

**The effect of pharmacological inhibition of
mitogen- and stress-activated protein kinase-1
(MSK1) on chemokine-induced neutrophil
recruitment**



**A Thesis Submitted to
The College of Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Department of Pharmacology
University of Saskatchewan**

By

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Abstract

Neutrophil recruitment to the site of acute inflammation is a multistep process regulated by specific signaling molecules. The signaling mechanisms that regulate neutrophil-endothelial cell interactions remain incompletely understood. p38 mitogen-activated protein kinase (MAPK) signalling was shown to regulate different steps of neutrophil migration in response to inflammatory stimuli. The mitogen- and stress-activated protein kinase-1 (MSK1) can be activated by either extracellular-signal-regulated kinase (ERK) 1/2 or p38 MAPK. The aim of the present study is to investigate the effects of pharmacological suppression of MSK1 by its specific inhibitor, SB747651A, on various steps of neutrophil recruitment. *In vivo* studies were conducted using real-time and time-lapsed intravital video microscopy of the cremaster microcirculation to determine the dynamic leukocyte-endothelial cell interactions. Intrascrotal injection of macrophage inflammatory protein-2 (MIP-2, 0.2 µg/mouse) decreased leukocyte rolling velocity which was significantly reversed by pre-treatment with SB747651A (intrascrotal injection of 3 mg/kg). SB747651A pre-treatment enhanced MIP-2-induced increase in neutrophil adhesion and emigration. To better understand the effect of SB747651A on different steps of neutrophil recruitment, we placed a small piece of MIP-2-containing agarose gel on the exposed cremaster muscle and studied directed migration of neutrophils in the postcapillary venule and in the tissue. Superfusion of SB747651A (5 µM) on cremaster muscle subjected to MIP-2 gradient significantly increased rolling velocity and adhesion, but decreased emigration of neutrophils in comparison to superfusion of normal saline

without SB747651A. SB747651A treatment significantly affected transmigration time, detachment time, intravascular crawling and the velocity of migration, but not the directionality of migrating neutrophils in tissue. The expression of intercellular adhesion molecule-1 (ICAM-1) in cultured endothelial cells was up-regulated by co-treatment with SB747651A and MIP-2 but not by MIP-2 alone. Flow cytometry analysis showed that co-treatment of bone marrow neutrophils with SB747651A and MIP-2 significantly decreased macrophage antigen-1 (Mac-1) but not lymphocyte function associated antigen-1 (LFA-1) expression as compared with MIP-2 treatment alone. Collectively, our findings demonstrate that pharmacological suppression of MSK1 by SB747651A affects multiple steps of MIP-2-induced neutrophil recruitment *in vivo*.

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Dedication

The thesis is dedicated to my family

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List of abbreviations

AGC kinases	The protein kinase A, G, and C families (PKA, PKC, PKG)
ATF-1	Activating transcription factor-1
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAMK	Calcium/calmodulin-dependent protein kinase
CBP	CREB-binding protein
CRE	cAMP-response element
CREB	cAMP-response element-binding protein
CTKD	C-terminal kinase domain
DMSO	Dimethyl sulfoxide
DUSP1	Dual specificity phosphatase 1
ECAM	Epithelial cell adhesion molecule
ERK	Extracellular signal-regulated kinase
ESAM	Endothelial cell-selective adhesion molecule
FITC	Fluorescein isothiocyanate
FMLP	Formyl-methionyl-leucyl-phenylalanine
GAGs	Glycosaminoglycans
GPCRs	G protein-coupled receptors
GSK3	Glycogen synthase kinase-3
H	Hour

HMG-14	High mobility group-14
HMGN1	High-mobility-group nucleosomal-binding protein 1
HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular adhesion molecule
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
INF- γ	Interferon- γ
i.p.	Intraperitoneal
i.sc.	Intrascrotal
JAM	Junctional adhesion molecule
JNK	c-Jun amino (N)-terminal kinase
KC	Keratinocyte-derived chemokine
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
LSP1	Leukocyte-specific protein 1
LTB4	Leukotriene B4
Mac-1	Macrophage antigen-1
MAPK	Mitogen-activated protein kinase
MEK1/2	MAPK/ERK kinase 1/2
MIP-1 α	Macrophage inflammatory protein-1 α
MIP-2	Macrophage inflammatory protein-2
MMP9	Matrix metalloproteinase 9

MPO	Myeloperoxidase
MSK	Mitogen-and stress-activated kinase
NF- κ B	Nuclear factor- κ B
NLK	Nemo-like kinase
NLS	Nuclear localization signal
NTKD	N-terminal kinase domain
p38	A class of mitogen-activated protein kinases
P300	A transcriptional co-activating protein
PBS	Phosphate-buffered saline
PECAM	Platelet endothelial cell adhesion molecule
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PSGL1	P-selectin glycoprotein ligand 1
PTEN	Phosphatase and tensin homolog
Rac	A member of Rho-GTPases
RBC	Red blood cell
Rho family	A family of Rho GTPases
RSK	Ribosomal protein S6 kinase
SCF	Stem cell factor

SDS	Sodium dodecyl sulfate (SDS)
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TCR	T cell receptor
TLR4	Toll-like receptor 4
TNF- α	Tumor necrosis factor- α
VAP-1	Vascular adhesion protein-1
VCAM-1	Vascular cell adhesion molecule-1
VLA4	Very late antigen-4
WT	Wild type

Introduction

1. Inflammation

Inflammation is a complex and defensive immune response which is normally under physiological regulation. It is characterized by the recruitment of leukocytes from blood to affected tissue in order to remove pathogens and start the process of repair in inflamed tissue (1). Inflammation can be divided into acute and chronic phases. Acute inflammation has a rapid onset and short duration, and is characterized by increased blood flow and vascular permeability along with the extravasation of plasma proteins and fluids, production of inflammatory mediators such as cytokines and emigration of leukocytes, especially neutrophils at the postcapillary venules into the injured or infected area (2-5). Acute inflammation is a defensive mechanism that aims to kill and to remove the infectious or injurious agents while still facilitating the resolution of the injured area. If the infectious or injurious agent is not eliminated during the acute phase of inflammation, the inflammation is prolonged and turns to chronic phase which manifests histologically by the existence of macrophages and lymphocytes, resulting in tissue damage and fibrosis (5).

Inflammation is a delicate immune response that is tightly regulated by our body, but abnormalities of inflammation cause many human diseases, for example, asthma and contact dermatitis which result from hyperreactive immune response to allergens. Defective immune system makes the individual more susceptible to infections (e.g., leukocyte adhesion deficiency disease (LADD) which is due to the absence of the β_2 integrin molecule on the surface of neutrophils called CD18 which plays an important

role in adhesion-dependent migration of neutrophils to the acute inflammation site) (6). Also, the persistent chronic inflammation causes many degenerative diseases such as atherosclerosis, rheumatoid arthritis (5). Uncontrolled inflammation causes many diseases that impairs the normal functions and life of the person. Therefore, it is very important to study the inflammation and mechanisms that are involved in its regulation.

2. Leukocyte recruitment

2.1 Neutrophil recruitment during inflammatory response

The recruitment of neutrophils from the blood flow to the affected site (infection or injury) is the fundamental feature of inflammation. This recruitment takes place in the post-capillary venules of the inflamed tissue. It can lead to the removal of a causative pathogen but can also cause inappropriate dysfunction of the tissue (8). The changes on the venular endothelial surface initiating neutrophil recruitment, are caused by the stimulation of inflammatory agents or mediators (including cysteinyl leukotrienes, histamine and cytokines) which are secreted from resident leukocytes or other tissue cells in the affected tissue when they are stimulated by invading pathogens (7). At post-capillary venules, there is a complicated process of cellular interactions triggered between neutrophils and endothelial cells during neutrophil recruitment to the site of inflammation. The cascade of neutrophil recruitment includes the following sequential steps in most tissues: neutrophils tethering and rolling, adhesion, intraluminal crawling on the luminal surface of the post-capillary venules, transendothelial migration across the venule and finally directional migration in extra-vascular tissue to reach the affected

site (injury or infection) under the control and guidance of a chemotactic gradient (chemotaxis) (7-12). The process of neutrophil recruitment is regulated by many molecules such as selectins, integrins, chemokines and chemokine receptors. Figure 1 presents a brief and schematic view of neutrophil recruitment cascade.

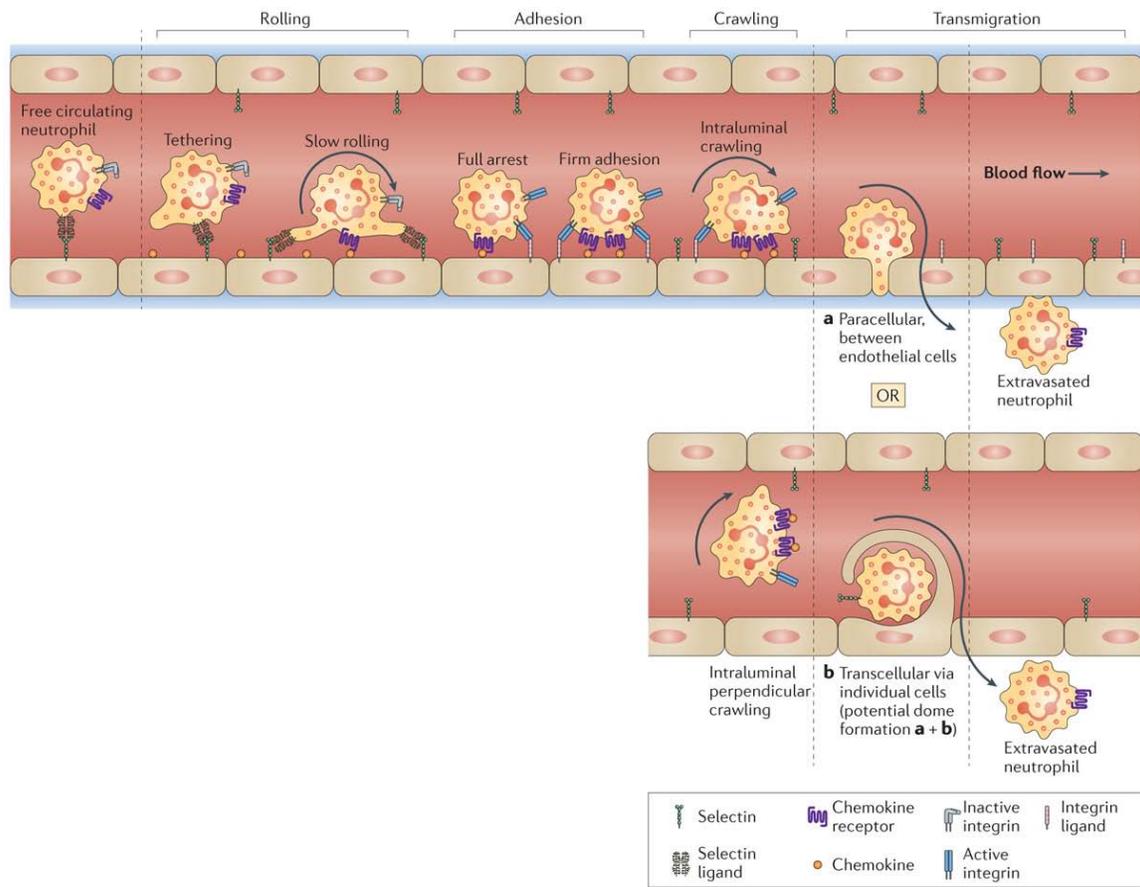


Figure 1. Neutrophil recruitment cascade. During inflammation, neutrophils in the blood vessels tether and roll along the endothelial surface, and then adhere to endothelium firmly. After that, neutrophils crawl to the sites of transmigration which can be either paracellular (between endothelial cells; **a**) or transcellular (through endothelial cells; **b**) (modified from Reference 7).

2.1.1 Neutrophil tethering, rolling and adhesion

Neutrophil tethering is the initial contact of neutrophils with the vascular endothelium and rolling is the following rotational movement along the endothelium. Neutrophil rolling is mediated by specific cell adhesion molecules called selectins. These selectins are one family of adhesion molecules belonging to calcium-dependent type I transmembrane glycoproteins (13,14). These selectins that mediate rolling of neutrophils are P-selectin, L-selectin, and E-selectin which are called by these names according to the type of tissues in which they were first discovered (platelet, leukocytes and endothelium, respectively) (13,14). The expression of P-selectin occurs constitutively in tissues and it is stored in secretory granules which are called Weibel-Palade bodies in endothelium or α -granules in platelets. Once endothelium is activated by inflammatory stimuli such as leukotrienes, thrombin and histamine, the Weibel-Palade bodies rapidly mobilize and merge with cell membrane resulting in the increased expression of P-selectins within minutes on the endothelial surface (21). In addition, P-selectins can be synthesized by the endothelial cells upon stimulation of cytokines and this type of P-selectin up-regulation involves protein synthesis and the process is slower (usually from > 30 min to several hours) (13,14). The up-regulation of the expression of E-selectins is through *de novo* protein synthesis in endothelium. When endothelial cells are activated by inflammatory mediators such as tumor necrosis factor- α (TNF- α), lipopolysaccharide (LPS) and interleukin-1 (IL-1), they express E-selectins on their surfaces. The expression of E-selectin on endothelium occurs within 2 hours after stimulation and declines in 24 hours. L-selectin was identified as the homing molecule

which allows lymphocytes to enter lymphoid tissues. L-selectin is expressed by most leukocytes (13-16,21). Leukocyte rolling is mediated by the interaction between selectins and P-selectin glycoprotein ligand 1 (PSGL1). It can also be mediated by other glycosylated ligands but PSGL1 is the dominant ligand for all these three selectins. PSGL1 is expressed by all leukocytes and by certain endothelial cells (11,18,19). E-selectins have other ligands which are E-selectin ligand 1, CD43, CD44 and L-selectin (11,13). Furthermore, it has been reported that some integrins are also involved in rolling step such as β_2 -integrin lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18, $\alpha_L\beta_2$ integrin) and α_4 -integrin very late antigen-4 (VLA4, $\alpha_4\beta_1$ integrin) (20). Rolling of neutrophils on endothelium facilitates their contact with chemokines or other chemoattractants on the endothelial surface.

The subsequent event is neutrophil adhesion. Triggering neutrophil arrest during rolling is mediated by chemoattractants and chemokines that are presented on the luminal surface of the endothelium (7). Neutrophil adhesion is mediated by the interaction of integrins on their surface with endothelial adhesion molecules in the family of immunoglobulin molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (11). The most relevant leukocyte integrins that mediate leukocyte arrest and adhesion are β_2 -integrins LFA-1 (CD11a/CD18, $\alpha_L\beta_2$ integrin) and macrophage antigen-1 (Mac-1, CD11b/CD18, $\alpha_M\beta_2$ integrin) and the β_1 -integrin VLA4 ($\alpha_4\beta_1$ integrin) (11). Both LFA-1 and Mac-1 mediate neutrophil adhesion but it has been reported that LFA-1 ($\alpha_L\beta_2$ integrin) plays a dominant role in neutrophil adhesion step whereas Mac-1 ($\alpha_M\beta_2$ integrin) plays a more important role in the

subsequent crawling step (17). Endothelial ligand for LFA-1 is ICAM-1 and ICAM-2. Mac-1 binds to ICAM-1 and to other ligands such as fibrinogen, fibronectin and complement fragment. VCAM-1 is the main endothelial ligand for $\alpha_4\beta_1$ -integrin VLA4 (13). VLA4-VCAM-1 interaction mediates the rolling and adhesion of mononuclear leukocytes (monocytes and lymphocytes) and eosinophils. There are a few studies showing that this $\alpha_4\beta_1$ -integrin, in addition to its support for neutrophil rolling, also mediates and enhances neutrophil adhesion (13,20,117,118).

Furthermore, shear flow is another crucial factor to initiate and to promote firm adhesion as it causes triggering and activation of β_2 -integrins through E-selectin signaling (21,22).

2.1.2 Neutrophil intraluminal crawling

The following newly identified step in neutrophil recruitment cascade is called intraluminal crawling. Intraluminal crawling means that the adherent leukocytes move from their initial adhesion site to the junctional extravasation site on the luminal surface of endothelium in response to chemokines in inflammation site (17,23). Schenkel *et al.* demonstrated that monocytes crawl from a site of firm adhesion to the nearest endothelial junction to transmigrate and this process was called locomotion. They found that blocking adhesion molecules such as β_2 integrins (LFA-1 and Mac-1) on monocytes and their integrin ligands on endothelium (ICAM-1 and ICAM-2) prevented monocytes from reaching transmigration site (24). Neutrophils in inflamed postcapillary venules

also crawl after adhesion to reach the optimal transmigration sites. Phillipson et al. demonstrated that in LFA-1-deficient mice few neutrophils adhered in response to chemokines but surprisingly, the few number of LFA-1-deficient neutrophils that adhered were able to crawl as efficiently as neutrophils from wild-type mice. In contrast, neutrophils with Mac-1-deficiency were able to adhere efficiently but showed a dramatical decrease in intraluminal crawling compared to wild-type neutrophils in response to endogenous or exogenous chemokines. They also discovered that inhibition of ICAM-1 but not ICAM-2 impaired intraluminal crawling. Phillipson et al. concluded that ICAM-1 but not ICAM-2 mediates Mac-1-dependent crawling, and they also concluded that the neutrophil adhesion is mainly dependent on LFA-1, the subsequent crawling step is mainly dependent on Mac-1 and, therefore, both LFA-1 and Mac-1 are important molecules for efficient neutrophil emigration out of blood vessel (17).

