The role of LSP1 in microvascular hyperpermeability during neutrophil recruitment *in vivo*

A Thesis Submitted to

The College of Graduate Studies & Research

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Pharmacology

University of Saskatchewan

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Abstract

Leukocyte-specific protein 1 (LSP1) is an F-actin- and Ca^{2+}-binding, intracellular cytoskeletal and nuclear phosphoprotein expressed in hematopoietic lineage and endothelial cells. It has been shown to play an important role in leukocyte motility and recruitment during inflammation when leukocytes interact with matrix proteins or the endothelial cells of postcapillary venules at the site of infection or injury. The role of LSP1 in microvascular hyperpermeability during neutrophil recruitment is the focus of this study. To induce neutrophil recruitment in the cremasteric microvasculature, mouse cremaster muscle was treated by superfusion with CXC chemokine KC (Keratinocyte-derived chemokine, CXCL1), MIP-2 (Macrophage inflammatory protein-2, CXCL2) or intrascrotal injection with the cytokine tumor necrosis factor-α (TNFα). Neutrophil recruitment was visualized and determined by intravital microscopy that measures neutrophil adhesion and emigration. The changes of microvascular permeability after chemokines or cytokine are simultaneously measured by the use of fluorescent intravital microscopy. I observed that both KC and TNFα induced similar increases in microvascular permeability in wild-type and LSP1-deficient mice. The emigration of neutrophils was significantly lower in response to KC and TNFα in LSP1-deficient mice than in wild-type mice. However, the permeability increases induced by each emigrated neutrophil in LSP1-deficient mice were significantly higher compared with that in wild-type mice. When the circulating neutrophils were depleted by >98% by using

anti-neutrophil antibodies, neutrophil rolling, adhesion and emigration and microvascular hyperpermeability in response to KC and TNFα were completely inhibited to the basal level in the inflamed venules. I conclude that neutrophil-endothelial cell interactions dictate the increases of microvascular permeability in inflamed tissues, and LSP1-deficient neutrophils contribute much more to the permeability increases than wild-type neutrophils do.

Acknowledgements

I would like to thank the many people who made this thesis possible. Foremost, I am deeply indebted to my supervisor Dr. Lixin Liu whose help, stimulating suggestions and encouragement helped me in all the time of research. I owe my most sincere gratitude to my committee members, Dr. John Gordon, Dr. J.Steven Richardson, Dr. Lily Wu, Dr. Kash Desai, Dr. Baljit Singh and Dr. Peter Yu, for their encouragement, detailed and constructive comments, and valuable advices. I am indebted to my colleagues in the lab for their support and help. And I wish to extend my warmest thanks to all those who have helped me with my work in Department of Pharmacology at the University of Saskatchewan. Last but not the least, I owe my loving thanks to my friends Bosong Gao and Lewei Rui, who keep me happy in Saskatoon.

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List of Abbreviations

AJs	Adherens junctions
BSA	Bovine serum albumin
CAMs	Cell adhesion molecules
CNF-1	Cytotoxic necrotizing factor 1
FP	Formyl peptide
FMLP	Formyl-methionyl-leucyl-phenylalanine
GAPs	GTPase-activating proteins
GEFs	Guanine nucleotide exchange factors
GPCRs	G protein-coupled receptors
HBP	Heparin-binding protein
ICAM	Intercellular adhesion molecule
IL	Interleukin
JAM	Junctional adhesion molecule
KC	Keratinocyte-derived chemokine
LPA	Lysophosphatidic acid
LSP1	Leukocyte-specific protein 1
MIP-2	Macrophage inflammatory protein-2
MLC	Myosin light chain
MPO	Myeloperoxidase

NAD	Neutrophil actin dysfunction
P AF	Platelet-activating factor
PECAM-1	Platelet endothelial cell adhesion molecule-1
ROS	Reactive oxygen species
SDF-1α	Stromal cell-derived factor-1α
TJs	Tight junctions
TNFα	Tumor necrosis factor-α
VAP-1	Vascular adhesion protein-1
VCAM-1	Vascular cell adhesion molecule-1
ZO	Zona occludens

Introduction

1. Inflammation

Inflammation is an extremely important process for our body to respond to harmful stimuli or injuries, and it is a complex immune response which is under very well control. Inflammation can be divided into acute and chronic inflammation. Acute inflammation is the initial response that is characterized by the increased movement of leukocytes and vascular hyperpermeability at the postcapillary venules in the inflamed tissues. When there are harmful stimuli or injuries, resident cells in the tissue will begin to release inflammatory mediators. In the inflamed area, vascular permeability increases and fluid and proteins leak out of blood vessel. These exudates contain complement proteins and antibodies which can opsonize or kill harmful microbes [1]. Meanwhile, leukocytes in blood flow are activated, and they interact with vascular endothelium and emigrate along the chemoattractant gradient to the inflamed site. Acute inflammation is a short process which lasts hours to days, and the process stops once the stimuli are removed. If the stimuli cannot be removed in a short time, inflammation is prolonged and turns to chronic inflammation which is characterized by destruction, repairing and healing of tissue.

Inflammation is sometimes regarded as a “double-edged sword”. In the healthy condition, every step in inflammation is under close regulation by our body,

but abnormalities during inflammation cause many human diseases. For example, allergy reaction is due to hypersensitive immune response to allergens. Dysfunctional leukocytes make patients vulnerable to infections (e.g., Chédiak–Higashi syndrome). Also chronic inflammation can cause many diseases such as atherosclerosis, rheumatoid arthritis, and even cancer. Inappropriate inflammatory reactions disrupt normal life of the person with inflammatory disorder. It is, therefore, important to study the mechanisms involved in regulating all aspects of inflammation.

2. Leukocyte recruitment

The hallmark feature of acute inflammation is the recruitment of neutrophils to the inflamed site. There are several sequential steps in this cellular process in which neutrophils move from the blood and finally reach the inflamed site. The initial interaction between neutrophils and endothelial cells that is critical in leukocyte recruitment cascades is when the flowing leukocytes start tethering and rolling on the endothelium [2]. This first important step is mainly dependent on one family of cell adhesion molecules (CAMs) called selectins which have three members, E-selectin, P-selectin and L-selectin. These three selectins were named after the cells where they were first discovered. They have been reported to have overlapping and distinctive functions. E-selectin is responsible for dramatic decreases in rolling velocity alone, but still P- or L-selectin is indispensable for the initial capture of neutrophils for their E-selectin-dependent slow rolling [3]. However, upon some other

inflammatory stimuli, P-selectin is required for rolling flux increases and velocity reductions [4]. L-selectin is important in the tethering efficiency between slow-rolling neutrophils and endothelial cells, but has no role in rolling velocity [5]. Some other studies suggest that the rolling and tethering processes can rely on other molecules in addition to selectins. Human vascular adhesion protein-1 (VAP-1) can mediate tethering and rolling, and α4-integrin supports the rolling of certain subsets of leukocytes during recruitment [6-8].

Integrins, which are another family of CAMs, contain two chains, α and β subunits. There are eighteen α subunits and eight β subunits in mammals, which are associated in different combinations to generate 24 integrins. Several integrins in the β2, β7, and β1 subfamilies are expressed in leukocytes [9]. The rolling leukocytes are activated through the interactions with chemokines or other chemoattractants present on the luminal surface of endothelial cells to become firmly adherent to the endothelium. The transition from rolling to adherent is dependent on integrins. It has been reported that neutrophil adherence requires the activation of β2 integrin, and the two CD11/CD18 integrins, CD11a/CD18 ($\alpha_L\beta_2$ integrin, also called LFA-1) and CD11b/CD18 ($\alpha_M\beta_2$ integrin, also called Mac-1), are the first discovered adhesive molecules [10]. By the use of LFA-1- and Mac-1-deficient mice, it has been reported that both LFA-1 and Mac-1 are important in neutrophil adhesion, and it seems LFA-1 plays a predominant role [11]. Henderson *et al.* reported that LFA-1 plays an important role in the initial adhesion, and Mac-1 supports the longer-lasting adhesion

[8]. Mac-1 seems more important during the transition between adhesion and transmigration. The α4-integrin, which can act like selectin to support rolling and tethering, also can perform the function of β2-integrin to mediate adhesion [8, 12].

A new step in leukocyte recruitment, called intraluminal crawling, was identified by Alan R Schenkel and his colleagues. They found that monocytes moved after firm adhesion to an endothelial junction to transmigrate, and that LFA-1 and Mac-1 both had a role in this movement [13]. Blockade of Intercellular adhesion molecule (ICAM)-1 or ICAM-2 prevented monocytes from moving to the junction [13]. Neutrophils in postcapillary venules also crawl to endothelial junctions to migration after adhesion. Phillipson *et al.* discovered that LFA-1-deficient neutrophils had problems in adhesion, but still could move properly in the venule. Mac-1-deficient neutrophils showed significantly decreased intraluminal crawling compared with wild-type neutrophils. The inhibition of ICAM-1 affected crawling, but blockade of ICAM-2 did not affect crawling [14]. The authors suggested that the LFA-1-dependent adhesion and Mac-1-dependent crawling are both important for the next recruitment step, neutrophil emigration [14].

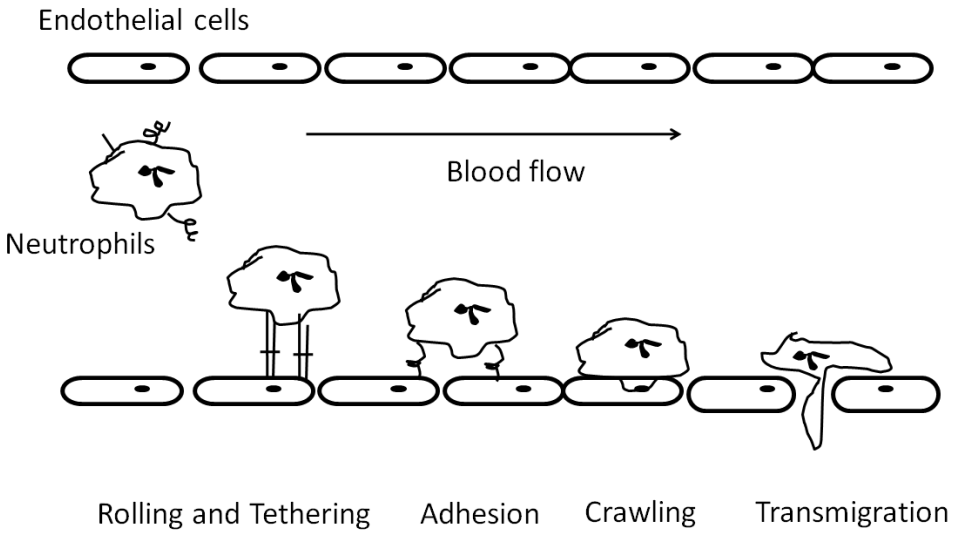


Figure 1. Leukocyte recruitment cascade. During inflammation, leukocytes in blood vessels tether and roll along the vessel lumen, and then adhere to endothelial cells. After firm adhesion, leukocytes crawl to optimal sites and begin to transmigrate.

After crawling to the optimal site, leukocytes immediately start migrating across the endothelium. Compared with other steps in leukocyte recruitment, leukocyte transendothelial migration is the least studied step. Many adhesion molecules are involved in this process, including ICAM-1, ICAM-2, junctional adhesion molecule (JAM)-A, JAM-C, platelet endothelial cell adhesion molecule-1 (PECAM-1) and CD99 [15]. There are two routes by which leukocytes emigrate across the endothelium, paracellular or transcellular. An *in vivo* study demonstrated that neutrophils prefer to migrate through endothelial cells predominantly rather than emigrate *via* endothelial junctions from venules in response to FMLP [16]. Yang *et al.* reported that a large number of neutrophils transmigrate *via* the transcellular route after 24-h treatment with TNFα, while after 4-h TNFα treatment, neutrophils still prefer the paracellular route [17]. They also found that lymphocytes transmigrating through paracellular or transcellular route are comparable [17]. It seems that the pattern of transmigration depends on the cell type, inflammatory stimuli and the time of assessment.

The leukocyte recruitment cascade, selectin-dependent rolling followed by integrin-dependent adhesion and a complex transmigration through the endothelium, is a generally accepted leukocyte recruitment paradigm that has been demonstrated in cremaster muscle, mesentery and many other tissues. But in some other organs the cascade may not be exactly the same [18]. In the liver, there are neither rolling leukocytes nor selectin expression within the sinusoids [19]. Adhesion is not

dependent on the rolling and tethering and still exists when β2- or α4-integrins are blocked. In the brain, platelets, which express P-selectin, play an important role in leukocyte recruitment. An endothelium-platelet–leukocyte bridge is formed during recruitment cascade [18]. A better understanding of the organ-specific mechanisms of leukocyte recruitment is important for the development of novel therapeutics for various inflammatory disorders.

2.1 Chemokines in leukocyte recruitment

Chemokines are a largest family of cytokines, the name of which comes from two words: chemotactic cytokines. Chemokines are involved in many processes in inflammation and immunity. They are small proteins that signal through binding to their G protein-coupled receptors (GPCRs) on target cells [20]. During inflammation, chemokines are generated by many types of cells, such as emigrated leukocytes, resident macrophages, and tissue cells including endothelial cells, epithelial cells, and fibroblasts. There are four subfamilies of chemokines CXC, CC, CX_3C and C chemokines named according to the position of the first two conserved cysteine residues [21]. As mentioned earlier, integrins play a very important role in leukocyte adhesion. Integrins mediate adhesion through binding to their ligands on endothelial cells or in interstitial tissues. Chemokines increase the adhesion of leukocytes by inducing rapid and transient high affinity binding of integrins to their ligands. For example, CXC and CC chemokines induce a change LFA-1 from a low affinity to a

high affinity state in a few seconds (rapid), after while LFA-1 returns to a low affinity state in minutes (transient) [22]. The affinity of α4-integrin is rapidly and transiently increased in monocytes stimulated with formyl peptide (FP) or stromal cell-derived factor-1α (SDF-1α, CXCL12) [23]. Chemokines induce changes not only in the affinity of integrins but also in the motility. After a cell is treated with a CXC chemokine, its LFA-1 is redistributed and clustered together in seconds [22]. However, chemokine-induced binding of integrins to their ligands varies in different cell types. SDF-1α rapidly increases integrin binding to ICAM-1 and not to vascular cell adhesion molecule-1 (VCAM-1) in lymphocytes. But in monocytes, SDF-1α also induces significant increases in VCAM-1 binding [22, 23].

