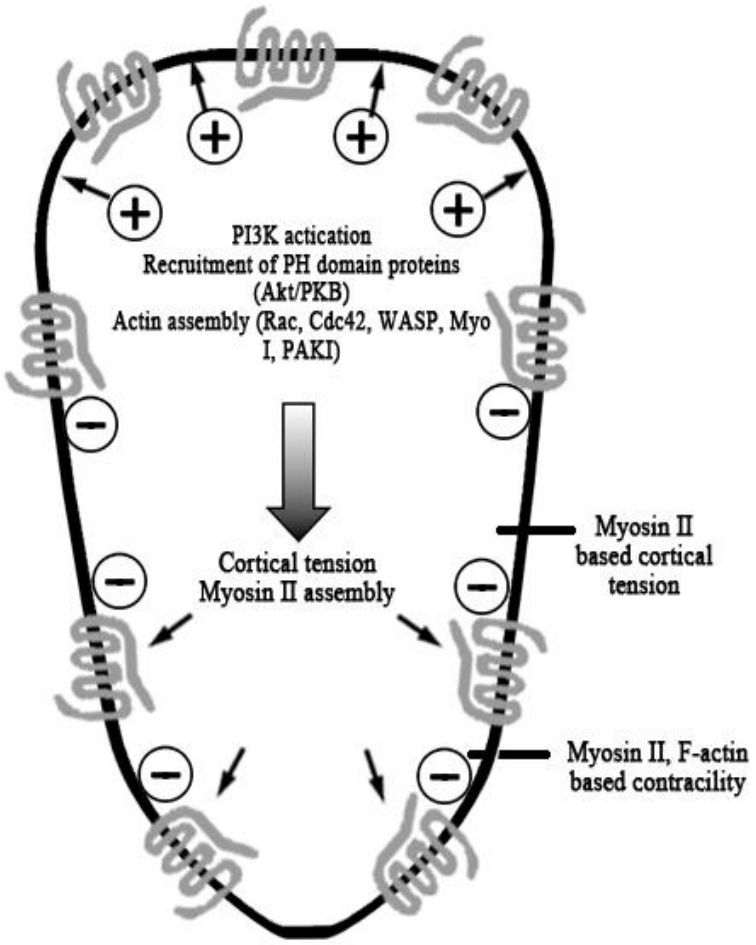


Fig. 1.4 The model for the formation of an activation domain in chemotaxing cells. Chemoattractant receptors, G-protein coupled receptors (GPCRs), are uniformly distributed around the cell membrane. On sensing a chemoattractant gradient, the activated phosphatidylinoside 3-kinase (PI3K) in the membrane closest to the chemoattractant source stimulates phosphatidylinositol (3,4,5) trisphosphate (PIP3) production [29]. The external chemoattractant gradient is amplified internally via positive feedback loops of PIP3 production at the front of cells (indicated by '+'), in combination with inhibition of pathways by dephosphorylation of PIP3 along

the trailing edges of the cells (indicated by '–') [29]. Multiple signaling molecules are preferentially localized at the leading edge, such as protein kinase B (Akt/PKB), cell division control protein 42 homolog (Cdc42), Wiskott–Aldrich syndrome protein (WASP), to produce an activation domain including PH-domain proteins [27]. F-actin localizes at the leading edge to induce protrusion of pseudopodia following the activation of small GTPases (Rac1/Cdc42) and their downstream effectors, including WASP, myosin I kinase and PAK1 in mammalian cells [27, 30]. Once activated, actin undergoes polymerization and induces cytoskeletal remodeling which results in cell protrusion, adhesion, de-adhesion and forward movement [27]. Myosin II is assembled at the trailing edges and lateral sides of cell. The main functions of myosin II provide rigidity to the polarized cells, helping uropod de-adhesion to the substrate and retraction in line with the chemoattractant gradient [27]. Abbreviations: Akt/PKB, protein kinase B; Cdc42, cell division control protein 42 homolog; F-actin, filamentous actin; PAK, p21-activated kinase; PH, pleckstrin homology; PI3K, phosphatidylinoside 3-kinase; PIP3, phosphatidylinositol (3,4,5) trisphosphate; WASP, Wiskott–Aldrich syndrome protein. (Modified from Reference 27)

1.2. Chemokines

Chemokines are a family of small 67-127 amino acid chemotactic cytokines that are either secreted or membrane-bound. Chemokines are chemoattractants and their major role is to induce cell chemotaxis. There are four subfamilies: CXC, CC, C, and CX_3C according to the spacing of their first two cysteine residues [31, 32]. The N-terminus of chemokines is important for their interactions with neutrophil chemokine receptors whereas the C-terminus has a high affinity for glycosaminoglycans (GAGs) and other negatively charged sugar moieties [33]. Chemokines induce signal transduction via binding to chemokine receptors. Chemokine receptors are a group of seven-transmembrane G-protein coupled cell membrane receptors (GPCRs) and divided into four subtypes (CXCR, CCR, CR and CX_3CR) based on the four chemokine subfamilies [33].

Chemokines function at all stages of neutrophil recruitment. During the process of neutrophil recruitment, chemokines must be immobilized on the venular lumen under shear flow to trigger rolling neutrophils to adhere [34]. Many chemokines can be immobilized on ECs via binding to GAGs and others can be co-immobilized with selectin ligands or integrin ligands [35]. It has been reported that chemokines are important for neutrophil adhesion by increasing the affinity of integrins [36-38]. A number of signaling pathways within neutrophils and the endothelium are involved in chemokine-induced transendothelial migration. It has been reported that CXC chemokine keratinocyte-derived chemokine (KC)-induced neutrophil recruitment *in vivo* is p38 MAPK signaling pathway-dependent [39]. In addition, it has been demonstrated that CXC chemokine KC and macrophage inflammatory protein-2 (MIP-2)-induced neutrophil recruitment is

eliminated by treating with a p38 MAPK inhibitor *in vitro* [40].

1.3. Leukocyte-specific protein 1 (LSP1)

Leukocyte-specific protein 1 (LSP1) is a 52-kDa intracellular Ca^{2+}- and F-actin-binding cytoskeletal and cytoplasmic protein [41-43] . LSP1 is phosphorylated as a result of chemokine stimulation, suggesting a role of LSP1 in myeloid cell activation and inflammation [44]. LSP1 was originally named lymphocyte specific protein-1 because of its initial identification in mouse and human B and T lymphocytes. It was subsequently renamed leukocyte-specific protein 1 to reflect its expression in macrophages/monocytes and neutrophils [45]. Furthermore, this protein was also discovered in dendritic cells and Langerhans' cells [44]. It has been reported that ECs also express LSP1 [46]. Mouse and human LSP1 have 67% identical sequences, which are highly conserved phosphoproteins of 330 and 339 amino acids, respectively [39, 47, 48]. It has also been shown that the basic region of LSP1 contains serine and threonine residues and that LSP1 is phosphorylated *in vivo* [49]. Murine LSP1 (Fig.1.5) has a highly acidic N-terminal domain with Ca^{2+}-binding regions, indicating its functions may be regulated by signal transduction [45]. LSP1 also has a basic amino acids C-terminal domain which is highly conserved and has high affinity regions for F-actin binding, indicating its importance for actin polymerization and cytoskeletal remodeling [45]. This C-terminal half of LSP1 also has several serine and threonine residues which are identified as potential phosphorylation sites by serine–threonine kinases such as MAPK-activated protein kinase-2 (MAPKAPK2, also abbreviated as MK2) and protein kinase C (PKC) [46].

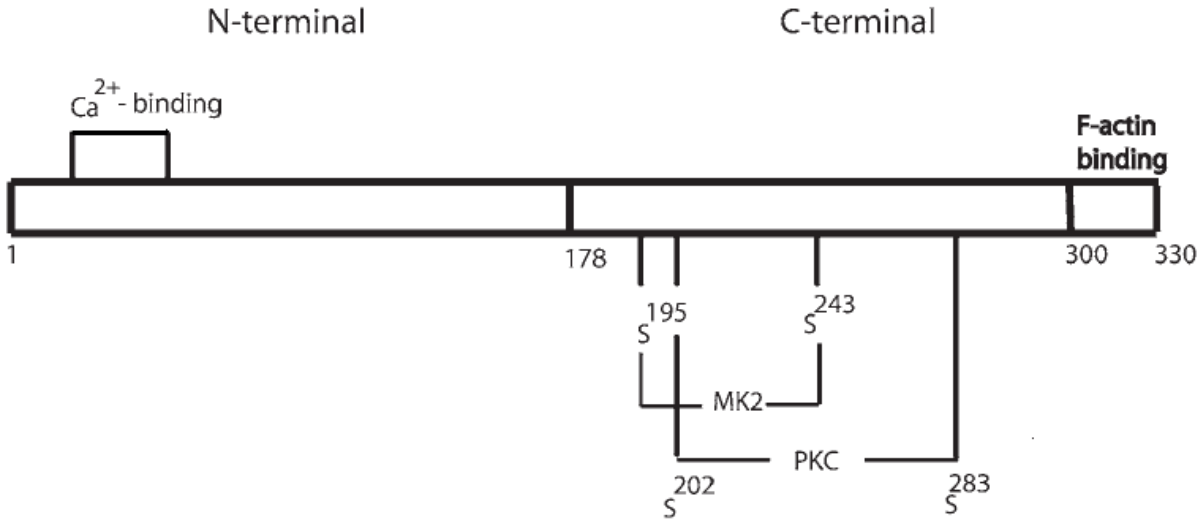


Fig. 1.5 The schematic presentation of mouse LSP1 protein [45]. The putative Ca^{2+} binding sites at the N-terminal region (1–178), the PKC, MK2 phosphorylation sites, and the F-actin–binding region within the C-terminal domain (178–330) are indicated. (Modified from Reference 45)

LSP1 is present at the cytoplasmic face of the plasma membrane in neutrophils and B lymphocytes [50, 51], whereas in ECs the majority of LSP1 is located in the nucleus with lower levels of LSP1 co-localizing with F-actin–rich microfilaments [46]. LSP1 rearranges polymerized F-actin to achieve cytoskeletal remodeling and cell motility during neutrophil chemotaxis [51]. Mouse LSP1 directly binds to F-actin via two high affinity F-actin binding regions [52], whereas human LSP1 contains four F-actin binding sites within its C-terminal domain [43].

LSP1 has been shown to be a major substrate of MK2 in the p38 MAPK signaling pathway [48]. MK2 and p38 MAPK have been reported to be necessary for neutrophil motility and chemotaxis [39, 53, 54], indicating that LSP1 may be important in transmigration and chemotaxis. In addition, LSP1 has also been shown to be a substrate for PKC [55, 56], which is another molecule involved in numerous neutrophil functions including adhesion, endothelial retraction and chemotaxis, suggesting that LSP1 may have multiple roles in neutrophil recruitment [46]. Furthermore, via this pathway, LSP1 has been shown to target proteins of the extracellular signal-regulated protein kinase (ERK)/MAPK pathway to specific sites and areas, indicating its complex functions in inflammation [57].

In summary, LSP1 is very important in neutrophil recruitment and chemotaxis because its main functions are directly related to intracellular signaling transduction, F-actin binding, cytoskeleton reorganization and cell motility.

1.4. Neutrophil Recruitment and LSP1

1.4.1. LSP1 negatively regulates neutrophil recruitment

Negative regulation of neutrophil migration by LSP1 has been shown in a variety of studies. One experimental model of inflammation induced in LSP1-deficient mice (*Lsp1*$^{-/-}$ mice) is the inflammation in the mouse knee joints by the injection of zymosan. The study showed that the number of resident synoviocytes in this inflammatory model was not affected by LSP1-deficiency [58]. The authors also observed that in *Lsp1*$^{-/-}$ mice, the number of neutrophils migrating into the mouse knee joint was increased in zymosan-induced acute inflammation [58]. By using time-lapse video analysis *in vitro*, they also found that the migration speed toward a gradient of fMLP in chemotaxis experiments was also increased in *Lsp1*$^{-/-}$ mice when compared with that in WT neutrophils [58], and the difference was observed when cells migrated on fibrinogen (the receptor for β2 integrin), but not on fibronectin (the receptor for β1 integrin). Furthermore, it has been shown that LSP1 negatively regulates the fMLP-induced adhesion to fibrinogen or ICAM-1, but not to ICAM-2, VCAM-1, or fibronectin, indicating that LSP1 is a functional modulator of Mac-1 during neutrophil adhesion [58]. In addition, fMLP-induced F-actin polarization was also increased in the absence of LSP1 when cells were layered on fibrinogen, but not on fibronectin [58]. These results suggest that LSP1 is a negative regulator of neutrophil adhesion, polarization, and migration via Mac-1 during acute inflammation.

Another experimental model of inflammation is the induced mouse peritonitis model mediated by the injection of thioglycollate. Thioglycollate-induced neutrophils was isolated and the neutrophil chemotaxis in *Lsp1*$^{-/-}$ mice was two to three times increased when compared with that of those WT control neutrophils in response to fMLP *in vitro*[59]. Furthermore, increased phorbol 12-myristate 13-acctate (PMA)-induced superoxide production was detected in thioglycollate-elicited *Lsp1*$^{-/-}$ neutrophils as compared to WT neutrophils [51]. The negative regulatory role of LSP1 was also supported by the fact that neutrophils isolated from a patient with neutrophil actin dysfunction syndrome (NAD47/89) exhibited increased levels of LSP1 and defects in migration, phagocytosis and spreading [60-62]. In addition, the over-expression of LSP1 in normally LSP1 negative, motile melanoma or U937 cells also demonstrated reduced motility [60, 63]. In conclusion, the research results from these studies suggest that high levels of LSP1 in the cytoplasm negatively regulated neutrophil motility and transmigration to inflamed sites via specific integrins such as Mac-1.

1.4.2. LSP1 positively regulates neutrophil recruitment

In a recent study using *Lsp1*$^{-/-}$ mice and control WT mice and their chimeric mice, it has been shown that endothelial LSP1 plays a positive role in the regulation of neutrophil transmigration *in vivo* [46]. By directly visualizing neutrophil rolling, adhesion, and emigration in post-capillary venules of cremaster muscle in *Lsp1*$^{-/-}$ mice using intravital microscopy, the authors found that LSP1 deficiency inhibited neutrophil recruitment in response to the chemokine KC as well as various cytokines (e.g. TNFα, IL-1β) [46]. By using LSP1 chimeric

mice which were generated via bone marrow transplantation, the authors found that in *Lsp1*$^{-/-}$ mice and WT→ *Lsp1*$^{-/-}$ mice, the number of neutrophils undergoing transendothelial migration was significantly decreased, although there was no effect on neutrophil rolling and adhesion in response to KC. In contrast, neutrophils of *Lsp1*$^{-/-}$ →WT mice and WT mice were able to transmigrate normally. Consistent with altered endothelial function, a reduced vascular permeability was observed in response to histamine in *Lsp1*$^{-/-}$ mice [46]. In addition, by using western blotting and immunofluorescence microscopy, LSP1 was confirmed to be present in WT mouse ECs and in human umbilical vein ECs but not in *Lsp1*$^{-/-}$ cells [46]. These studies demonstrated that LSP1 positively regulates neutrophil recruitment *in vitro* and that LSP1 in ECs is critical for neutrophil TEM. In addition, it has been reported that LSP1-deficient neutrophils show abnormal morphology and impaired migration speed in response to KC when compared to WT mice *in vitro* [51].

1.4.3. LSP1 is involved in cytoskeletal reorganizations and signal transduction

It is well known that rearrangement of actin filaments has an important role in cytoskeletal reorganizations and cell motility and that LSP1 plays a role in cytoskeletal remodeling. Normally, neutrophils which express LSP1 do not have hair-like projections unless the LSP1 protein level is abnormally high (example, 4-5 fold above basal) [64]. The over-expression of a 47kDa protein (identified as LSP1), and reduced expression of a 89kDa protein, are named as neutrophil actin dysfunction (NAD) 47/89. Studies showed that over-expression of LSP1 in neutrophils of NAD patients lead to recurrent bacterial infections [60-62]. NAD neutrophils fail

to increase actin polymerization in response to chemotactic factors, display numerous thin, hair-like, F-actin-rich filamentous projections on their plasma membrane surfaces and have defects in chemotaxis, phagocytosis and spreading on glass [62]. In ECs, although cytoplasmic LSP1 expression was low, LSP1 had an agonistic effect in neutrophil emigration by interacting with actin filaments [46].