2.1.3 Neutrophil transendothelial migration

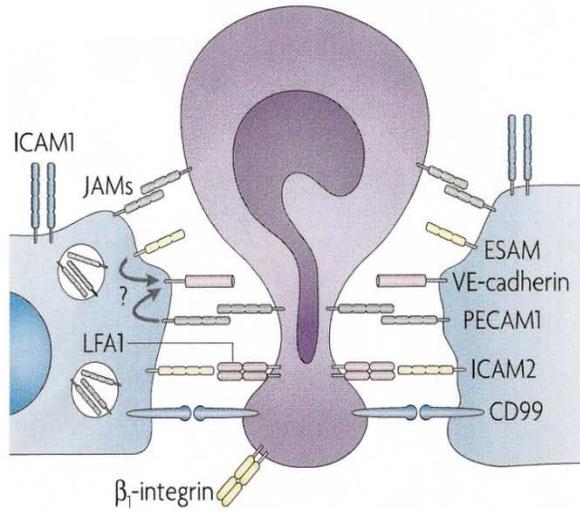
The subsequent fundamental step is the transmigration through the vascular wall that enables the leukocytes to leave the vasculature and move along chemoattractant gradient in inflamed tissue (7). The leukocyte during its transmigration through the vascular wall faces three different layers which are endothelial cells, the endothelial basement membrane and pericytes subsequently. The emigration of leukocytes through the layer of endothelial cell is fast (<2-5 minutes), but they spend much longer time to penetrate basement membrane of endothelial cell (>5-15 minutes) (11). Leukocytes transendothelial migration occurs either through endothelial junctions,

where the leukocytes pass between the adjacent endothelial cells and this is called paracellular transmigration route, or directly through the endothelial cells and this is called transcellular transmigration route (25,26). The factors determining which route is preferred are still unknown, but the route of transmigration may differ between different organs, different inflammatory stimuli and different types of emigrating leukocyte (25). The paracellular transmigration is the main route taken by neutrophils, as it has been reported that about 90% of neutrophil transmigration occurs paracellularly (17,27-31). However, transcellular pathway is also used by emigrating neutrophils but it is less efficient because it takes longer time (20-30 minutes) (7,25). Since all adherent neutrophils crawl in Mac-1-dependent mechanism to reach to their optimal sites of transmigration which are thought to be at the endothelial junctions, Mac-1-deficient neutrophils transmigrate predominantly transcellularly in comparison to wild-type neutrophils that predominantly use the paracellular route (17). Transmigration process is mediated by integrins (VLA4, LFA-1 and Mac-1), immunoglobulin family cell adhesion molecules (VCAM-1, ICAM-1 and ICAM-2) and a variety of junctional proteins such as junctional adhesion molecules (JAMs), platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31), epithelial cell adhesion molecule (ECAM), CD99, leukocyte-specific protein-1 (LSP-1) and vascular adhesion protein-1 (VAP-1) (7,10,11,32).

The paracellular migration of leukocytes occurs at endothelial cell junctions. During the paracellular migration, the vascular endothelial cadherin (VE-cadherin) is released to facilitate neutrophil transmigration through endothelial junctions and it is enhanced by endothelial-cell junctional molecules such as PECAM-1, JAM-A, ESAM, ICAM-1/2 and

CD99. Neutrophil paracellular migration is also mediated by increased levels of intracellular endothelial calcium which triggers endothelial cells contraction through activation of myosin light-chain kinase. The contraction of endothelial cells causes widening of endothelial junctions and facilitates neutrophil paracellular transmigration (11). At the thin parts of endothelial cell-lining, the transcellular migration occurs, therefore neutrophils cross less distance. Ligation of ICAM-1 stimulates cytoplasmic signaling events that cause transportation of apical ICAM-1 to F-actin- and caveolae-rich parts and subsequently transportation of ICAM-1 with caveolin-1 to the basal part of plasma membrane. All these events will lead to formation of channels inside endothelial cell called vesiculo-vacuolar organelles (VVOs) through which the neutrophils migrate (11). During the transcellular migration, endothelial cells extend projections that moves up forming cup-like structure around the adherent leukocytes. The endothelial projections that form dome-like structures (transmigratory cups) are rich in ICAM-1 and VCAM-1 that bind to LFA-1 and VLA4 on leukocyte surface (7,25). Figure 2 demonstrates the paracellular and transcellular routes of neutrophil transendothelial migration.

(A) Paracellular route



(B) Transcellular route

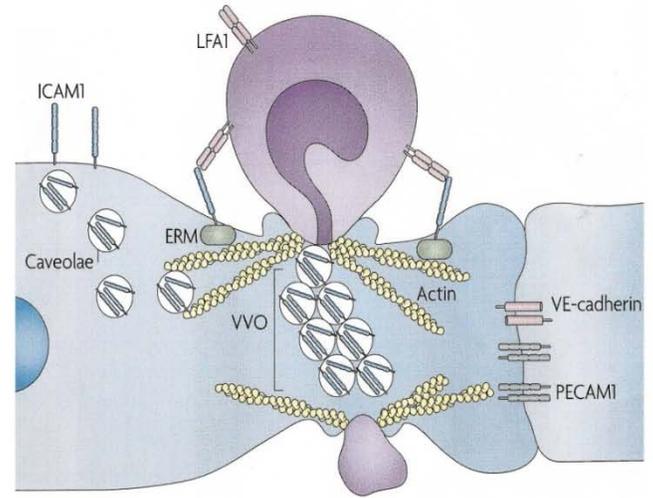


Figure 2. Transmigration pathways of neutrophils through the vascular routes. **(A)** Paracellular migration occurs at endothelial cell junctions. **(B)** Transcellular migration occurs through endothelial cells (modified from Reference 11).

2.1.4 Neutrophil chemotaxis in tissue

The capacity of a cell to sense the gradient of extracellular chemical signals (chemoattractant) and respond by directional migration towards their sources is a vital cellular mobile process called chemotaxis (33).

After neutrophils cross the vascular wall and reach the extra-vascular tissue, they start directional movement toward the chemoattractant source at the injury or infection site. Neutrophil chemotaxis is a dynamic process that involves a combination of directional sensing, cell polarization, cell adhesion and motility (34-37). Neutrophils have an extraordinary ability to detect and respond to very low chemoattractant gradient (as low as 2% difference across their length) (33,39,40). They perform this function through their ability to amplify the chemotactic gradient signals via conversion of small changes in the extracellular chemoattractant concentration into highly polarized intracellular signaling events through activation of many downstream signal transduction pathways which lead to dramatic redistribution of cytoskeletal components that ultimately lead to directed migration (33). Sensing of chemotactic stimuli is mediated by the stimulation and activation of G protein-coupled receptors (GPCRs) on the surface of neutrophils during binding of chemoattractants to GPCRs (23). Once GPCRs are activated, they transmit the signals to many downstream signaling pathways causing cell polarization and morphological changes in neutrophils to enable them to migrate towards the chemoattractant gradient. The cell fronts (leading edge) of the polarized neutrophils have constantly protruding and retracting motile membranous structures which are

called lamellipodia or filopodia. These filopodia are consistently oriented in the direction of chemotaxis. The rear of the cell (trailing edge or tail) is termed uropod (41). Neutrophil polarization is mediated by significant redistribution of cytoskeletal elements. It involves localization of F-actin and many actin binding proteins at the leading edge (the front of the cell) and localization of myosin II and actin filaments at the back (trailing edge) and at the sides of polarized neutrophils (42). This structural front-rear polarization is required for efficient translocation and movement of neutrophils toward chemokine gradient (41).

When chemoattractants bind to transmembrane GPCRs, several signaling pathways are activated to mediate neutrophil cell polarization and to enhance chemotaxis process towards chemoattractant stimuli that might be endogenous stimuli (IL-8, LTB₄) derived from host cells such as macrophages, endothelial cells and neutrophils or exogenous stimuli derived from bacteria such as formyl-methionyl-leucyl-phenylalanine (fMLP)(34). The phosphatidylinositol 3-kinase (PI3K) pathway is the most important pathway involved in neutrophil chemotaxis process. The role of PI3K and Rho-family GTPases in neutrophil polarization is illustrated in Figure 3 (43).

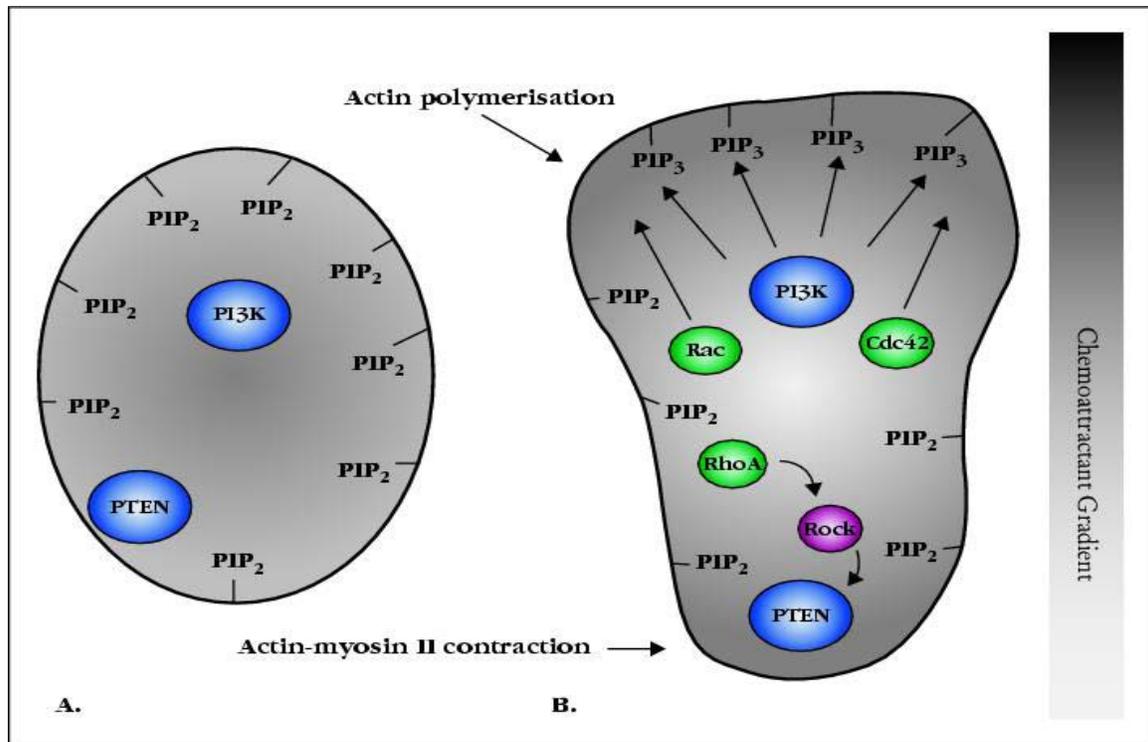


Figure 3. The role of PI3K and Rho-family GTPases in neutrophil polarization (modified from Reference 43). **A)** Unstimulated, resting leukocytes have a spherical shape. PI3K is localized in the cytoplasm and phosphatase and tensin homolog (PTEN) at the cell membrane. **B)** Binding of chemoattractant to GPCRs on leukocyte cell surface stimulates the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) by activation of PI3K in the membrane closest to the chemoattractant source. The external chemoattractant gradient is magnified inside the cell by upregulation of PIP₃ production at the leading edge and by PIP₃ dephosphorylation by PTEN at the rear of the cell. The localized accumulation of PIP₃ promotes activation of Rho-family GTPase Rac at the leading edge. Subsequently, active Rac catalyzes remodeling of actin cytoskeleton, enhancing cell protrusions and formation of lamellipodia or filopodia at the leading edge. At the rear of the cell, RhoA activation catalyzes remodeling of actinomyosin cytoskeleton which

enhances uropod contraction. Furthermore, RhoA activation promotes PTEN activation at back of the cell which enhances dephosphorylation of PIP3 and this will lead to asymmetrical distribution of PIP3 at cell fronts, this allows stabilization of cell polarity and enables the cell orientation toward chemoattractant source (43).

2.2 Chemokines in leukocyte recruitment

Chemokines are chemotactic cytokines that mediate leukocyte chemotaxis in inflammation and in immunological responses. They are small proteins with a molecular weight between 8kd to 14kd (44-46). There are about 50 chemokines in humans (with 70% homology in amino acid sequence) which are classified into four subfamilies depending on the number and position of the first two N-terminal cysteine residues in their structure (47,48). These four subfamilies include C, CC, CXC, CX3C. The C subfamily contain one cysteine residue at N-terminal side while the CC subfamily has two adjacent N-terminal cysteine residues. In the CXC and CX3C subfamilies, the first two N-terminal cysteine residues are separated by amino acids, one amino acid in CXC group and three amino acid in CX3C group (49,50). Chemokines are produced by various types of cells during inflammation such as leukocytes, endothelial cells, macrophages and fibroblasts. Chemokines are able to conduct the signals to their target cells through their ability to bind to and stimulate their seven-transmembrane GPCRs (8,51-53). The chemokine receptors are also categorized into four groups which are CR, CCR, CXCR and CX3CR similar to their corresponding chemokine subfamilies. Chemokine receptors are expressed by leukocytes, neurons, endothelial cells and epithelial cells (49). Chemokines plays a crucial role in neutrophil migration to the site of inflammation by creating a concentration gradient that directs neutrophils and by activating adhesion molecules on neutrophil surface (8). The produced chemokines in the site of inflammation are immobilized on the luminal surface of endothelium through their ability to bind to glycosaminoglycans (GAGs) and other molecules on endothelial surface

such as selectin ligands and integrin ligands (8). Subsequently, the immobilization of chemokines on endothelial surface enhances neutrophil recruitment and by triggering the rolling neutrophils to adhere firmly to the endothelium while the soluble chemokines are unable to induce neutrophil adhesion but can induce transendothelial migration by establishing chemokine concentration gradient surrounding the stimulus (8). It has been reported that the immobilized chemokine on endothelial cells binds to its receptor on neutrophil surface and activates the integrins on the surface of neutrophils allowing them to bind to their ligands on endothelial surface (48). This interaction leads to a switch from selectin-mediated neutrophil rolling to integrin-mediated neutrophil firm adhesion (48). Chemokines increase the adhesion of neutrophils by activation of integrins via two different mechanisms, the first, chemokines induce rapid and transient high affinity binding of integrins to their endothelial ligands and the second, chemokines increase the avidity of integrins for their ligands (44,8).

3. Neutrophils in inflammation

Neutrophils are a type of polymorphonuclear leukocytes which have a major role in acute inflammation. They are the first leukocytes that are recruited to the inflammation site to eliminate pathogens. In human, neutrophils have a short half life in the circulation about eight to ten hours. Human neutrophils constitute 50-70% of circulating leukocytes and also constitute 95% of circulating granulocytes. They are produced continuously from the bone marrow. In the blood, mature neutrophils are spherical cells

with approximately 7 μ m in diameter. They have a segmented nucleus and their cytoplasm contains various types of granules (7). Neutrophils have three different types of granules which contain pro-inflammatory proteins. Azurophilic granules also called primary granules are rich in myeloperoxidase (MPO), specific granules known as secondary granules are filled with lactoferrin, and gelatinase granules also named as tertiary granules are rich in matrix metalloproteinase 9 (MMP9). Neutrophils also have secretory vesicles which are mobile vesicles, therefore, they have the ability to carry their contents and to incorporate them into plasma membrane surface. During activation of neutrophils, the secretory vesicles carry β_2 integrins to neutrophil surface which enhance neutrophil adhesion to endothelium while gelatinase granules carry proteases which disrupts vascular basement membrane and extracellular matrix thus facilitating neutrophil emigration (7,54).

Neutrophils are the first immunological protection line against pathogens in acute inflammation. They are able to synthesize and secrete pro-inflammatory cytokines such as TNF α and IL-1 β , chemokines for example IL-8 and macrophage inflammatory protein-1 α (MIP-1 α), and other inflammatory mediators such as leukotrienes and prostaglandins (55). These inflammatory mediators produced by neutrophils play an important role in the pathogenesis of acute inflammation and in elimination of invading pathogens. Normal body neutrophils are essential for healthy life as they maintain balanced innate immunity. Individuals become extremely susceptible to bacterial infection if they have neutrophil disorders such as neutropenia or leukocyte adhesion deficiency (LAD). On the

other side, hyperactive neutrophils also cause several pathologies such as vasculitis and glomerulonephritis (56).

4. Mitogen- and stress-activated protein kinase-1 (MSK1)

4.1 MSK structure, tissue expression and activation

Mitogen-activated protein kinases (MAPKs) constitute a vital network of signaling pathways that are involved in various biological functions in cells such as survival, proliferation, apoptosis, differentiation and neuronal and immunological functions (38,57,58). Abnormal MAPK cascade causes several pathologies such as autoimmune disorders, cancer and neurodegeneration. There are fourteen MAPKs in mammalian and they are classified into seven groups. Classical MAPKs include p38 isoforms (α , β , γ and δ), extracellular signal-regulated kinases 1/2 (ERK1/2), ERK5 and c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3) while the atypical or non-classical MAPKs consists of ERK3/4, ERK7, and nemo-like kinase (NLK) (59-61).