2.2 Leukocyte transendothelial migration

There are two routes for endothelial transmigration and many adhesion molecules are involved in the process [15]. In this chapter, I will introduce what and how these molecules are involved.

2.2.1 ICAM-1

Intercellular adhesion molecule (ICAM)-1 is a transmembrane protein and belongs to the immunoglobulin (Ig) superfamily. ICAM-1 is constitutively expressed in endothelial cells at a low concentration, but its expression can be significantly upregulated after cytokine stimulation [24]. An *in vitro* study showed that blockade of ICAM-1 expression by antibody inhibited neutrophil transmigration

[25]. Yang *et al.* reported that high levels of ICAM-1 in endothelial cells enhanced both trans- and para-cellular transmigration of neutrophils, but did not have a role in T-lymphocyte transmigration [17]. During transmigration, ICAM-1 tends to relocalize and is enriched in the endothelial border, where these molecules form a cup-like structure that surrounds the site of transmigration [26, 27]. This ICAM-1-rich projection accompanies the whole process of transmigration, and inhibition of this structure decreased neutrophil transmigration [27]. ICAM-1 engagement activates endothelial Pyk2 and Src, which are endothelial signaling molecules that are important in neutrophil transmigration [28].

2.2.2 JAMs

Junction adhesion molecule (JAM) is an immunoglobulin gene superfamily member and is expressed and concentrated in intercellular junctions of endothelial and epithelial cells [29]. JAM-A is also expressed in circulating cells, such as neutrophils, monocytes and platelets [30]. JAM-A is a ligand of LFA-1 and also contributes to LFA-1-dependent transmigration of neutrophil [31]. In JAM-A-deficient mice, neutrophil transmigration induced by LTB_4 or platelet-activating factor (PAF) is comparable with that in WT mice, but in response to tumor necrosis factor (TNF)-α and interleukin (IL)-1β, inhibition or deficiency of JAM-A significantly decreases neutrophil transmigration [32, 33]. Like JAM-A, JAM-C is also expressed in monocytes and platelets of human, but it is not expressed

in mouse leukocytes [30]. Blockade of JAM-C by antibodies *in vitro* or *in vivo* both significantly decreased neutrophil transendothelial migration [34], and overexpression of JAM-C in mice enhanced neutrophil transmigration [35]. It seems JAM-C is one molecule that is involved in this process. Another research group reported that functional blockade of JAM-C had no effect on neutrophil transmigration under shear flow, but JAM-C did contribute to the formation of a small gap at sites where neutrophils transmigrate [36].

2.2.3 PECAM-1

Platelet endothelial cell adhesion molecule-1 (PECAM-1), also called CD31, is a member of the Ig superfamily. It is expressed on the surface of circulating cells and is rich in endothelial cell intercellular junctions [37]. A polyclonal antibody against PECAM-1 inhibited neutrophil transmigration by about 20%; interactions between leukocyte and endothelial PECAM-1 are crucial in this process [38]. By the use of PECAM-1-deficient mice, PECAM-1 was found to be involved in neutrophil transmigration induced by IL-1β, but not TNFα [39]. Endothelial PECAM-1 can relocalize to the cellular borders to form a “passive ligand” for neutrophil PECAM-1 for their interactions during the transmigration [40] .

2.2.4 CD99

CD99 is a small membrane glycoprotein with unique properties, and it has no homology with any other protein family. Mouse CD99 is expressed in both

endothelial cells and leukocytes [41]. Blockade of CD99 by antibody significantly decreases neutrophil transmigration, and blockade of CD99 in either endothelial cells or neutrophils inhibits neutrophil transmigration. Similar to PECAM-1, the homophilic CD99 interactions between endothelial cells and neutrophils is critical for this process [42]. Unlike PECAM-1, the role of CD99 is not dependent on the type of stimulus (both IL-1β and TNFα) [42]. The reduction of transmigration *in vitro* is due to trapping of neutrophils between endothelial junctions and their inability to complete the transmigration process [43]. CD99L2 is a novel protein related to CD99, and similar to CD99, it is also involved in neutrophil transmigration [43].

2.2.5 VE-cadherin

VE-cadherin is rich in inter-endothelial cell contacts and is important in maintaining the integrity of endothelial cells [44]. Unlike other molecules, VE-cadherin is a negative regulator of neutrophil transmigration. It has been shown that using a monoclonal antibody against mouse VE-cadherin increased the number of transmigrated neutrophils by more than three times compared to that in the control mice [44]. During transmigration, there is a transient gap formation between VE-cadherins which reseals in 5 min after transmigration [45].

3. Neutrophils in inflammation

In humans, neutrophils constitute the majority of leukocytes in the blood

(60%-70%), and also the majority of granulocytes (95%). Neutrophils are produced in bone marrow and have a short half-life (8-10 h). In healthy people, neutrophils are found in the circulation in a resting state. During inflammation, neutrophils are activated and induced to degranulate. There are four types of neutrophil granules [46], including primary (azurophil or peroxidase-positive) and secondary (specific or peroxidase-negative) granules. Gelatinase (tertiary) granules are gelatinase-rich peroxidase-negative granules. Finally, secretory vesicles have unique properties which make them different from the other three granules [47]. Signaling through selectin binding to its ligand can mediate the mobilization of secretory vesicles, which can induce neutrophils to present β2-integrins on its surface, and further enhance neutrophil adhesion. The gelatinase granules can support neutrophil emigration because gelatinase disrupts the vascular basement membrane. Azurophilic granules, which contain myeloperoxidase (MPO), elastase and other bactericidal mediators, have important role in neutrophil phagocytosis [48].

Upon activation, neutrophils secrete many inflammatory mediators, including leukotrienes, prostaglandins, cytokines and chemokines. In response to different inflammatory stimuli, neutrophils secrete proinflammatory cytokines, such as TNF-α and IL-1β [49]. Neutrophils are also a source of chemokines. IL-8 (CXCL8) is the first chemokine proved to be synthesized by neutrophils [50]. To date many chemokines have been demonstrated to be synthesized by neutrophils. Activated neutrophils are reported to produce leukotrienes and prostaglandins, which

are derived from arachidonic acid [51]. At the site of inflammation, leukotrienes and prostaglandins have effects on vasodilation, and permeability increases in postcapillary venules. Leukotrienes have been reported to have the ability to increase neutrophil chemotaxis and adhesion through integrins [52]. With the activation of neutrophils, NADPH oxidase is also activated to produce reactive oxygen species (ROS), which are critical for the destruction and killing of bacteria [53].

4. Vascular permeability

4.1 Normal permeability

The endothelium is a thin cellular monolayer on the interior surface of a blood vessel; it regulates the exchange of protein, water and even the movement of whole cells [54]. Vascular permeability is the sum of mechanisms that control the stability of barrier function and signal pathway. Transcellular permeability (transcytosis) and paracellular permeability are two routes for these molecular exchanges.

4.1.1 Paracellular permeability

To maintain the normal paracellular permeability, the inter-endothelial junctions present in endothelium, composed of adherens, tight and gap junctions, play an important role. Adherens junctions (AJs) and tight junctions (TJs) control endothelial cell-to-cell adhesion, whereas gap junctions are responsible for the

delivery of water, iron or other molecules from one cell to another [55]. Also, many proteins are located in the inter-endothelial junctions, such as JAM, claudins and PECAM-1, many of which are involved in the formation of junctions and the control of endothelial permeability [56].

(1) Adherens junctions (AJs):

VE-cadherin is the major component of AJs. It is a transmembrane adhesion protein specifically expressed at the endothelial cellular borders. VE-cadherin is responsible for homophilic adhesion of adjacent endothelial cells and further mediates permeability changes. Vascular permeability significantly increases after the blockade of VE-cadherin by a monoclonal antibody [57]. The extracellular domain of VE-cadherin is 5 cadherin-like repeats which bind to the homophilic VE-cadherin on the adjacent cell, whereas the intracellular domain binds to the intracellular linker proteins α-catenin, β-catenin, and plakoglobin. The catenin-cadherin complex links AJs to the actin cytoskeleton [58, 59]. But one research group reported that α-catenin cannot bind to β-catenin-cadherin complexes and the actin cytoskeleton simultaneously [60]. One actin-binding protein called EPLIN is demonstrated to fill this gap and it can link the catenin-cadherin complex and the actin cytoskeleton at the same time [61]. p120 catenin is also important in AJ integrity. The juxtamembrane domain of VE-cadherin binds directly to p120, which is reported to promote cadherin clustering and increase adhesion [62]. Furthermore, p120 controls the expression level of VE-cadherin. Inhibition of p120 dramatically

decreases the cellular level of VE-cadherin [62].

(2) Tight junctions (TJs):

TJs only constitute about 20% of total junctions in endothelial cells, but still have important roles in maintaining normal permeability. TJs are formed by claudins, occludin and JAMs [63]. Occludin is the first-identified transmembrane protein in TJs [64]. Occludin is associated with zona occludens (ZO)-1 and ZO-2 is also bound to occludin through ZO-1. This bound is the linkage of actin cytoskeleton and occluding [65]. Different domains of occludin have effects on the regulation of paracellular permeability [66]. Like occludin, claudin is a transmembrane protein containing four transmembrane domains. Claudin also binds ZO-1 and then linked to the actin cytoskeleton [63]. Claudin forms homotypic adhesive plaques to improve cell-cell adhesion [67]. JAMs present in endothelial cells are up-regulated during inflammation. JAM-A, a member of the JAMs family, colocalizes with ZO-1 and A-6 in the TJs, which directly links JAM-A to F-actin [68]. JAMs have also been reported to play a role in neutrophil transendothelial migration; JAMs redistribute in this process.

4.1.2 Transcellular permeability

Transcellular permeability is regulated by transcytosis, which mainly depends on the function of caveolae in endothelial cells. Caveolae can transport macromolecules from inside the blood vessel to the outside. Caveolae are abundant

in endothelial cells and accounts for about 20% of endothelial cell volume. Caveolin-1 is the protein that is responsible for caveolae assembly [69]. Caveolin-1 has a domain rich in cholesterol, and the expression of caveolin-1 is dependent on cellular cholesterol level [70]. By using caveolin-1-knockout mice, a research group found that the microvascular permeability is significantly increased in these mice. When the expression of caveolin-1 is inhibited by siRNA, the lung vascular permeability dramatically increased [71, 72]. In caveolin-1-knock-out mice, the tight junctions in endothelial cells are much smaller than those in wild-type mice [72]. Caveolin-1 is a very important regulator of vascular permeability. It seems that there is a cross-talk between paracellular permeability and transcellular permeability through caveolin-1.

4.1.3 Actin cytoskeleton:

The actin cytoskeleton, a dynamic subcellular structure, is a critical component in endothelial cell permeability [73]. The actin cytoskeleton is linked to the junctional proteins, such as cadherin in AJs and ZO in TJs [73]. In response to proinflammatory stimuli, inter-endothelial junctions fall apart and form a gap. It has been shown that inter-endothelial gap formation needs the contraction of endothelial cells, which is mediated by the actin cytoskeleton. Gap formation and actin reorganization require myosin light chain (MLC) phosphorylation, which is regulated by MLC kinases [74]. Many inflammatory mediators have been reported

to increase vascular permeability via endothelial cell contraction or actin organization to form gaps between endothelial cells [75]. Some mediators, such as histamine, thrombin or bradykinin, are known to signal through GPCRs. The activation of GPCRs causes an intracellular Ca^{2+} increase which can cause MLC kinase activation and myosin phosphorylation [76]. Tyrosine kinases are important for the effect of some other mediators on vascular permeability increases, and VEGF is one of these mediators. VEGF-induced permeability increases have been reported to play a role in tumor development and ischemia/reperfusion injury [76].

4.2 Regulation of vascular permeability

Many molecules and mechanisms are involved in the maintenance of the integrity of endothelial barrier functions and the regulation of vascular permeability.

4.2.1 Rho GTPases:

Rho GTPases are a family of small signaling G proteins, which are able to switch between active and inactive forms. The activation of this protein is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Activated RhoGTPases induce the changes of activities of the downstream factors to mediate some effects [77]. Three members of the RhoGTPase family are well-studied, Rac1, RhoA and Cdc42, and are involved in permeability control [78]. The role of RhoGTPases in endothelial barrier function was studied by using toxin B to inactivate the protein. Administration of toxin B results in the loss of endothelial

barrier function, which is accompanied by the formation of gaps between endothelial cells and decreases in F-actin [79]. Inhibition of Rac1 by lethal toxin has similar effects, such that permeability is significantly increased. Specifically, adhesion of endothelial cells through VE-cadherin is reduced and the AJs are disrupted, causing the opening of inter-endothelial cell junctions and an increase in gap formation [80]. This evidence indicated that Rac1 is important in maintaining endothelial barrier integrity under resting condition. Besides its role in the resting condition, Rac1 was been demonstrated to reduce the increase of permeability caused by TNF-α. Increased activation of Rac-1 blocks gap formation and endothelial barrier breakdown induced by TNF-α [81]. Activation of Rac1 by a protein activator cytotoxic necrotizing factor 1 (CNF-1) increases F-actin levels in the endothelial cells to strengthen the endothelial barrier. Increased activation of Rac1 also reduces PAF-induced permeability increases [82]. Similar to Rac1, Cdc42 is also critical in endothelial permeability maintenance [82]. Cdc42 was shown to have the ability to regulate the association of α-Catenin and VE-cadherin [83]. Besides the maintenance of endothelial barrier, Cdc42 seems to have a role in permeability restoration. After disassembly of AJs by thrombin, AJs can start reassembly and Cdc42 is activated during this process. The inhibition of Cdc42 delays the reassembly of AJs [84]. RhoA is a negative regulator of endothelial barrier. The inhibition of RhoA improves the cell-cell adhesion and enhanced endothelial function [85]. The RhoA is activated in response to thrombin or lysophosphatidic acid (LPA) and is involved in

reorganization of the cytoskeleton and increases of permeability [86, 87].

4.2.2 Cytosolic calcium:

Calcium is an important second messenger involved in many cellular functions. Increases in intracellular calcium levels can be induced by many inflammatory mediators and these cause significant increases in microvascular permeability. It has been found that inhibition of calcium levels in endothelial cells block 50% of thrombin-induced permeability increases [88]. Further studies showed that F-actin cytoskeleton in endothelial cells was increased and reorganized after the increases in intracellular calcium concentration, and this was regulated by Ins(1,4,5)P_3 levels [89]. The activation of MLC kinase is dependent on calcium levels, which induce MLC phosphorylation [90]. Cytosolic calcium levels are also critical in vascular permeability control.