LSP1 has been shown to be a major substrate of the MK2 in the p38 MAPK pathway [48]. MK2 and p38 MAPK were reported to be essential for neutrophil motility and chemotaxis in response to fMLP [53]. Inhibition of p38 MAPK by SB203580 and PI3K inhibitor wortmanin inhibited the phosphorylation of p38 MAPK, MK2 and LSP1 in fMLP-treated human neutrophils, respectively, suggesting that PI3K was an upstream regulator of the p38 MAPK pathway in neutrophils [65]. Studies also showed that fMLP-induced LSP1 phosphorylation was important for its negative effects which were associated with neutrophil adhesion, polarization, crawling and chemotaxis [54, 66]. For the structural characteristics of LSP1, it can be phosphorylated by PKC and MK2 at distinct sites. It has been shown that PKC was not a regulator of the fMLP-induced p38 MAPK/MK2 pathway [56]. However, LSP1 directly interacted with PKCβI but not with PKCα or PKCβII. The PKCβI was involved in a larger signaling complex, ERK signaling pathway [45]. A hypothetical model for LSP1 function in the transmigration of neutrophils is illustrated in Fig. 1.6 below.

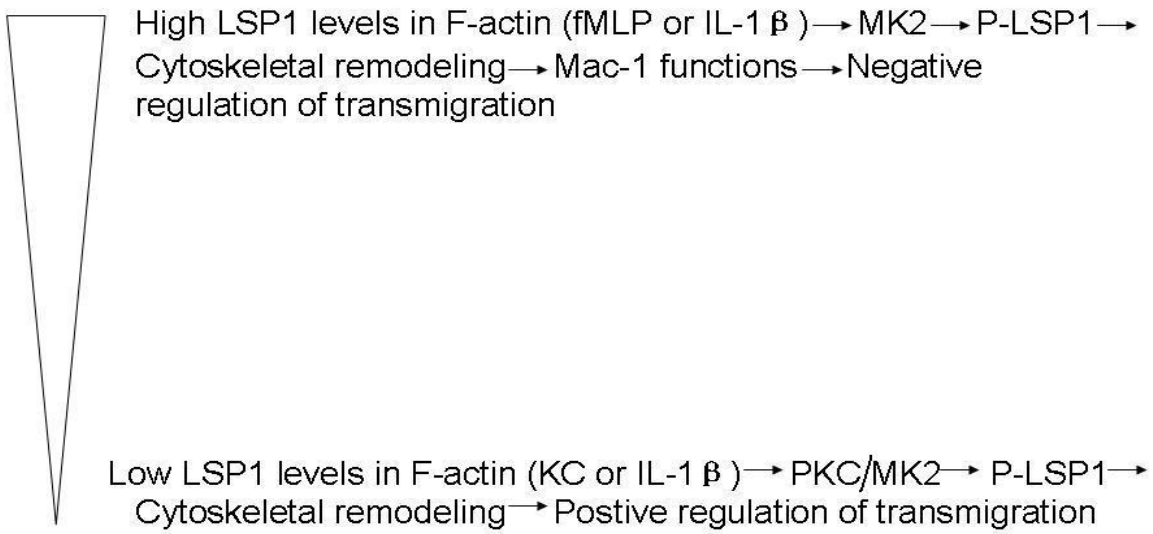


Fig. 1.6 A hypothetical model for LSP1 function in the transmigration of neutrophils. In neutrophils with high levels of LSP1 on the cytoskeleton, neutrophil recruitment is negatively regulated by LSP1 through MK2 signaling pathways in response to inflammatory stimuli such as fMLP or IL-1β. Once activated, the cascade of MK2 signaling pathways, LSP1 is phosphorylated and subsequently induces cytoskeletal remodeling via the integrin Mac-1 [58]. On the other hand, in the case of ECs where LSP1 levels in the cytoskeleton are low, phosphorylated LSP1 induces cytoskeletal remodeling and results in positive modulation of neutrophil transmigration through PKC or MK2 signaling pathways in response to KC [46].

2. Rationale for the study

As mentioned above, neutrophil recruitment is a complex and sequential process and each step during neutrophil recruitment will be affected by the previous one. Although LSP1 is involved in positive regulation of transmigration in CXC chemokine KC-induced neutrophil emigration and chemotaxis both *in vivo* and *in vitro* [46, 51], the exact role of LSP1 in each step of neutrophil recruitment is still as yet incompletely understood. It has been reported that another CXC chemokine MIP-2 can induce chemotaxis through a PI3K-dependent mechanism and the PI3K signaling pathway is important in actin polarization, cell motility and migration [67]. However, whether and how LSP1 is involved in MIP-2-induced neutrophil recruitment is currently unknown. LSP1 is expressed both in neutrophils and in ECs [45] and endothelial LSP1 is important for neutrophil emigration in response to KC [46]. However, the functional differences of neutrophil LSP1 and endothelial LSP1 in neutrophil recruitment are incompletely understood. In addition, it has been established that p38 MAPK signaling pathway is involved in KC-elicited neutrophil recruitment [39], whether it is involved in MIP-2-induced neutrophil recruitment is unclear. Furthermore, if p38 MAPK-activation is involved in MIP-2-induced neutrophil recruitment, whether the effects are achieved through LSP1 or not is another question.

3. Working Hypothesis

Based on the above, the following working hypotheses are formulated:

a. LSP1 regulates MIP-2-and KC-induced neutrophil recruitment and chemotaxis through different mechanisms.

b. LSP1 expressed in endothelium is crucial in regulating neutrophil recruitment and chemotaxis in tissue in response to the neutrophil chemokines, MIP-2 and KC.

c. MIP-2-induced neutrophil recruitment is dependent on the p38 MAPK signaling pathway.

4. Experimental Objectives

a. To determine the role of LSP1 in neutrophil recruitment in the response to MIP-2 and KC *in vivo.*

b. To identify the role of neutrophil and endothelial LSP1 in neutrophil chemotaxis assay *in vivo*.

c. To determine if the p38 MAPK pathway is involved in MIP-2-induced neutrophil recruitment.

5. Materials & Methods

5.1. Animals

Control, wild-type (WT) 129/SvJ mice were originally from The Jackson Laboratory (Bar Harbor, ME). Jongstra-Bilen and colleagues generated the *Lsp1*$^{-/-}$ mice on the 129/SvJ background by homologous recombination as described previously [59]. Both strains of mice were obtained from the lab of Dr. Paul Kubes at the University of Calgary and transferred to the University of Saskatchewan. The mice were bred at the University of Saskatchewan Animal Resource Centre and used in experiments when they were between 6 and 12 weeks of age.

Four types of bone marrow (BM) chimeric mice were generated following the standard protocols from Dr. Paul Kube's laboratory [68]. The BM cell suspensions from 6-8-wk-old donor *Lsp1*$^{-/-}$ and 129/SvJ WT mice were injected into the tail vein of *Lsp1*$^{-/-}$ and 129/SvJ WT mice respectively to generate *Lsp1*$^{-/-}$→ *Lsp1*$^{-/-}$, *Lsp1*$^{-/-}$→WT, WT→ *Lsp1*$^{-/-}$, WT→WT mice. After bone marrow transplantation (BMT), these mice were housed in specific pathogen-free facilities for 6–10 wk to allow full humoral reconstitution before use in experiments. All animal protocols and procedures were approved by the University Committee on Animal Care and Supply at the University of Saskatchewan and conformed to Canadian Council for Animal Care Guidelines.

5.2. Bone Marrow Transplantation (BMT)

The 6-8-wk-old recipient mice were taken to the γ source (Gammacell, ^{137}Cs γ-irradiation

source). To get the 500-525 rad/min radiation, the exact time was calculated according to the current activity of the γ source. Recipient mice were irradiated for the specified length of time and put back in cages with food and water. A second irradiation was performed after a 3-hour interval.

Bone marrow cells were isolated using an aseptic technique and cell samples were prepared in insulin syringes. The irradiated recipient mice were placed into a prepared injection tube (50 ml Falcon, with hole in lid and air holes down sides, sterilized in ethanol) and Kimwipes was put into it to minimize the mouse movement. The tail was brought out through the hole in the lid and sterilized with 70% ethanol. The tail was warmed up with warm water and i.v. injected with at least 8×10^6 cells in 0.2 ml physiological saline with a 27G needle. The anti-bacterial solution enrofloxacin, 1ml in 250ml of distilled water (Bayer Inc., Animal Health, Toronto, Ontario, Canada) was prepared daily as drinking water for the number of cages for the first two weeks after BMT.

5.3. Mouse Preparation for Intravital Microscopy

A mixture of 10 mg/kg xylazine (Bayer Inc., Animal Health, Toronto, Ontario, Canada) and 200 mg/kg ketamine hydrochloride (Bioniche, Animal Health, Belleville, Ontario, Canada) were injected i.p. to anesthetize the adult male mice. The area over the right-side external jugular vein and the anterior aspect of the scrotum were shaved with an electric razor. An incision was made on the neck to expose the jugular vein, which was catheterized with a PE-10 tubing (Becton Dickinson, Canada) filled with 100 U/ml heparin saline (Sigma, Canada). For all protocols, the

right-side jugular vein was cannulated to administer additional anesthetic or drugs when necessary.

5.4. Intravital Microscopy of Cremaster Muscle

The mouse cremaster muscle preparation was used to study the behavior of neutrophil recruitment in the microcirculation and adjacent connective tissue. Briefly, the preparation involved an incision in the scrotal skin to expose the left cremaster muscle, which was then carefully dissected to free it from the associated fascia. The cremaster muscle was then cauterized longitudinally. The testicle and the epididymis were disassociated from the underlying muscle and moved into the abdominal cavity. The muscle was secured along the edges with 5–0 suture and bicarbonate-buffered physiological saline (131.9 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 20 $NaHCO_3$, in mM, pH 7.4, 37°C) superfused over the exposed tissue. The muscle was held flat and covered with a 22 × 22 mm glass coverslip on an optically clear viewing pedestal of a home-made cremaster muscle board. The cremasteric microvasculature was visualized using an intravital microscope (Carl Zeiss, Germany) with a 16× objective lens and a 10x eyepiece. A CCD video camera (DC220, Dage-MIT, USA) was used to project the images onto a monitor. The images were recorded with a high definition HD-DVD recorder for subsequent playback real-time analysis to determine neutrophil rolling, adhesion and transmigration and time-lapse analysis to observe and measure neutrophil intraluminal crawling, transendothelium migration and chemotaxis in tissue.

5.5. Induction of Neutrophil Recruitment in Cremaster Muscle

To induce neutrophil recruitment independent of cytokines, an agarose gel containing MIP-2 (R&D Systems, Inc., Minneapolis, USA) or KC (Peprotech, Rocky Hill, USA) was used. The 2% agarose gel in PBS was prepared by adding 10 ml of 2 × PBS to a boiling concentrated agarose solution (4% in 10 ml of distilled water). A 110-µl aliquot of this solution was added into the lid of a 1.5-ml Eppendorf tube containing MIP-2 or KC and mixed to achieve a final concentration of 0.5 µM. A small amount of India ink was added to the lid in order to visualize the gel on the cremaster muscle. For the dose-response studies, MIP-2 or KC at 0.05, 0.5, 1.5 µM prepared in agarose gel was used to induce neutrophil recruitment in mice. After 30 min recording of the image of the selected post-capillary venule, the superfusion was stopped and the coverslip was removed from the muscle. A 1 mm^3 piece of MIP-2 or KC-containing gel was punched out of the lid using the cut tip end of a 200-µl pipette and the gel is carefully placed on the surface of the cremaster muscle in a preselected area 350 µm from the observed post-capillary venule. A 22 × 22 mm glass coverslip was added to the muscle to hold the gel in place and the tissue was superfused beneath the coverslip at a very slow rate (≤10 µl/min) to allow the establishment of a gradient of chemoattractant that is slowly released from the gel. The images were recorded for 90 min after the addition of chemoattractant containing gel.

5.6. Inhibition of p38 MAPK

The animals were treated with one of the two different p38 MAPK inhibitors: SB203580 or

SKF860002. SB203580 (EMD Chemicals, Gibbstown, USA) was given i.v. 30 min before administration of MIP-2 in a dose of 20 mg/kg based on previously reported optimal concentrations [69]. SKF86002 (EMD Chemicals, Gibbstown, USA) was given locally by superfusion at a dose of 0.7 mM based on previously reported optimal concentrations [70] 30 min before or after administration of MIP-2, respectively.

5.7. Evaluation of the Neutrophil Recruitment Parameters

The number of rolling, adherent, and emigrated neutrophils was determined offline during real-time video playback analysis as described [46]. The transmigration time, detach time, and data of intraluminal crawling and chemotaxis in tissue were obtained from time-lapse movie playback analysis.

5.7.1. Analysis of rolling velocity, rolling flux, adhesion and emigration cell

Neutrophil rolling velocity was measured from the first 20 neutrophils which entered the field of view at the recording time. It was calculated from the time which was required for an average neutrophil to roll along a 100-μm length of venule and expressed as μm/sec. The rolling flux was measured as the number of rolling neutrophils passing by a given point in the observed venule per min (averaged from 3 min). Neutrophil was defined as adherent if it remained stationary for at least 30 sec, and the total neutrophil adhesion was measured as the number of adherent cells within a 100-μm length of venule in 5 min. Neutrophil emigration was defined as the number of

emigrated neutrophils in the extravascular tissue within a 200 × 300-μm area (0.06 mm^2) adjacent to the observed venule.

5.7.2. Tracking cell movement both in vessel and tissue with time-lapse movies

5.7.2.1. Analysis of time-lapse video using ImageJ

The recorded video was imported into a computer and transformed to a time-lapse movie in AVI format with computer software bitRipper and Windows Movie Maker. The neutrophil movement was manually tracked in the lumen, during transendothelial migration, and in muscle tissue one by one and frame by frame with software ImageJ (downloaded from http://rsb.info.nih.gov/ij/download.html). The cell tracking results were saved in Microsoft Excel automatically.

5.7.2.2. Intraluminal crawling

a. Crawling distance: the total distance the cell crawls from the initial adherent site to the transmigration site (μm).

b. Crawling chemotaxis distance: the distance the cell crawls in x-axis in the lumen (μm)

c. Intraluminal crawling time: the total time the cell crawls from the initial adherent site to the transmigration site (min)

d. Crawling velocity: crawling distance divided by intraluminal crawling time (μm/min)

e. Crawling chemotaxis index: the ratio achieved by dividing the chemotaxis distance by the crawling distance in the lumen.

5.7.2.3. Transendothelial migration

a. Transmigration time: from the time the cell starts to transmigrate across endothelium to the time the cell body is just outside the venule (min).

b. Detachment time: from the time the cell body is just outside the venule (immediately after transmigration) to the time point when the cell loses contact with the venule (the tail retracts) (min).

5.7.2.4. Chemotaxis in tissue

a. Migration distance: the sum of the migration distance the cell moves from the start point to the end point of the migration in tissue (μm)

b. Chemotaxis distance: the distance the cell migrates in x-axis in tissue (μm)

c. Chemotaxis velocity: the chemotaxis distance/time (μm/min)

d. Chemotaxis index: the ratio achieved by dividing the chemotaxis distance by the migration distance in tissue.

5.8. Genotyping

The genomic DNA from samples of mouse blood and tail clips was extracted by using Wizard Genomic DNA Purification Kit (Promega, Madison, USA) following the manufacture's procedures. Using GoTaq Flexl DNA polymerase (Promega, Madison, USA), we analyzed the genotypes of LSP1 chimeric mice according to the manufacture's procedures. Primer sequences of WT, *Lsp1*$^{-/-}$ and hybrids used were (P123 and P124 for *Lsp1*$^{-/-}$genotype, 159bp; P123 and P125 for WT genotype, 333bp):

P123: AGA AAC ACC AGG AGC CAT AAC AAG

P124: AGA ACG AGA TCA GCA GCC TCT G

P125: CAG TCC CTT CAC ACC AAG CAT C

The PCR parameters were set as following: one cycle for 5 min at 95°C; 35 cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec; one cycle for 7 min at 72°C and hold samples at 4°C until loading. PCR samples were then run on 1% agarose gel for visualization (PCR product 333bp for WT; 159bp for *Lsp1*$^{-/-}$; both 333bp and 159bp for hybrid).