The mitogen- and stress-activated kinases 1 and 2 (MSK1, MSK2) are two kinases with high structural similarity and they are downstream kinases of p38 and ERK1/2 MAPKs. MSK1 and MSK2 consist of two kinase domains which are N-terminal domain and C-terminal domain. The N-terminal kinase domain belongs to the protein kinase A, G, and C families (AGC kinases) while the C-terminal kinase domain belongs to the calmodulin kinases (CAMK) (38) (Fig.4). The upstream MAPKs (p38 and ERK1/2) activate MSKs via phosphorylation process which occurs on at least three sites. This phosphorylation leads

to the activation of the C-terminal domain which results in autophosphorylation of MSKs and thereby activation of the N-terminal domain. Once N-terminal domain is activated, it acts on phosphorylation of MSK target substrates. The sites which are phosphorylated on MSK by p38 MAPK are the same sites which are phosphorylated by ERK1/2 MAPK. The effects of activation of the ERK1/2-MSK and p38 α -MSK signaling pathways on the cell functions may be various, depending on different stimuli and different cell types used (38,62-64).

The interaction of ERK1/2 and p38 MAPKs with MSKs occurs at specific binding site on MSKs which is called MAPK-binding domain. This MAPK binding site is located close to the C-terminal end of the MSKs. Human MSK1 and MSK2 are 63% identical to each other. MSK1 and MSK2 also have a functional nuclear localization signal (NLS) at their C-terminal end. Presence of NLS segment in MSKs makes them exclusively localized intranuclearly in both quiescent and stimulated states of the cell. In accordance with this localization, MSK1 and MSK2 have been found to influence and modulate the nuclear events mainly (59).

Analytical studies about the expression of MSKs in various tissues demonstrated that MSK 1 and MSK2 have ubiquitous expression, and they are predominantly expressed in brain, skeletal muscles, heart and placenta (59). Moreover, it has been reported that MSK1 was expressed in human endothelial cells and human neutrophils (102, 113). Figure 4 illustrates domain structure of MSKs.

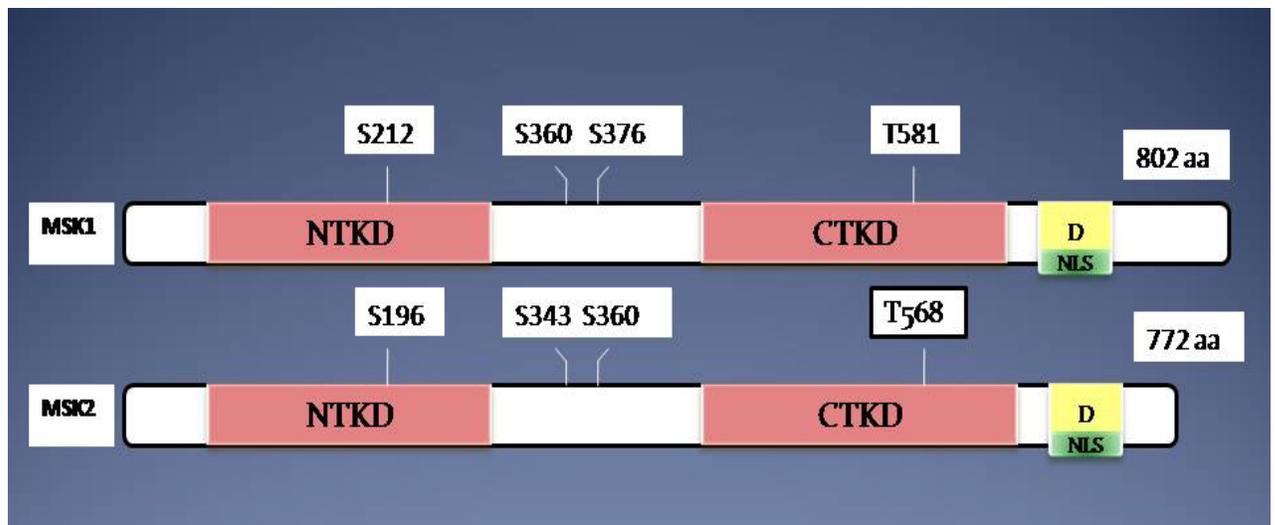


Figure 4. The schematic presentation of the MSK1 and MSK2 structures. MSK1 and MSK2 consist of two different kinase domains: the NTKD is a member of the AGC kinases while CTKD belongs to the CAMK kinases. The composition of the amino acids and the number of phosphorylation sites in both MSK1 and MSK2 are referred to the human nomenclature. NLS, nuclear localization signal; D, D domain or MAPK docking site; NTKD, N-terminal kinase domain; CTKD, C-terminal kinase domain (modified from Reference 59).

4.2 Physiological roles of MSK

4.2.1 Regulation of gene transcription

MSKs phosphorylate the transcriptional factor cAMP-response element-binding protein (CREB) in response to the mitogen and or stress-induced activation of its two upstream MAPKs, ERK1/2 and p38 α MAPK, in different cells, such as neurons, embryonic stem cells, macrophages and fibroblasts. In accordance with this, the MSK1/2 double-knockout cells demonstrated dramatic decrease in the mitogen and or stress-induced transcription of immediate early (IE) genes such as c-fos and nur77 which are dependent on cyclic AMP-response element (CRE) (38,66,67). CREB is a subfamily of transcription factors which also include activating factor 1 (ATF1). Similar to CREB, phosphorylation of ATF1 in response to mitogens and cellular stresses is mediated by MSK (65).

The nuclear factor-kappa B (NF- κ B) is a transcriptional factor which regulates transcription in response to various stimuli. The activity of NF- κ B is regulated by posttranslational modification. MSKs phosphorylate p65 subunit of NF- κ B at Ser276 and this phosphorylation enhances the ability of p65 to interact with the cofactors CBP and p300 that results in acetylation of histones and NF- κ B bound to promoters (59,68,69). It has been reported that MSK1/2-knockout fibroblasts showed reduction in TNF- α -mediated activation of NF- κ B indicating that activation of MSKs is crucial for NF- κ B-dependent transcription (59,70).

4.2.2 Regulation of nucleosomal response

The MSKs act as primary mediators of the nucleosomal response in order to promote gene relaxation and activation. Nucleosomal response includes the phosphorylation of histone H3 on Ser10 and of high-mobility-group nucleosomal-binding protein 1 (HMGN1) on Ser6 which takes place during induction of immediate early genes (59,71). Histone H3 is one of the components of nucleosome, and the post-translation modification occurs at its C-terminal. Post-translation modification includes phosphorylation, acetylation and methylation. It has been reported that MSKs are the essential kinases involved in the phosphorylation process of histone H3 downstream of ERK1/2 and p38 α MAPK cascade activation. Consistent with this, MSKs may regulate transcriptional process of some non-CRE-dependent IE-genes. Histone H3 phosphorylation in response to various stimuli can be completely inhibited in MSK1/2-knockout fibroblasts (59,72).

MSKs also phosphorylate HMGN1 (HMG-14) which is chromatin-associated protein but it is not the core composition in the nucleosome structure. HMGN1 causes suppression of phosphorylation of histone H3 resulting in reduction in expression of IE-genes. When HMGN1 is phosphorylated by MSKs, the interaction of HMGN1 with nucleosome is reduced and this in turn allows MSKs to phosphorylate core histone H3 (59,73,74).

4.2.3 MSKs in immunity

4.2.3.1 T cell development

It has been reported that both of MAPK activation and CREB signaling are involved in T cell development and T cell receptor (TCR) induces CREB phosphorylation through

ERK1/2 signaling pathway. TCR- and IL-2-induced CREB phosphorylation is significantly impaired in MSK1/2-knockout mice (38,65). However, CREB-knockout mice showed significant decreases in the number of embryonic T cells and impaired T cell development (65,109). MSKs knockout mice have normal T cell number and development. Furthermore, the number of T cells in spleen was slightly decreased in MSKs-knockout mice (65).

4.2.3.2 Role of MSKs in different models of inflammation

Some studies demonstrated that MSKs regulate the production of cytokines during immunological responses to inflammatory stimuli (65). Recently, it has been reported that MSK1 and MSK2 have a negative regulatory role during acute inflammation (75,76). The studies demonstrated that MSK1 and MSK2 play a negative regulatory role on toll-like receptor4 (TLR4) signaling pathway. Therefore, they reduce and limit TLR4-mediated production of many inflammatory cytokines such as IL-6, IL-12 and TNF- α . Moreover, it has been shown that MSK1 and MSK2 achieve this negative regulatory effect via their ability to induce both of the anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist (IL-1ra) and the MAPK phosphatase dual specificity phosphatase-1 (DUSP-1)(75,76). IL-10 is an anti-inflammatory cytokine which suppresses the production of proinflammatory cytokines and induces the production of the anti-inflammatory cytokine IL-1ra by macrophages (76-78). IL-1ra is a naturally occurring inhibitor of IL-1 function, it has similar structure to IL-1 (homology), and thereby it binds to IL-1 receptor but without activating it (77). Furthermore, some studies revealed that

DUSP1 was able to inhibit the p38 MAPK leading to reduction in the level of numerous proinflammatory cytokines such as IL-6, IL-12 and TNF- α (75,79). These results reveal that although ERK1/2 and p38 MAPK have a fundamental role in inflammatory process pathogenesis, they also induce the activation of negative feedback systems that are important in suppression of inflammation. The presence of hyper-activated proinflammatory signals causes many chronic inflammatory diseases. Therefore, the negative feedback systems which were reported to regulate inflammation are extremely important in damping the inflammatory process. Recent studies reported that both MSK1/2 and p38 α are involved in coordination and regulation of anti-inflammatory reactions and responses (75,80).

Bertelsen *et al.* studied the role of MSK1 and MSK2 in chronic skin inflammation, and they used an oxazolone to induce allergic contact dermatitis in wild-type mice and MSK1/2-deficient mice (75). Their results revealed that there was a significant increase in inflammation in MSK1/2-deficient mice in comparison to wild-type mice. This was evaluated by an increase in ear thickness and in skin neutrophil infiltration. Moreover, they discovered that there was a significant elevation in the levels of the proinflammatory cytokines IL-1 β and IL-6 and TNF- α at mRNA level as well as at protein level in MSK1/2-deficient mice in comparison to wild-type mice. Their findings demonstrated that the negative feedback systems that suppress skin inflammation induced by oxazolone were activated by MSK1 and MSK2, and that is why in MSK1/2-deficient mice, there was a dramatic increase in inflammation over wild-type mice (75).

Ananieva et al. reported that animals with MSK1/2-knockout are extremely sensitive to endotoxic shock induced by LPS, and they also reported that the inflammation in MSK1/2-knockout animals continued for much longer time period than that of wild-type animals in a model of toxic contact eczema (76). This study explained that the deficiency in MSK1 and MSK2 caused a reduction in the expression of DUSP1 and IL-10 which are physiologically participated in the negative feedback systems that inhibit the immune response (59). The study presented by Adam D. Bachstetter *et al.* showed the role of MSKs in reducing proinflammatory cytokine production, thereby reducing neuronal damage and degeneration (81).

In contrast, Funding *et al.* demonstrated that high level of activated MSK1 and MSK2 in psoriatic skin lesion compared with normal non-affected skin which indicates that MSK1 and MSK2 play an important role in pathogenesis of psoriasis and they can be the therapeutic target of psoriasis (82,83). While L. Reber reported that MSK1 is involved in pathogenesis of murine asthma through the activation of NF- κ B which enhances over-expression of mast cell growth factor called stem cell factor (SCF) in inflammation (84). Therefore, MSK1 can be valuable therapeutic target to reverse mast cell related inflammation especially in conditions such as asthma (84).

Furthermore, Kawaguchi *et al.* reported that IL-17F is involved in pathogenesis of asthma through induction of IL-11 over-expression and release in bronchial epithelial cells (85). IL-11 plays a crucial role in airway remodeling and inflammation in patients

with asthma. They found that ERK1/2-MSK1-CREB is a novel and important signaling pathway mediating IL17-F-induced asthma (85).

The study reported by Song *et al.* demonstrated that ERK/p38-MSK1-CREB is the major signaling pathway that is involved in IL-1 β - and TNF- α -induced mucin hyper-secretion in human airway epithelial cells during inflammation (86).

According to all previously mentioned studies, the biological role of MSKs in inflammation may be heterogenous depending on tissue type, stimulus type and type of inflammation model used. However, the role of MSK1 in neutrophil recruitment remains elusive.

4.3 MSK inhibitors

Many compounds have been shown to inhibit MSKs *in vivo*. H89 and RO-31-8220 were the first inhibitors found to block MSK activity. However, neither compound is very specific for MSKs (65,110). In addition to MSKs inhibition, H89 was able to inhibit other kinases such as protein kinase A (PKA), protein kinase B (PKB), ribosomal protein S6 kinase (RSK) and others (65,111). RO-31-8220 was originally developed as a protein kinase C (PKC) inhibitor, and it was subsequently found to inhibit MSK1, RSK and glycogen synthase kinase-3 (GSK3) with a similar potency to PKC (65,112). While both these compounds have been used to study MSK in cells, care must be taken in the interpretation of the results for two reasons. Both inhibitors also target other kinases,

including RSK, and both have also been shown in some, but not all, circumstances to inhibit the activation of the ERK1/2 cascade (65). Both MEK1/2 and p38 inhibitors can be used to block MSK activation, but both have to be used to block both MAPK pathways at the same time to completely inhibit MSK activation (59). Figure 5 demonstrates the stimuli and MAPK pathways that activate MSK and the pharmacological inhibitors of MAPK modules used to inhibit MSK (88).

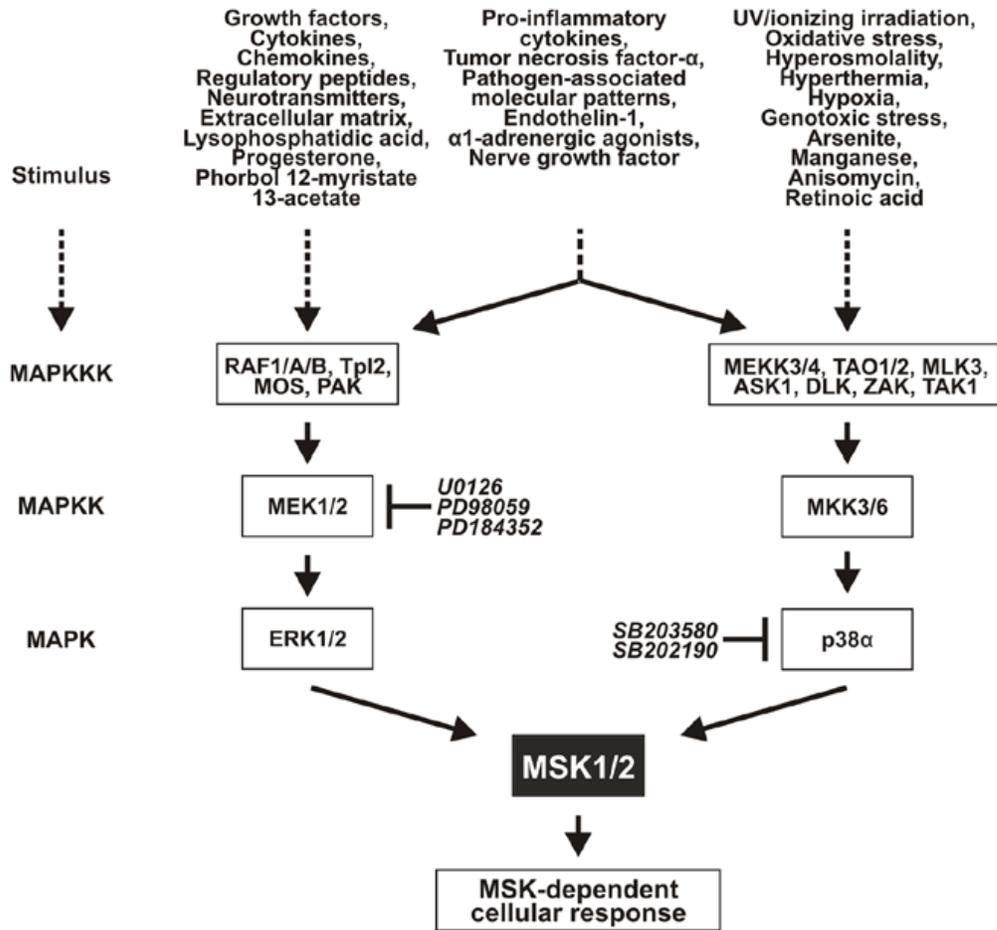


Figure 5. Stimuli and MAPK pathways that activate MSK. The three sequential organization of the MAPK cascade module is indicated to the left. Selective pharmacological inhibitors of MAPK module components that have been used to dissect MSK upstream signaling are shown in italics (modified from Reference 88).