5. Leukocyte-specific protein 1 (LSP1)

Leukocyte-specific protein 1 (LSP1) is a 52kDa intracellular Ca^{2+}- and F-actin-binding protein [91]. LSP1 was first found in lymphocytes and named as lymphocyte-specific protein 1 [92]. However, more recent studies found that LSP1 is expressed in all mouse and human leukocytes, including neutrophils, macrophages and lymphocytes [93, 94]. Therefore, LSP1 was renamed as leukocyte-specific protein 1 [95]. Mouse LSP1 contains 330 amino acids whereas its human homologue LSP1 is 339 amino acids protein [96, 97]. Both human and mouse LSP1 are

phosphoproteins and their sequences are highly conserved and largely identical. Eighty-five percent of the C-terminal in human LSP1 is identical compared to that of mouse LSP1. The Ca^{2+}-binding sites in the two proteins are not conserved. Human LSP1 has a highly conserved basic domain which contains most of the serine and threonine residues which might be the targets of protein kinases [96]. LSP1 in lymphocytes is located in the cytoplasmic face of the plasma membrane and also associates with the cytoskeleton [92, 97]. Further studies showed that LSP1 binds to F-actin through the highly conserved COOH-terminal domain in both humans and mice [98]. Zhang *et al.* found in 2000 that there are four F-actin binding sites in human LSP1 [99]. LSP1 has been identified as one of the major substrates for mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK2) in human neutrophils [100].

5.1 LSP1 in leukocyte transendothelial migration

To study the role of LSP1, Jongstra-Bilen *et al.* generated LSP1-deficient (*Lsp1*$^{-/-}$) mice on a 129/SvJ (WT) background and also indicated that LSP1 deficiency in mice had no effect on myeloid development [101]. First, they induced inflammation in the peritoneum and found that the total number of infiltrating leukocytes, macrophages and neutrophils was significantly increased in *Lsp1*$^{-/-}$ mice compared with that in WT mice. Also, the total number of neutrophils reached maximal levels at 8 h in *Lsp1*$^{-/-}$ mice while WT mice took 24 h. Then the emigrated

neutrophils in the peritoneum were isolated and stimulated with FMLP. Chemotaxis of neutrophils from *Lsp1*$^{-/-}$ mice was about two-fold higher than that of neutrophils from WT mice [101]. In another inflammatory model, LSP1 had a similar role. Significantly increased number of neutrophils moved into knee joints of *Lsp1*$^{-/-}$ mice in response to zymosan, whereas chemotaxis of *Lsp1*$^{-/-}$ neutrophils on fibrinogen was increased [102]. This evidence demonstrated that LSP1 is a negative regulator of leukocyte transendothelial migration. This negative role of LSP1 was further proved in neutrophil actin dysfunction (NAD) patients where LSP1 is overexpressed [103]. But another research team in a vitro experiment showed that *Lsp1*$^{-/-}$ neutrophils had significantly lower speed in chemotaxis in the KC gradient. During chemotaxis, WT neutrophils formed lamellipodia and protrusions which were rarely seen in *Lsp1*$^{-/-}$ neutrophils. Unlike chemotaxis, superoxide production induced by phorbol 12-myristate 13-acetate (PMA) was significantly higher in *Lsp1*$^{-/-}$ neutrophils than in WT neutrophils [104]. Li *et al.* found that the role of LSP1 in locomotion was biphasic by using three types of monocyte-differentiated U937 cells, U937 cells with no LSP1, with normal LSP1 and with 4-fold LSP1 overexpression. The cells with no LSP1 or normal LSP1 remained round and had smooth surfaces, whereas overexpression of LSP1 in cells caused the formation of projection on the surface which contained F-actin. Compared with cells with normal LSP1, the other two types both showed impaired motility. So the level of LSP1 in cell is important in regulation of cell motility [105]. Another group used intravital microscopy which can visualize

leukocytes and their interactions with the endothelium in the postcapillary venule to study the role of LSP1. They found that *Lsp1*$^{-/-}$ mice had significantly lower number of emigrating leukocytes in cremasteric microvasculature in response to TNFα, IL-1β or the chemokine KC [106]. Obviously, LSP1 has a very important role in leukocyte transendothelial migration.

5.2 LSP1 in endothelial cells

Liu *et al.* have demonstrated that LSP1 is expressed in mouse and human endothelial cells [106]. Endothelial LSP1 is mainly in the nucleus and moved to the cytoplasm to carry out its function [106, 107]. They also reported that the chimeric mice with normal endothelial cells but *Lsp1*$^{-/-}$ leukocytes emigrated as effectively as WT mice. The WT leukocytes had difficulty emigrating through *Lsp1*$^{-/-}$ endothelial cells [106]. Endothelial cells had been reported to form a dome-like structure to help leukocyte transmigration [107]. It seems that endothelial LSP1 also has a role in leukocyte transendothelial migration.

5.3 LSP1 in cytoskeleton remodeling

In the NAD patient, neutrophils are abnormal in motility and actin polymerization. Neutrophils in these patients cannot form pseudopods or F-actin-rich projections and these abnormalities are associated with significant increases in LSP1 [108]. Regulation of the actin cytoskeleton seems to be one of the physiological functions of LSP1. The high level expression of LSP1 in cells induced formation of

hair-like projections whereas, at normal levels of LSP1, the cells remain round [109]. And the elevated LSP1 in cells was mainly because of the increase of LSP1 binding to actin [110]. Therefore, morphological changes of actin cytoskeleton induced by LSP1 are dependent on the amount of this protein binding to F-actin. It is well known that actin cytoskeleton organization is crucial in cell movement. After attaching on the substrate, the cells spread and started organizing actin to form filopodia and lamellipodia [111].

5.4 LSP1 in endothelial permeability

It is clear that cytoskeleton remodeling is a critical component in endothelial cell permeability [73]. LSP1 is an F-actin binding protein that is involved in cytoskeletal remodeling. So LSP1 may play a role in regulation of endothelial permeability. In an *in vivo* study, *Lsp1*$^{-/-}$ mice had significantly lower permeability than WT mice after treatment with histamine [106]. Very few studies are done on the role of LSP1 on vascular permeability changes [112]. Further studies are required to find out the mechanisms by which LSP1 mediates endothelial permeability changes.

6. Leukocyte–endothelial cell interactions and vascular permeability

6.1 Cell adhesion molecules (CAMs)

There are at least three families of CAMs involved in leukocyte-endothelial interactions, selectins, integrins and integrin ligands. As I have

introduced in previous sections, selectins and integrins have been demonstrated to participate in leukocyte rolling and adhesion. The major integrin ligands belong to the Ig superfamily and are expressed on endothelial cells [113]. The most important members in this family are ICAM-1 and ICAM-2, which act as ligands for β_2-integrins LFA-1 and Mac1. ICAM-1 is expressed in many types of cells including endothelial cells, and the levels of ICAM-1 expression can be increased by proinflammatory stimuli, such as TNFα or LPS [114, 115]. ICAM-2 is mainly expressed on endothelial cells, and its expression is not increased in response to cytokines. During transmigration, ICAM-1 tends to cluster and form a cup-like structure that surrounds the site of transmigration [26, 27]. One study showed that this ICAM-1 engagement caused a permeability increase which was mediated by Src phosphorylation of caveolin-1 both *in vivo* and *in vitro* [116]. Also ICAM-1 was found to co-localize with F-actin in endothelial cells and to trigger the remodeling of the actin cytoskeleton by forming a ring-like structure [117]. In addition to ICAM-1 and ICAM-2, some other adhesion molecules are also involved in leukocyte-endothelial cell interactions, such as VCAM-1 and PECAM-1 [118].

6.2 Leukocyte-endothelial cell interactions and microvascular permeability

The leukocyte-endothelial cell interaction is a crucial process in inflammation. It is necessary for the circulating leukocytes to transmigrate through

the endothelial cells to inflammatory sites. The transendothelial migration process causes cell shape changes and actin remodeling which also contribute to vascular permeability increases. To date, whether the interaction between leukocytes and endothelial cells directly causes the increase of microvascular permeability is still controversial. Some studies have shown that the leukocyte-endothelial cell interaction is a critical component that leads to vascular dysfunction. In the ischemia model, neutrophil adhesion was associated with an increase in vascular permeability. And prevention or depletion of neutrophils significantly prevented the permeability increases [119, 120]. But inhibition of neutrophil adhesion did not completely eliminate the vascular dysfunction [121]. It seems that this interaction between leukocytes and endothelial cells is an important factor but not the only factor in microvascular permeability increases. In the postcapillary venule, the ICAM-1 ligation, which is required by the leukocyte-endothelial cell interaction, causes permeability increase. And leukocyte adhesion, not leukocyte rolling is involved in the TNFα-mediated permeability increases [122]. During leukocyte transmigration, in endothelial cells, there is an elevation of cytosolic free calcium concentration which is required for transendothelial migration and causes increases in endothelial permeability [123, 124]. Neutrophil adhesion has been proved to trigger the disorganization of AJs and degradation of endothelial catenins which is a component of AJs [125, 126]. It is known that leukocytes adherent to endothelial cells are a major source of ROS, which is critical for the destruction and death of bacteria in

inflammation [127, 128]. ROS also can cause gap formation, shape change and actin reorganization of endothelial cells [128]. All of these changes contribute to microvascular permeability increases.

However, some studies found that leukocyte adhesion and emigration are uncoupled with vascular permeability. Blocking leukocyte adhesion had no effect on leakage of venules in response to histamine, wherein some leaky venules there were no adherent leukocytes [129]. Another group reported that sites of leukocyte adhesion and migration were not associated with vascular leakage [130]. In a wound model, both permeability and neutrophil recruitment increased over 24h, but permeability reached peak levels at 6 h, faster than neutrophils (18 h). Inhibition of neutrophil influx by 90% had no effect on vascular permeability at 24 h. These research data demonstrated that leukocyte adhesion and emigration are temporally and spatially uncoupled with the increases of vascular permeability.

Hypothesis

Leukocyte-specific protein 1 (LSP1) is an F-actin- and Ca^{2+}-binding, intracellular cytoskeletal phosphoprotein expressed in hematopoietic lineage and endothelial cells. It has been shown to play an important role in neutrophil motility and recruitment during inflammation when neutrophils interact with the endothelial cells of postcapillary venules at the site of infection or injury. Some studies have shown that the interactions between leukocytes and endothelial cells could couple with microvasculature permeability increases. My hypothesis is that LSP1 is important in microvascular hyperpermeability during neutrophil recruitment in microvasculature *in vivo*.

Objectives

- To identify the role of LSP1 in microvascular permeability increases induced by different inflammatory stimuli.
- To identify the role of leukocyte-endothelial cell interactions in microvascular permeability increases induced by different inflammatory stimuli.

Materials and methods

1. Animals

Three types of mice were used in this study: C57BL/6, 129/SvJ and LSP1-deficient (*Lsp1*$^{-/-}$) mice (Background mice are 129/SvJ mice). All animal protocols were approved by the University Committee on Animal Care and Supply (UCACS) at the University of Saskatchewan and met the standards of the Canadian Association of Animal Care.

2. Intravital microscopy

Intravital microscopy is a very powerful tool to visualize movement of cells. An upright microscope (model BX61WI, Olympus) was connected with a 3CCD color video camera (DXC-900, Sony) for bright-field intravital microscopy. A HD-DVD video recorder (model LRH-890, LG Electronics Inc.) was connected with this camera to record video images for playback analysis and the captured images were projected onto a TV monitor (model 22LG30-UA, LG Electronics Inc.). A monochrome deep-cooled CCD digital camera (Retiga™ SRV, QImaging) was also connected to the microscope port for fluorescence intravital microscopy, the images from which were directly processed by a computer. A 20 × Olympus objective lens was used in the experiments.

3. Preparation of cremaster muscle for intravital microscopy

Male mice were anesthetized with an i.p. injection of a mixture of 10 mg/kg xylazine and 200 mg/kg ketamine hydrochloride. The mouse cremaster muscle preparation was used to study microvascular permeability changes and leukocyte recruitment in the microcirculation and tissue.

3.1 Mouse jugular vein catheterization

After anesthesia, the area over the right external jugular vein of the neck was shaved with an electric razor. The mouse was placed on its back on a surgical plate with the head oriented towards the experimenter. The four limbs of the mouse were fixed with tape. A horizontal incision (~1 cm) was made above the jugular vein anterior in the shaved area. A small curved forceps was used to create a subcutaneous pocket on anterior side of incision. The fat tissue was gently teased away from the incision site until the jugular vein was visualized. The jugular vein was isolated by placing small curved forceps under the vein. Two pieces (approximately 6 cm each) of 4-0 suture were fed under the vein. The anterior suture was tied with forceps to occlude vein. The catheter was prepared by attaching a PE-10 tubing to a needle with a 1cc syringe filled with 100 U/ml heparin saline solution. A small incision was made with needle in the vein between the anterior and posterior sutures making sure not to cut through the vessel. Using forceps to hold the catheter, the end was fed into the lumen of the vessel. The catheter was fed into the vessel about 2-4 mm, ensuring that

blood from the vein could flow freely in and out of the catheter. The catheter was fixed by tying off the posterior suture.

3.2 Preparation of cremaster muscle

The anesthetized mouse was placed ventral side up on the cremaster muscle board. To maintain physiological temperature of the mouse and cremaster muscle tissue, the board was connected to a water circulator and circulated with 37°C-water. The cremaster muscle board was also connected to a peristaltic pump that perfused the exposed muscle tissue with 37°C-warmed bicarbonate-buffered saline (131.9 NaCl, 4.7 KCl, 1.2 MgSO4, 20 NaHCO3, in mM, pH 7.4) to keep the exposed muscle moist. An incision was made on the scrotal skin using a scissor. The left testicle was exposed and the underlying connective tissue was then carefully separated from the cremaster muscle. Using a cautery and 4-0 sutures, the cremaster muscle was cauterized longitudinally, spread and secured on the glass pedestal of the board. A glass coverslip (22×22 mm) was used to cover the muscle, and then the muscle was ready for viewing under the microscope.

A single postcapillary venule, 25–40 μm in diameter, was selected, and was moved to the middle of the screen. The number of rolling, adherent, and emigrated neutrophils was determined during video playback analysis. Rolling neutrophils were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. The flux of rolling neutrophil was measured as the number of rolling

neutrophils passing by a given point in the venule per minute. Neutrophil rolling velocity was measured for the first 20 neutrophils entering the field of view at the time of recording and calculated from the time required for a leukocyte to roll along a 100 μm length of venule. A neutrophil was considered to be adherent if it remained stationary for at least 30 s, and total neutrophil adhesion was quantified as the number of adherent cells within a 100-μm length of venule in 5 min. Neutrophil emigration was defined as the number of cells in the extravascular space (220×210 μm^2) adjacent to the observed venule.