5.9. Statistical analysis

The data are expressed as means ± SEM. One-way ANOVA followed by post hoc comparisons was applied to compare the statistical difference within two groups. A P-value of < 0.05 or 0.01 was considered statistically significant or very significant, respectively, and the number of each group was at least three mice.

6. Results

6.1 The role of LSP1 in chemokine-induced neutrophil recruitment

6.1.1. Dose-response curve of MIP-2- and KC-induced neutrophil recruitment

To obtain an optimal dosage and time point, recruitment responses of neutrophils from 129/SvJ mice to both MIP-2 and KC at 0.05 μM, 0.5 μM, or 1.5 μM doses or saline in agarose gel were assessed. The responses were recorded for 120 min: 30 min for the control treatment (no agarose gel) and 90 min for the MIP-2, KC or saline-containing gel.

Fig. 6.1 shows that in response to MIP-2, the different doses induced similar responses in neutrophil rolling velocity and rolling flux, but significant increased neutrophil adhesion and emigration when compared to the saline-containing gel controls. The 0.5 μM dose was found to be the optimal one: the number of emigrating cells were substantially increased compared to a 0.05 μM dose at 90 min; with increasing time, the adhesion in response to 0.5 μM remained in plateau after 30 min while the adhesion tendency of 1.5 μM was reduced after 30 min; and the number of emigrating cells at 0.5 μM was much greater than that at 1.5 μM at 60 min and 90 min. At 90 min, the number of adherent cells at the 0.5 μM dose showed a slight increase while emigrating cells exhibited a significant increase compared to 60 min. As a result, the optimal time point for neutrophil emigration was at 90 min for the MIP-2 treatment.

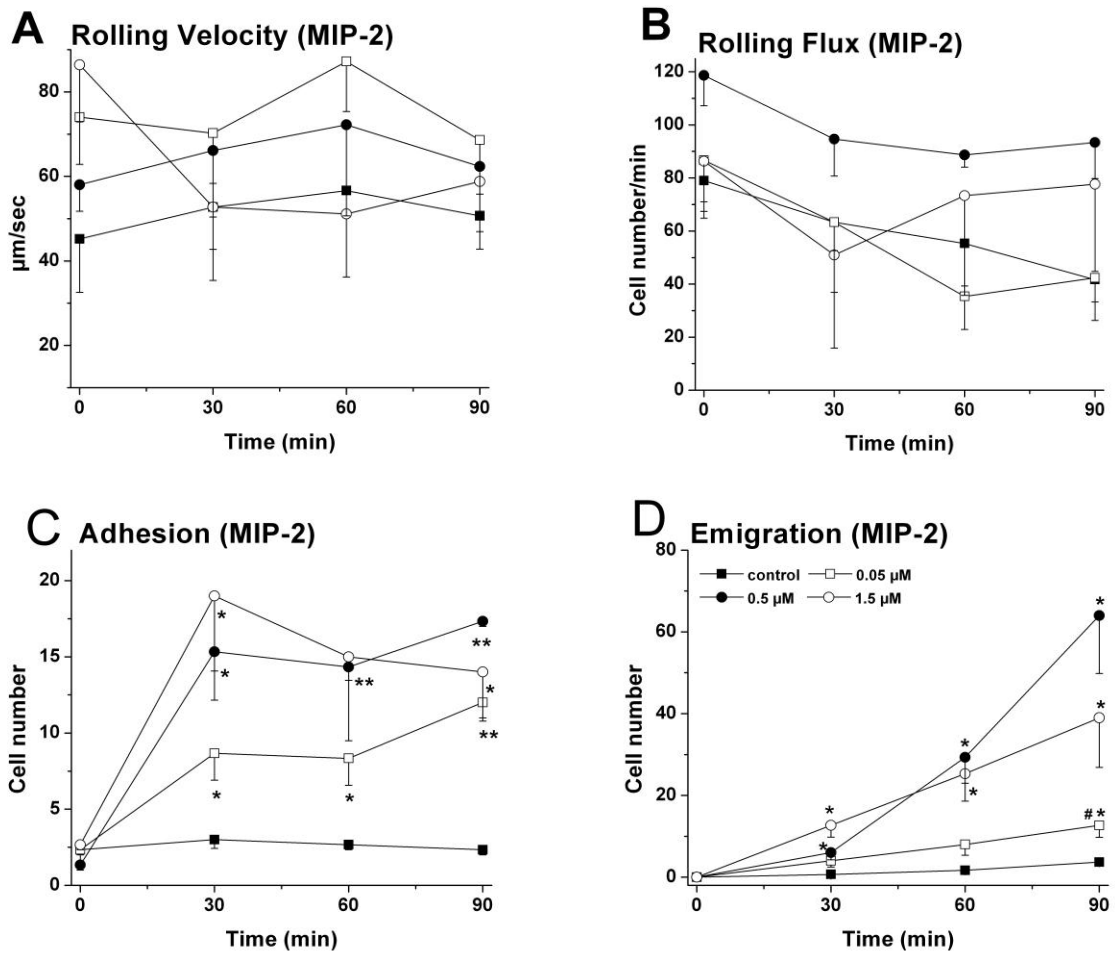


Fig. 6.1 **The dose-response and kinetics of neutrophil recruitment induced by MIP-2 in agarose gel**. (A) The velocity of rolling neutrophils, (B) the flux of rolling neutrophils, (C) neutrophil adhesion, and (D) neutrophil emigration in cremaster post-capillary venules of 129/SvJ mice were determined by intravital microscopy at 30, 60 and 90 min after the addition of 0.05 (open squares), 0.5 (solid circles) or 1.5 (open circles) μM MIP-2 or saline (solid squares) in agarose gel (n = 3 in each group). **P* < .05 and ***P* < .01 as compared with each value in control mice. #*P* < .05 means 0.5 μM MIP-2-in-gel compared with 0.05 μM MIP-2-in-gel. Error bars represent means ± SEM.

The dose response curve of KC was similar to that of MIP-2 although MIP-2 was more potent in inducing neutrophil recruitment than was KC (Fig. 6.1 & Fig. 6.2). In conclusion, neutrophil recruitment is similar in response to MIP-2- or KC-in-gel treatment and 0.5 μM MIP-2 or KC for 90 min elicits the highest number of emigrated neutrophils

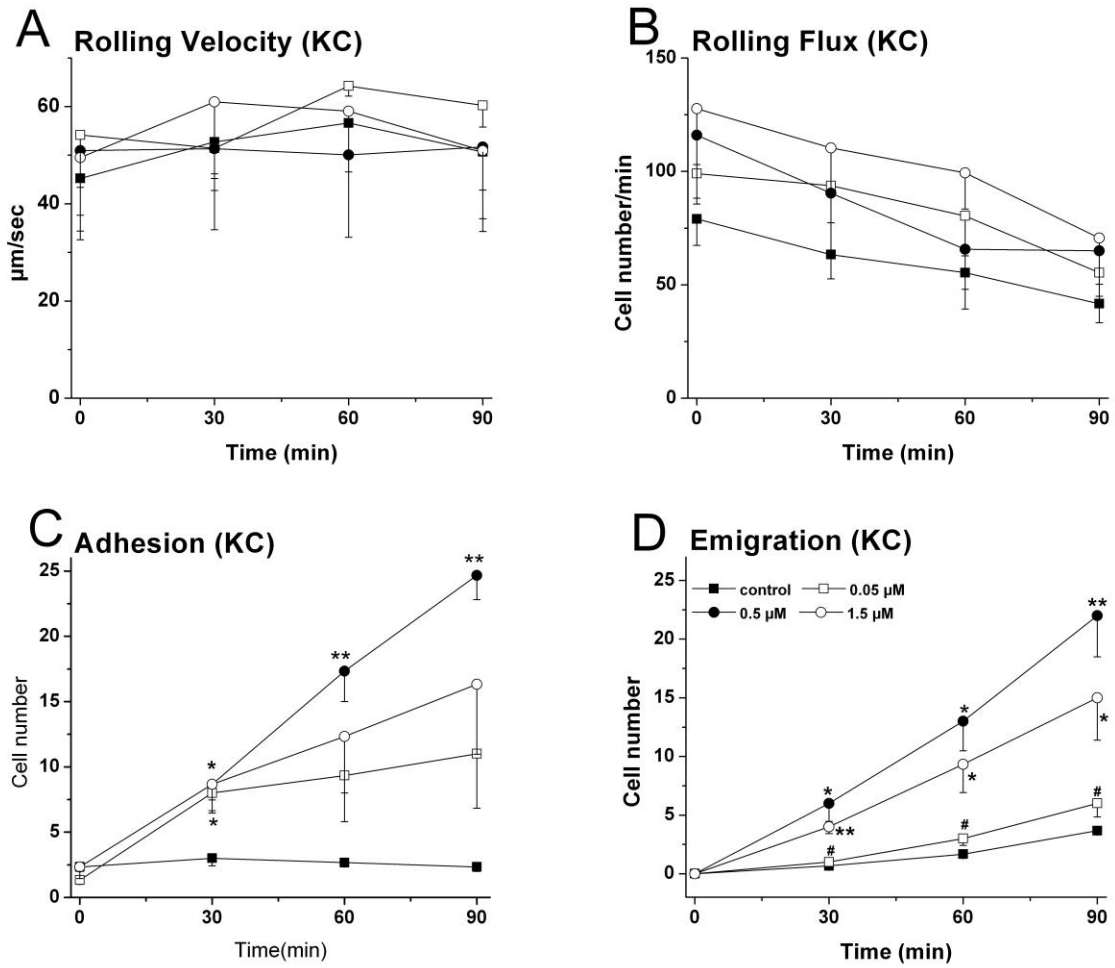


Fig. 6.2 **The dose-response and kinetics of neutrophil recruitment induced by KC in agarose gel**. (A) The velocity of rolling neutrophils, (B) the flux of rolling neutrophils, (C) neutrophil adhesion, and (D) neutrophil emigration in cremaster post-capillary venules of 129/SvJ mice were determined by intravital microscopy at 30, 60 and 90 min after the addition of 0.05 (open squares), 0.5 (solid circles) or 1.5 (open circles) μM KC or saline (solid squares) in an agarose gel (n = 3 in each group). **P* < .05 and ***P* < .01 as compared with each value in control mice. #*P* < .05 means 0.5 μM KC-in-gel compared with 0.05 μM KC-in-gel. Error bars represent means ± SEM.

6.1.2. The role of LSP1 in MIP-2 and KC-induced neutrophil recruitment

To determine the role of LSP1 in neutrophil recruitment in response to MIP-2 or KC, I used intravital microscopy to observe neutrophil rolling velocity, rolling flux, adhesion and emigration. The data for MIP-2- and KC-induced neutrophil recruitment in *Lsp1*$^{-/-}$ mice were compared with the data from WT mice.

In response to MIP-2, *Lsp1*$^{-/-}$ mice exhibited similar responses in neutrophil rolling flux and velocity, but a decrease of neutrophil adhesion and more significant decrease of emigration in neutrophil recruitment compared to WT mice (Fig. 6.3). At 90 min, the number of adherent cells in *Lsp1*$^{-/-}$ mice decreased to about eight cells, about half the number of that in WT mice. The number of emigrating cells in *Lsp1*$^{-/-}$ mice was significantly lower as early as 30 min and about half of that in WT mice at 60 min, and only thirty percent at 90 min. As a result, the role of LSP1 in MIP-2-induced neutrophil recruitment is effective mainly in the step of emigration and slightly effective during neutrophil adhesion but only at 90 min.

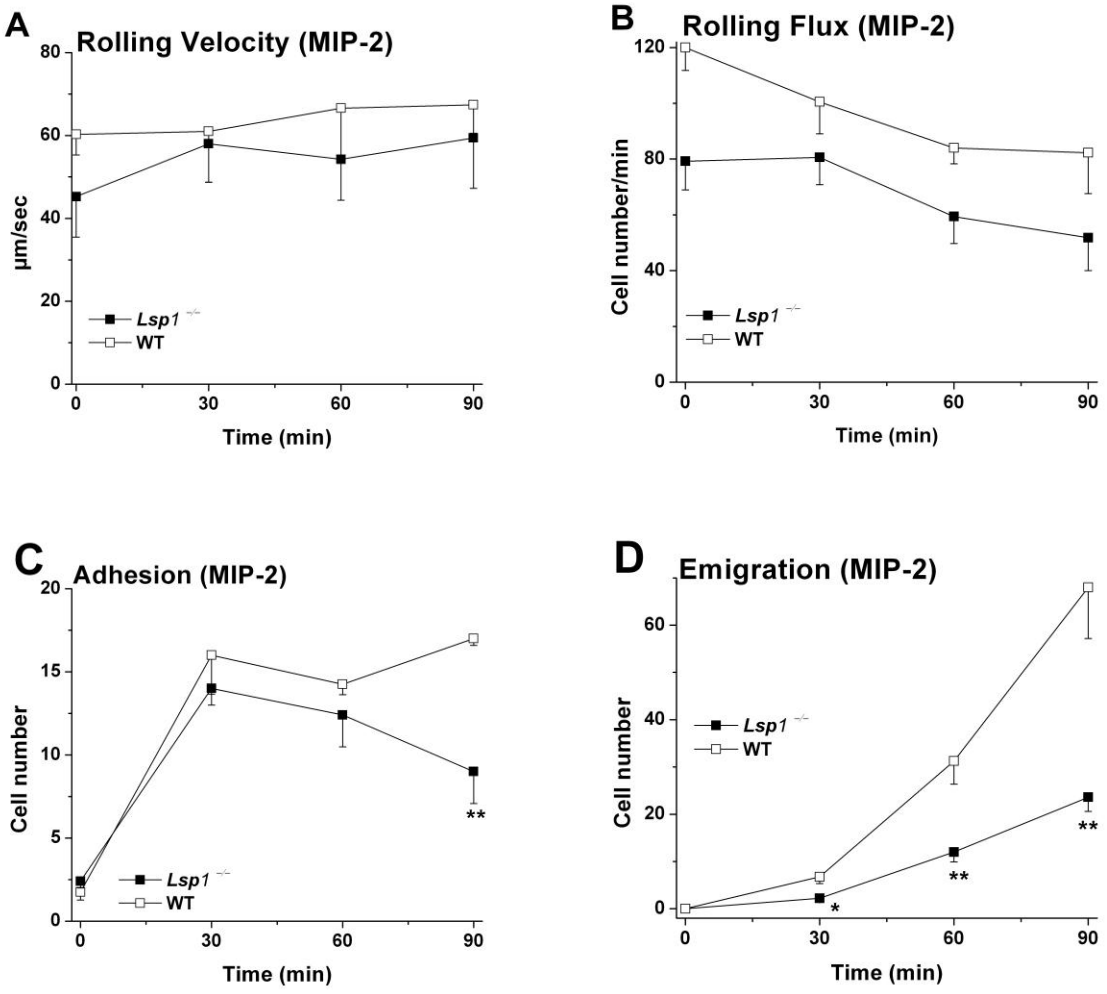


Fig. 6.3 **The role of LSP1 in MIP-2-containing gel-induced neutrophil recruitment.** (A) The velocity of rolling neutrophils, (B) the flux of rolling neutrophils, (C) neutrophil adhesion, and (D) neutrophil emigration in cremaster post-capillary venules of *Lsp1*$^{-/-}$ (open squares) and WT (solid squares) mice are shown (n = 5 in each group). After measurement of basal neutrophil recruitment (at time 0), neutrophil recruitment was induced by the addition of 0.5 μM MIP-2-in-gel. **P* < .05 and ***P* < .01 as compared with each value in WT control mice. Error bars represent means ± SEM.

Neutrophil recruitment induced by chemokine KC was similar to that induced by MIP-2 and the crucial steps where LSP1 played a role was the neutrophil emigration (Fig. 6.4). Consequently, for further study, the data of neutrophil recruitment in the MIP-2 containing gel treated mice were shown in the following text.