Bamford *et al.* have reported the synthesis of a group of novel compounds of imidazo(4,5-c)pyridines that were able to inhibit MSK1, and the synthesis of these compounds resulted in inhibitors with selectivity for MSK over GSK3 and RSK *in vitro* (65,103). One of these newly discovered inhibitors is SB-747651A which has become available in the market from the beginning of 2013. SB-747651A is an imidazo(4,5-c)pyridine derivative with improved selectivity for MSK over H89 and Ro-31-8220 and therefore represents a useful tool to study MSK function in cells (87). In cells, it fully inhibits MSK activity at 5-10 μ M (87). SB-747651A was found to inhibit the production of the anti-inflammatory cytokines IL-10 in wild-type, but not in MSK1/2-knockout, macrophages following LPS stimulation. Both SB-747651A treatment and MSK1/2-knockout resulted in elevated production of pro-inflammatory cytokines by LPS-stimulated macrophages (87). SB-747651A is a potent ATP-competitive inhibitor of MSK1 *in vitro* and it inhibits the activity of the N-terminal kinase domain of MSK1. SB-747651A also inhibits other kinases such as PKA, PKB and RSK but despite these off-target activities, SB-747651A provides a significant improvement over the previous alternatives. Both H89 and Ro-318220 have been used as MSK inhibitors, but SB-747651A shows a better selectivity profile for MSK1 compared with these two previous alternatives. Thus, it is a very useful tool to study MSK function in cells (87,104).

Rationale for the present study

The kinase MSK1 has been shown in various studies to play either pro- or anti-inflammatory role in various animal models. However, there are no reports in the current literature showing the effect of its pharmacological suppression on leukocyte-endothelial cell interactions *in vivo*. Based on the above, the following working hypothesis has been formulated.

Hypothesis

The hypothesis for this study is that pharmacological suppression of MSK1 by the specific inhibitor, SB747651A, modulates inflammation and leukocyte-endothelial cell interactions by affecting multiple steps of MIP-2-induced neutrophil recruitment *in vivo*.

Experimental objectives

To test the above hypothesis, the following specific experimental objectives have been put forward :

1. To identify the effect of MSK1 inhibition by SB747651A on MIP-2-induced neutrophil rolling, adhesion and emigration *in vivo*.
2. To identify the effect of MSK1 inhibition by SB747651A on MIP-2-induced neutrophil intraluminal crawling, transendothelial migration and chemotaxis in cremasteric muscle tissue *in vivo*.

To achieve the above two objectives, mouse cremasteric muscle preparation and intravital microscopy were used to identify the effect of MSK1 inhibition by SB747651A on MIP-2-induced neutrophil recruitment parameters in cremasteric post-capillary venules and tissue.

3. To identify the cell specific effect of MSK1 inhibition by SB747651A on neutrophils and endothelial cells during MIP-2-induced neutrophil recruitment. Isolated primary murine neutrophils and cultured murine microvascular SVEC4-10EE2 endothelial cell line cells were used to identify the effect of MSK1 inhibition by SB747651A on the expression of neutrophil integrins (Mac-1 and LFA-1) and endothelial ICAM-1 respectively (*in vitro*).
4. To identify if MSK1 has a role in neutrophils during MIP-2-induced neutrophil recruitment.

Isolated primary murine neutrophils were used to identify the effect of MIP-2 on MSK1 expression in murine neutrophils (*in vitro*).

Materials and methods

1. Animals

Two types of mice were used in this study: C57BL/6 and 129/SvJ mice. Male mice aged 8-16 week-old were used in this study. 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 Council on Animal Care.

2. Preparation of mouse 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 intra-peritoneally (i.p.) to anesthetize adult male mice. An incision was made on the neck to expose the left 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 left jugular vein was cannulated to administer additional anesthetic or drugs when necessary (89,100).

3. Preparation of cremaster muscle for intravital microscopy

The mouse cremaster muscle preparation was used to study the behavior of leukocytes in the microcirculation and adjacent connective tissue. Briefly, an incision was made in the scrotal skin to expose the left cremaster muscle, which was then carefully dissected to free from the associated fascia. The cremaster muscle was then cut longitudinally

with a cautery. The testicle and the epididymis were separated from the underlying muscle and moved into the abdominal cavity. The muscle was secured along the edges with 4–0 suture. The exposed muscle was held flat and superfused with bicarbonate-buffered physiological saline (131.9 NaCl, 4.7 KCl, 1.2 MgSO₄, 20 NaHCO₃, in mM, pH 7.4, 37°C) 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 (Nikon Canada Inc., Mississauga, ON, Canada) with a 20× objective lens and a 10x eyepiece. A digital colour video camera (DC220, Dage-MIT, USA) was used to project the images onto a monitor. The images were recorded with a DVD video recorder for subsequent playback real-time analysis to determine leukocyte rolling, adhesion and emigration and time-lapse analysis to observe and measure neutrophil intraluminal crawling, transendothelium migration and chemotaxis in tissue (89,90,99).

Single venules (25–40 µm in diameter) were selected. The number of rolling, adherent, and emigrated leukocytes was determined offline during video playback analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. The flux of rolling leukocytes was measured as the number of rolling leukocytes passing by a given point in the venule per minute. Leukocyte rolling velocity was measured for the first 20 leukocytes entering the field of view at the time of recording and calculated from the time required for a leukocyte to roll along a 100-µm length of venule. A leukocyte was considered to be adherent if it remained stationary for at least 30 s, and total leukocyte adhesion was quantified as the

number of adherent cells within a 100- μm length of venule in 5 min. Leukocyte emigration was defined as the number of cells in the extravascular space within a 200 x 265- μm area (0.053 mm^2), adjacent to the observed venule (89,99,100).

4. Induction of neutrophil recruitment in cremaster muscle

To induce neutrophil recruitment in cremaster muscle, the CXC keratinocyte-derived chemokine (KC/CXCL1, 0.2 μg ; Peprotech, Rocky Hill, USA) or macrophage inflammatory protein-2 (MIP-2/CXCL2, 0.2 μg ; R&D Systems, Inc., Minneapolis, USA) in 100 μl of saline was injected intrascrotally (i.sc.) into C57BL/6 WT mice. The leukocyte rolling flux, rolling velocity, adhesion and emigration were measured in post capillary venule of cremaster muscle at 3.5, 4.0, and 4.5 h after KC or MIP-2 injection.

To induce neutrophil recruitment independent of cytokines, an agarose gel containing MIP-2 was used. The agarose gel was prepared by adding 10 ml of phosphate-buffered saline (2 \times 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 and mixed to achieve a final concentration of 0.5 μM . In order to visualize the gel on the cremaster muscle, a small amount of India ink was added to the preparation. After 30 min recording the images 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 containing gel was punched out of the lid using the cut tip end of a 200- μl pipette tip and the gel was carefully placed on the surface of the cremaster muscle in a preselected area 350- μm from the observed post-capillary venule. A 22 \times 22 mm glass

coverslip was used to hold the gel in place and the tissue was superfused beneath the coverslip at a very slow rate ($\leq 10 \mu\text{l}/\text{min}$) to allow formation of a chemotactic gradient which is necessary to stimulate leukocyte emigration and chemotaxis. The images were recorded for 60 min after the addition of chemoattractant-containing gel. The number of rolling, adherent and emigrated neutrophils were determined as described previously during offline playback analysis of the recorded real-time video (89-91).

Cell tracking and analysis of neutrophil intraluminal crawling, transmigration and chemotaxis in cremasteric vasculature were measured by using ImageJ software (NIH, Bethesda, MD, USA) and the time-lapsed movie which was converted from the real-time video recording of the experiment (90-92). The following recruitment parameters were quantified from tracking and analyzing at least 35 cells for each treatment group:

- a) Crawling distance: the total distance the neutrophil crawled from the initial site of adhesion to transmigration site (μm).
- b) Crawling time: the time spent by the neutrophil to crawl from the initial adhesion site to the transmigration site (min).
- c) Velocity of crawling: the crawling distance in the vessel lumen divided by crawling time ($\mu\text{m}/\text{min}$).
- d) Directional crawling index: the ratio of the crawling distance in the direction toward the MIP-2 gel to the total crawling distance the cell crawled in the lumen of the venule.
- e) Transmigration time: from the time the neutrophil started to transmigrate across

- endothelium to the time the whole cell body was just outside the venule (min).
- f) Detachment time: from the time the neutrophil body was just outside the venule to the time when the cell body lost contact with the venule (min).
 - g) Migration distance: the sum of the distance the neutrophil moved from the start point after detachment from the venule to the end point of the migration in tissue at 60 min of MIP-2 treatment or to the end point of the field of view (μm).
 - h) Velocity of migration: migration distance in tissue divided by migration time ($\mu\text{m}/\text{min}$).
 - i) Chemotaxis index in tissue: the ratio of total chemotaxis distance, i.e., the distance in the direction toward the MIP-2 gel, to the total migration distance the cell moved in tissue.

5. Inhibition of MSK1

The mice were treated with one of the two different inhibitors of MSK1: Ro-31-8220 and SB747651A. Ro-31-8220 (Cayman Chemical, Michigan, USA) was given i.p. 1 h before administration of KC in a dose of 6 mg/kg based on previously reported optimal concentrations (93). SB747651A (Axon Medchem BV, Groningen, Netherlands) was superfused locally at a dose of 5 μM based on previously reported optimal concentration (87) and it was superfused 30 min prior to placement of MIP-2 containing gel and its superfusion remained for the whole duration of MIP-2 treatment (60 min). In another experiment, SB747651A compound was injected i.sc. 1 h before administration

of MIP-2 in a dose 3 mg/kg based on a dose-response concentrations which were done in our laboratory.

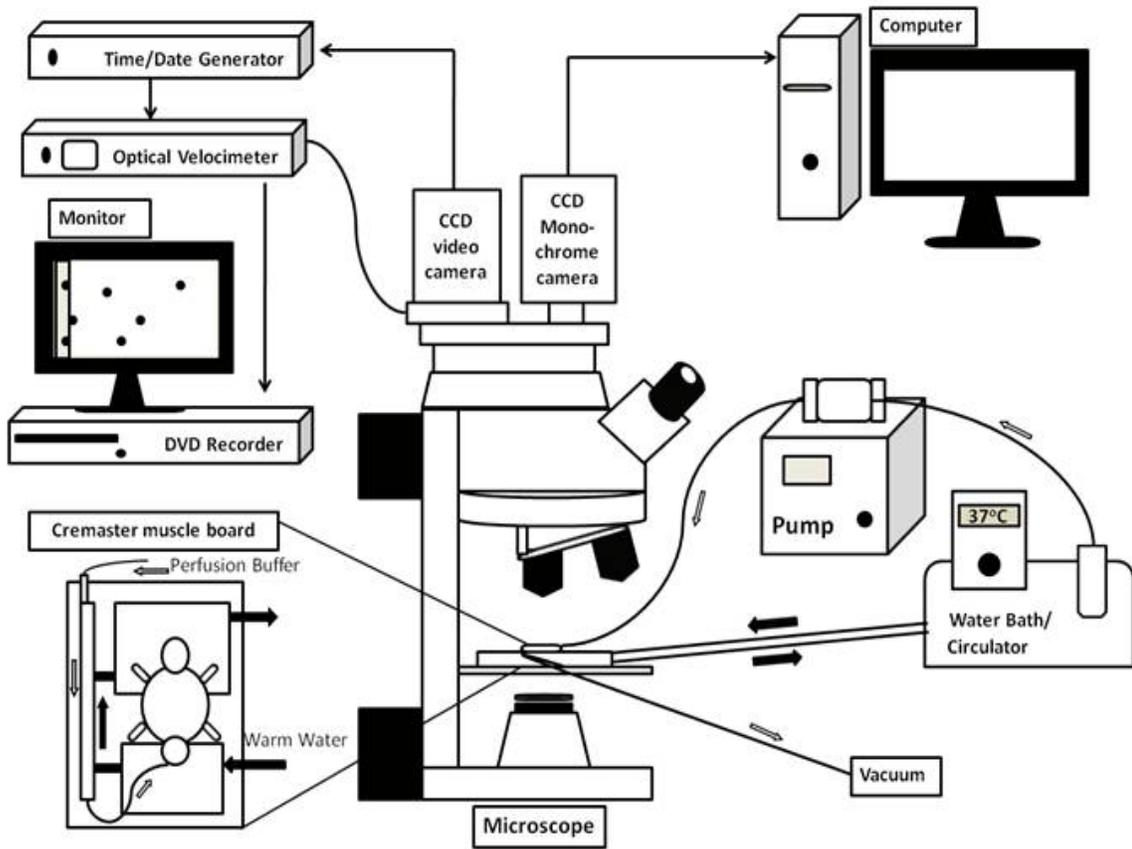
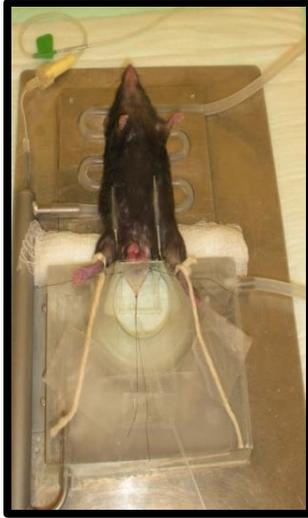


Figure 6. The intravital video microscopy system. An upright microscopy is linked to video camera which projects the images to a DVD recorder and a TV monitor. The prepared cremaster muscle was placed on the cremaster muscle board warmed with 37°C circulating water and the muscle was superfused with 37°C-warmed bicarbonate-buffered saline (modified from Reference 90).

6. Isolation of mouse bone marrow neutrophils

The femurs and tibias from C57BL/6 WT mice were isolated and their tips were dissected. After that, the bone marrow flushed with ice-cold Ca^{2+} - and Mg^{2+} -free phosphate buffered saline (PBS) solution. Percoll (GE Healthcare, Uppsala, Sweden) gradient (72%, 64%, and 52%) was used to isolate neutrophils by layering the collected marrow on the top of these three percoll gradient layers followed by centrifugation at $1060 \times g$ for 30 min. After that, the collected neutrophils were washed with PBS (94).

7. Flow cytometry analysis of neutrophil integrin Mac-1 and LFA-1 expression

The expression of Mac-1 and LFA-1 on neutrophils was measured by flow cytometry. After lysis of RBC, the isolated bone marrow neutrophils were incubated in the presence or absence of 5 μM SB747651A for 30 min at 37°C. Then, the cells were stimulated with 30 nM CXCL2/MIP-2 and incubated for 10 min at 37°C. The neutrophil samples at 1×10^6 /ml were washed in ice-cold PBS containing 1% bovine serum albumin (BSA). The neutrophils were stained with either a fluorescence anti-Mac-1 antibody (anti-mouse CD11b FITC; clone M1/70; eBioscience, San Diego, CA) or a fluorescence anti-LFA-1 antibody (anti-mouse CD11a FITC; clone M17/4; eBioscience) or their respective isotype controls which are rat IgG2bκ FITC (eBioscience) for Mac-1 and rat IgG2aκ FITC (eBioscience) for LFA-1, and incubated at dark at 4°C for 30 min. Next, all samples were

centrifuged and washed twice with ice-cold PBS containing 1% BSA and then analysed using FL-1 channel of an Epics XL flow cytometer (Beckman Coulter, Miami, FL) (95).

8. Culture of endothelial cell line cells

Murine microvascular SVEC4-10EE2 endothelial cell line cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Cellgro, Manassas, VA) which contains 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) in a 5% CO₂ incubator with maximum humidity at 37°C (96).

9. Western blotting

Following the treatment of murine endothelial cells for indicated length of time with 100 nM MIP-2 or 5 µM SB747651A and following treatment of murine neutrophils with 30 nM MIP-2, the cells were lysed in a lysis buffer (pH 8.0) which contains 150 mM NaCl, 50 mM Tris, 1% nonidet P-40 and protease and phosphatase inhibitor cocktails. For measurement of endothelial ICAM-1 expression, the lysate was centrifuged (10,000 × g, 4°C, 10 min) and the supernatant was collected. While for measurement of neutrophil MSK1 expression, the lysate was sonicated for 10 sec to get whole cell lysate. Next, the collected lysate of samples were mixed with 4 X sample loading buffer which contained

200 mM Tris-HCl, 50% glycerol, 0,04% bromophenol blue, 2% SDS and 20% β -mercaptoethanol. Then the mixture was boiled for 5 min. After determination of protein concentration in cell lysate of all samples, the same amount of protein were used from all samples and separated on 7.5% SDS-PAGE and transferred electrically to a nitrocellulose membrane. The membrane was blocked with 5% non fat milk in TBS-Tween buffer for 1.5 h at room temperature and incubated overnight at room temperature with the primary antibodies against MSK1 (1:200; Santa Cruz, CA), β -actin (1:2000; Santa Cruz, CA) and against ICAM-1 (1:1000; Abcam). After that, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Following washing extensively, the bands were detected with enhanced chemiluminescence reagents and with X-ray to expose the bands on X-ray film (97,101).