4. Induction of neutrophil recruitment in cremaster muscle

To induce neutrophil recruitment in cremaster muscle, recombinant mouse TNFα (0.1 μg; R&D Systems, MN, USA) in 100 μl of saline was injected intrascrotally into C57BL/C, 129/SvJ or *Lsp1*$^{-/-}$ mice 4 h before each experiment. In some mice, exposed, untreated cremaster muscles were superfused with control superfusion buffer, 5 nM KC or MIP-2 (both from R&D Systems, MN, USA) for 1 h.

5. Microvascular permeability measurement

FITC-labeled bovine serum albumin (BSA) at 25 mg/kg (Sigma-Aldrich, MO, USA) was injected to the mice i.v. at the start of the experiment, and FITC-derived fluorescence (excitation at 495 nm, and emission at 525 nm) was detected using a monochrome deep-cooled CCD digital camera. The selected venule (25–40 μm in diameter) was kept in the middle of the screen and pictures taken at

different time points were saved in a computer. Image analysis software, METAMORPH (MetaMorph®, Molecular Devices Inc., PA, USA), was used to determine the intensity of FITC-albumin–derived fluorescence within the lumen of the venule and in the adjacent perivascular tissue. Background was defined as the fluorescence intensity before FITC-albumin injection. The index of vascular albumin leakage, permeability index, at different time points was determined according to the following ratio expressed as a percentage: (mean interstitial intensity - background)/ (venular intensity - background).

6. Depletion of circulating neutrophils in mice

Mice were injected (i.p.) with 200 μg/mouse anti-neutrophil antibodies (anti-mouse Ly-6G FG purified RB6-8C5, also called anti-GR-1, eBioscience, CA, USA) for 24 h. This treatment depleted circulating neutrophils by >98% [131]. We have confirmed the effect of this treatment by examination of peripheral blood cell count and differential cell count on a blood smear after 24-h anti-mouse Ly-6G treatment.

7. Statistical analysis

Data in this thesis represent mean ± SEM. Data were analyzed using One-way ANOVA, post hoc comparison or Student's t test. $P<0.05$ and $P<0.01$ were considered as statistically significant and very significant, respectively.

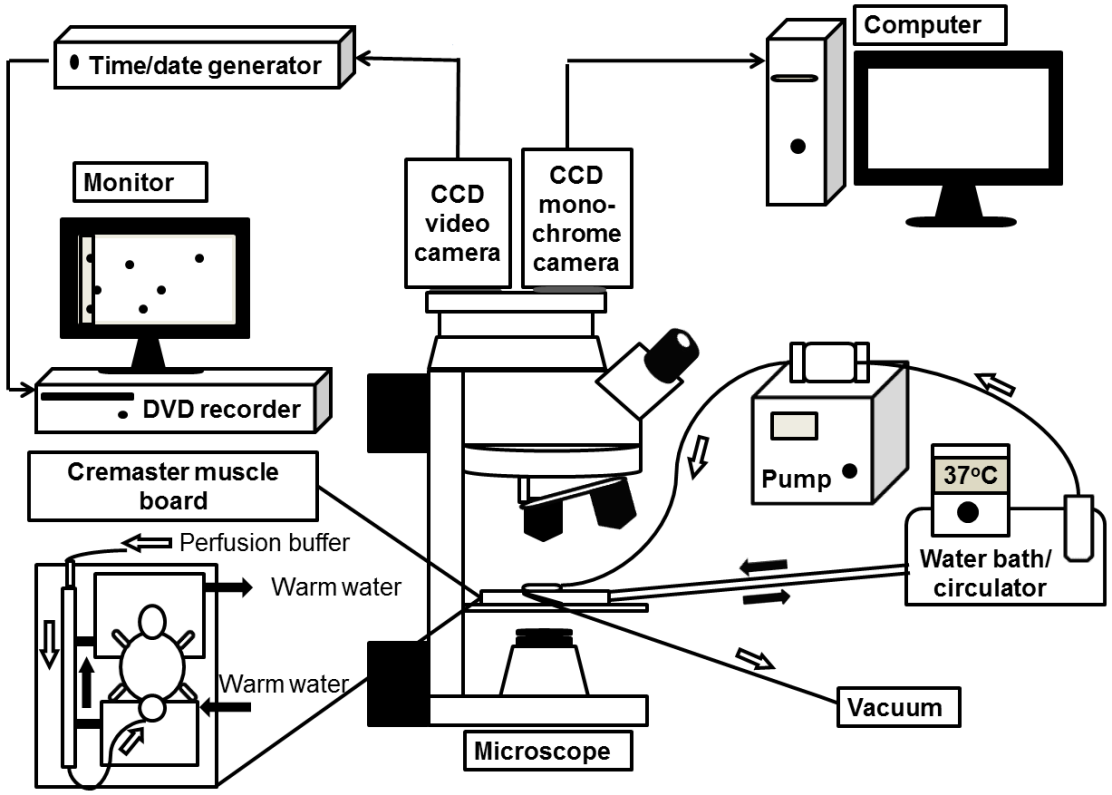


Figure 2. An intravital microscope system. An Olympus BX61WI up-right microscope is connected with two types of cameras, and the images are projected to a HD-DVD recorder and a TV monitor, and to a computer to record the images. A peristaltic pump and a 37°C-water circulation bath were used to maintain physiologic environment for the exposed cremaster muscle and for the anesthetized mouse.

Results

1. The effect of KC and MIP-2 on microvascular permeability changes

1.1 Both KC and MIP-2 increases neutrophil adhesion and emigration

MIP-2 and KC are two CXC chemokines that are expressed at the site of acute inflammation. These two chemokines have been shown to be involved in inducing neutrophil recruitment in microvasculature [132]. In my experiment, KC or MIP-2 was superfused on the exposed cremaster muscle to induce neutrophil recruitment in local postcapillary venules. Without KC or MIP-2 treatment, there were no chemokine-induced adherent and emigrated neutrophils in postcapillary venule. The baseline adherent and emigrated leukocytes, were always kept at 0-3 cells (per 100 μm-length venule over 5 min for adhesion and per field of view for emigration), a minimal level that is acceptable for intravital microscopy studies. After superfusion of KC or MIP-2 on the exposed cremaster muscle for 60 min, the adherent and emigrated neutrophils were determined. Figure 3 demonstrated that the numbers of adherent neutrophils in the cremasteric venules was significantly increased after KC or MIP-2 superfusion. Also a large number of neutrophils were found emigrated into the tissue. The induction of adhesion by MIP-2 was slightly higher than that by KC, whereas the emigration in response to MIP-2 was a little lower compared to KC. These differences are not significant, and the effects of MIP-2 and KC on neutrophil recruitment are similar.

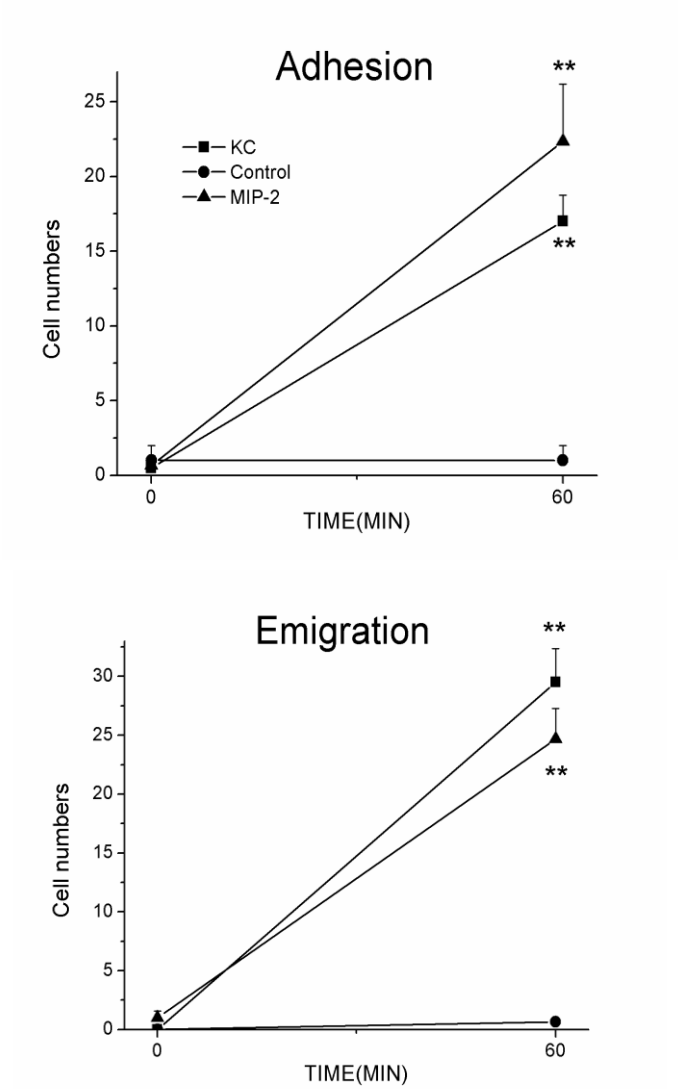


Figure 3. Effect of superfusion of KC or MIP-2 on neutrophil adhesion and emigration in cremasteric postcapillary venules of WT mice. KC or MIP-2 was dissolved in superfusion buffer at 5 nM and warmed to 37°C before superfusion. The control mice were superfused only with 37°C superfusion buffer. Data represent mean ± SEM from four mice per group. **, $P<0.01$, KC or MIP-2 compared with the control group.

1.2 The effect of KC and MIP-2 on venular permeability changes is similar

During 60 min superfusion of KC or MIP-2, the permeability index of postcapillary venule was measured at every 5 min. Without any treatment, vascular permeability was stable and normal. At 60 min, superfusion of KC or MIP-2 both caused a significant increase in microvascular permeability (Figure 4). Comparing the two curves representing KC and MIP-2 in Figure 4, we noticed that the increases of permeability in response to KC tended to be higher than MIP-2. In response to KC, permeability increases of postcapillary venules began to reach significant level at early time point of 20 min. The permeability index kept increasing and finally was about 30% increased over that in the control group. Under MIP-2 treatment, the increases of permeability were not significant until 55 min. The final increase was about 15% over that at the starting point and that in the control group. But there was no significant difference between the KC and MIP-2 groups ($P>0.05$). So we concluded that both KC and MIP-2 significantly increase microvascular permeability, and that the effect of KC and MIP-2 on permeability changes is similar.

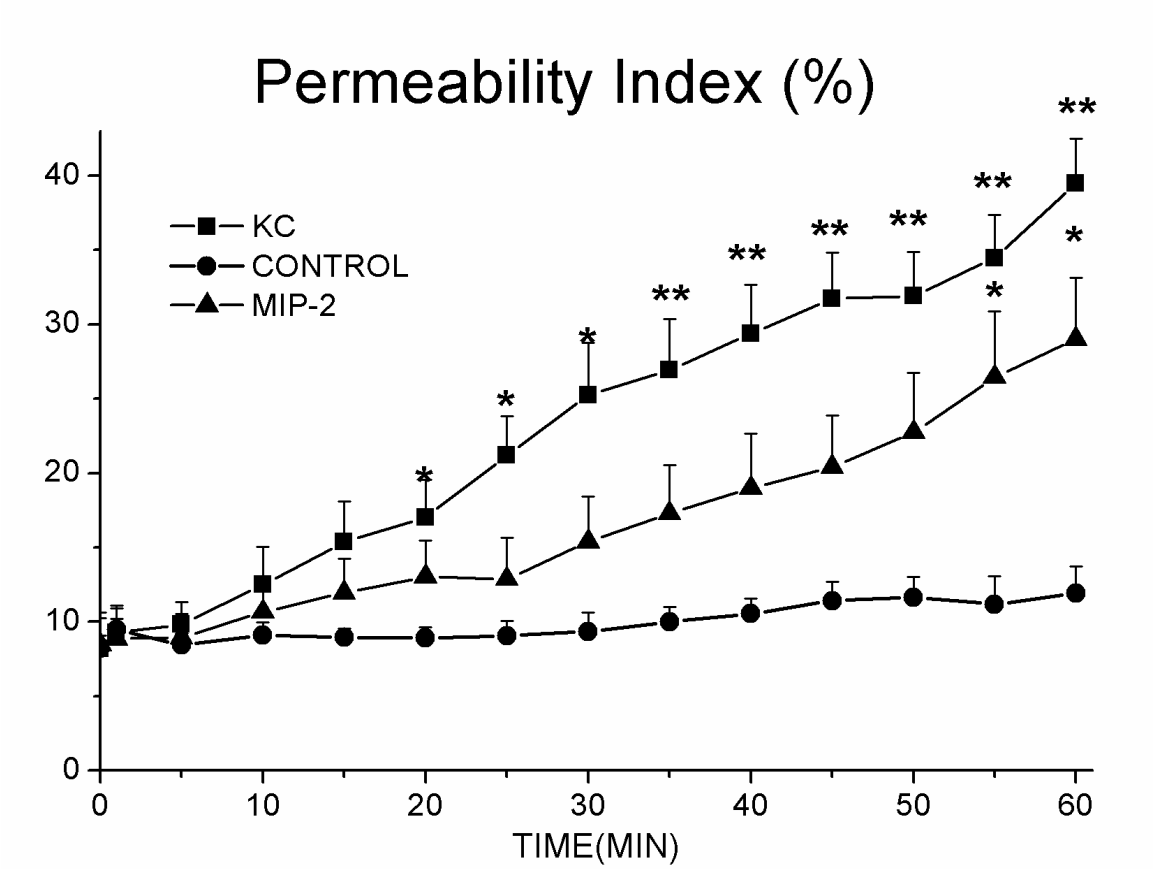


Figure 4. The increases of microvascular permeability after KC or MIP-2 superfusion in cremasteric postcapillary venules in WT mice. The protocol of KC or MIP-2 superfusion was described in the legend of Figure 3. Data represent mean ± SEM of the permeability index from four mice per group. *, $P<0.05$, **, $P<0.01$, KC or MIP-2 compared with the control group.

2. The effect of TNFα on microvascular permeability changes

TNFα has been known as a proinflammatory cytokine that is produced during acute inflammation and activates various inflammatory cells. The local intrascrotal injection of TNFα induces acute inflammation at the site. After 4 h of TNFα treatment, I began to measure the permeability index and neutrophil recruitment at postcapillary venules in cremaster muscle. Figure 5 shows that the increases in permeability reached significant levels at 15 min after 4 h TNFα treatment. At 60 min, the increase of permeability was about 35% over that in untreated control group. Therefore, TNFα treatment significantly increases permeability of postcapillary venule. Also, the adherent and emigrated neutrophils were significantly increased. More than 20 cells adhered to the venule and about 22-23 cells already migrated through the endothelial cells to the tissue (Figure 6).