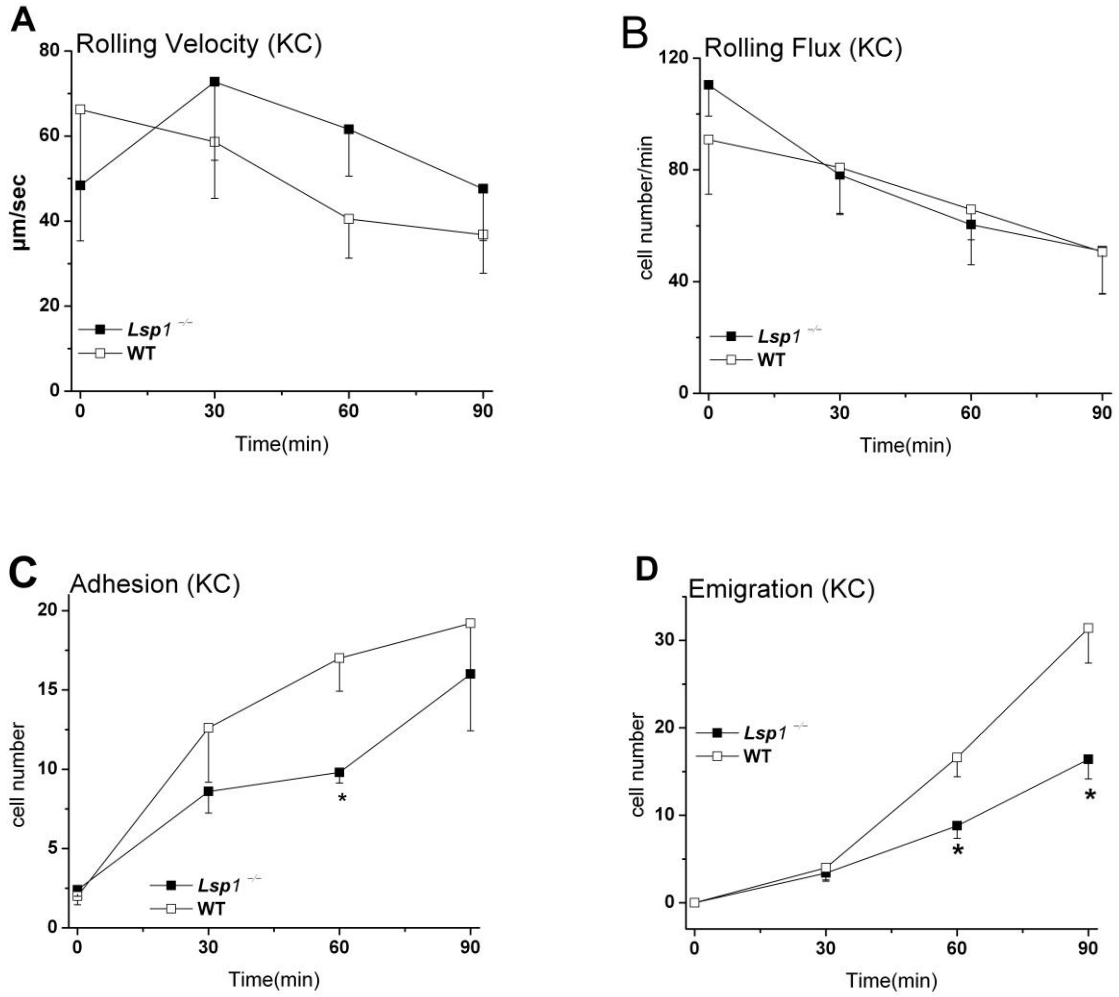
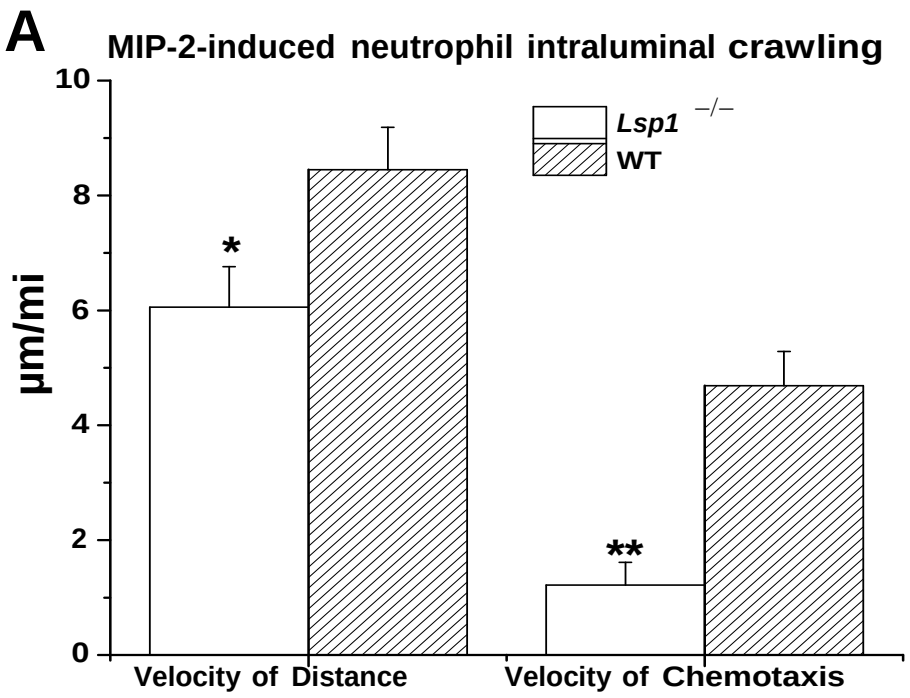
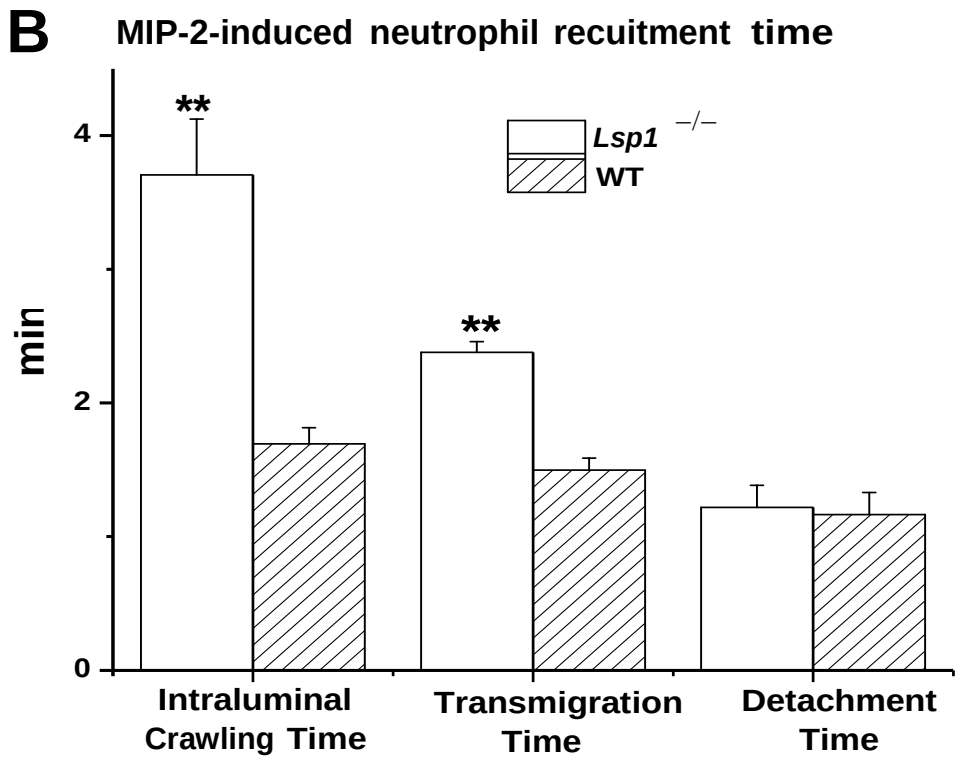
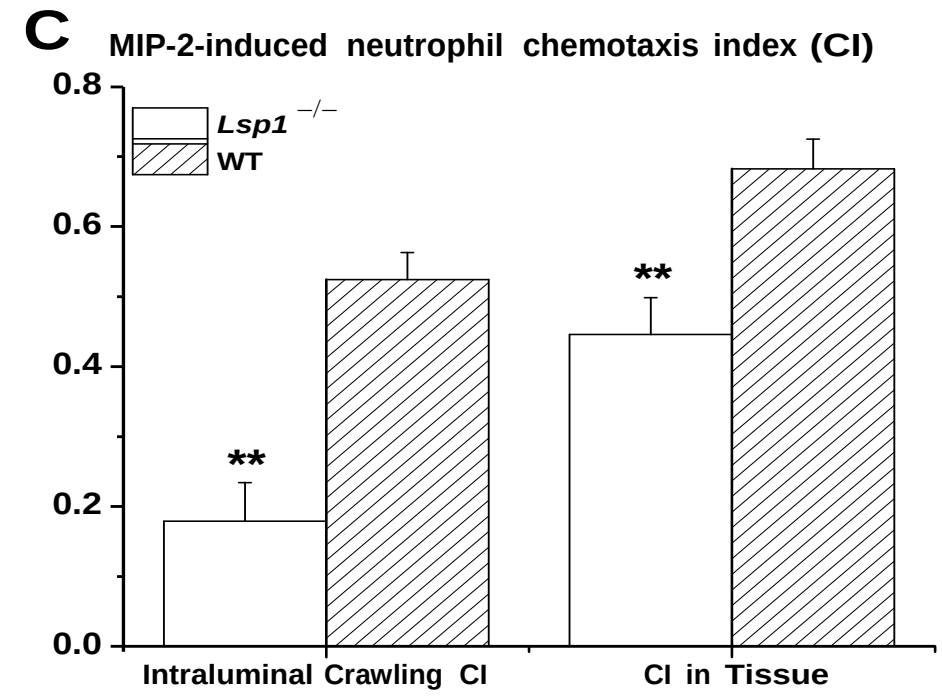


Fig. 6.4 **The role of LSP1 in KC-containing gel-induced neutrophil recruitment.** (A) The velocity of rolling neutrophils, (B) the flux of rolling neutrophils, (C) neutrophil adhesion, and (D) neutrophil emigration in cremaster post-capillary venules of *Lsp1*$^{-/-}$ (open squares) and WT (solid squares) mice are shown (n = 5 in each group). After measurement of basal neutrophil recruitment (at time 0), neutrophil recruitment was induced by the addition of 0.5 μM KC-in-gel. **P* < .05 and ***P* < .01 as compared with each value in WT control mice. Error bars represent means ± SEM.

To determine the exact functions of LSP1 in each step of neutrophil recruitment, we combined intravital microscopy with time-lapse video photography to analyze neutrophil recruitment in much more details (Fig. 6.5). During intraluminal crawling, neutrophils in *Lsp1*$^{-/-}$ mice exhibited defects both in the crawling distance and the crawling chemotaxis in x-axis. The velocity of crawling distance, velocity of crawling chemotaxis (Fig. 6.5A), intraluminal crawling chemotaxis index (Fig. 6.5C) were all significantly decreased and the crawling time (Fig. 6.5B) was significantly increased in *Lsp1*$^{-/-}$ mice compared to these parameters in WT mice. After crawling to the optimal sites, neutrophils underwent transendothelial migration and detached from the cremaster post-capillary venules. Neutrophils in *Lsp1*$^{-/-}$ mice substantially displayed longer transendothelial migration times but showed no defect on their detachment times (Fig. 6.5B). During chemotaxis in extravascular tissue, neutrophils in *Lsp1*$^{-/-}$ mice exhibited lower velocity of migration, lower velocity of chemotaxis and a depressed chemotaxis index (Fig. 6.5C&D). The velocity defected in x-axis movement was much more obvious than that of total movement of neutrophils in *Lsp1*$^{-/-}$ mice, when compared to these parameters in WT mice both in the lumen and in tissue (Fig. 6.5A&D), suggesting an important role of LSP1 in chemotaxis. In addition, the chemotaxis index data showed that neutrophils in *Lsp1*$^{-/-}$ mice migrated more randomly while neutrophils in WT mice migrated in a more directed fashion toward the chemokine both in the lumen and in tissue (Fig. 6.5C). Thus, LSP1 is essential for neutrophil-endothelial cell interactions, especially for intraluminal crawling and transendothelial migration, and regulates the subsequently directional migration in tissue in response to MIP-2.


A
MIP-2-induced neutrophil intraluminal crawling
μm/mi
10
8
6
4
2
0
*
**
Lsp1 −/−
WT
Velocity of Distance
Velocity of Chemotaxis


B
MIP-2-induced neutrophil recuitment time
min
4
2
0
**
**
Lsp1 −/−
WT
Intraluminal Crawling Time
Transmigration Time
Detachment Time


C
MIP-2-induced neutrophil chemotaxis index (CI)
0.8
0.6
0.4
0.2
0.0
Lsp1 −/−
WT
**
**
Intraluminal Crawling CI
CI in Tissue

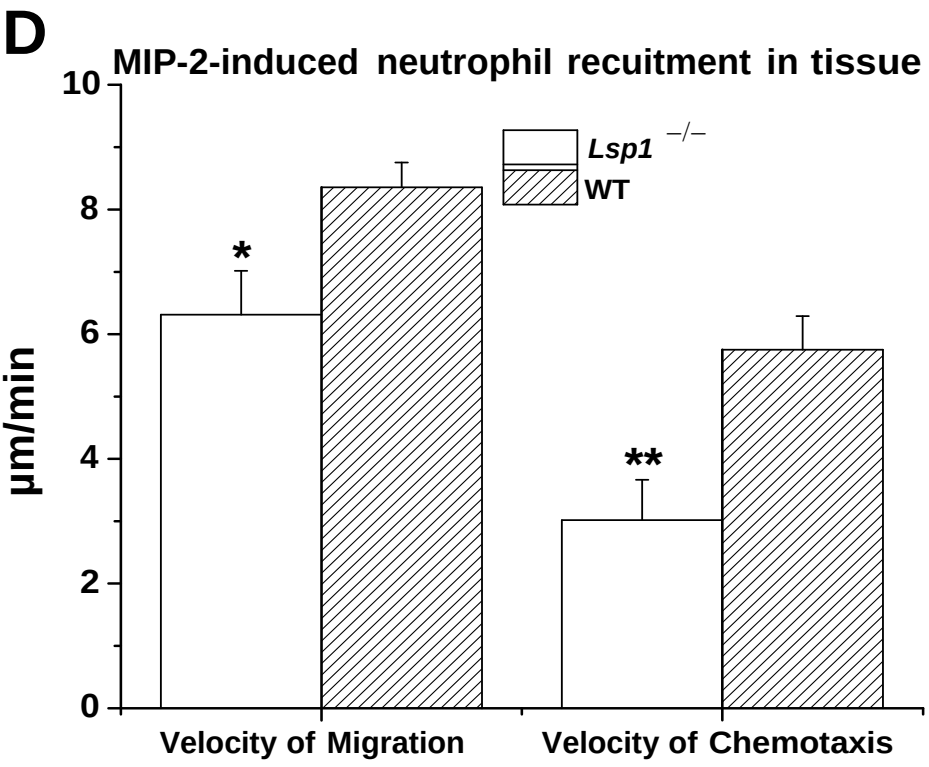

D
MIP-2-induced neutrophil recuitment in tissue
10
8
6
4
2
0
μm/min
Lsp1 −/−
WT
*
**
Velocity of Migration
Velocity of Chemotaxis

Fig. 6.5 **The role of LSP1 in MIP-2-containing gel-induced neutrophil intraluminal crawling, transendothelial migration, and chemotaxis in tissue.** (A) The velocity of crawling distance and crawling chemotaxis, (B) the intraluminal crawling time, transmigration time and detachment time, (C) crawling chemotaxis index and chemotaxis index in tissue, and (D) velocity of migration and chemotaxis in extravascular tissue of $Lsp1^{-/-}$ (open bars) and WT (hatched bars) mice are shown (n = 6 or 7, total > 40 cells in each group). Neutrophil recruitment was induced by the addition of 0.5 μM MIP-2-in-gel. $*P < .05$ and $**P < .01$ as compared with each value in WT control mice. Error bars represent means ± SEM.