10. Statistical analysis

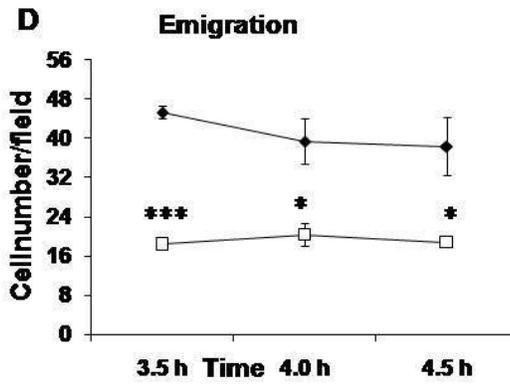
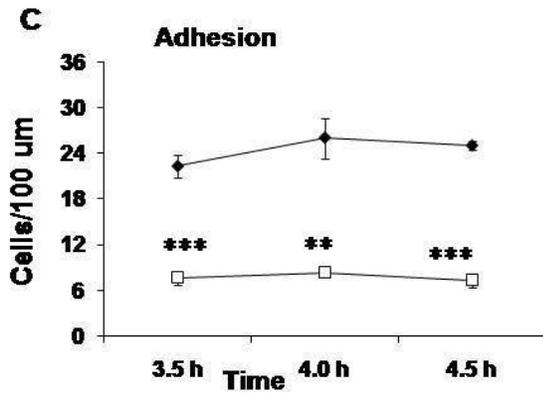
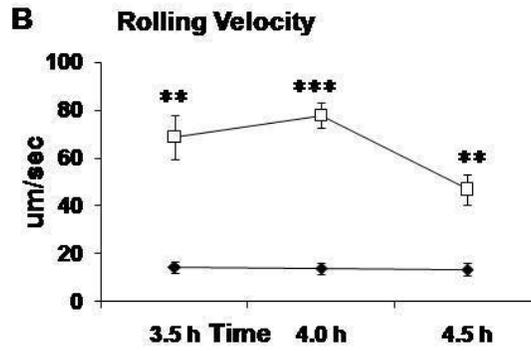
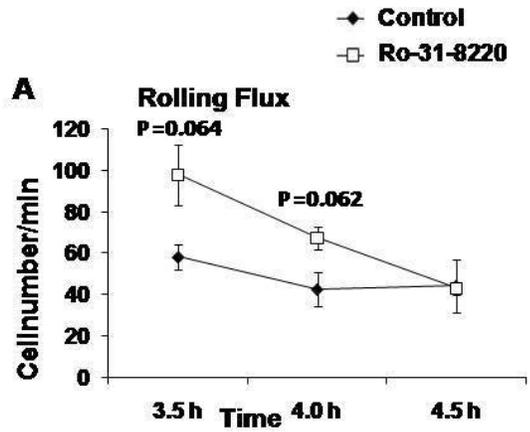
Data in this study are expressed as mean \pm SEM. Statistical analysis was performed using Student's *t* test or one way ANOVA. *n* indicates the number of mice used per experiment. Values of $P < 0.05$ were considered statistically significant.

Results

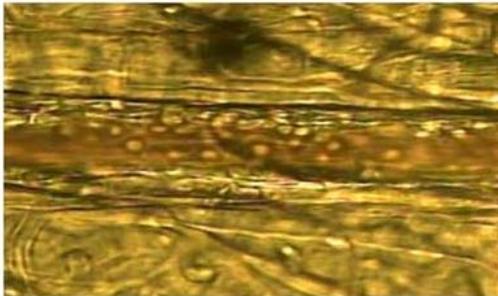
1. Effect of Ro-31-8220 on KC-induced neutrophil recruitment

1.1 Pretreatment with Ro-31-8220 increases rolling velocity significantly while no significant change in rolling flux

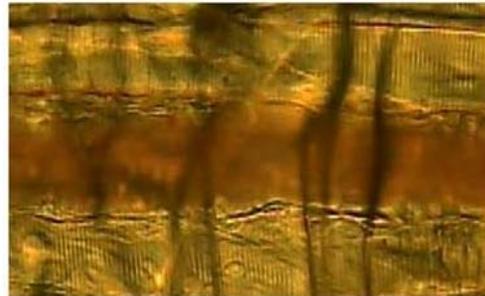
KC/CXCL1, a murine CXC chemokine, is produced in the site of inflammation and selectively induces mouse neutrophil recruitment (98). In this experiment, C57BL/6 mice were injected i.sc. with 0.2 µg KC/CXCL1 to induce neutrophil recruitment in post capillary venules of cremaster muscle. The control mice group were injected i.p. with DMSO in 100 µl saline 1 h before KC administration at the same amount of DMSO in which Ro-31-8220 was dissolved. To assess the effect of Ro-31-8220 on KC-induced neutrophil recruitment, another group, the Ro-31-8220 group, were injected with Ro-31-8220 i.p. (6 mg/kg) 1 h before KC injection. Next, leukocyte rolling flux, rolling velocity, adhesion and emigration were determined at 3.5, 4.0 and 4.5 h after KC injection. Figure 7 (A and B) demonstrates that KC-decreased leukocyte rolling velocity was significantly reversed by the pretreatment with Ro-31-8220 at all time points ($P < 0.01$) whereas there was no significant difference in leukocyte rolling flux between the two groups although it tends to be higher in Ro-31-8220-treated group at 3.5 h and 4 h ($P > 0.05$).



E



Control group



RO-31-8220 group

Figure 7. Effect of of Ro-31-8220 on KC-induced neutrophil rolling flux, rolling velocity, adhesion and emigration in cremasteric post-capillary venules of C57BL/6 WT mice. Control mice were injected intraperitoneally with DMSO in 100 μ l saline 1 h before KC injection while Ro-31-8220 mice group were injected intraperitoneally with 6 mg/kg of Ro-31-8220 1 h before KC injection. Both groups were injected with 0.2 μ g KC intrascrotally. Data represents mean \pm SEM from three mice per group. *: P<0.05, **: P<0.01, ***: P<0.001, Ro-31-8220 group compared with the control group.

1.2 Both KC-induced neutrophil adhesion and emigration are significantly reduced by pretreatment with Ro-31-8220

Regarding the effect of Ro-31-8220 on KC-induced neutrophil adhesion and emigration, Figure 7 (C,D,E and F) demonstrates that pretreatment with Ro-31-8220 decreased KC-induced adhesion and emigration significantly at all time points ($P < 0.05$, $P < 0.01$ or $P < 0.001$).

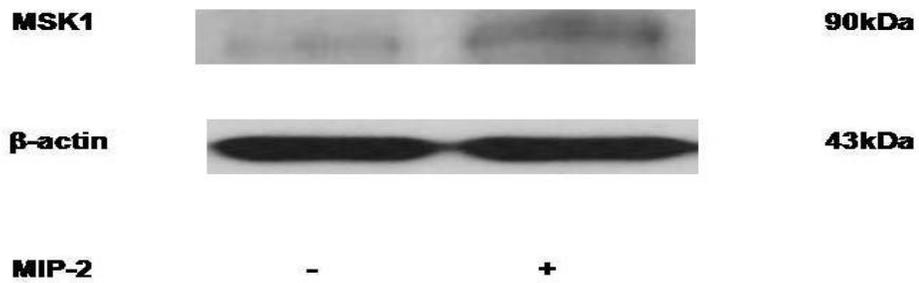
The previous studies demonstrated that Ro-31-8220 has poor selectivity because it inhibits MSK1 with similar potency to PKC, RSK and GSK3 (65,87). For this reason, I used the more potent and selective inhibitor of MSK1, SB747651A, to study the effect of MSK1 inhibition on chemokine-induced neutrophil recruitment in the following experiments (87,104). Furthermore, it has been reported that 99% of recruited leukocytes in response to CXC chemokine MIP-2 were neutrophils and therefore, it is more potent neutrophil chemoattractant than KC chemokine (17). That is why, I used MIP-2 chemokine instead of KC chemokine to study the effect of MSK1 suppression by SB747651A on neutrophil recruitment.

2. Effect of MIP-2 on MSK1 expression in murine neutrophils

It has been reported that MSK1 was expressed in human neutrophils, and stimulation of human neutrophils with LPS increased MSK1 expression (113). In this study, to assess the effect of MIP-2 stimulation on MSK1 expression in murine neutrophils, we treated

the isolated murine neutrophils with 30 nM MIP-2 for 1 h. Figure 8 reveals that stimulation of murine neutrophils with MIP-2 caused up-regulation of MSK1 expression.

A



B

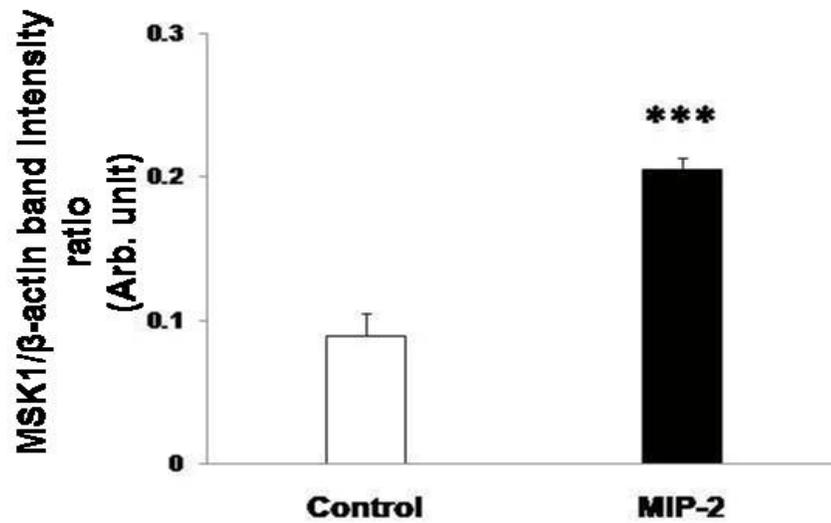


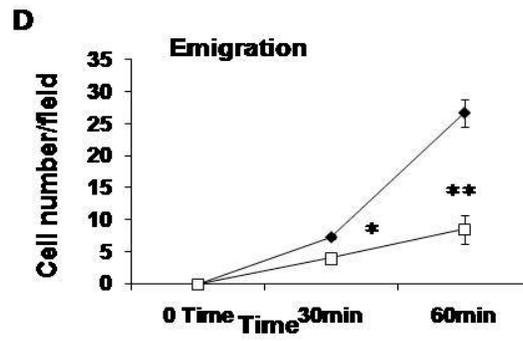
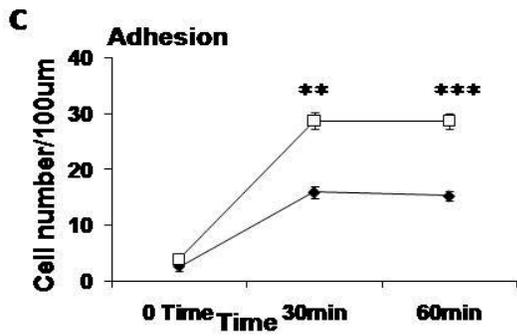
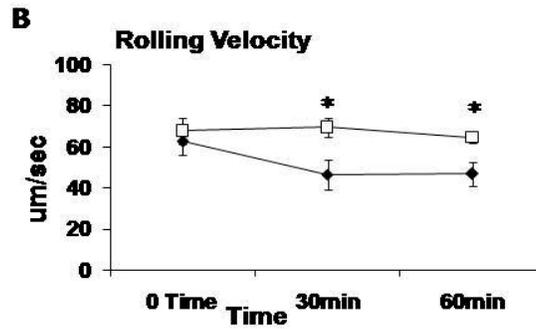
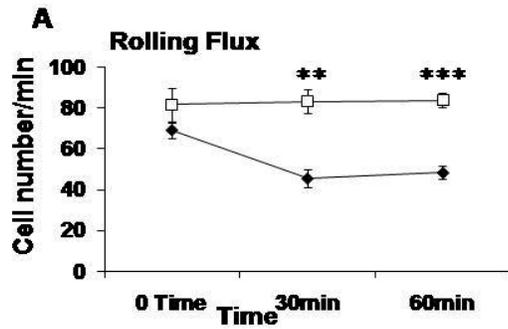
Figure 8. Effect of MIP-2 on MSK1 expression in murine neutrophils. Murine neutrophils were incubated without (Control) or with MIP-2 (30 nM) for 1 h. **A**: Original immunoblots (representative of 3 similar experiments) of MSK1 expression in the Control and MIP-2-treated murine neutrophils. **B**: Densitometric analysis of MSK1 expression in the Control and MIP-2-treated group of murine neutrophils. The values represent means \pm SEM (n = 3). ***: P<0.001, indicates significant difference from the control neurophils without MIP-2 treatment.

3. Effect of MSK1 specific inhibitor SB747651A on MIP-2-induced neutrophil recruitment

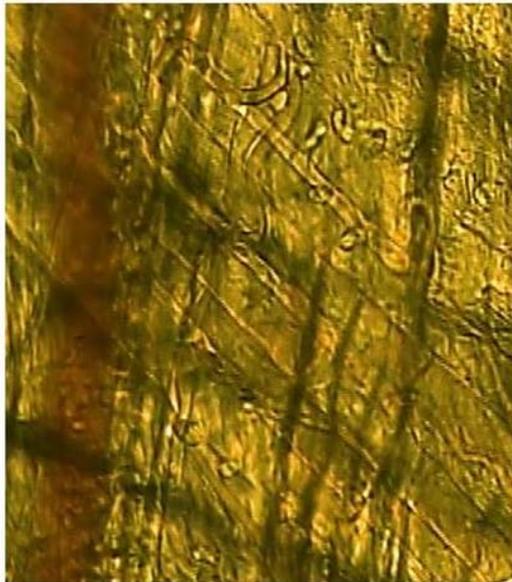
3.1 Pretreatment with SB747651A restored MIP-2-reduced rolling flux and rolling velocity, enhanced MIP-2-induced adhesion but impaired MIP-2-induced emigration at earlier time points

To demonstrate the effect of MSK1 specific inhibitor SB747651A on different steps of neutrophil recruitment, a piece of 0.5 μM MIP-2-containing-agarose gel (1mm^3) was placed on cremaster muscle surface at 350 μm distance and parallel to the selected post capillary venules to induce neutrophil recruitment. In the control group, the muscle was superfused with bicarbonate-buffered saline for 30 min prior to and for 60 min after placing MIP-2 gel while in SB747651A group, the muscle was superfused with 5 μM SB747651A compound for 30 min prior to the placement of MIP-2 gel and the SB747651A superfusion was remained for the duration of MIP-2 treatment (60 min). Figure 9 illustrates that superfusion of SB747651A (5 μM) on murine cremaster muscle subjected to MIP-2 gradient significantly increased rolling velocity, rolling flux and adhesion, but decreased emigration of neutrophils as compared to superfusion with normal saline without SB747651A.

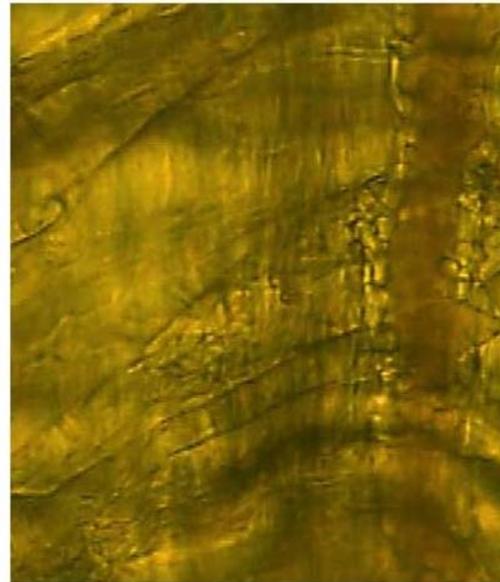
◆ Control
 □ SB747651A



E



Control group



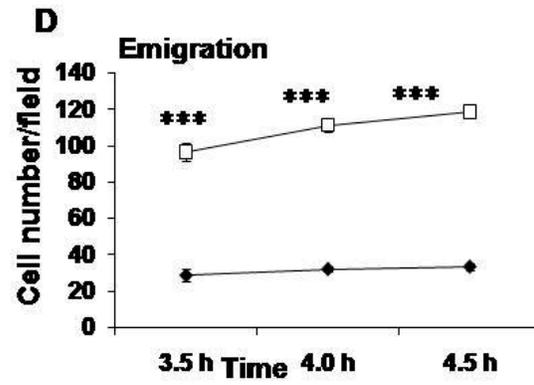
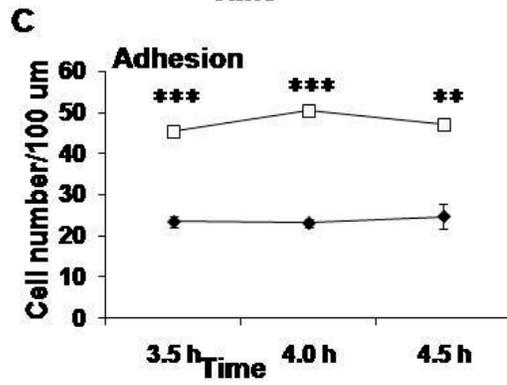
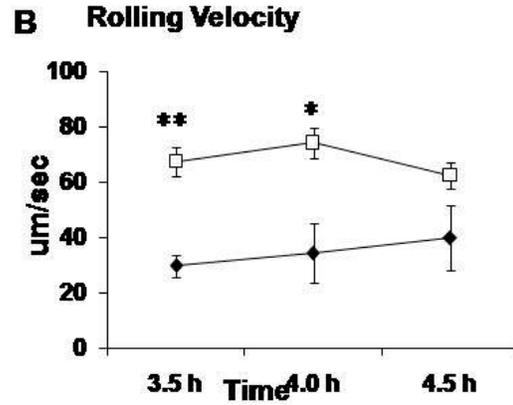
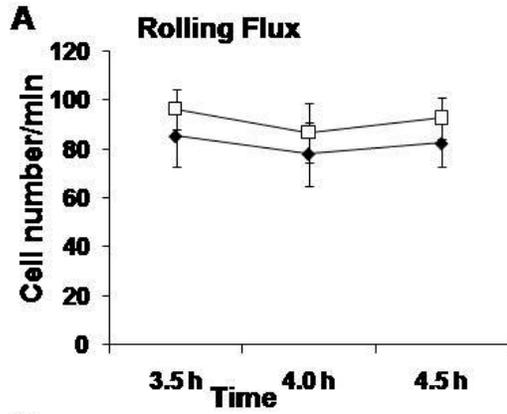
SB747651A group

Figure 9. Effect of SB747651A superfusion (5 μ M) on MIP-2 gel (0.5 μ M)-induced neutrophil rolling flux, rolling velocity, adhesion and emigration in cremasteric post-capillary venules of 129/SvJ WT mice. The muscles of control mice were superfused with bicarbonate-buffered saline while the muscles of SB747651A mice group were superfused with SB747651A for 30 min prior to and for 60 min after MIP-2 gel placement. Data represents mean \pm SEM from four mice per group. *: P<0.05, **: P<0.01, ***: P<0.001, SB747651A group compared with the control group.