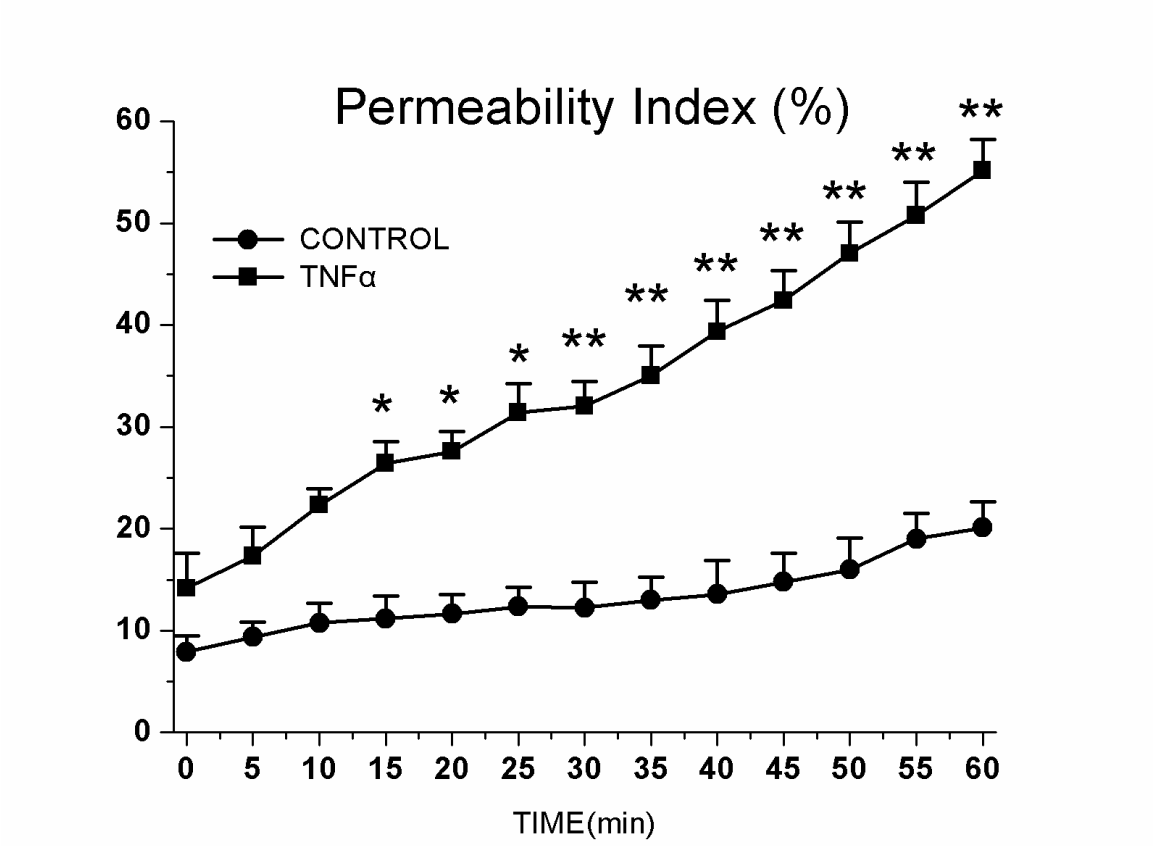


Figure 5. The increases of microvascular permeability in cremasteric postcapillary venules after 4 h TNFα treatment in WT mice. The mice were given intrascrotal injections of TNFα (100 ng/mouse in 100 μl sterile saline), and the microvascular permeability index was determined by intravital microscopy after 4 h TNFα treatment. The control mice were injected with saline alone. Data represent mean ± SEM of permeability index from three mice per group. *, $P<0.05$, **, $P<0.01$, TNFα compared with the control group.

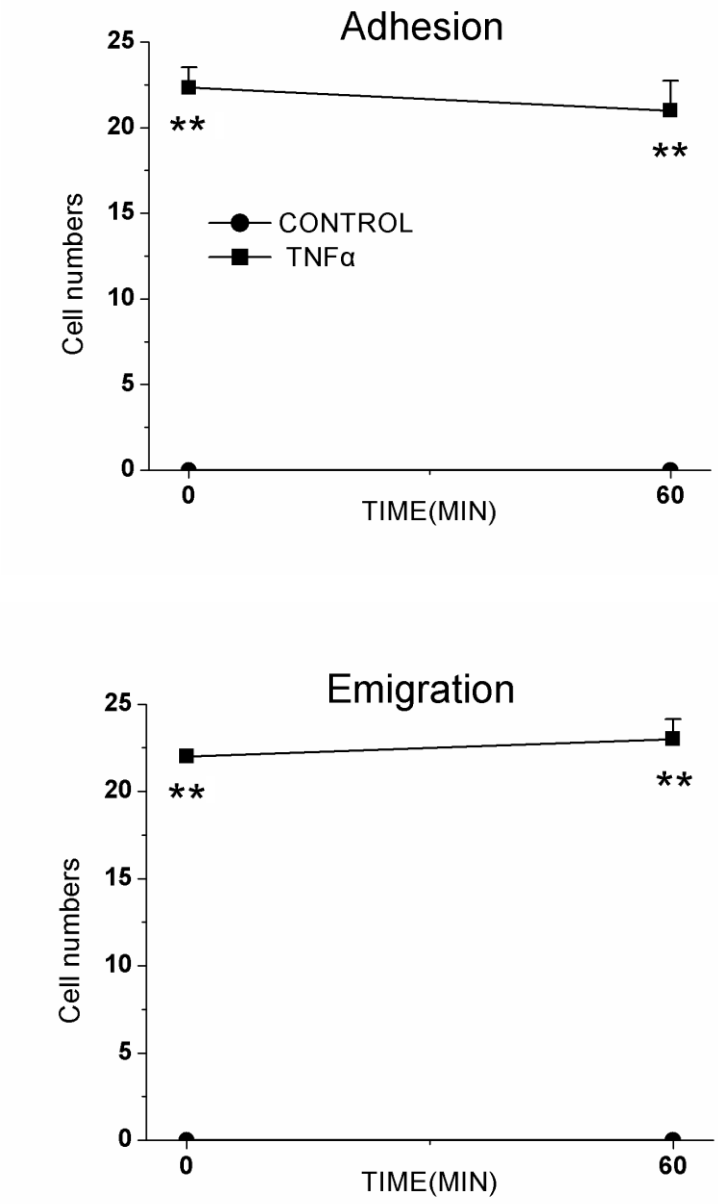


Figure 6. Effect of injection of TNFα on neutrophil adhesion and emigration in cremasteric postcapillary venules of WT mice after 4 and 5 h of TNFα injection. The protocol of TNFα injection was described in the legend of Figure 5. Data represent mean ± SEM from three mice per group. **, $P<0.01$, TNFα compared with the control group.

3. The role of LSP1 in hyperpermeability changes in microvasculature

3.1 The role of LSP1 in KC-induced hyperpermeability changes

To study the role of LSP1 in CXC-chemokine induced permeability changes, we used two types of mice 129/SvJ (WT) and *Lsp1*$^{-/-}$ mice in my experiments. Because I have shown that chemokine KC and MIP-2 have similar role in permeability change and neutrophil recruitment, I only used KC in my following experiments. Without any treatment, the deficiency of LSP1 did not affect permeability index compared with WT mice (Figure 7). At the resting condition of venule, LSP1 had no role in endothelial permeability. When KC was applied to cremaster muscle, the permeability index in WT and *Lsp1*$^{-/-}$ mice was significantly higher than the control group of each genotype. But there were no statistically significant differences in the permeability index between the two genotypes of mice after KC superfusion ($P>0.05$). The increases of permeability in both types of mice reached significant levels at 20 min. The increases in permeability lasted until at least 60 min. At 60 min, the increases of permeability in response to KC were similar between WT mice and *Lsp1*$^{-/-}$ mice (Figure 7).

After 60 min exposure of cremaster muscle to KC, I measured neutrophil adhesion and emigration cells in the venules. Figure 8 demonstrates a significant inhibition of neutrophil adhesion and emigration in *Lsp1*$^{-/-}$ mice compared with WT mice in response to KC. LSP1 is important for neutrophil

adhesion and emigration in response to KC superfusion.

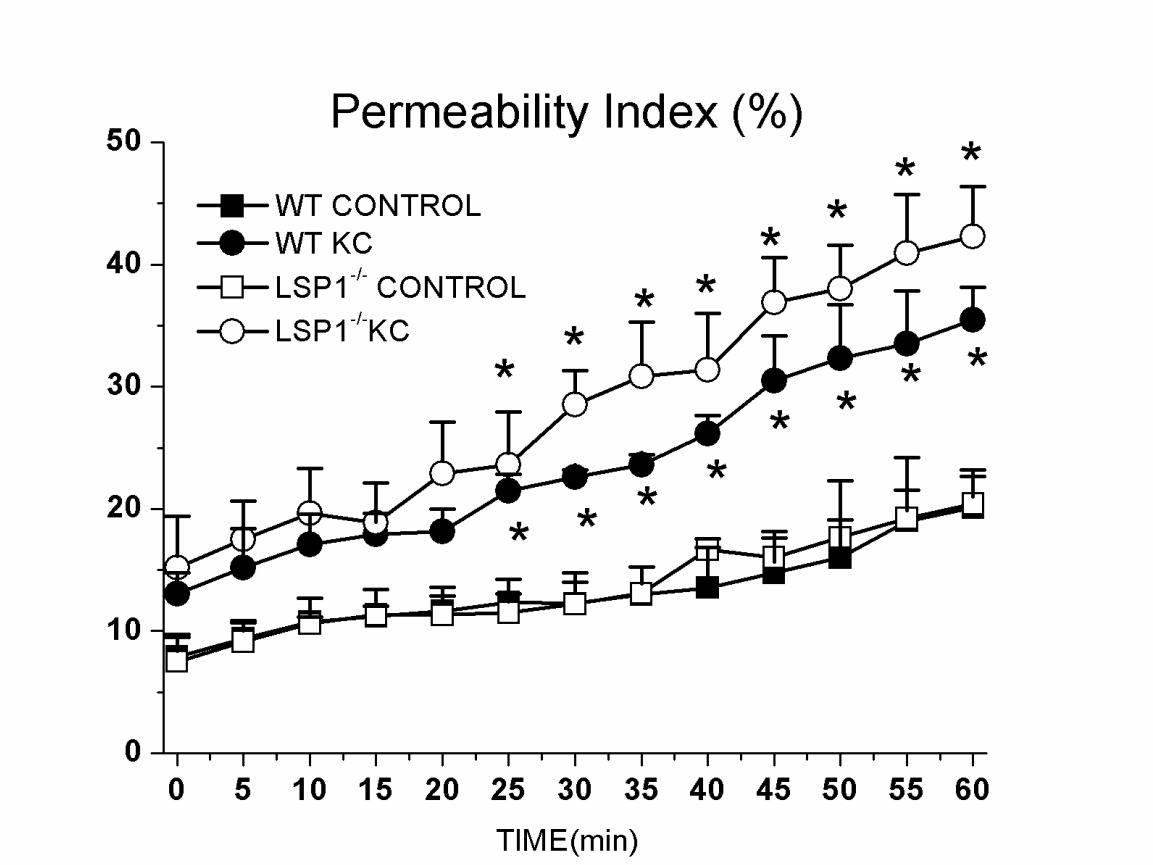


Figure 7. Change of microvascular permeability after KC superfusion in cremasteric postcapillary venules in WT and *Lsp1*$^{-/-}$ mice. The protocol of KC superfusion was described in the legend of Figure 3. Data represent mean ± SEM of permeability index from three mice per group. *, P<0.05, KC-superfused group compared with the control group.

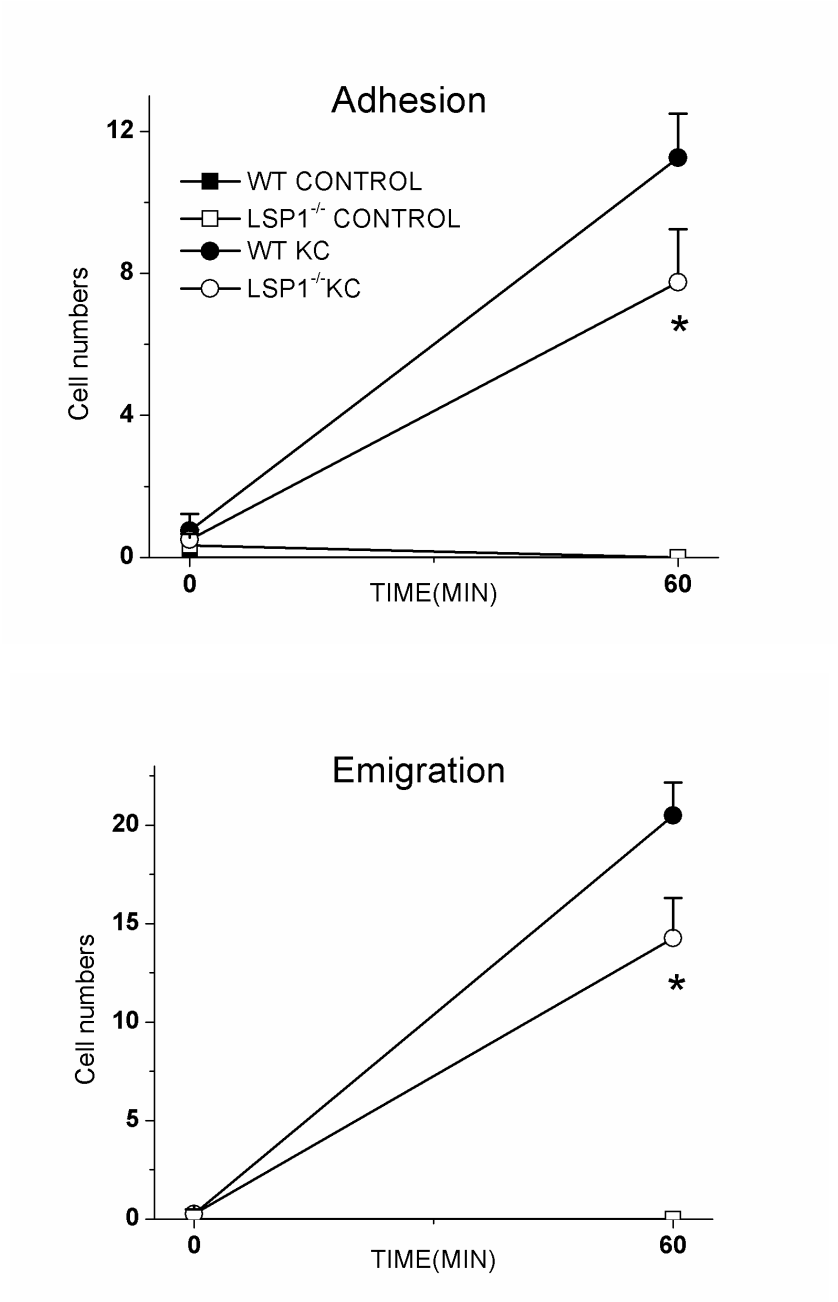


Figure 8. Effect of superfusion of KC on neutrophil adhesion and emigration in cremasteric postcapillary venules of WT and *Lsp1*$^{-/-}$ mice. The protocol of KC superfusion was described in the legend of Figure 3. Data represent mean ± SEM from three mice per group. *, P<0.05, WT mice compared with *Lsp1*$^{-/-}$ mice.