6.2 The role of neutrophil LSP1 (N-LSP1) and endothelial LSP1 (E-LSP1) in MIP-2-induced neutrophil recruitment in chimeric mice

Although the role of LSP1 in MIP-2-induced neutrophil recruitment has been investigated, the respective role of neutrophil LSP1 versus endothelial LSP1 is still unknown. Therefore, four types of chimeric mice (*Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$, *Lsp1*$^{-/-}$→WT, WT→*Lsp1*$^{-/-}$, WT→WT) were generated and the same intravital microscopy experiments were performed to identify the cellular site of functional LSP1. By using PCR and the primers listed in Materials & Methods, we verified the genotypes of these generated chimeric mice. We confirmed, by analyzing the genotypes of blood samples and tail clips from chimeric mice, that the genotypes of the four types of chimeric mice were indeed as we anticipated, and these mice were used in the subsequent experiments.

In response to MIP-2, the four types of chimeric mice exhibited similar responses in terms of neutrophil rolling velocity and rolling flux (Fig. 6.6). Although there was a decrease of adhesion in *Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$ mice compared to WT→WT mice at 90 min, no significant differences were observed between other groups (*Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$ *vs* *Lsp1*$^{-/-}$→WT; *Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$ *vs* WT→*Lsp1*$^{-/-}$; WT→WT *vs* *Lsp1*$^{-/-}$→WT; WT→WT *vs* WT→*Lsp1*$^{-/-}$), indicating that only when both N-LSP1 and E-LSP1 are deficient and at 90 min after MIP-2 treatment does LSP1 show a minor role in MIP-2-induced neutrophil adhesion (compare Fig. 6.6C to Fig. 6.3C). However, neutrophil emigration was inhibited in WT→*Lsp1*$^{-/-}$ mice as well as in *Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$ mice but not in *Lsp1*$^{-/-}$→WT mice or WT→WT mice (Fig. 6.6D). This indicates that, similarly to KC-induced neutrophil recruitment [46], MIP-2-induced neutrophil emigration is also endothelial

LSP1-dependent. In addition, the responses of neutrophil recruitment in WT→WT and *Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$ chimeric mice were identical to those of WT and *Lsp1*$^{-/-}$ mice (compare Fig. 6.3 to Fig. 6.6), and the results of genotyping (data not shown) confirmed that our bone marrow transplantations had successfully taken place and were functional.

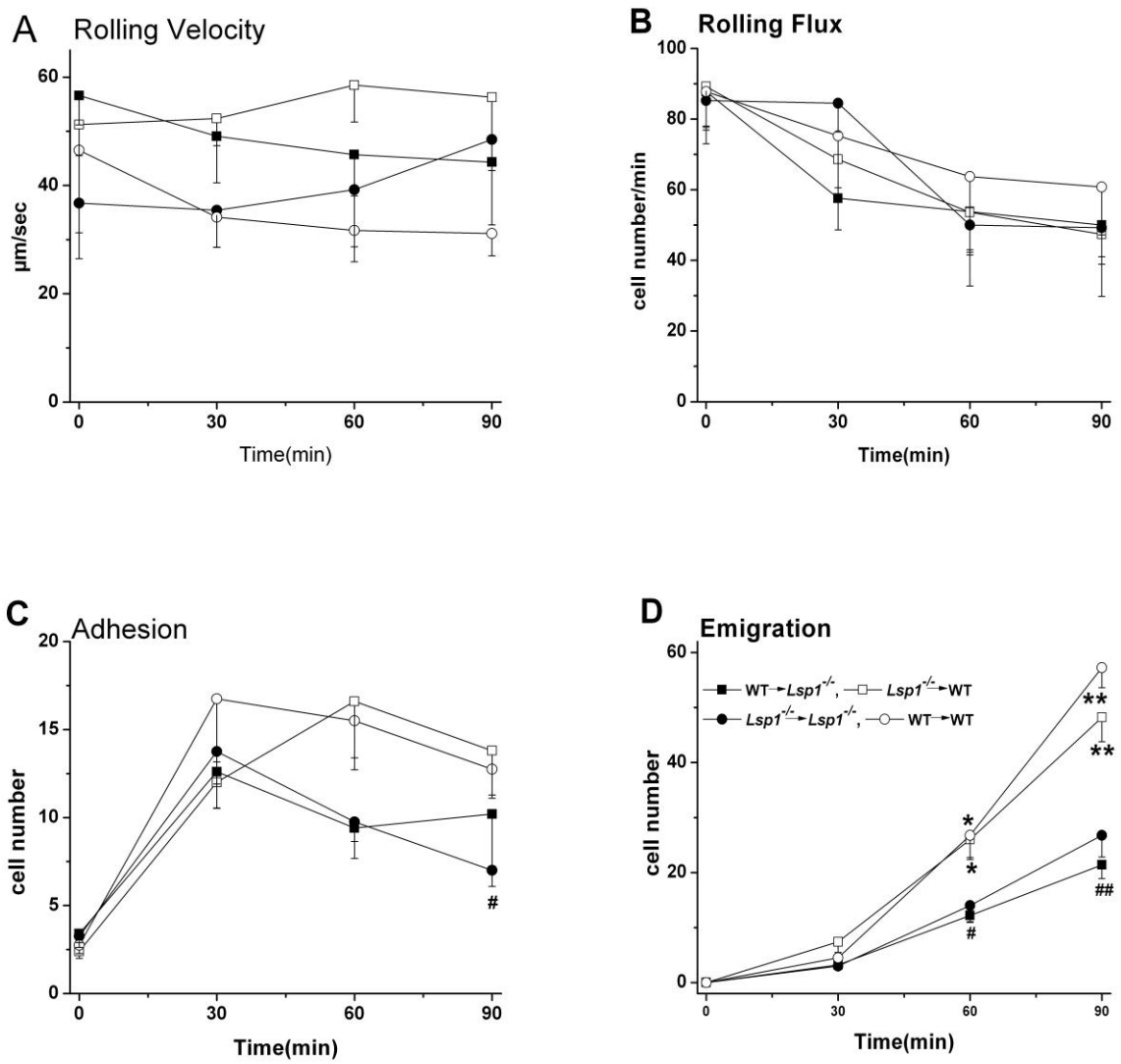
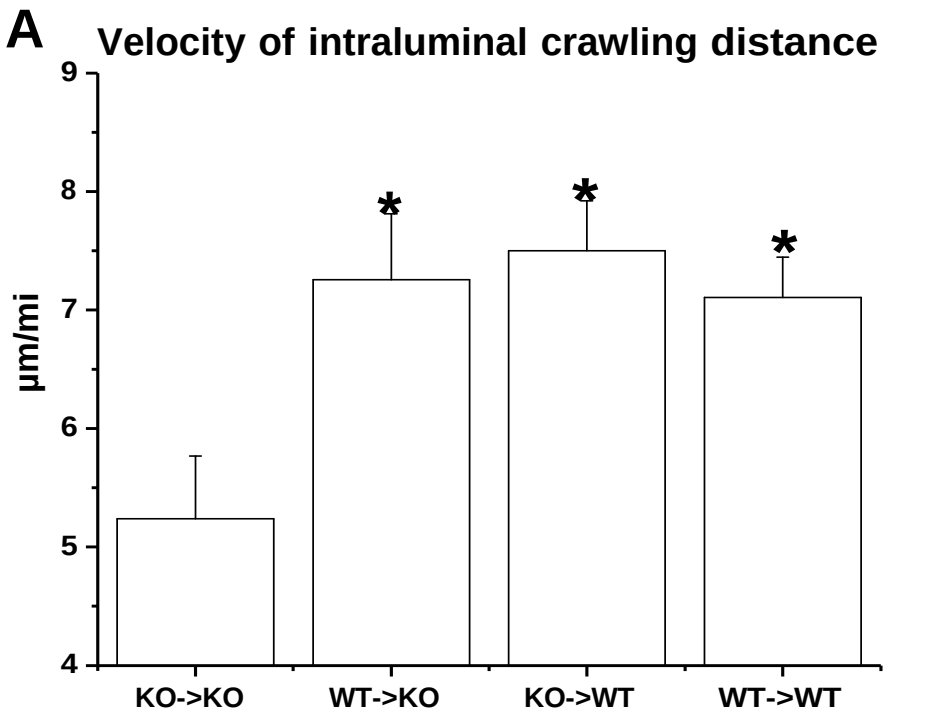
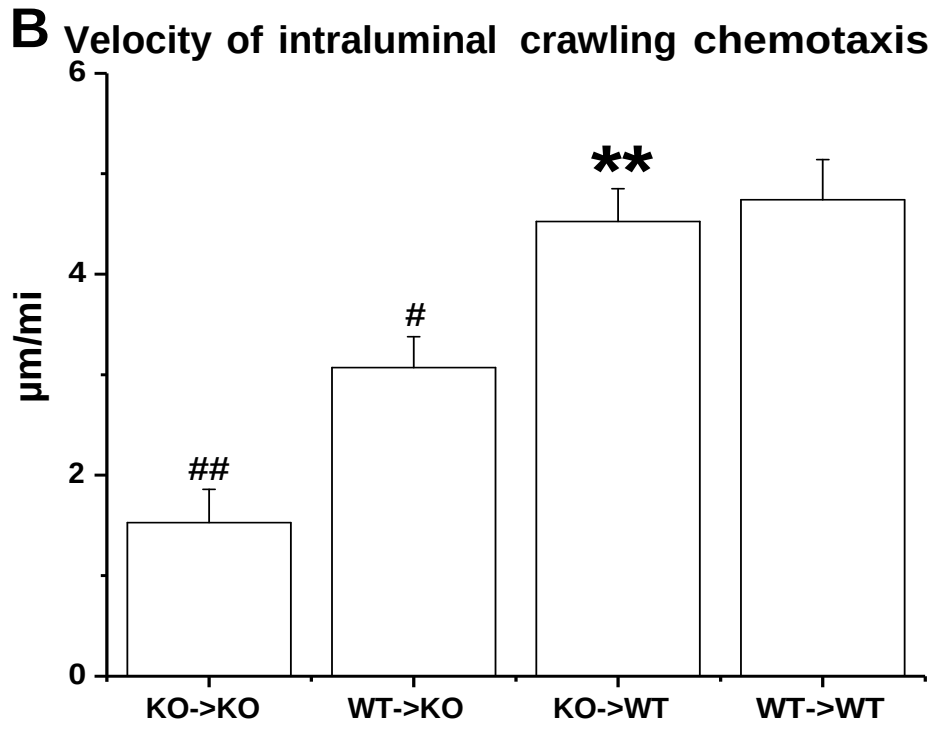


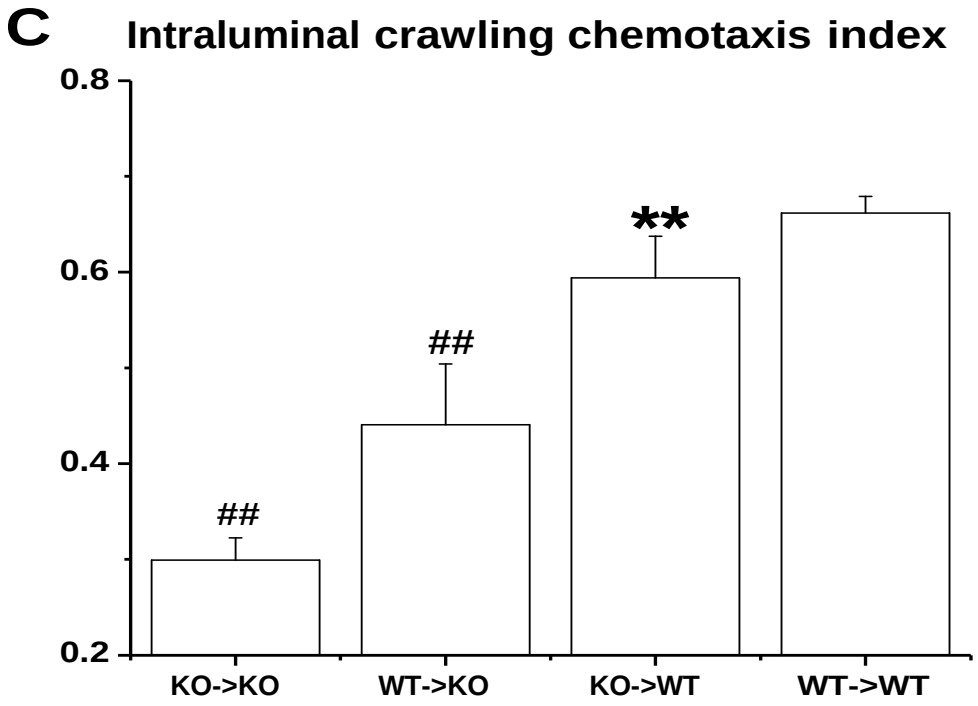
Fig. 6.6 **The role of neutrophil LSP1 (N-LSP1) and endothelial LSP1 (E-LSP1) in MIP-2-gel-induced neutrophil recruitment**. (A) The velocity of rolling neutrophils, (B) the flux of rolling neutrophils, (C) neutrophil adhesion, and (D) neutrophil emigration in cremaster post-capillary venules of four types of chimeric mice are shown (n = 4 in each group). *Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$ (solid circles): both the BM donor and recipient are *Lsp1*$^{-/-}$ mice; WT→*Lsp1*$^{-/-}$ (solid squares): the BM donor is 129/SvJ mice and recipient is *Lsp1*$^{-/-}$ mice; *Lsp1*$^{-/-}$→WT (open

squares): the BM donor is *Lsp1*$^{-/-}$ mice and recipient is 129/SvJ mice; WT→WT (open circles): both the BM donor and recipient are 129/SvJ mice. Neutrophil recruitment was induced by the addition of 0.5 μM MIP-2-in-gel. *P < .05 and **P < .01 as compared with each value in *Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$ mice. #P < .05 and ##P < .01 as compared with each value in WT→WT mice. Error bars represent means ± SEM.

For further study, I observed each step of neutrophil recruitment in these LSP1 chimeric mice by using time-lapse video photography. During intraluminal crawling (Fig. 6.7), the velocity of intraluminal crawling distance in $Lsp1^{-/-} \rightarrow Lsp1^{-/-}$ mice significantly decreased compared to the other three types of chimeric mice (Fig. 6.7.A), suggesting only when both N-LSP1 and E-LSP1 are absent has LSP1 an impact on the velocity of intraluminal crawling. However, we found that the velocity of chemotaxis was dependent on E-LSP1 (Fig. 6.7.B). Additionally, the intraluminal crawling chemotaxis index was completely E-LSP1-dependent (Fig. 6.7.C). On the other hand, either N-LSP1 deficiency or E-LSP1 deficiency led to increased crawling times (Fig. 6.7.D). As a result, both N-LSP1 and E-LSP1 are involved in neutrophil intraluminal crawling, while E-LSP1 plays a more important role in neutrophil directional crawling towards the chemoattractant.