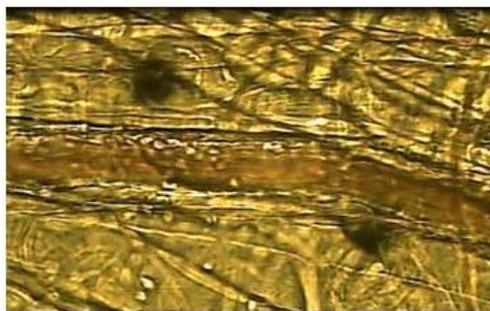
3.2 Pretreatment with SB747651A reversed MIP-2-reduced velocity and enhanced MIP-2-induced adhesion and emigration at later time points

To demonstrate the effect of MSK1 specific inhibitor SB747651A on neutrophil recruitment at later time points, in this experiment, the control group mice were injected i.sc. with 100 μ l saline 1 h before MIP-2 injection, and after that they were given 0.2 μ g MIP-2 i.sc. injection for 4 h while the SB747651A-treated group mice were injected i.sc. with SB747651A (3 mg/kg) 1 h before i.sc. injection of MIP-2 (0.2 μ g, 4 h). In both groups, neutrophil recruitment parameters were determined at 3.5, 4.0, and 4.5 h after MIP-2 injection. Figure 10 demonstrates that pre-treatment with SB747651A significantly reversed MIP-2-reduced leukocyte rolling velocity at 3.5 and 4 h after MIP-2 treatment while there was no significant difference in rolling velocity at 4.5 h although it still tended to be higher in SB747651A-treated group. SB747651A pre-treatment further increased MIP-2-induced increased adhesion and emigration significantly at all time points.

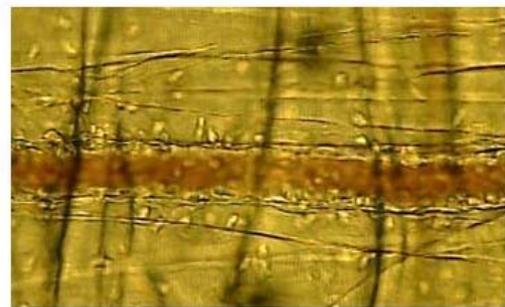
◆ Control
 □ SB747651A



E



Control group



SB 747651A group

Figure 10. Effect of of intrascotal injection of SB747651A on MIP-2-induced neutrophil rolling flux, rolling velocity, adhesion and emigration in cremasteric post-capillary venules of C57BL/6 WT mice. The control mice were injected intrascrotally with 100 μ l saline 1 h before MIP-2 administration while SB747651A mice group were injected with 3 mg/kg of SB747651A intrascrotally 1 h before MIP-2 injection. Both groups were injected intrascrotally with 0.2 μ g of MIP-2. Data represents mean \pm SEM from three mice per group. *: P<0.05, **: P<0.01, ***: P<0.001, SB747651A group compared with the control group.

4. Effect of MSK1 specific inhibitor SB747651A on MIP-2 gel-induced neutrophil intraluminal crawling, transmigration and chemotaxis in cremaster muscle

I used Image J software to analyze time-lapsed video for determining neutrophil intraluminal crawling, transmigration and chemotaxis in microcirculation of cremaster muscle. The following results demonstrated the effect of SB747651A on neutrophil intraluminal crawling, transmigration and chemotaxis in tissue.

4.1 Pretreatment with SB747651A compound impaired MIP-2-induced neutrophil intraluminal crawling

To assess the effect of specific inhibitor of MSK1 on MIP-2-induced neutrophil intraluminal crawling, 5 μ M of SB747651A was superfused for 30 min prior to and for 60 min after placing 0.5 μ M MIP-2 gel on cremaster muscle, while the control group was superfused with bicarbonate-buffered saline. Figure 11 demonstrates that both of intraluminal crawling distance and time tended to be higher by pretreatment with SB747651A although there was no significant difference from the control group. Accordingly, velocity of neutrophil intraluminal crawling is significantly reduced by the pretreatment with SB747651A in comparison to the treatment with MIP-2 alone in control group.

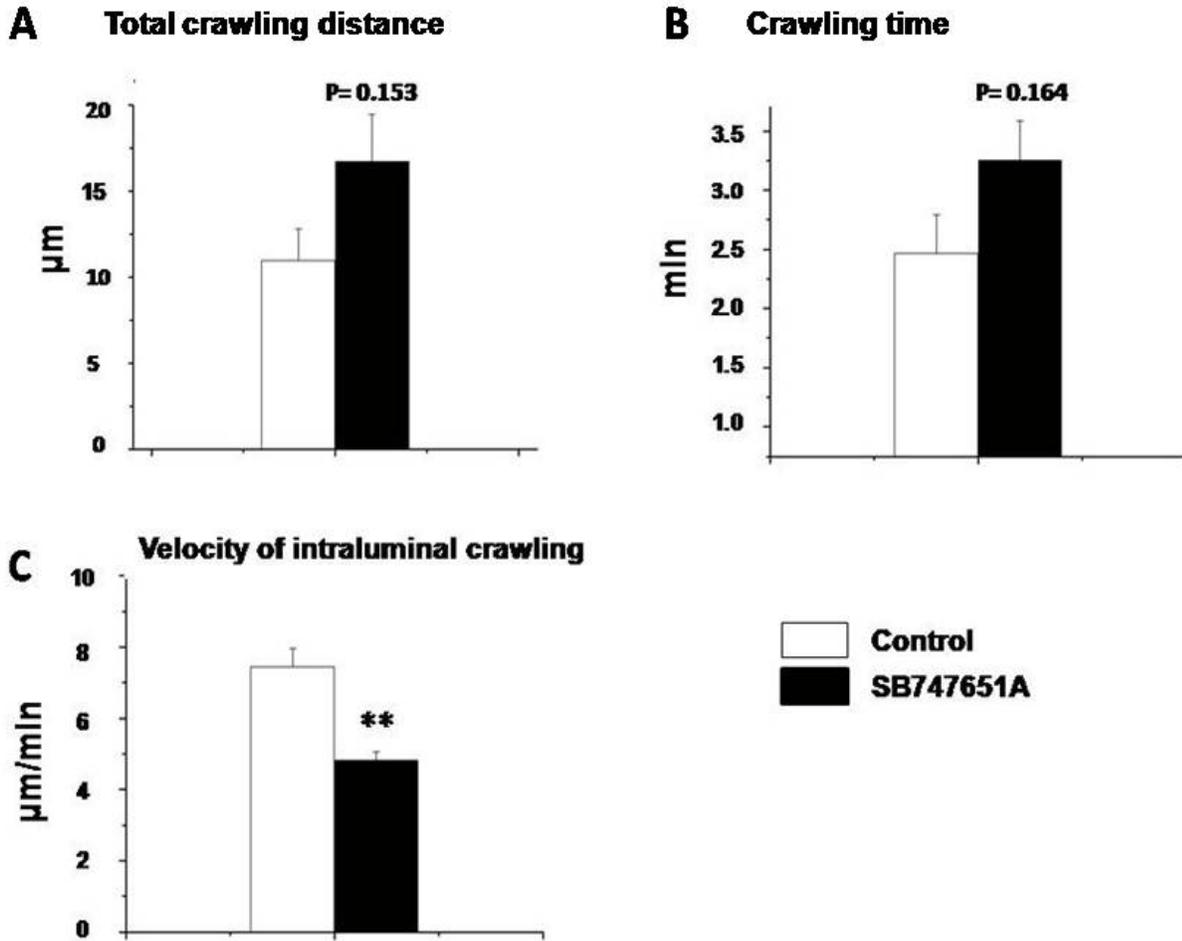


Figure 11. Effect of SB747651A superfusion (5 μM) on MIP-2 gel (0.5 μM)-induced neutrophil intraluminal crawling in cremasteric post-capillary venules of 129/SvJ WT mice. The muscles of the control mice were superfused with bicarbonate-buffered saline while the muscles of SB747651A-treated mice were superfused with SB747651A for 30 min prior to and for 60 min after MIP-2 gel placement. Data represents mean \pm SEM from four mice per group. **: $P < 0.01$, SB747651A-treated group compared with the control group.

4.2 Pretreatment with SB747651A compound impaired MIP-2-induced neutrophil transmigration through endothelium

Once the neutrophils stop crawling, they start transmigration through the endothelium to reach the extra-vascular space where it emigrates toward the chemokine gradient in tissue. Figure 12 reveals that both transmigration time and detachment time of neutrophils in response to MIP-2 gel were increased significantly by the pretreatment with SB747651A compound in comparison to treatment with MIP-2 alone in control group. These results indicate the impairment of neutrophil migration across endothelial layer after MSK1 is inhibited by SB747651A.

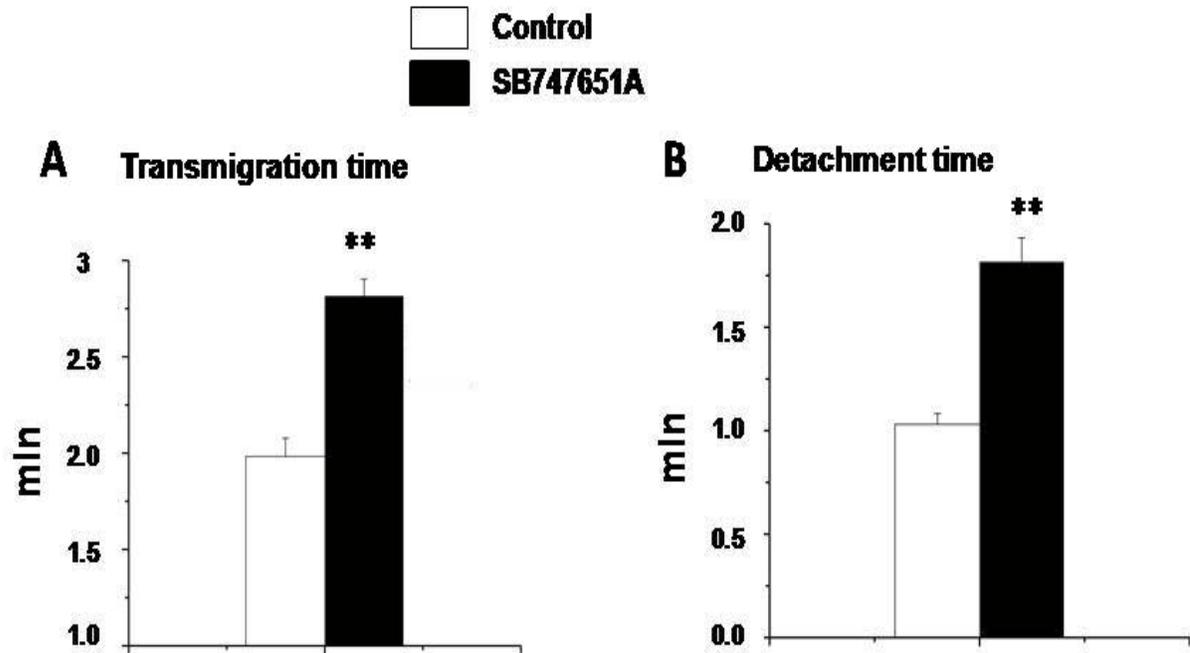


Figure 12. Effect of SB747651A superfusion (5 μ M) on MIP-2 gel (0.5 μ M)-induced neutrophil transmigration time and detachment time in post-capillary venules of 129/SvJ WT mice. The muscles of the control mice were superfused with bicarbonate-buffered saline while the muscles of SB747651A-treated mice were superfused with SB747651A for 30 min prior to and for 60 min after MIP-2 gel placement. Data represents mean \pm SEM from four mice per group. **: $P < 0.01$, SB747651A-treated group compared with the control group.

4.3 Pretreatment with SB747651A compound impaired MIP-2-induced neutrophil migration velocity but not chemotaxis index in cremateric tissue

The next step of neutrophil recruitment after neutrophil transmigration across the endothelium is to start its directional movement toward MIP-2 chemokine gradient and this event is termed chemotaxis. The results in Figure 13 demonstrate that the velocity of neutrophil migration in cremateric tissue adjacent to post capillary venule was significantly reduced by the pretreatment with SB747651A compound in comparison to the treatment with MIP-2 alone in control group while there was no significant difference in the directional parameter chemotaxis index between the two groups.

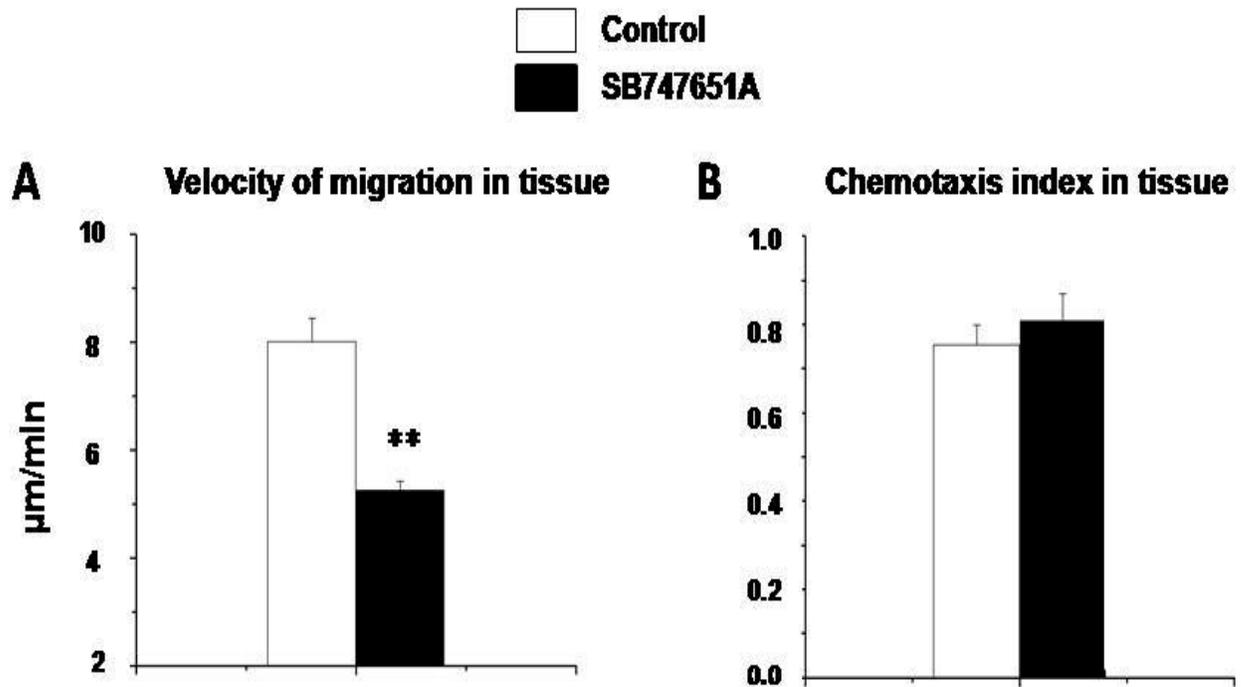


Figure 13. Effect of SB747651A superfusion (5 μ M) on MIP-2 gel (0.5 μ M)-induced neutrophil migration and chemotaxis in cremasteric tissue adjacent to post-capillary venules of 129/SvJ WT mice. The muscles of the control mice were superfused with bicarbonate-buffered saline while the muscles of SB747651A-treated mice were superfused with SB747651A for 30 min prior to and for 60 min after MIP-2 gel placement. Data represents mean \pm SEM from four mice per group. **: $P < 0.01$, SB747651A-treated group compared with the control group.