3.2 The role of LSP1 in TNFα-induced vascular hyperpermeability changes

To study the role of LSP1 in TNFα-induced vascular hyperpermeability, we have used TNFα intrascrotal injection for 4-5 h in *Lsp1*$^{-/-}$ and WT mice. Previous results in this thesis demonstrated that TNFα caused significant increases in vascular permeability. In this part, I studied the role of LSP1 in this vascular permeability change induced by TNFα. Similar to KC, the increases of permeability in both WT and *Lsp1*$^{-/-}$ mice in response to TNFα were comparable (Figure 9), but the deficiency of LSP1 had no effect on adhesion (Figure 10). Both types of mice had more than 20 cells adhering to the venule in 5 min at 4 h and 5 h after TNFα treatment. Very significant inhibition of emigration (~50%) was noted in *Lsp1*$^{-/-}$ mice compared to that in WT mice in response to TNFα (Figure 10). This suggested that LSP1 is important for neutrophil emigration but does not play a role in neutrophil adhesion to postcapillary venules in response to 4-5 h TNFα treatment.

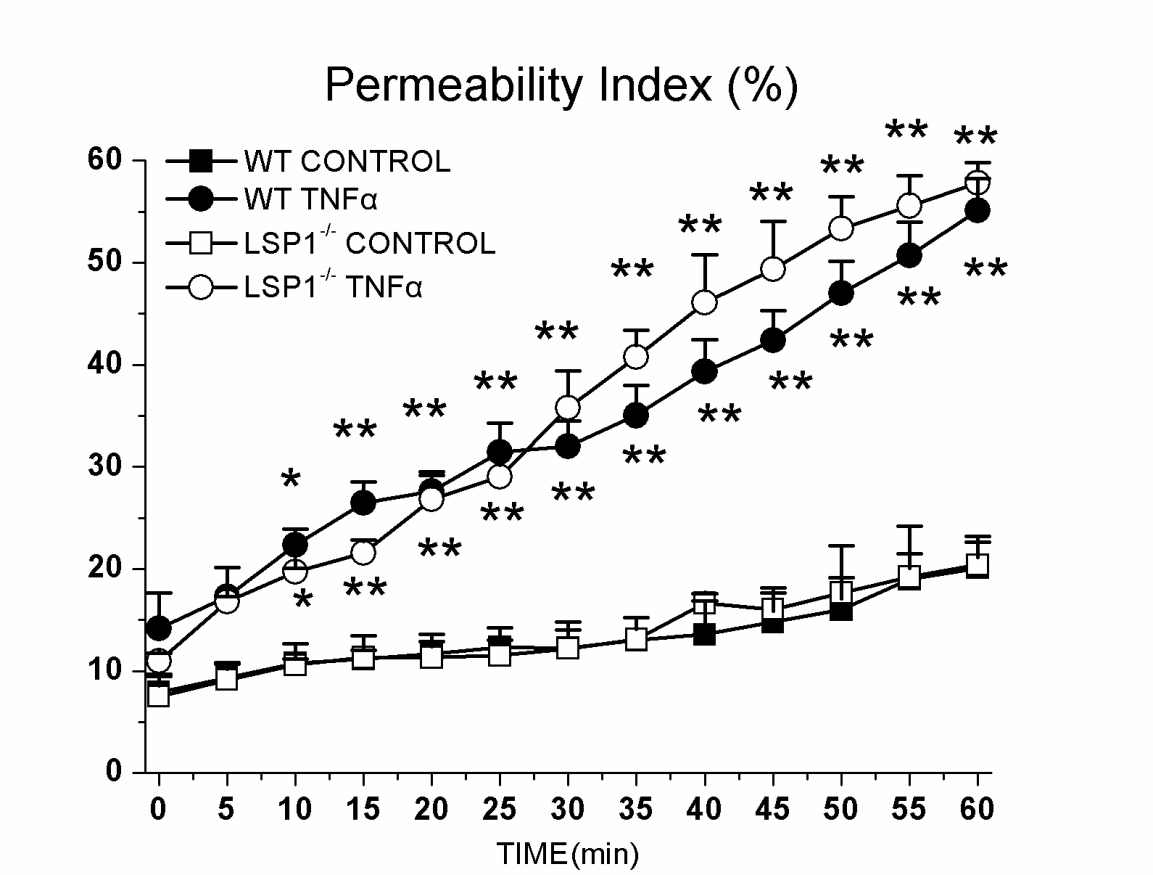


Figure 9. Change of microvascular permeability after TNFα injection in cremasteric postcapillary venules in WT and *Lsp1*$^{-/-}$ mice. The protocol of TNFα injection was described in the legend of Figure 5. Data represent mean ± SEM of permeability index from three mice per group. *, P<0.05; **, P<0.01, TNFα compared with the control group.

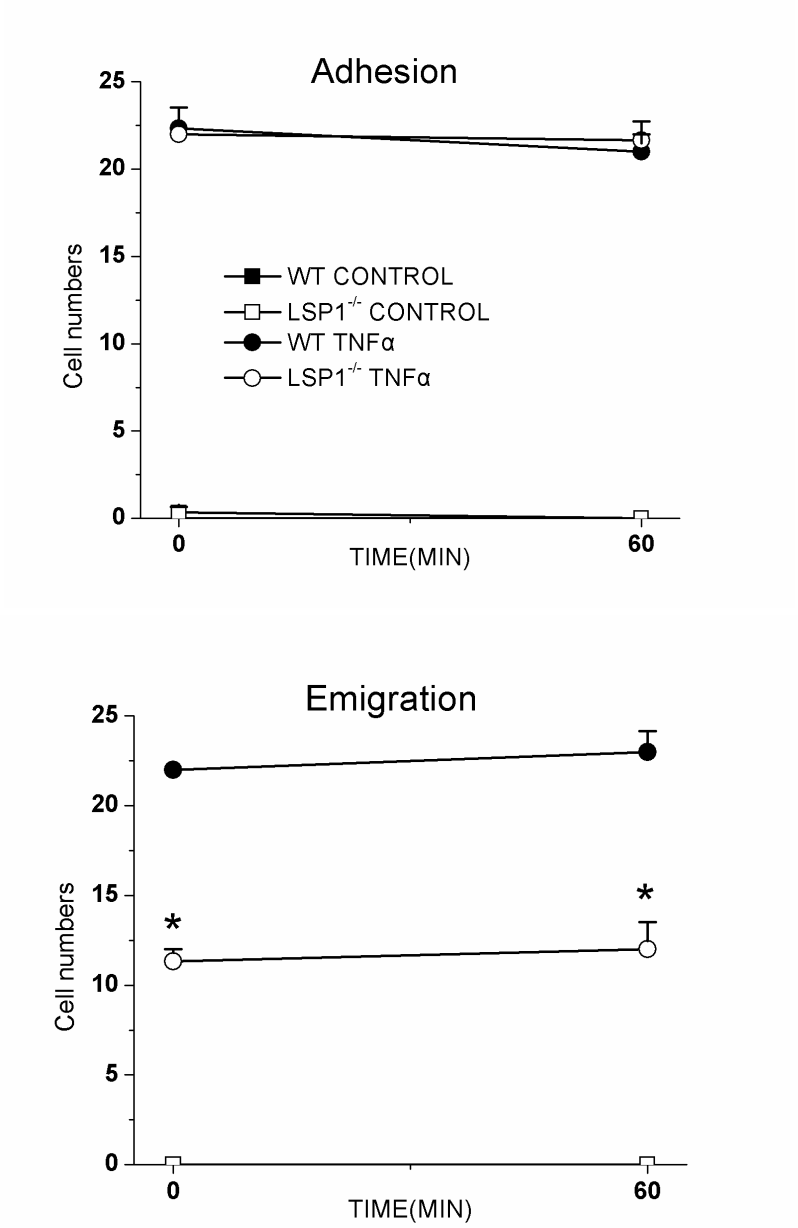


Figure 10. Effect of TNFα injection on leukocyte adhesion, and emigration in cremasteric post-capillary venules of WT and *Lsp1*$^{-/-}$ mice. The protocol of TNFα injection was described in the legend of Figure 5. Data represent mean ± SEM from three mice per group. *, P<0.05, *Lsp1*$^{-/-}$ mice compared with WT mice.

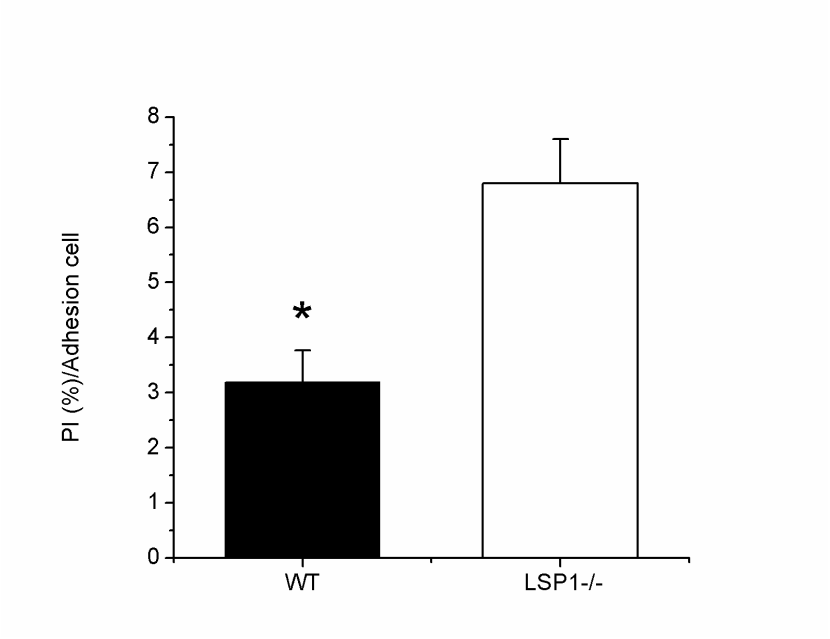
3.3 Loss of LSP1 impairs neutrophil emigration without changing microvascular permeability

3.3.1 Adhesion and permeability increases

The role of LSP1 in neutrophil adhesion induced by TNFα is different from that induced by KC. The deficiency of LSP1 had no effect on adhesion in response to TNFα as measured between 4-5 h after treatment, but reduced the adhesion in response to KC as measured across 1 h after treatment. However, under both treatment conditions the increases of permeability in *Lsp1*$^{-/-}$ mice were still comparable with that in WT mice. I calculated the permeability index per adherent neutrophil ratio at 60 min to determine the contribution of each adherent cell to the permeability increases. In response to KC, each adherent *Lsp1*$^{-/-}$ neutrophil contributed much more than each adherent WT neutrophil to the permeability index increases (Figure 11). But under TNFα stimulation, there was no difference of the contribution to permeability increases in each adherent *Lsp1*$^{-/-}$ or WT neutrophil (Figure 12). This result suggested that LSP1 has a role in KC-induced adhesion of neutrophils to the postcapillary venules and has an important impact on KC-induced, neutrophil adhesion-related vascular permeability increases. This also suggested that LSP1 does not play a role in TNFα-induced neutrophil adhesion to the inflamed venule and has no impact on TNFα-induced, neutrophil adhesion-related venular permeability increases.

3.3.2 Emigration and permeability increases

After 60 min of KC superfusion and 5 h of TNFα treatment, the neutrophil emigration in WT mice was significantly higher compared with *Lsp1*$^{-/-}$ mice in response to both KC and TNFα (Figure 8 and 10), and also there was no difference in permeability increases between two types of mice after the treatment (Figure 7 and 9). Comparing the value of permeability index per emigrated neutrophil, it is obvious that the contribution of each emigrated *Lsp1*$^{-/-}$ neutrophil was significantly higher than each emigrated WT neutrophil (Figure 11 and 12). These results confirmed that LSP1 has an important role in the regulation of neutrophil emigration induced by either cytokine or chemokine and indicated that LSP1 contributes to the integrity of the endothelium during neutrophil emigration; deficiency of LSP1 substantially increases the vascular permeability induced by each transmigrating neutrophils.