A
Velocity of intraluminal crawling distance
µm/mi
9
8
7
6
5
4
*
*
*
KO->KO
WT->KO
KO->WT
WT->WT


B
Velocity of intraluminal crawling chemotaxis
µm/mi
6
4
2
0
##
#
**
KO->KO
WT->KO
KO->WT
WT->WT


C
Intraluminal crawling chemotaxis index
0.8
0.6
0.4
0.2
##
##
**
KO->KO
WT->KO
KO->WT
WT->WT

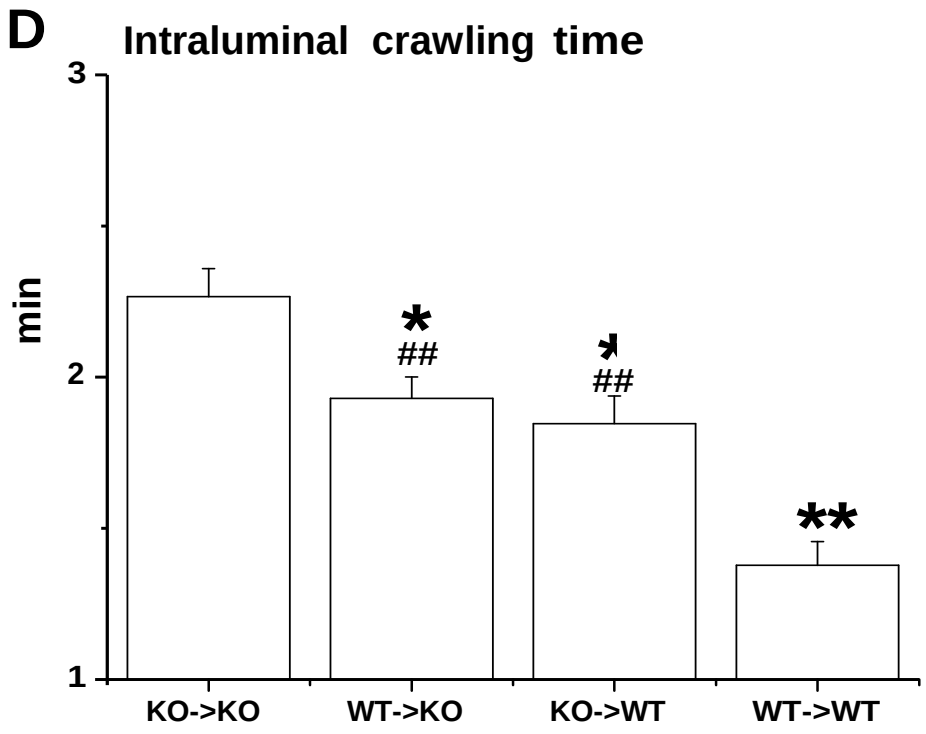

D
Intraluminal crawling time
min
3
2
1
*
##
*
##
**
KO->KO
WT->KO
KO->WT
WT->WT

Fig. 6.7 **The role of neutrophil LSP1 (N-LSP1) and endothelial LSP1 (E-LSP1) in MIP-2-induced neutrophil intraluminal crawling in cremaster post-capillary venules of chimeric mice.** (A) The velocity of intraluminal crawling distance, (B) the velocity of intraluminal crawling chemotaxis, (C) intraluminal crawling chemotaxis index, and (D) intraluminal crawling time of four types of chimeric mice are shown (n = 5 or 6, total > 40 cells in each group, KO means $Lsp1^{-/-}$). Neutrophil recruitment was induced by the addition of 0.5 μM MIP-2-in-gel. **P* < .05 and ***P* < .01 as compared with each value in $Lsp1^{-/-} \rightarrow Lsp1^{-/-}$ mice. #*P* < .05 and ##*P* < .01 as compared with each value in WT→WT mice. Error bars represent means ± SEM.

After crawling to the optimal sites, neutrophils undergo the transendothelial migration and after transmigration, then detach from the cremaster post-capillary venules. My transmigration data showed that both N-LSP1 and E-LSP1 had effects on neutrophil transmigration (Fig. 6.8). Either N-LSP1 or E-LSP1 must be present to have a normal transmigration time. Four types of chimeric mice exhibited no significant differences in detachment time (data not shown).

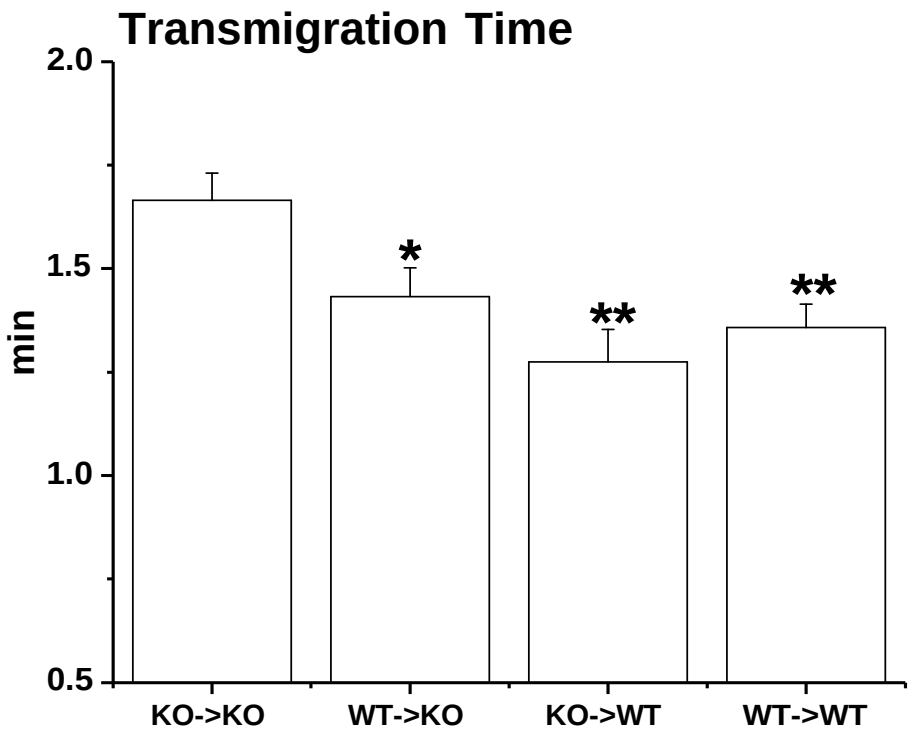
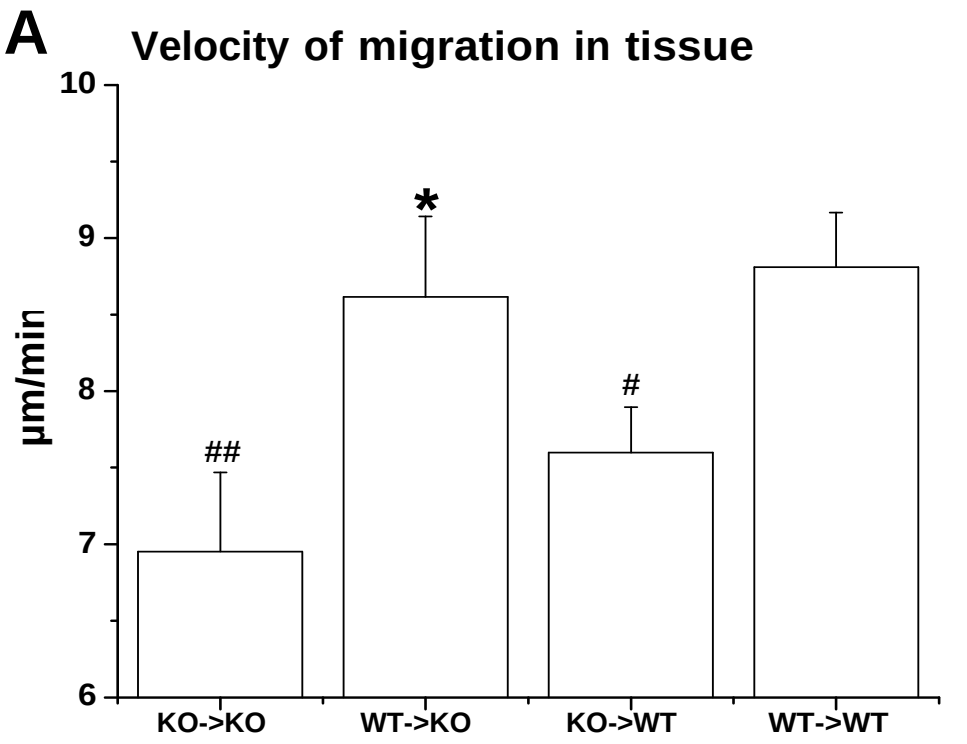
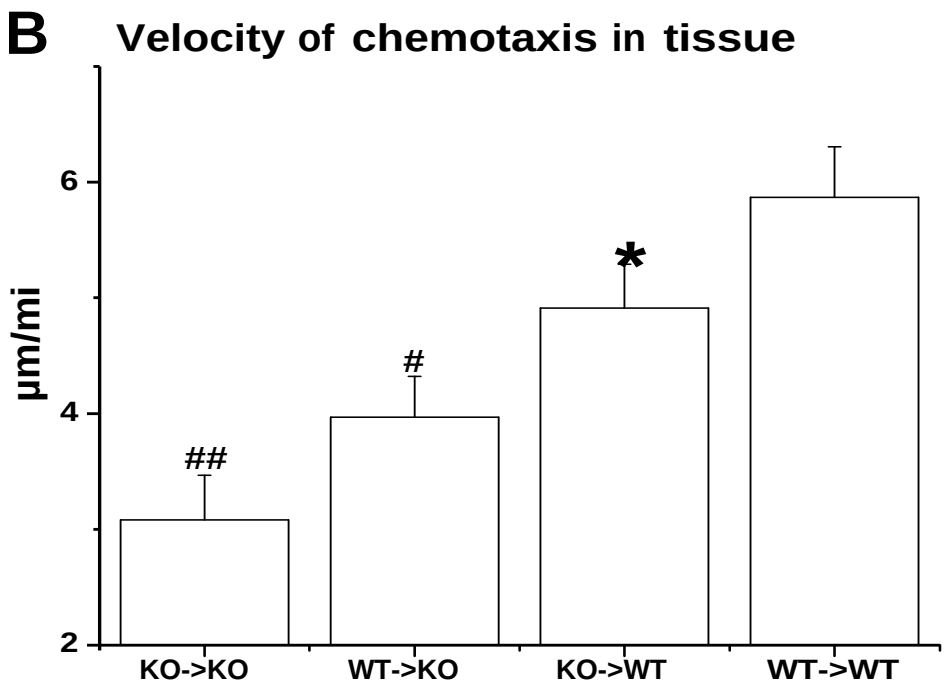


Fig. 6.8 **The role of neutrophil LSP1 (N-LSP1) and endothelial LSP1 (E-LSP1) in MIP-2-induced transendothelial migration from cremaster post-capillary venules of chimeric mice** (n = 5 or 6, total > 40 cells in each group, KO means $Lsp1^{-/-}$). Neutrophil recruitment was induced by the addition of 0.5 μM MIP-2-in-gel. **P* < .05 and ***P* < .01 as compared with each value in $Lsp1^{-/-} \rightarrow Lsp1^{-/-}$ mice. Error bars represent means ± SEM.

Following transmigration, neutrophils migrate towards the source of chemoattractant within the tissue (Fig. 6.9). We found that the velocity of neutrophil migration in tissue was much more dependent on N-LSP1 (Fig. 6.9.A). However, the situation of the velocity of chemotaxis and chemotaxis index were different (Fig. 6.9.B&C). It is interesting to note that, there was a significant decrease in neutrophil chemotaxis index in tissue in *Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$ mice and WT→*Lsp1*$^{-/-}$ mice but not in *Lsp1*$^{-/-}$→WT mice, indicating that N-LSP1 is not crucial in determining neutrophil chemotaxis index in response to MIP-2 in tissue. It also suggested that E-LSP1 may play a more important role in determining the subsequent neutrophil chemotaxis in tissue. Therefore, N-LSP1 has effects on the velocity of neutrophil migration in tissue whereas E-LSP1 may have a crucial role in neutrophil chemotaxis in issues in response to MIP-2.


A
Velocity of migration in tissue
µm/min
10
9
8
7
6
*
#
##
KO->KO
WT->KO
KO->WT
WT->WT


B
Velocity of chemotaxis in tissue
µm/mi
6
4
2
*
#
##
KO->KO
WT->KO
KO->WT
WT->WT

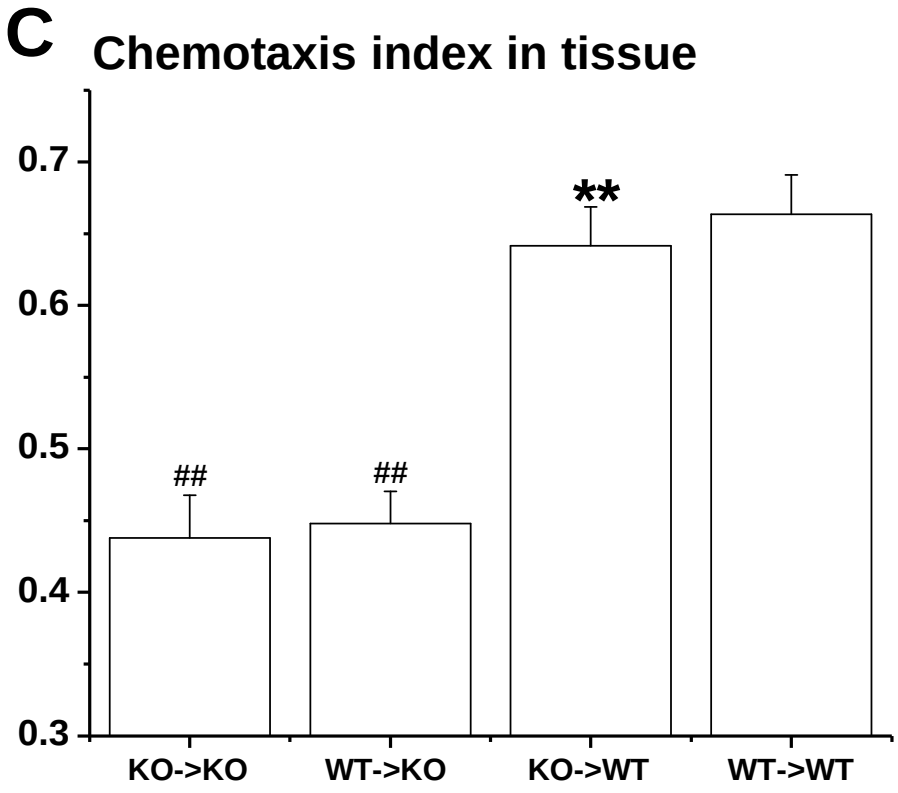


Fig. 6.9 **The role of neutrophil LSP1 (N-LSP1) and endothelial LSP1 (E-LSP1) in MIP-2 induced neutrophil migration and chemotaxis in tissue after their transmigration across cremaster post-capillary venules of chimeric mice.** (A) The velocity of migration in tissue, (B) the velocity of chemotaxis in tissue, and (C) chemotaxis index in tissue of four types of chimeric mice are shown (n = 5 or 6, total > 40 cells in each group, KO means $Lsp1^{-/-}$). **P* < .05 and ***P* < .01 as compared with each value in $Lsp1^{-/-}$→$Lsp1^{-/-}$ mice. Neutrophil recruitment was induced by the addition of 0.5 μM MIP-2-in-gel. #*P* < .05 and ##*P* < .01 as compared with each value in WT→WT mice. Error bars represent means ± SEM.

These data showed that the E-LSP1 is critical for MIP-2-induced neutrophil emigration. Both N-LSP1 and E-LSP1 have effects on neutrophil intraluminal crawling, transendothelial migration and migration in tissue. However, the directional neutrophil movement towards MIP-2 is much more dependent on the functions of E-LSP1 during neutrophil-endothelial cell interactions in the lumen. In addition, neutrophil chemotaxis in tissue seems to be E-LSP1-dependent.

6.3 Role of p38 MAPK in MIP-2-induced neutrophil recruitment

It was reported that p38 MAPK is involved in KC-induced neutrophil recruitment [39]. To investigate the role for p38 MAPK in MIP-2-induced neutrophil recruitment, I performed the same neutrophil recruitment assay using MIP-2-in-gel, but with p38 MAPK inhibitor SB203580 or SKF86002 treatments in 129/SvJ mice. Firstly, the 129/SvJ mice were treated by i.v. injection of SB203580 (20 mg/kg) 30 min before MIP-2-in-gel addition. My results revealed that this systematic treatment with SB203580 completely abolished neutrophil recruitment (data not shown). As a result, we conclude that, similar to the role of p38 MAPK in KC-induced neutrophil recruitment [39], MIP-2-induced neutrophil recruitment is indeed dependent on p38 MAPK pathway.

Next, I performed a local inhibition study by superfusion of the cremaster muscle with SKF86002 (0.7 mM). The mice were treated with SKF86002 superfusion 30 min before MIP-2-in-gel administration. The results showed that compared with saline-treated control mice, the SKF86002 pretreated mice exhibited similar responses in rolling velocity and rolling flux, indicating that p38 MAPK may not play an important role in neutrophil rolling in the venule. The number of adherent cells was reduced to about 50% of control values, whereas emigration was completely abolished at all time points tested (Fig. 6.10). My data indicated that MIP-2-induced neutrophil emigration is definitely dependent on the functions of p38 MAPK, whereas neutrophil adhesion in the venules is only partly dependent on this kinase.