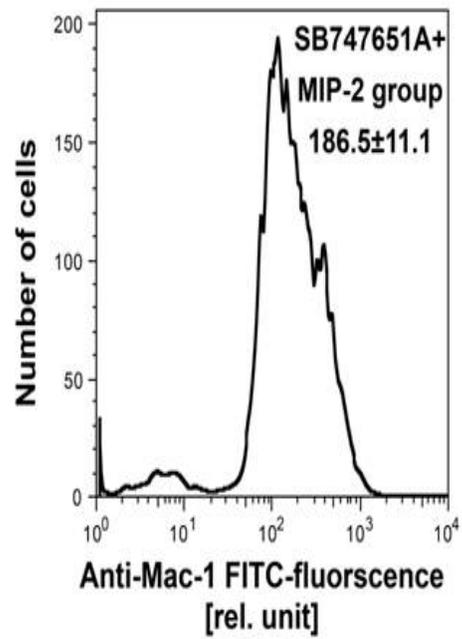
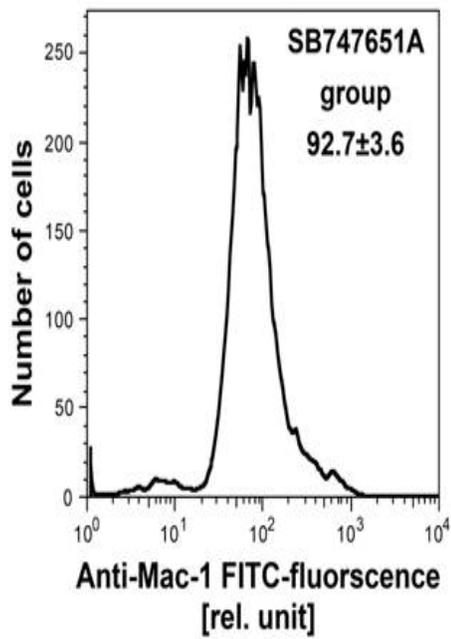
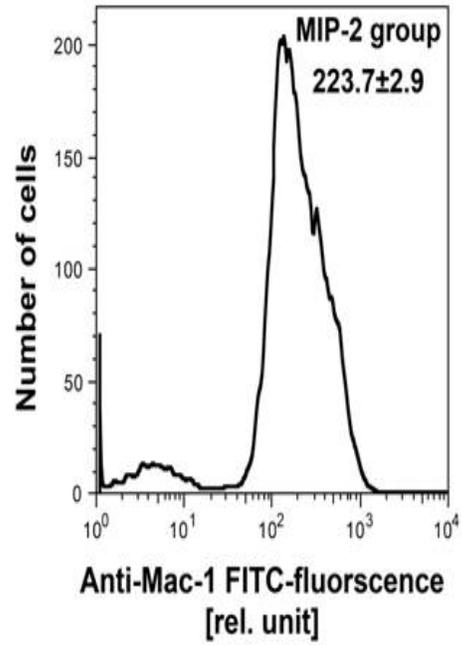
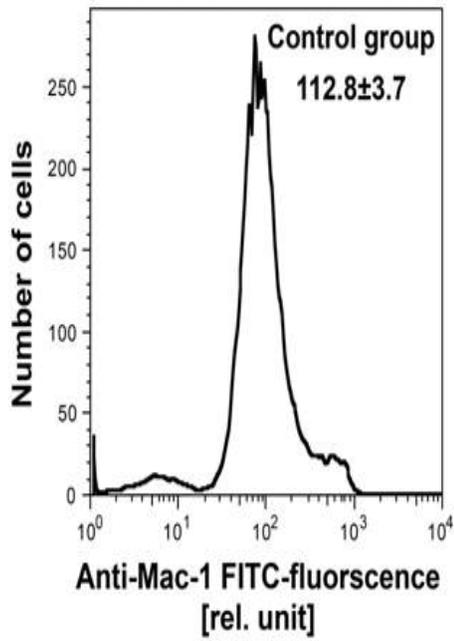
5. Effect of MSK1 specific inhibitor SB747651A on MIP-2-induced expression of β_2 -integrins on neutrophils

The β_2 -integrins Mac-1 and LFA-1 are expressed on neutrophil surface and are critical adhesion molecules in neutrophil recruitment during inflammation. It has been reported that LFA-1 is important for adhesion of neutrophils to the endothelial cells while Mac-1 is important for neutrophil intraluminal crawling and subsequent efficient transmigration across endothelial layer (17). In this study, the isolated murine bone marrow neutrophils were treated with 30 nM MIP-2 for 10 min to induce β_2 -integrin expression. To assess the effect of MSK1 specific inhibitor SB747651A on Mac-1 and LFA-1 expression on neutrophil surface, these isolated neutrophils were treated with 5 μ M of SB747651A for 30 min prior to MIP-2 treatment. The following results were obtained.

5.1 SB747651A compound significantly decreased MIP-2-induced Mac-1 expression in murine neutrophils

Figure 14 reveals that co-treatment of murine bone marrow neutrophils with SB747651A and MIP-2 significantly reduced MIP-2-induced up-regulation of Mac-1 expression as compared with MIP-2 treatment alone.

A



B

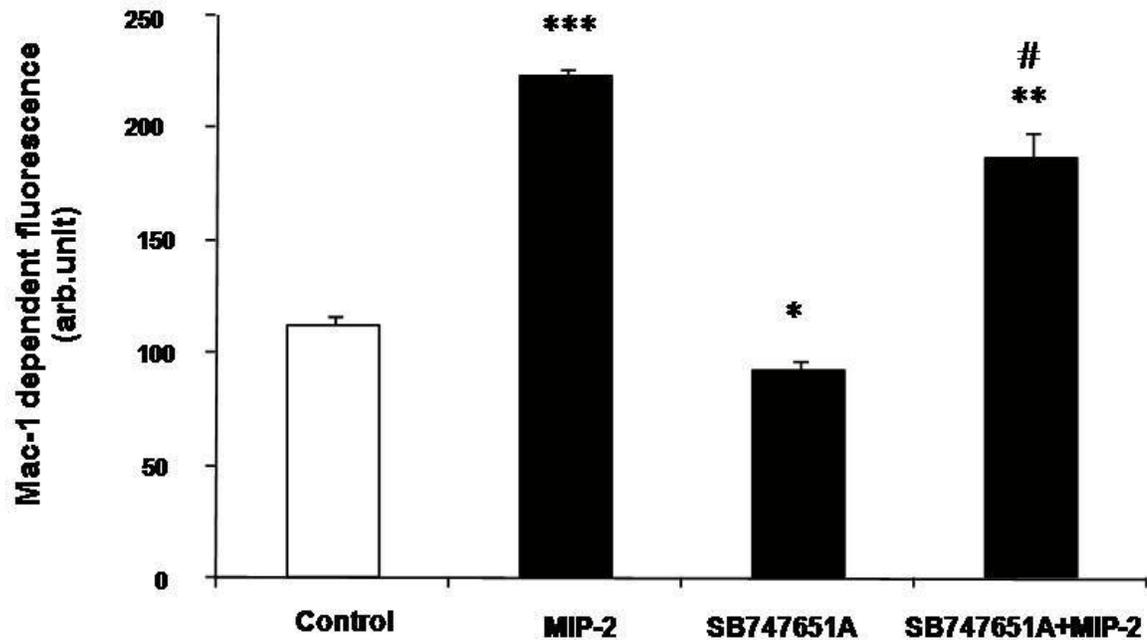
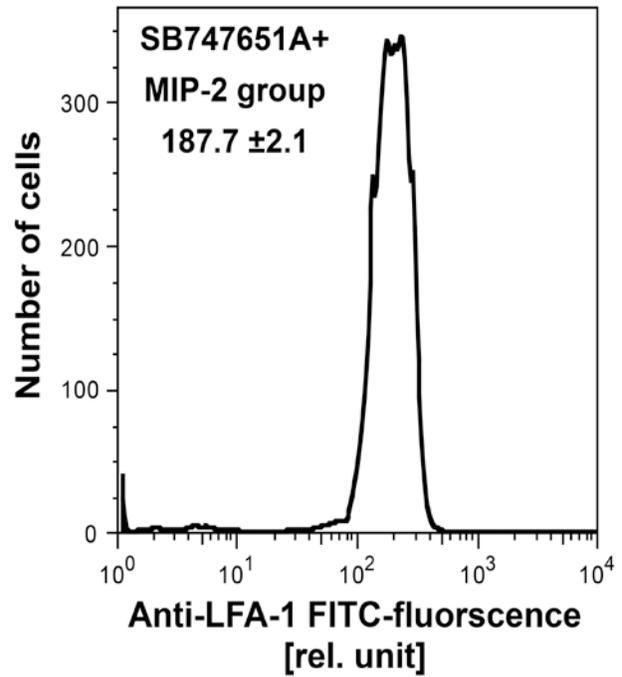
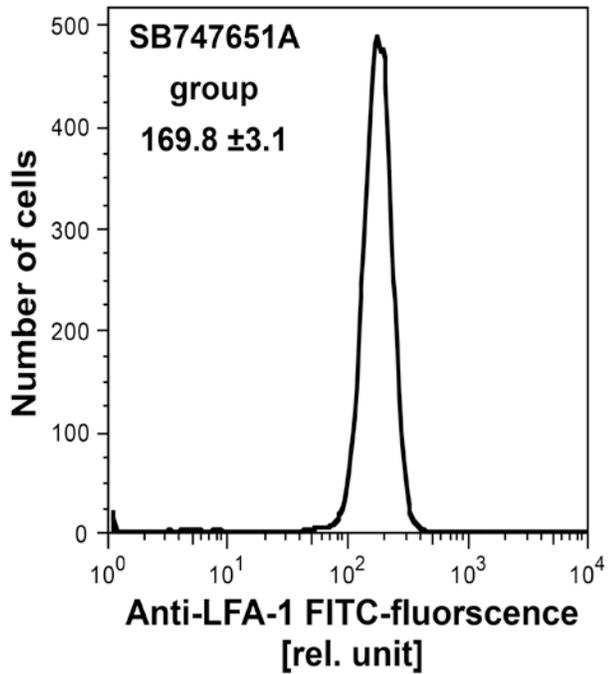
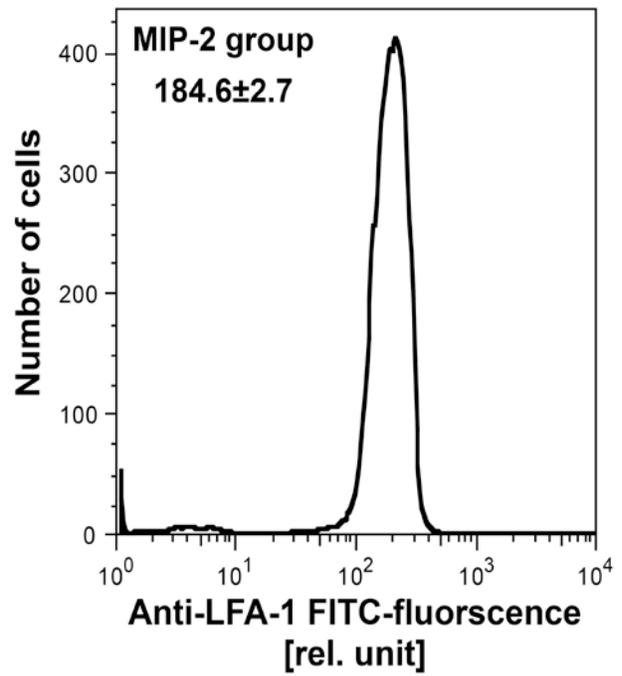
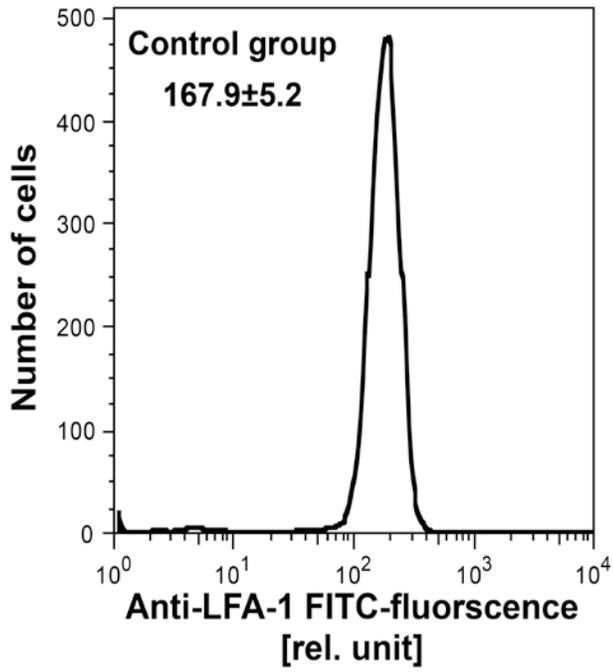


Figure 14. Effect of SB747651A on MIP-2-induced up-regulation of neutrophil Mac-1 expression *in vitro*. **A.** Representative original histograms of Mac-1-dependent fluorescence in bone marrow neutrophils in four different groups: neutrophils of control group did not receive any treatment, MIP-2 neutrophils were treated with MIP-2 only (30 nM for 10 min), neutrophils of SB747651A group were treated with SB747651A only (5 μ M for 30 min), and the neutrophils of the last group were treated with SB747651A for 30 min prior to MIP-2 treatment. **B.** Data are means \pm SEM (n = 3) of Mac-1-dependent fluorescence expressed as geomeans in the four groups. # or *: P<0.05, **: P<0.01, ***: P<0.001. * indicates significant difference from the control neutrophils without MIP-2 stimulation and # indicates significant difference from the MIP-2 stimulation without SB747651A pretreatment.

5.2 SB747651A compound did not change MIP-2-induced LFA-1 expression in murine neutrophils

Figure 15 reveals that pre-treatment of murine bone marrow neutrophils with SB747651A did not affect on MIP-2-induced LFA-1 expression on neutrophils.

A



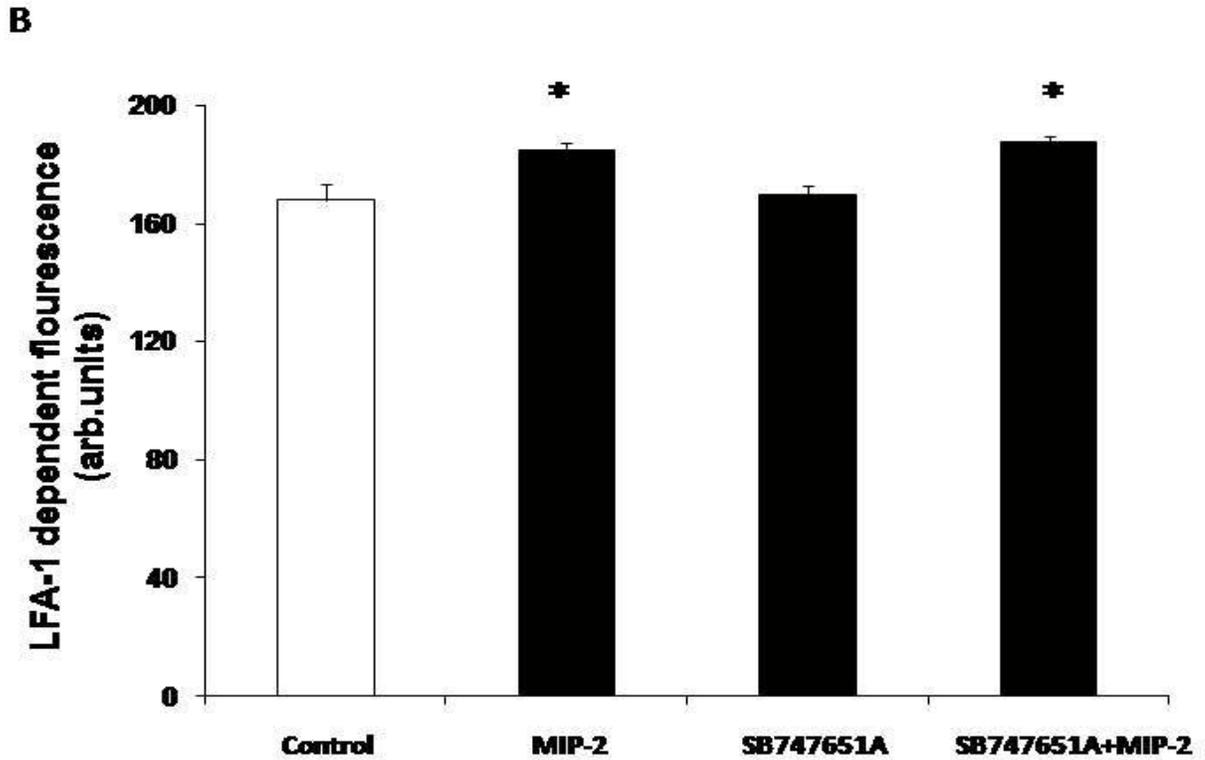


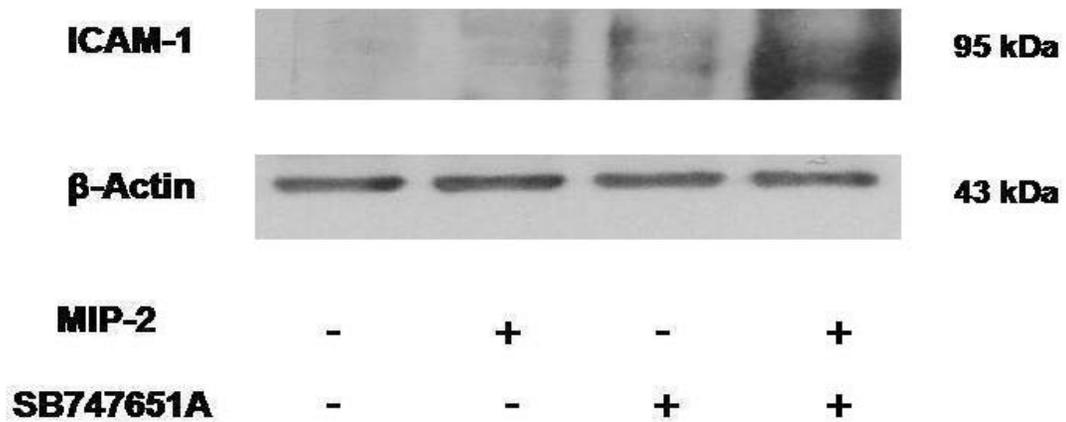
Figure 15. Effect of SB747651A on MIP-2-induced neutrophil LFA-1 expression in vitro.