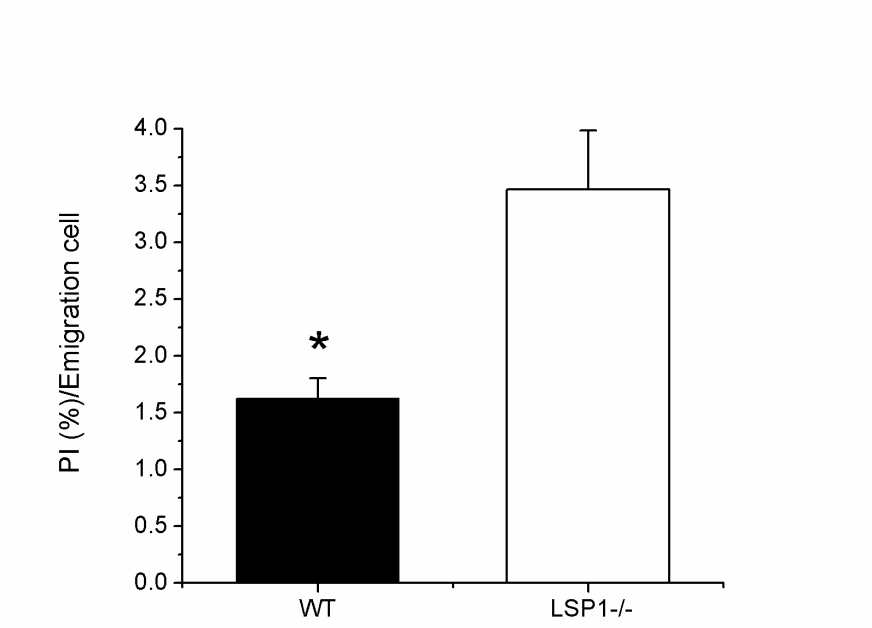
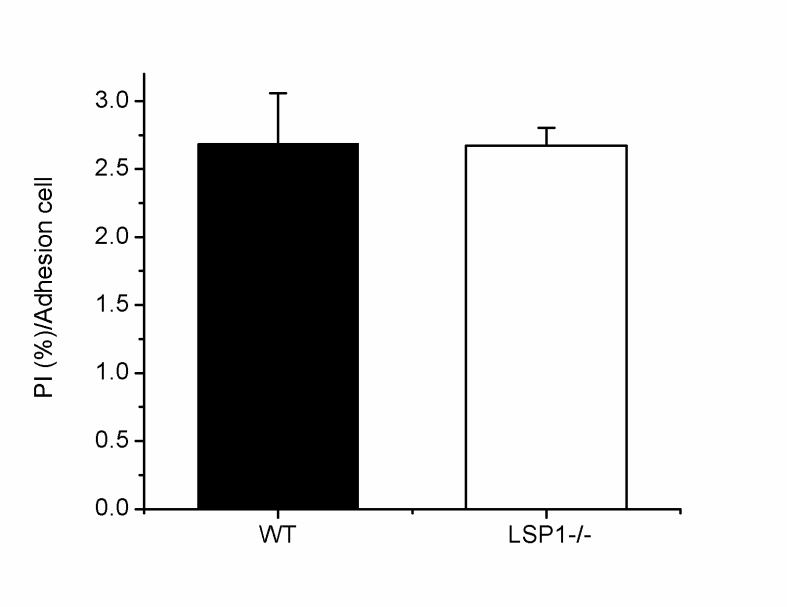


Figure 11. Permeability index per adherent and migrating neutrophil in response to KC superfusion for 60 min. Data represent mean ± SEM from three mice per group. *, P<0.05, WT mice compared with *Lsp1*$^{-/-}$ mice.



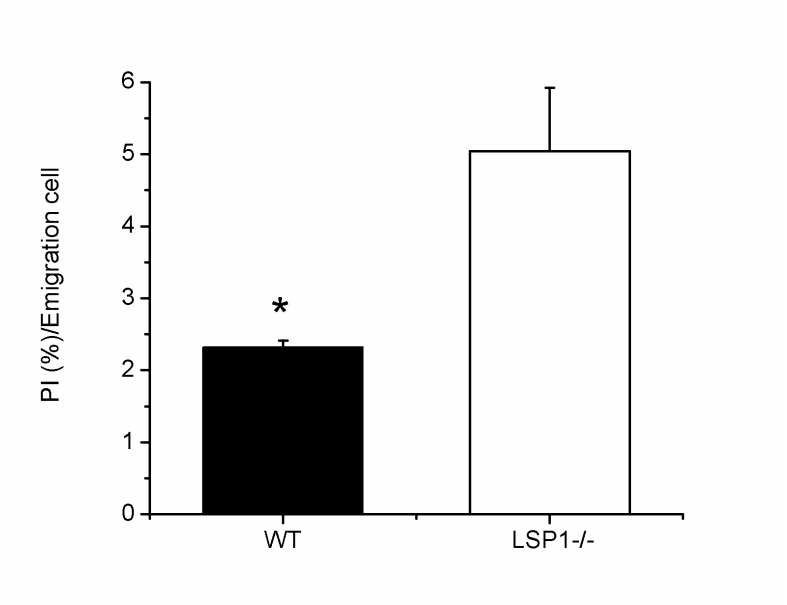


Figure 12. Permeability index per adherent and emigrated neutrophil in response to TNFα 5 h treatment. Data represent mean ± SEM from three mice per group. *, P<0.05, WT mice compared with *Lsp1*$^{-/-}$ mice.

4. The role of neutrophil-endothelial interactions in microvascular permeability changes

It was reported that at 24 h after the injection of anti-neutrophil antibody anti-Gr-1 into mice, more than 98% neutrophils in the circulation are depleted [131]. We have confirmed the effect of this antibody in the depletion of circulating neutrophils. After KC or TNFα administration in these neutrophil-depleted mice, there were almost no rolling neutrophils in the venules. Consequently, the number of adherent and emigrating neutrophils was extremely low (Figure 13 and 14). This depletion of neutrophils in circulation completely diminished the interactions between neutrophils and endothelial cells. The depletion of neutrophils also reduced the permeability increases induced by KC to the baseline in both mice strains (Figure 15). This suggested that the increases of permeability were basically caused by neutrophil-endothelial interactions in response to KC in both types of mice. Similar results were obtained in response to 4 to 5 h TNFα treatment (Figure 16). The depletion of neutrophils also significantly inhibited the permeability increases induced by TNFα. My data indicated that, at 4-5 h after TNFα (100 μg) local treatment, it is the neutrophil-endothelial cell interaction induced by TNFα, but not the activation of endothelium by TNFα treatment that is important in microvascular permeability increases in WT and *Lsp1*$^{-/-}$ mice.

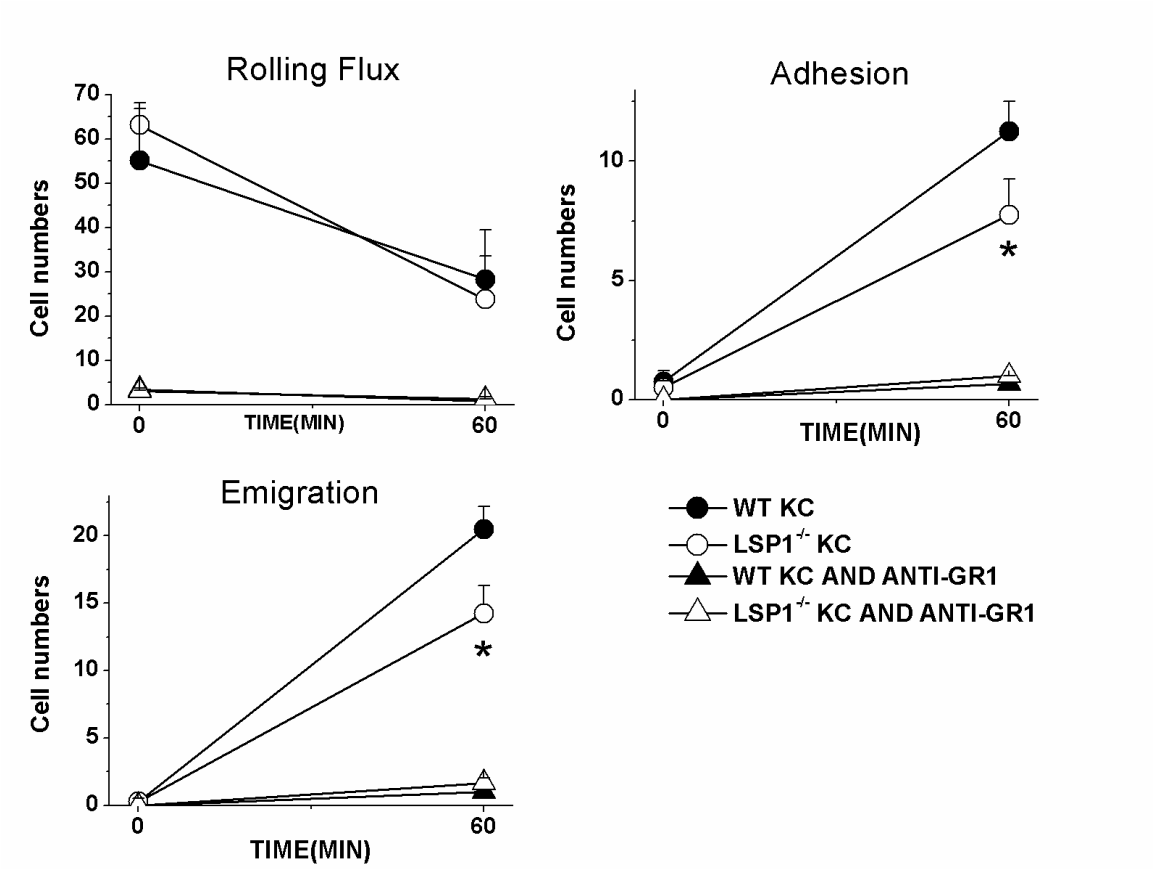


Figure 13. Effect of superfusion of KC on leukocyte rolling flux, adhesion, and emigration in cremasteric postcapillary venules of WT and *Lsp1*$^{-/-}$ mice after 24 h anti-neutrophil antibody i.p. injection. Because the leukocyte rolling flux was close to zero at 24 h after anti-Gr-1 treatment, leukocyte rolling velocity could not be determined. The protocol of KC superfusion was described in the legend of Figure 3. Data represent mean ± SEM of from three mice per group. *, $P<0.05$, WT with KC compared with *Lsp1*$^{-/-}$ mice with KC.

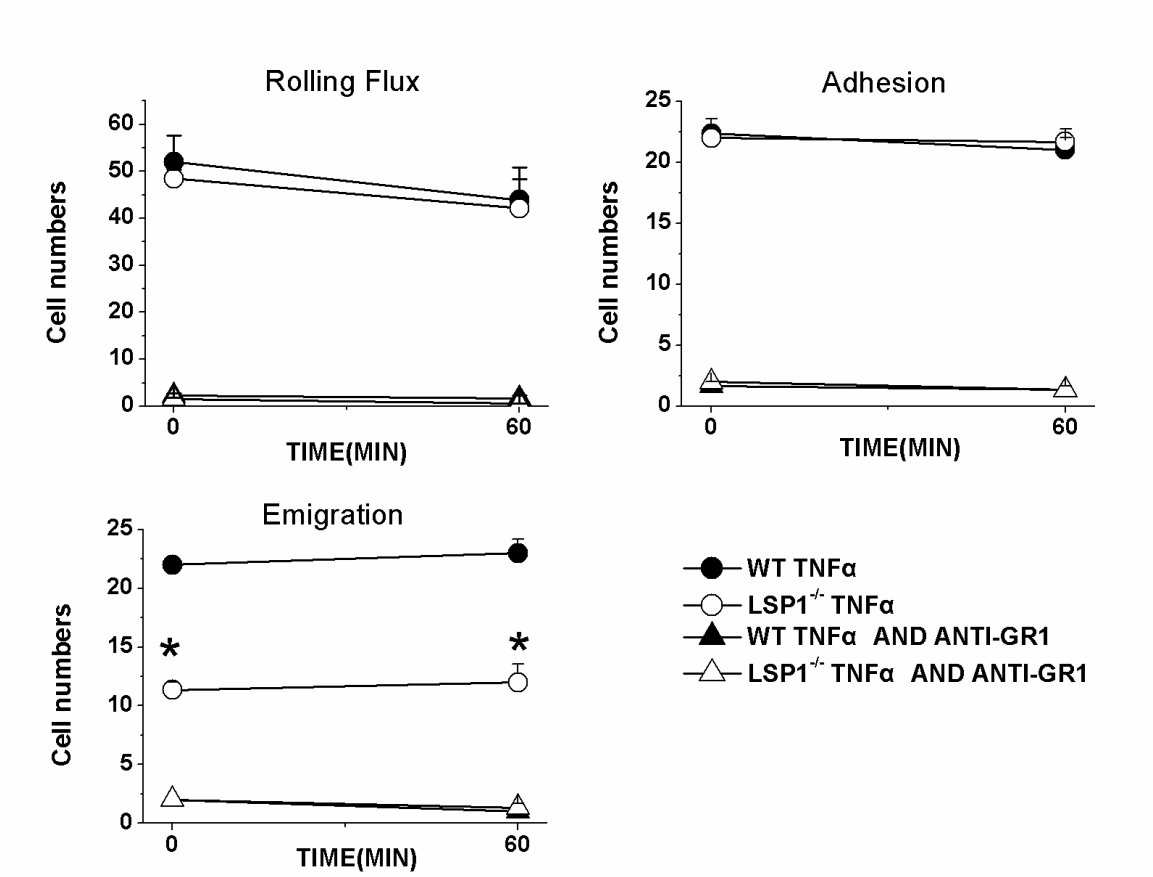


Figure 14. Effect of TNFα injection on leukocyte rolling flux, adhesion, and emigration in cremasteric postcapillary venules of WT and *Lsp1*$^{-/-}$ mice after 24 h anti-neutrophil antibodies i.p. injection. Because the leukocyte rolling flux was close to zero at 24 h after anti-Gr-1 treatment, leukocyte rolling velocity could not be determined. The protocol of TNFα injection was described in the legend of Figure 5. Data represent mean ± SEM of from three mice per group. *, P<0.05, WT with TNFα compared with *Lsp1*$^{-/-}$ mice with TNFα.