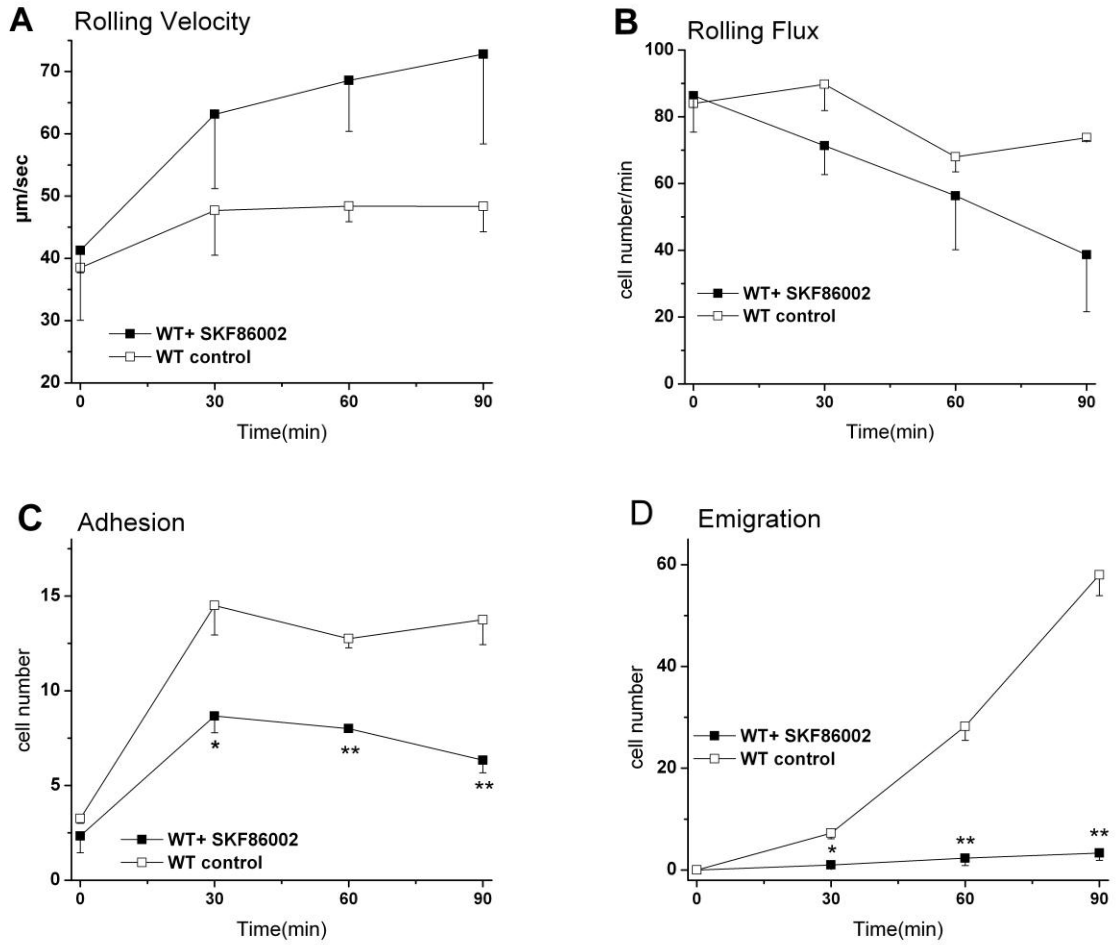


Fig. 6.10 **The role of p38 MAPK in MIP-2-containing gel-induced neutrophil recruitment.** (A) The velocity of rolling neutrophils, (B) the flux of rolling neutrophils, (C) neutrophil adhesion, and (D) neutrophil emigration in cremaster post-capillary venules of SKF86002 pretreated mice (open squares) and untreated WT (solid squares) mice are shown (n = 3 in each group). The cremaster muscle was treated with or without 30 min 0.7 mM SKF86002 superfusion before MIP-2-in-gel administration, and the muscle remained superfused with SKF86002 or saline after addition of MIP-2-in-gel. **P* < .05 and ***P* < .01 as compared with each value in control mice. Error bars represent means ± SEM.

To further investigate the role of p38 MAPK in neutrophil migration and chemotaxis in tissue, we superfused the cremaster muscle with SKF86002, after 30 min MIP-2-in-gel treatment when some neutrophils had transmigrated and just begun to chemotaxis in tissue (Fig. 6.11). In response to MIP-2, SKF86002-treated mice exhibited a similar rolling velocity to that in control mice. The rolling flux and adherent cells were significantly decreased after 30 min of SKF86002 superfusion. Neutrophil emigration displayed substantial inhibition after superfusion for 60 min. These results suggest that p38 MAPK plays an important role in neutrophil rolling flux, adhesion and emigration in response to MIP-2.

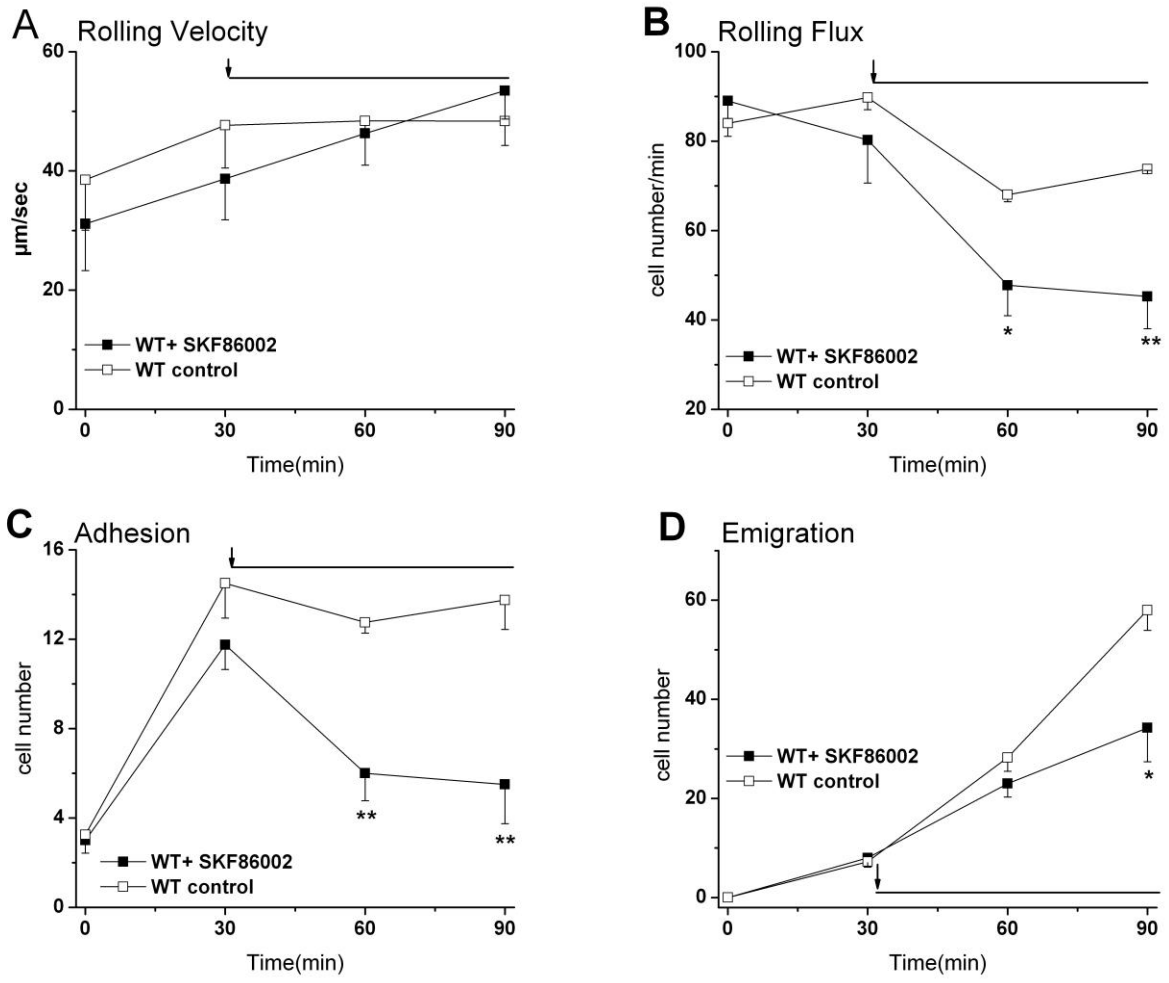


Fig. 6.11 **The role of p38 MAPK in MIP-2-containing gel-induced neutrophil recruitment.** (A) The velocity of rolling neutrophils, (B) the flux of rolling neutrophils, (C) neutrophil adhesion, and (D) neutrophil emigration in cremaster post-capillary venules of SKF86002 treated mice (open squares) and saline-treated WT (solid squares) mice are shown (n = 3 in each group). The cremaster muscle was treated with or without 60 min 0.7 mM SKF86002 superfusion (indicated as an arrow and solid line in the figures), after 30 min MIP-2-in-gel administration when some neutrophils had transmigrated and just begun to chemotaxis in tissue. **P* < .05 and ***P* < .01 as compared with each value in control mice. Error bars represent means ± SEM.

To determine the role of p38 MAPK in neutrophil migration and chemotaxis in tissue, I tracked the movement and measured the directionality of those transmigrated neutrophils after SKF86002 superfusion of cremaster muscle where the MIP-2-in-gel had been applied to induce neutrophil recruitment 30 min before SKF86002 superfusion (Fig. 6.12). The velocity of neutrophil migration, velocity of chemotaxis and chemotaxis index in tissue were all decreased in SKF86002-treated mice compared to SKF86002 untreated mice. As a result, neutrophil migration and chemotaxis in tissue are dependent on p38 MAPK-activation.

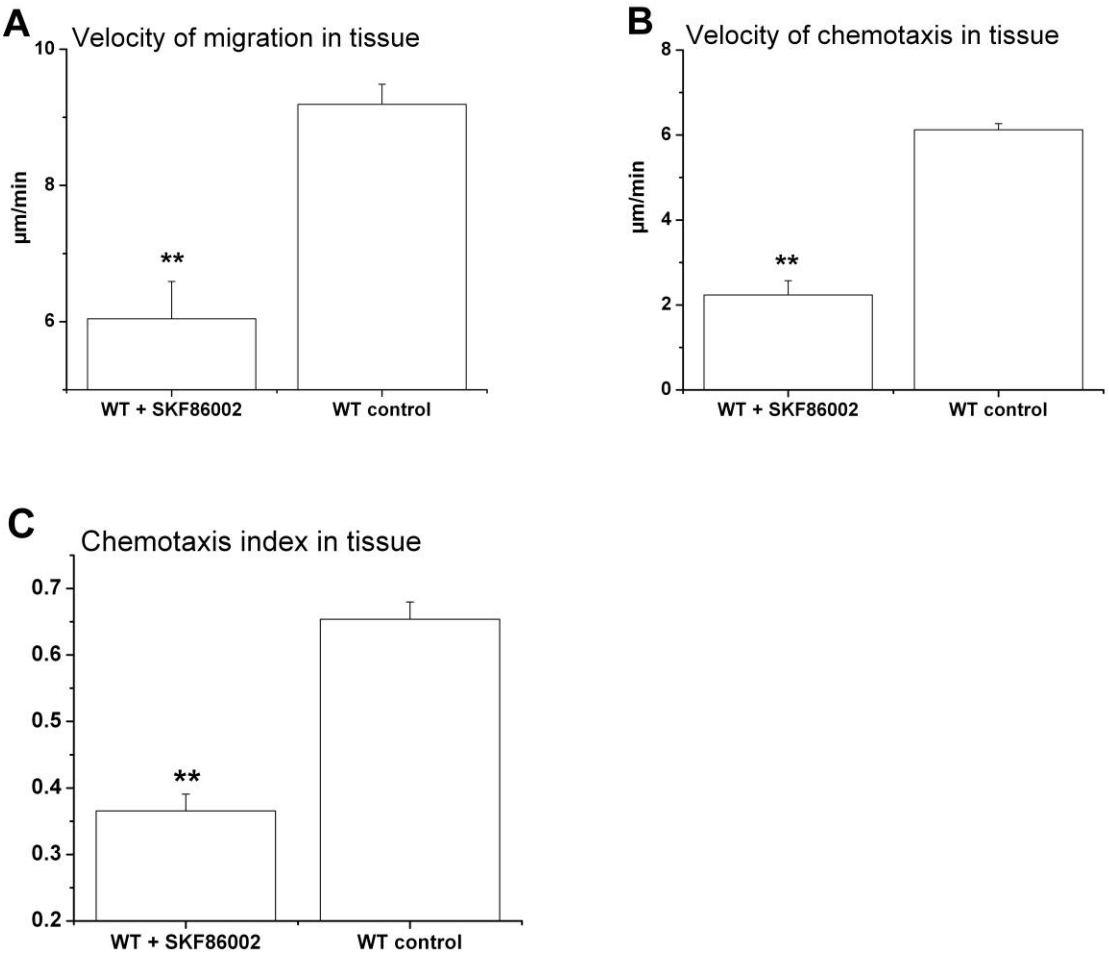


Fig. 6.12 **The role of p38 MAPK in MIP-2-induced neutrophil migration and chemotaxis in tissue in 129/SvJ mice.** (A) The velocity of migration in tissue, (B) the velocity of chemotaxis in tissue, and (C) chemotaxis index in tissue in cremaster post-capillary venules of SKF86002 treated and untreated WT mice (n = 3, total> 20 cells in each group). After 30 min of MIP-2-in-gel addition when some neutrophils had transmigrated across endothelium, SKF86002 (0.7 mM) was superfused on the muscle and the experiment was continued for additional 60 min to record the cellular movement in tissue after transmigration. ***P* < .01 as compared with each value in control mice. Error bars represent means ± SEM.

In neutrophils, LSP1 is one of the downstream effector molecules in the p38 MAPK signaling pathway [71], and the results from Part B indicate that neutrophil chemotaxis in tissue is E-LSP1-dependent but N-LSP1-independent. To further study the role of p38 MAPK in neutrophil chemotaxis in tissue, I performed the same assay using *Lsp1*$^{-/-}$→WT chimeric mice to determine if LSP1 is involved in the inhibitory effect of p38 MAPK inhibitor (Fig. 6.13). Interestingly, the velocity of migration, the velocity of chemotaxis and chemotaxis index of N-LSP1-deficient neutrophils were all significantly decreased after the treatment with SKF86002 when compared to the group without p38 MAPK inhibition (Fig. 6.13). These results suggest that: (1) in response to MIP-2-in-gel, *Lsp1*$^{-/-}$ neutrophils in WT mice had a defect in migration in tissue indicating that N-LSP1 plays an important role in determining the velocity of neutrophil migration in tissue (Fig. 6.9A). In these mice, SKF86002 further decreased *Lsp1*$^{-/-}$ neutrophil migration in tissue (Fig. 6.13A), suggesting that in addition to LSP1, neutrophils rely on another signaling pathway that is also downstream of p38 MAPK to mediate some migration response in tissue; (2) I demonstrated in Fig. 6.9B&C that *Lsp1*$^{-/-}$ neutrophils in WT mice had a normal chemotaxis response in tissue, suggesting that neutrophil chemotaxis in tissue in response to MIP-2 is E-LSP1-dependent and N-LSP1-independent. Here, my results in Part C (Fig. 6.13B&C) revealed that these *Lsp1*$^{-/-}$ neutrophils still rely on the functions of p38 MAPK for chemotaxis in tissue, suggesting the fundamental importance of p38 MAPK in neutrophil chemotaxis, and the existence of another signaling pathway downstream of p38 MAPK that is involved in neutrophil

chemotaxis in tissue. Overall, my results suggest that MIP-2-induced neutrophil chemotaxis in tissue is p38 MAPK dependent but N-LSP1 independent.