A. Representative original histograms of LFA-1-dependent fluorescence in bone marrow neutrophils in four different groups: neutrophils of control group did not receive any treatment, MIP-2 neutrophils were treated with MIP-2 only (30 nM for 10 min), neutrophils of SB747651A group were treated with SB747651A only (5 μ M for 30 min), and the neutrophils of the last group were treated with SB747651A for 30 min prior to MIP-2 treatment. **B.** Data are means \pm SEM (n = 3) of LFA-1-dependent fluorescence expressed as geometric means in the four groups. *: P<0.05, * indicates significant difference from the control neutrophils without MIP-2 stimulation.

6. Effect of MSK1 specific inhibitor SB747651A on endothelial ICAM-1 expression

ICAM-1 is an important endothelial cell adhesion molecule which is expressed at basal level on the surface of resting endothelial cells. Its expression is up-regulated in acute inflammation and it mediates many steps of neutrophil recruitment. ICAM-1 mediates neutrophil adhesion to endothelium through the interaction with LFA-1 and it mediates neutrophil intraluminal crawling through the interaction with Mac-1 on neutrophil surface (7). In this study, we assessed the effect of MSK1 specific inhibitor SB747651A on endothelial ICAM-1 expression. The results in Figure 16 demonstrates that the expression of ICAM-1 in cultured murine endothelial cells was up-regulated by the treatment with SB747651A alone but not by MIP-2 treatment alone. Co-treatment of endothelial cells with SB747651A and MIP-2 further significantly increased ICAM-1 expression as compared with those treated with SB747651A alone or MIP-2 alone.

A



B

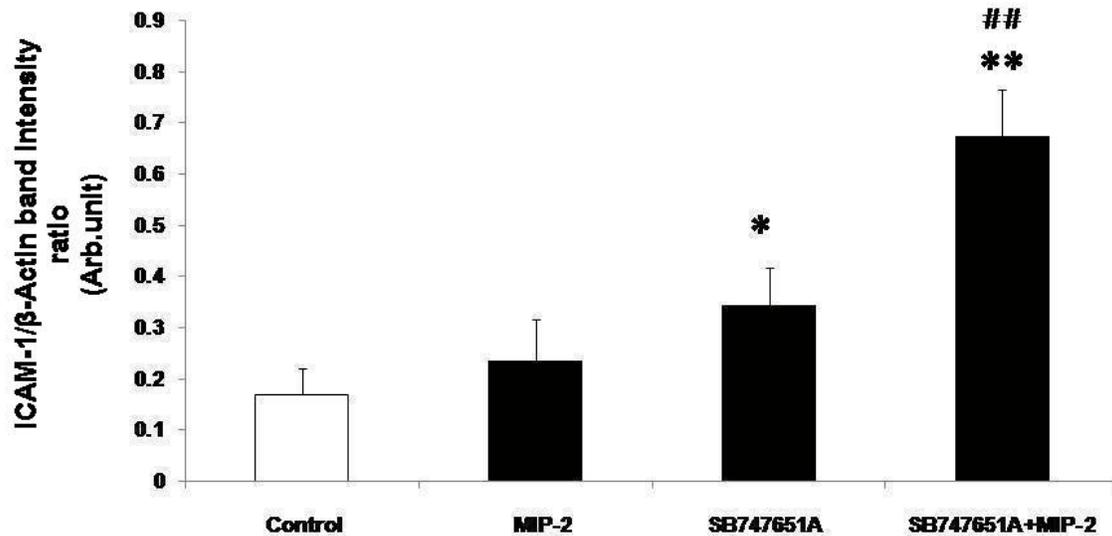


Figure 16. Effect of SB747651A on endothelial ICAM-1 expression. Four different treatment groups were included: control group (blank), MIP-2 group (treated with MIP-2, 100 nM for 4 h), SB747651A group (treated with 5 μ M of SB747651A for 5 h), and the last group SB747651A+MIP-2 (treated with SB747651A compound 1 h prior to and

during 4 h MIP-2 treatment). **A:** Original immunoblots (representative of 3 similar experiments) of ICAM-1 expression in four different treatment groups of cultured murine endothelial cells. **B:** Densitometric analysis of ICAM-1 expression in four different treatment groups of cultured murine endothelial cells. The values represent means \pm SEM (n = 3). *: P<0.05, ## or **: P<0.01. * indicates significant difference from the control endothelial cells without MIP-2 or SB747651A treatment and # indicates significant difference from the MIP-2 stimulation without SB747651A pretreatment.

Discussion

Neutrophil recruitment to the site of inflammation is a crucial feature of acute inflammation (17). It is a multistep process regulated by specific signaling molecules. The signaling mechanisms that regulate neutrophil-endothelial cell interactions are still incompletely understood. p38 MAPK signaling was shown to regulate various steps of neutrophil migration in response to different inflammatory stimuli (91). MSK1 can be activated by either upstream ERK1/2 or p38 MAPK (64,65). To date, the role of MSK1 in neutrophil recruitment has not been characterized yet. Therefore, in this study, we investigated the effect of pharmacological inhibition of MSK1 by the more potent and selective MSK1 inhibitor SB7478651A on various steps of MIP-2-induced neutrophil recruitment. We used the CXC chemokine MIP-2 because previous studies reported that 99% of recruited leukocytes in response to MIP-2 were neutrophils and it is more potent than another CXC chemokine KC (17). Because this study is focusing exclusively on neutrophil recruitment, the neutrophil selective CXC chemokine MIP-2 is the best choice to study the effect of MSK1 suppression by SB747651A on neutrophil recruitment.

At the beginning of this study, I demonstrated the effect of Ro-31-8220 on various steps of neutrophil recruitment. I found that *in vivo* treatment with Ro-31-8220 reversed KC-reduced neutrophil rolling velocity and decreased both KC-induced adhesion and emigration. Actually, the results obtained from this study can not be interpreted well because it has been found that in addition to the inhibition of MSK1, Ro-31-8220 was

able to inhibit other kinases such as PKC, RSK and GSK3 with similar potency to MSK1 (65). The kinases PKC and GSK3 are known to be involved in leukocyte recruitment (114-116). Accordingly, the data obtained from this inhibitor, Ro-31-8220, can not be interpreted as to whether the inhibition was resulted from the inhibition of MSK1 or other kinases. Subsequently, in the following experiments, I used the more selective and specific inhibitor of MSK1, SB747651A, to study the effect of MSK1 on neutrophil recruitment. SB747651A is newly developed inhibitor which targets the N-terminal kinase domain of MSK1 which belongs to AGC kinases (87). It has been reported that SB747651A acts as potent and selective inhibitor of MSK1 and it has 300 times selectivity for MSK1 over RSK (103,104).

The data of this study reveal that pharmacological inhibition of MSK1 by SB747651A modulates several steps of neutrophil recruitment. *In vivo* treatment with SB747651A significantly increased MIP-2-induced adhesion at all time points after MIP-2 treatment but the effect of SB747651A on neutrophil emigration seems to be time-dependent. Interestingly, I found that SB747651A impairs MIP-2-induced emigration at earlier time (30-60 min after MIP-2 treatment) points but enhanced MIP-2-induced emigration at later time points (3.5-4.5 h after MIP-2 treatment). To better understand the effect of SB747651A on different steps of neutrophil recruitment, I assessed its effect on neutrophil intraluminal crawling in post capillary venules, transmigration through the endothelium and chemotaxis in tissue of cremaster muscle toward the MIP-2 chemokine. As previously reported and now generally accepted, intraluminal crawling is the intermediate step that enables neutrophil transition from adhesion to emigration. In

other words, after firm adhesion to endothelium, neutrophils start crawling from adhesion site to the optimal transmigration site which is mostly through endothelial junctions (17). In this study, pharmacological inhibition of MSK1 by SB747651A impaired MIP-2-induced neutrophil intraluminal crawling because it caused significant reduction in velocity of crawling and there was tendency of incremental changes in crawling time and distance although not reaching statistical significance. Interestingly, both of transmigration time and detachment time of neutrophils were prolonged significantly in the presence SB747651A. In addition, although the directionality of neutrophil chemotaxis in tissue toward MIP-2 chemokine was not affected by SB747651A, the velocity of migration of neutrophils in cremasteric tissue was reduced significantly in the presence of SB747651A. The previous studies revealed that all adherent neutrophils crawl to reach to the optimal transmigration sites (mostly through endothelial junctions) and impairment of crawling blocks the adherent neutrophils from moving to the optimal transmigration sites and subsequently the neutrophils start to transmigrate through non or less optimal sites (directly through endothelial cell, transcellular route) causing the cells to spend much more time in transmigration and thus leading to dysfunctional emigration process which is characterized by decreased and delayed emigration at earlier time point (17). Interestingly in my study, impairment of MIP-2-induced intraluminal crawling in the presence of pharmacological inhibitor of MSK1, SB747651A, and the subsequent prolonged transmigration process which finally caused the reduction in the number of emigrated neutrophils at the earlier time points supported the previous reports regarding the effect of impaired emigration due to impaired

crawling. Since my data showed increased adherent neutrophils in the presence of SB747651A and the expected result from increased adherent neurophils would be the increased number of emigrated neutrophils. But in fact, my data show that the number of emigrated neutrophils at earlier time points (30-60 min after MIP-2 stimulation) was decreased in the presence of SB747651A and this decrease in number of emigrated neutrophils may be due to dysfunctional crawling as a result of SB747651A effect. The impaired crawling in the presence of SB747651A causing a dramatic decrease in emigration at earlier time points may be due to the reason that the adherent neutrophils transmigrate through non optimal sites prolonging their time in transmigration. Although these increased adherent neutrophils spent much more time in crawling and transmigration, they eventually migrate out of the venule which was evident as the increase in the number of emigrated neutrophis at later time points (3.5-4.5 h) in my study.

Furthermore, to demonstrate molecular mechanism through which the pharmacological inhibitor of MSK1, SB747651A, affects neutrophil recruitment, I studied the effect of SB747651A on MIP-2-induced β_2 -integrin expression on the surface of neutrophils. β_2 -integrins include Mac-1 (CD11b/CD18, $\alpha_M\beta_2$ integrin) and LFA-1 (CD11a/CD18, $\alpha_L\beta_2$ integrin). Handerson *et al.* demonstrated that LFA-1 is more important in neutrophil adhesion whereas Mac-1 is more fundamental in neutrophil migration, and their results were based on using Mac-1 blocking antibodies that caused dramatic decrease in neutrophil migration without affecting adhesion step (20). Furthermore, Phillipson *et al.* reported that neutrophil adhesion to endothelium is mainly dependent on LFA-1

interaction with endothelial ICAM1/2 whereas intraluminal crawling is mediated predominantly by Mac-1 interaction with endothelial ICAM-1 (17). The previous study revealed that using Mac-1-deficient neutrophils or monoclonal antibody against Mac-1 resulted in failure of crawling of majority of neutrophils in response to MIP-2 chemokine (17). It was less than 30% of Mac-1-deficient neutrophils that showed the ability of crawling and although they crawled, they took much longer time than the wild-type neutrophils indicating that impairment of crawling in Mac-1-deficient neutrophils (17). It has been demonstrated that neutrophils from wild-type mice transmigrated predominantly via endothelial junctions (86%) in contrast to neutrophils from Mac-1-deficient mice, only minor portion of them (39%) migrated through the endothelial junction while majority of them emigrated mostly through non junctional sites and this subsequently caused impairment of emigration process in Mac-1-deficient neutrophils as a result of loss of ability to reach to optimal junctional emigration sites (17). It also has been reported that LFA-1 deficiency resulted in few number of adherent neutrophils but those few adherent neutrophils were able to crawl efficiently (17). Conclusively, the previous studies confirmed that Mac-1 and LFA-1 have different and distinct roles in two sequential steps of neutrophil recruitment because neutrophil adhesion was mainly dependent on LFA-1 whereas intraluminal crawling was mainly dependent on Mac-1. The data of my study revealed that pharmacological inhibition of MSK1 by SB747651A suppressed MIP-2-induced up-regulation of Mac-1 expression in murine neutrophils but it did not affect on MIP-2-induced up-regulation of LFA-1 expression. Since pretreatment with SB747651A caused reduction in MIP-2-induced

Mac-1 expression and based on the reported effect of Mac-1 deficiency on efficiency of intraluminal crawling and subsequent transmigration and emigration process (17), I conclude that the impairment of neutrophil crawling, transmigration and the subsequent emigration at early time frame after SB747651A treatment in my study was likely due to Mac-1 suppressive effect of MSK1 specific inhibitor SB747651A. The reduction in Mac-1 caused inability of neutrophils to crawl to the optimal junctional endothelial sites for transmigration with subsequent prolonged transmigration through the non-optimal sites which finally caused the reduction in emigrated neutrophils at earlier time points, 30-60 min after MIP-2 stimulation.

Previous studies have highlighted the role of ICAM-1 in several steps of neutrophil recruitment. ICAM-1, an endothelial cell adhesion molecule, is categorized under immunoglobulin superfamily. It is expressed constitutively at basal level on endothelial cells and its expression is increased in response to several inflammatory cytokines such as IL-1, TNF- α , INF- γ and LPS (105-108). ICAM-1 is one of major ligands of β_2 -integrins LFA-1 and Mac-1 which are expressed in neutrophils, therefore, ICAM-1 mediates leukocyte-endothelial cell interactions in inflammation (106). It is well-known that ICAM-1 mediates neutrophil firm adhesion through its interaction with LFA-1 and mediates neutrophil efficient intravascular crawling through its interaction with Mac-1 on neutrophil surface (17). In our study, we demonstrated the positive effect of the pharmacological inhibitor of MSK1, SB747651A, on endothelial ICAM-1 expression. Although MIP-2 alone did not cause significant increase in ICAM-1 expression, we observed that endothelial ICAM-1 expression after MIP-2 stimulation was significantly

up-regulated in the presence of MSK1 Inhibitor SB747651A. This result suggests that increased MIP-2-induced adhesion in the presence of SB747651A can be attributed at least partly to enhancing effect of SB747651A on endothelial ICAM-1 expression.

Conclusion

In this study, we revealed that MSK1 is expressed in murine neutrophils and its expression was up-regulated significantly after MIP-2 stimulation. We found that MSK1 is one of downstream kinases that plays a role in MIP-2-induced neutrophil recruitment. Our study concludes that the more potent and selective pharmacological inhibitor of MSK1, SB747651A, enhances MIP-2 induced neutrophil adhesion likely through its positive effect on endothelial ICAM-1 expression but impairs intraluminal crawling, transmigration and subsequent emigration of neutrophils at earlier time points, and these effects may be mediated through the suppressive effect of SB747651A on MIP-2-induced up-regulation of Mac-1 expression. Collectively, our study demonstrates that pharmacological inhibition of MSK1 by SB747651A affects multiple steps of MIP-2 induced neutrophil recruitment *in vivo*.

Significance of this study

The current study is the first attempt to understand the role of MSK1 in leukocyte-endothelial interactions *in vivo*. Although other studies have shown the participation of MSK1 in inflammation, its role in an acute, chemokine-induced inflammatory response has not been shown. Several studies have reported the role of p38 MAPK signaling in

inflammation but these studies have not addressed the role of the downstream molecule MSK1. We, here, show the significance of MSK1 in different steps of leukocyte recruitment by using intravital microscopy which enables us to study real-time cell behavior and dynamics of leukocyte recruitment. Furthermore, by using time-lapsed video microscopy we were able to analyze the different parameters of neutrophil recruitment such as intraluminal crawling, transmigration and extravascular chemotaxis which are not observed at real-time. The present research shall become the basis for future investigations on the role of MSK1 in innate immunity.

Limitation of study

This study for the first time identifies the role of MSK1 in different neutrophil recruitment steps during neutrophil-endothelial cell interactions. Despite these merits, there are certain limitations which may preclude us to draw safe conclusions. We have based our entire conclusions on the data of using the inhibitor alone and not using MSK1 transgenic mice. Secondly, the inhibitor has been confirmed to be highly specific to MSK1 suppression *in vivo*. However, due to lack of extensive *in vivo* and *in vitro* studies on its safety and non toxicity, it does not warrant any clinical use at this stage. Because the leukocyte-endothelial cell interaction is a complex process and MSK1 is expressed in both leukocytes and endothelial cells, it is best to use chimeric mice generated by bone marrow transplantation to study the cell-specific effects of MSK1 in each cell type in the future.

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