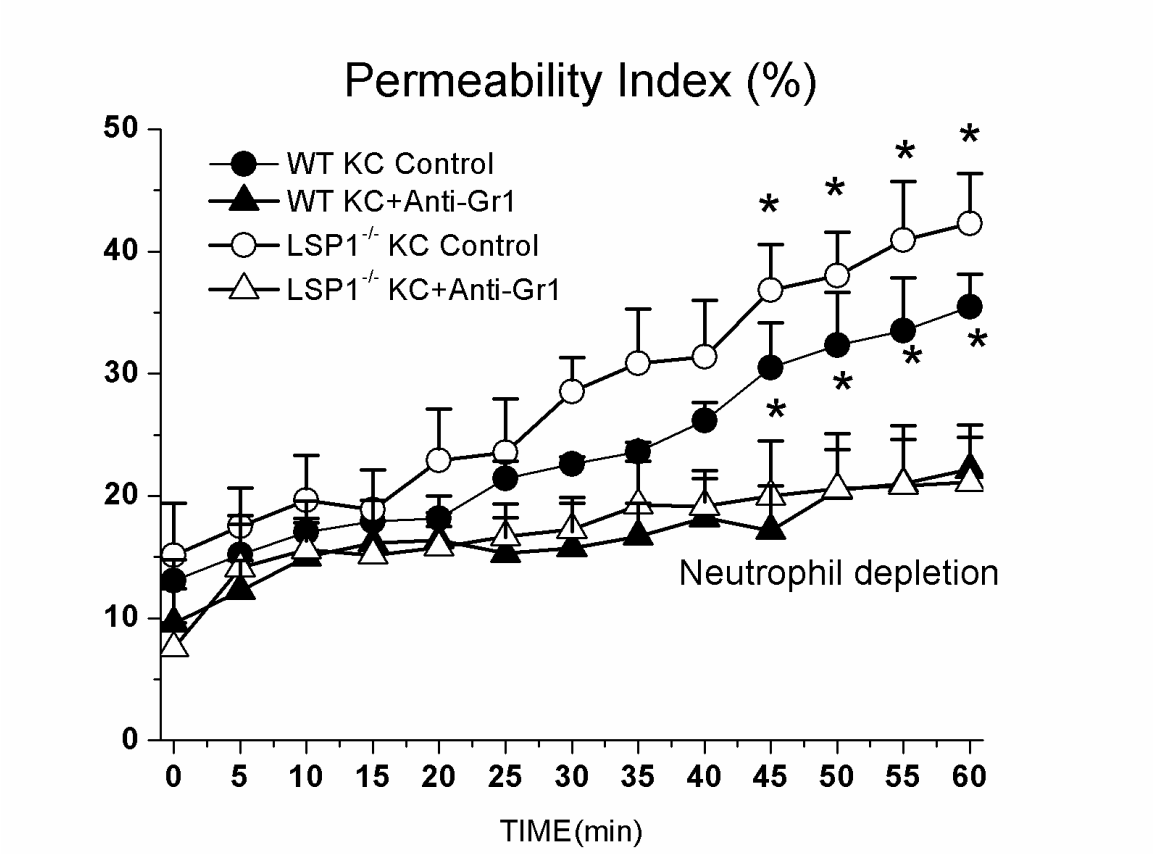


Figure 15. The changes in microvascular permeability after KC superfusion in cremasteric postcapillary venules in WT and *Lsp1*$^{-/-}$ mice after 24 h anti-neutrophil antibody i.p. injection. The protocol of KC superfusion was described in the legend of Figure 3. Data represent mean ± SEM of permeability index from three mice per group. *, $P<0.05$, KC compared to KC with anti-Gr-1.

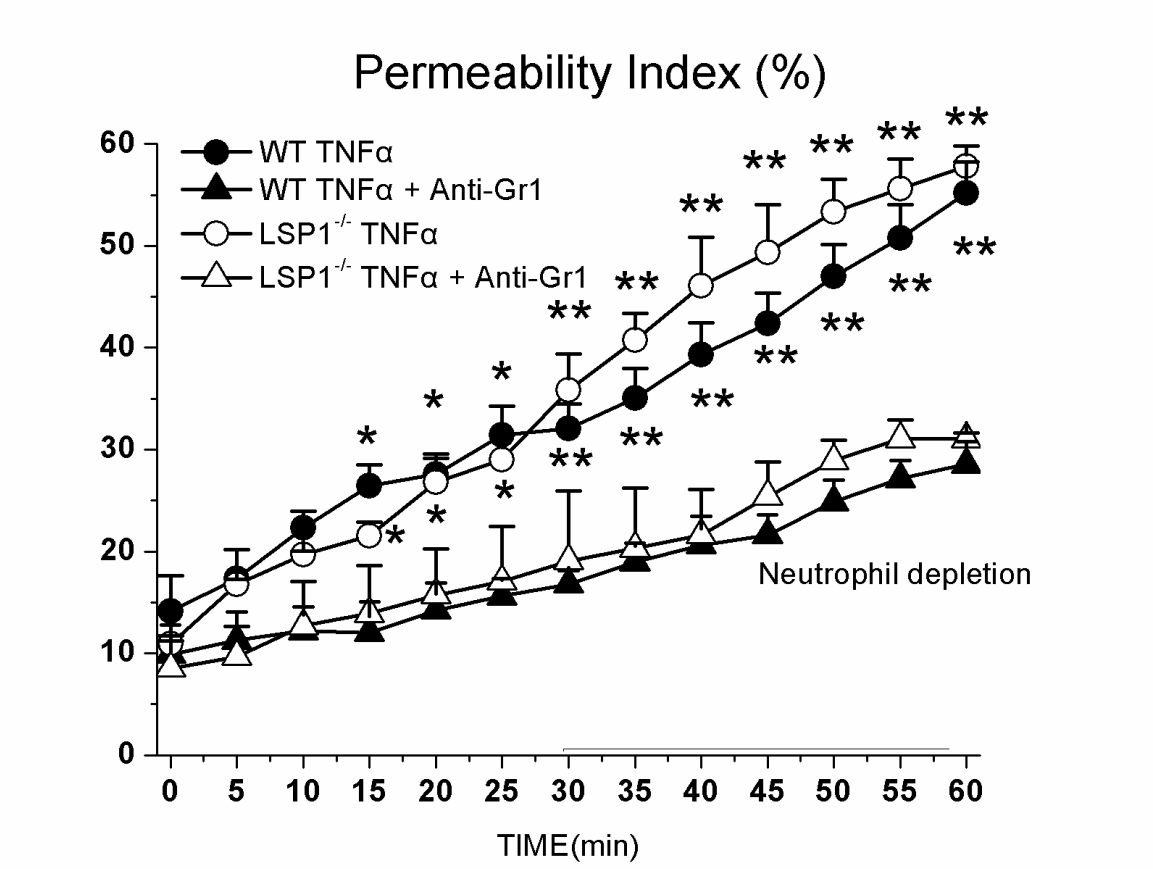


Figure 16. The changes in microvascular permeability after TNFα injection in cremasteric postcapillary venules in WT and *Lsp1*$^{-/-}$ mice after 24 h anti-neutrophil antibody i.p. injection. The protocol of TNFα injection was described in the legend of Figure 5. Data represent mean ± SEM of permeability index from three mice per group. *, P<0.05, and **, P<0.01, TNFα compared to TNFα with anti-Gr-1, respectively.

Discussion

Acute inflammation is characterized by the recruitment of leukocytes and vascular hyperpermeability at the postcapillary venules. In the inflamed tissue, microvascular permeability increases and fluid and proteins leak out of blood vessel to the extravascular interstitial space. These exudates contain complement proteins and antibodies which can kill harmful invading microorganisms [1]. Meanwhile, leukocytes in blood stream are stimulated and activated, and they emigrate along the chemoattractant gradient to the inflamed site. Some studies have shown that leukocyte recruitment and permeability increases in microvasculature are two coupled events [119, 120], but some other studies have indicated there is a disassociation between permeability changes and leukocyte recruitment [129, 130]. Therefore, there is still a controversy. LSP1, a 52kDa intracellular Ca^{2+} and F-actin binding protein[91], has been shown to have an important role in leukocyte transendothelial migration induced by CXC chemokine KC and cytokines TNFα and IL-1β and in the permeability increases induced by histamine [106]. But the role of LSP1 in permeability changes induced by chemokines and cytokines has not been studied. The primary goal in my study in this thesis is to investigate the role of LSP1 in neutrophil recruitment and microvascular permeability changes during neutrophil-endothelial cell interactions in the neutrophil recruitment process.

In this study, we found that during neutrophil recruitment, microvascular permeability in postcapillary venules was increased, and that after depletion of circulating

neutrophils, therefore abolishing neutrophil-endothelial cell interactions, there was no microvascular permeability increases. Thus, at dosages of KC and TNFα that we tested in the induction of neutrophil recruitment, the hyperpermeability in postcapillary venules is due to neutrophil-endothelial cell interactions. Interestingly, LSP1 alone does not contribute to the hyperpermeability in postcapillary venules and the role of LSP1 in hyperpermeability is through neutrophil-endothelial cell interactions. Loss of LSP1 causes each adherent or transmigrating neutrophil to contribute much more to the hyperpermeability during neutrophil recruitment.

In this study, depletion of neutrophils by anti-neutrophil antibody anti-GR-1 in the circulation almost totally diminished the interactions between neutrophils and endothelial cells in the inflamed tissues. Without neutrophil-endothelial cell interactions, there was no increase in vascular permeability in the postcapillary venules after KC superfusion or after 4-5 h TNFα treatment. Therefore, the hyperpermeability in postcapillary venule induced by KC or TNFα was due to neutrophil-endothelial cell interactions. How neutrophil-endothelial interactions increase vascular permeability is not completely understood, but there may be several possible mechanisms that are involved. Firstly, transendothelial migration of neutrophils disrupts junctional molecules which are important in maintaining endothelial barrier functions. VE-cadherin, which is the major component of AJs, is located in endothelial cell contacts when there is no neutrophil trafficking. With neutrophil adherence, VE-cadherin is redistributed and is no longer in the intercellular space. The β-catenin linking to AJs is also redistributed. This

disruption of AJs causes permeability increases [126, 133] . A research group reported that activated neutrophils can induce phosphorylation of two major AJ proteins, VE-cadherin and β-catenin, which is accompanied by the distribution of these two proteins [134]. Neutrophil transmigration can also disrupt TJs between endothelial cells. During neutrophil transmigration, ZO-1 and occludin disappear from the endothelial border, and ZO-2 expression level is significant reduced. Occludin was found to be cleaved to a smaller fragment [135]. Some other junctional molecules have also been reported to have roles in neutrophil transmigration, such as PECAM-1 and JAMs. Therefore, it is reasonable that the disruption in the integrity of endothelial junctions causes increases in endothelial permeability.

Secondly, the interactions between neutrophils and endothelial cells activate endothelial cells to further activate certain signal pathways in the two cell types. Gautam *et al.* reported that cross-linking β2-integrins between neutrophils and endothelial cells causes increases in endothelial cell cytosolic calcium concentrations and F-actin content, and subsequently leads to permeability increases [136]. Further studies showed that activated neutrophils promote endothelial cell MLC phosphorylation which causes endothelial cell retraction and permeability increases [137, 138]. The adhesion of neutrophils to endothelial cells causes an elevation of the cytosolic free calcium concentrations in endothelial cells which is required for subsequent neutrophil transendothelial migration and is important for the increases in endothelial permeability [123, 124].

Finally, activated neutrophils release various compounds which may induce vascular permeability increases. Activated neutrophils have been reported to be a source of ROS and the production of ROS is important for vascular permeability increases [139] . The production of ROS triggers the internalization of VE-cadherin in endothelial junctions and increases endothelial permeability [140]. Heparin-binding protein (HBP), a member of the neutrophil cationic proteins, was reported to increase endothelial permeability. The secretion of HBP can be triggered by cross-linking of β2 integrin, and this secretion induces the rearrangement of the endothelial cell cytoskeleton. HBP acts as a mediator for neutrophil adhesion-induced permeability increases [141]. It is also reported that activated neutrophils produce leukotrienes and prostaglandins, which increase endothelial permeability [51, 142].

By using *Lsp1*$^{-/-}$ mice in this study, we reported that loss of LSP1 causes each adherent or transmigrating neutrophil to contribute more to the hyperpermeability during recruitment. After depletion of neutrophils in the circulation of WT or *Lsp1*$^{-/-}$ mice, LSP1 had no role in permeability changes, suggesting that the role of LSP1 in the hyperpermeability in postcapillary venules is through the neutrophil-endothelial cell interactions. It is well known that endothelial cells play an active role in neutrophil transmigration. During neutrophil-endothelial cell interactions, endothelial ICAM-1 relocalizes and is enriched in endothelial borders, forming a cup-like structure that surrounds the site of transmigration [26, 27]. This cup-like structure *in vitro* was described as the dome-like structure formation of endothelial cells *in vivo* [143]. It was

reported in a recent study that LSP1 is expressed in mouse and human endothelial cells and that endothelial LSP1 is important in neutrophil emigration induced by the CXC chemokine KC and the cytokine TNFα [106]. Petri and his colleagues reported very recently that after stimulation with TNFα, endothelial LSP1 association with the cytoskeleton region was significantly increased [112]. In WT mice, endothelial cells formed domes completely covering the transmigrating neutrophil. But in *Lsp1*$^{-/-}$ mice, endothelial cells could not form complete domes, and only covered the transmigrating neutrophil pseudopodia. A lack of LSP1 decreases the motility of endothelial cells during neutrophil transmigration [112]. The impaired motility of endothelial cells decreased the number of neutrophils crossing the endothelium, whereas incomplete dome formation in *Lsp1*$^{-/-}$ mice disrupted the integrity of the postcapillary venules leading to increased vascular permeability. In a previous study, LSP1 had been reported to have an important role in the hyperpermeability in postcapillary venules at 60 min treatment of histamine which did not induce neutrophil emigration [106]. LSP1 could directly affect hyperpermeability without involving neutrophil-endothelial cell interactions. Therefore, LSP1 may have different roles in vascular permeability increases under various inflammatory conditions.

As we demonstrated in this study, LSP1 has a role in hyperpermeability elicited by KC-induced neutrophil adhesion and emigration, and by TNFα-induced neutrophil emigration, but does not appear to be involved in the hyperpermeability in TNFα-stimulated neutrophil adhesion. This suggests the possibility that TNFα stimulates

an LSP1-independent signaling mechanism that is important for neutrophil adhesion-related regulation of microvascular permeability increases. A research group found that TNFα-induced neutrophil adhesion could be inhibited by Src, epidermal growth factor receptor (EGFR), and PI3K/Akt inhibitors, suggesting that TNFα induces neutrophil adhesion through Src, EGFR, and PI3K/Akt [144]. The mechanisms of TNFα-induced neutrophil adhesion are still under investigation.

In this study, I demonstrated that in response to KC or TNFα, the hyperpermeability in postcapillary venules is due to neutrophil-endothelial cell interactions, but mechanisms of neutrophil-induced hyperpermeability remain incompletely understood. Therefore, I raised some possibilities in this discussion on the possible mechanisms. My study demonstrated that LSP1 contributes to the integrity of the postcapillary venule through interactions between neutrophils and endothelial cells during neutrophil recruitment. The role of LSP1 in the hyperpermeability in postcapillary venules is still under investigation because it appears that LSP1 has different roles in hyperpermeability under various stimuli. Also, the mechanisms of TNFα-induced neutrophil adhesion require more studies. In this study, we only tested the effect of 4-5 h TNFα treatment on permeability increases and neutrophil recruitment. These time points are most commonly used for the study of mechanisms of inflammation and neutrophil recruitment when TNFα is applied. It could be better to examine the effect of TNFα treatment at different time points to gather more information on the role of LSP1 and the effect of neutrophil-endothelial cell interactions in mcirovascular permeability changes

during neutrophil recruitment. In future work, more advanced microscopy technologies such as spinning-disk confocal intravital microscopy and two-photon microscopy may be used to directly visualize and determine the whole process of neutrophil transendothelial migration and the increases of endothelial permeability in greater details.

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