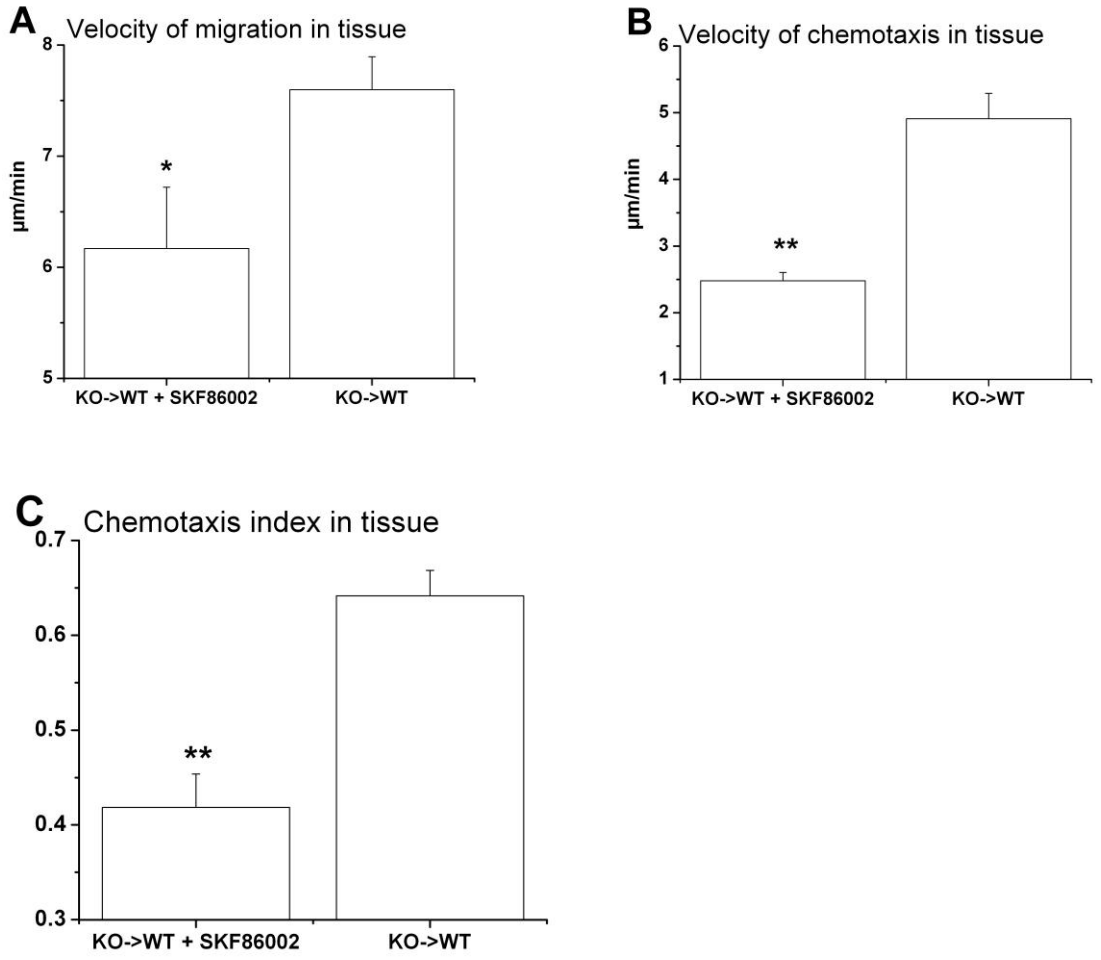


Fig. 6.13 **The role of p38 MAPK in MIP-2-induced *Lsp1*$^{-/-}$ neutrophil migration and chemotaxis in tissue in *Lsp1*$^{-/-}$→WT chimeric mice.** (A) The velocity of migration in tissue, (B) the velocity of chemotaxis in tissue, and (C) chemotaxis index in tissue of the cremaster post-capillary venules of SKF86002 treated and untreated *Lsp1*$^{-/-}$→WT chimeric mice (n = 3, total> 20 cells in each group). Neutrophil recruitment was induced in and p38 MAPK inhibitor SKF86002 was applied to the cremaster muscle of *Lsp1*$^{-/-}$→WT chimeric mice as described in the legend of Fig 6.12. $^{**}P < .01$ as compared with each value in control mice. Error bars represent means ± SEM.

7. Discussion

7.1. LSP1 positively regulates neutrophil recruitment in response to MIP-2

The Ca^{2+} and F-actin binding intracellular phosphoprotein LSP1 has been shown to play an important role in neutrophil recruitment through its mediation of signal transduction and its involvement in cytoskeleton rearrangement. Neutrophil recruitment is a very dynamic process and a hallmark feature of inflammatory response. Many stimuli, such as fMLP, IL-8 and KC have been shown to initiate multiple intracellular effects that induce neutrophil recruitment. Both *in vivo* and *in vitro* studies have confirmed that LSP1 plays an important role in CXC chemokine KC-induced neutrophil emigration and chemotaxis [46, 51]. However, the mechanism of this positive regulation of LSP1 is still unclear. Furthermore, another CXC chemokine, MIP-2, induce neutrophil migration in a PI3K-dependent manner [67] and the PI3K pathway is known to be important in actin polarization, cell motility and migration. However, whether LSP1 is involved in MIP-2-induced neutrophil chemotaxis is currently unknown. In addition, the functional differences of neutrophil LSP1 (N-LSP1) and endothelial LSP1 (E-LSP1) in neutrophil intraluminal crawling, TEM and chemotaxis in tissue are incompletely understood. These create a need to extend our understanding of the role of E-LSP1 and N-LSP1 in chemokine MIP-2- and KC-induced neutrophil recruitment.

It is well known that neutrophil directional migration can be induced by chemokines or other chemoattractants both *in vivo* and *in vitro*. Phillipson *et al.* recently showed that in the presence of shear flow *in vivo*, neutrophils exhibited intraluminal crawling in a perpendicular direction to the

vessel lumen by the regulation of Vav1, a major regulator of the organization of the actin cytoskeleton during neutrophil polarization and migration [72]. Menezes *et al.* demonstrated that a chemokine gradient was formed intravascularly and neutrophils crawled towards the chemoattractant [5]. My intraluminal crawling data demonstrated that neutrophils in WT mice crawled towards MIP-2 and suggested the existence of a chemokine gradient and chemotaxis in the lumen. This suggests that once a neutrophil starts to crawl in the lumen, it in effect becomes a "chemotaxing" cell. The "chemotaxing" cells undergo polarization, cytoskeletal reorganization, shape changes and rapid low-affinity crawling to move cell body towards the chemoattractant [25]. The regulation of cytoskeletal remodeling by LSP1 has been intensely investigated [58, 60-63, 73]. The *in vitro* study by Hannigan *et al.* revealed the impacts of the defects on migration speed and chemotaxis in *Lsp1*$^{-/-}$ neutrophils in response to KC, and concluded that these were due to the abnormal morphologies in these cells, indicating that LSP1 was important for the modulation of F-actin bundle and the cell stability during chemotaxis [51]. As a result, the major functions of N-LSP1 appear to be the modulation of F-actin binding, cytoskeleton reorganization and cell motility towards the chemokine gradient. These findings appear to explain my *in vivo* data: the decreased velocity of crawling migration, crawling chemotaxis and migration in tissue and prolonged crawling time and transmigration time in *Lsp1*$^{-/-}$→ *Lsp1*$^{-/-}$ mice can be rescued to some extent in WT→ *Lsp1*$^{-/-}$ mice.

Similar responses to MIP-2- and KC-induced neutrophil recruitment in my study indicated that the role of LSP1 in MIP-2- and KC-elicited chemotaxis appears to be the same. Liu *et al.* showed

that, in response to KC-in-gel, *Lsp1*$^{-/-}$ mice exhibited significantly decreased emigration in neutrophil recruitment and this impairment was also observed in WT→*Lsp1*$^{-/-}$ mice but not in *Lsp1*$^{-/-}$→WT mice [46]. This is consistent with my data following both MIP-2- and KC-in-gel administration and demonstrated a dominant role for E-LSP1 in neutrophil emigration. In my study, I examined the role of LSP1 in further detailed steps of neutrophil recruitment and my data showed E-LSP1 had a role in chemokine-induced neutrophil intraluminal crawling, transendothelial migration and chemotaxis in extravascular tissue. My data also revealed that during intraluminal crawling in E-LSP1-deficient mice, neutrophils exhibited decreased velocity of chemotaxis, chemotaxis index and prolonged crawling time in response to MIP-2. One possible reason for the phenomenon could be the absence of an intravascular chemokine gradient, due to an inability of *Lsp1*$^{-/-}$ ECs to transport and present chemokines to neutrophils. By using intravital and confocal microscopy, Menezes *et al.* recently showed that in response to MIP-2-in-gel, a chemokine gradient formed intravascularly, and influenced intraluminal neutrophil crawling [5]. Chemokines were found to bind to the endothelial heparin sulfate (HS) chains to be immobilized on the luminal surface of endothelium. Thereafter, chemokines were transported from basolateral to apical of endothelium and finally transported to neutrophils via endothelial heparin sulfate proteoglycans (HSPGs) binding to L-selectins to induce the effective intraluminal crawling and chemotaxis in the lumen [5]. Many of the intracellular transportation processes are related to the functions of actin and F-actin may be involved in chemokine transportation. Because of LSP1 has F-actin binding sites and has an impact on F-actin functions, E-LSP1 may play an important role

on chemokine transportation.

In this study, my data showed that during transendothelial migration,E-LSP1-deficient mice exhibited increased transmigration time and reduced transmigration cells in response to MIP-2. The failure of dome structure formation is one possible mechanism. Petri *et al.* provided evidence that some LSP1 in ECs is translocated to the cytoskeleton from the nucleus to induce dome structure formation, which is a subendothelial complex for neutrophil-endothelial cell interactions that is critical for neutrophil recruitment [74]. Another reasonable mechanism may be the impairment of endothelial retraction in E-LSP1-deficient mice. Studies by Liu *et al.* suggested that LSP1 may affect endothelial retraction to regulate vascular permeability and promote neutrophils transmigration [46]. As a result, E-LSP1 may be critical for dome structure formation and endothelial retraction during neutrophil transmigration.

Neutrophil chemotaxis in extravasular tissue is, to a large extent, dependent on multiple functions of integrins, which affect adhesion, spreading, contractility and retraction [75]. My results demonstrated the important role of E-LSP1 in neutrophil chemotaxis in tissue and one possible mechanism for the role of LSP1 may involve the functions of integrins. It has been reported that after TEM, the phenotype and functions of neutrophils will change such as the transfer of membrane proteins from ECs to the migrating neutrophils [76]. During TEM, the endothelium forms dome structures to facilitate neutrophil migration [11] and LSP1 is important for dome structure formation [74]. Therefore, one possibility is that LSP1 involves in the changes of neutrophil phenotype and functions during TEM. Lee *et al.* recently demonstrated that the

extracellular matrix (ECM) protein lumican regulates neutrophil chemotaxis in tissue through regulating β_2 integrins (especially Mac-1) directly and β_1 integrins indirectly [77]. Interestingly, they demonstrated that lumican is expressed on endothelium but not on bone marrow or peripheral blood neutrophils. However, lumican exists on the surface of emigrated peritoneal neutrophils, indicating that neutrophils must acquire lumican during the migration across the endothelium in order to migrate effectively towards chemoattractant in the gradient in extravascular tissue [77]. As a result, the impairments of velocity of neutrophil chemotaxis and chemotaxis index in tissue after the transmigration across *Lsp1*$^{-/-}$ ECs in my data may be due to the disability of ECs to transfer endothelium proteins such as lumican to neutrophils, leading to the low affinity of integrins on the transmigrated neutrophils and the reduced chemotaxis of these neutrophils in tissue.

7.2. MIP-2-induced neutrophil chemotaxis in tissue is dependent on p38 MAPK signaling pathway

LSP1 has been shown to be a major substrate of the MK2 in the p38 MAPK signaling pathway [48]. MK2 and p38 MAPK were reported to be essential for neutrophil motility and chemotaxis in response to fMLP [53]. Cara *et al.* demonstrated that intravenous treatment with the p38 MAPK inhibitor SB203580 (20 mg/kg) for 30 min before the administration of KC-in-gel blocked about 70% of emigrating cells and inhibited neutrophil chemotaxis in tissue [39]. They also showed that 0.7 mM p38 MAPK inhibitor SKF86002 superfusion for 60 min after KC-in-gel

treatment restricted neutrophil chemotaxis in tissue, suggesting that KC-induced neutrophil recruitment is via the p38 MAPK pathway [39]. My data showed that both p38 MAPK inhibitors SB203580 and SKF86002 blocked MIP-2-induced neutrophil recruitment and chemotaxis in tissue, indicating MIP-2-induced neutrophil recruitment is also dependent on p38 MAPK-activation. In addition, my data showed that E-LSP1 is critical for neutrophil chemotaxis in tissue and SKF86002 treatment inhibited the chemotaxis of neutrophils after their transendothelial migration. My data revealed that although the chemotaxis of *Lsp1*$^{-/-}$ neutrophils, after transmigration across WT ECs, were almost normal and LSP1-independent (Fig. 6.9), LSP1 regulation of MIP-2-induced neutrophil recruitment and chemotaxis in tissue is completely dependent on p38 MAPK pathway, suggesting the existence of an LSP1-independent signaling mechanism in chemotaxing neutrophils in tissue that functions under the control of or downstream of p38 MAPK activity during their chemotaxis.

8. Summary

My first objective of this study was to determine the role of LSP1 in MIP-2- or KC-in-gel-induced neutrophil recruitment by using intravital microscopy *in vivo*. Mouse cremaster muscle treated with 0.5 μM MIP-2- or KC-in-gel for 90 min exhibited similar rolling velocity, rolling flux responses in WT mice and *Lsp1*$^{-/-}$ mice, but a lower adhesion and significantly decreased emigration in *Lsp1*$^{-/-}$ mice. Time-lapse video photography analysis revealed that LSP1 regulated neutrophil intraluminal crawling, transendothelial migration and chemotaxis in tissue. In addition, the recruitment responses to MIP-2 and KC were similar, suggesting that the two CXC chemokines may induce neutrophil recruitment via the same signaling pathway.

To further understand the functional role of neutrophil LSP1 (N-LSP1) and endothelial LSP1 (E-LSP1) in neutrophil recruitment, intravital video microscopy and time-lapse video photography were performed using four types of chimeric mice that we generated. Emigration in neutrophil recruitment was significantly inhibited in WT→*Lsp1*$^{-/-}$ mice as well as in *Lsp1*$^{-/-}$→*Lsp1*$^{-/-}$ mice but not in *Lsp1*$^{-/-}$→WT mice. The results suggested that N-LSP1 promoted intraluminal crawling, transmigration and migration in tissue maybe by the modulation of cytoskeletal remodeling and that E-LSP1 played more important roles in MIP-2-induced neutrophil recruitment maybe through regulating the intravascular chemokine gradient, dome structure formation, ECs retraction and the integrin affinity of chemotaxing neutrophils in tissue.

Finally, I examined the role of p38 MAPK signaling pathway in MIP-2-induced neutrophil

recruitment and found that LSP1 regulation of MIP-2-induced neutrophil emigration and chemotaxis in tissue is completely p38 MAPK pathway dependent and there exists a signaling mechanism that is important in the chemotaxing neutrophils, is downstream of or under the control of p38 MAPK and in parallel but independent of LSP1 signaling pathway for the neutrophil migration and chemotaxis *in vivo*.

9. Conclusion

In general, this study provides the first evidence that E-LSP1 appears to play a critical role in neutrophil directional migration both in the lumen and in extravascular tissue, especially for chemotaxis in tissue in response to MIP-2-in-gel-induced neutrophil recruitment. Additionally, blockade of p38 MAPK pathway locally or systematically inhibit neutrophil emigration and chemotaxis in tissue and this inhibitory effect is partially achieved through LSP1.

10. Future studies

Based on the experiments described in this thesis, the following studies are proposed.

a. To study the role of E-LSP1 in neutrophil-endothelial cell interactions and neutrophil transendothelial migration *in vitro* with *in vitro* transwell system

b. To identify the functional molecules which influence the chemotaxis of transmigrated neutrophils in *Lsp1*$^{-/-}$→WT mice in tissue with flow cytometry.

c. To study LSP1-involved signaling mechanisms *in vitro*.

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