ABSTRACT

ECKERT, RACHAEL ELIZABETH. Molecular Mechanisms of Neutrophil Migration. (Under the direction of Samuel L. Jones.)

This work is an investigative look behind the mechanisms of neutrophil migration. Each of three chapters involves exploration into a different signaling pathway important for migration downstream of chemoattractant stimulation through inhibition of a kinase or disruption of the function of an effector and examination of the effects on migration, adhesion, and actin reorganization in primary human or equine neutrophils. Chapter II examines the requirement for the signaling molecule p38 Mitogen Activated Kinase (MAPK) in equine neutrophil chemotaxis through use of the p38 specific inhibitor SB203580. SB203580 reduced LTB_4- and PAF-induced migration and disrupted the ability of cells to polarize, but did not affect β2 integrin-dependent adhesion or surface β2 integrin expression. Chapter III is a comprehensive inquiry into the regulation of the phosphorylation of serine 157 of the cytoskeletal protein Vasodilator-stimulated Phosphoprotein (VASP). The rapid and transient phosphorylation of VASP serine 157 corresponded with F-actin levels in chemoattractant-stimulated human neutrophils. fMLF-induced serine 157 phosphorylation was abolished by pretreatment with the PKA inhibitor H89 and the adenylyl cyclase inhibitor SQ22536. In contrast, fMLF-induced serine 157 phosphorylation was unaffected by PKC inhibitors, PKG inhibitors, and the CamKII inhibitor KN-62. Inhibition of adhesion did not alter fMLF-induced VASP phosphorylation or dephosphorylation. This study demonstrated that chemoattractant stimulation of human neutrophils induces a rapid and transient PKA-dependent and adhesion-independent VASP serine 157 phosphorylation. Chapter IV probed into the function of the actin binding protein and PKC substrate Myristoylated Alanine-Rich

C-kinase Substrate (MARCKS) through utilization of a cell permeant peptide derived from the MARCKS myristoylated aminoterminus (MANS peptide). Treatment of isolated human neutrophils with 50 μM MANS, but not a scrambled control peptide, significantly inhibited their migration and adhesion in response to fMLF, IL8, or LTB_4. MANS significantly reduced F-actin content in neutrophils 30s after fMLF-induced polymerization, but did not alter the ability of cells to polarize, spread, or upregulate surface β2 integrin expression. These data provided evidence that MARCKS, via its myristoylated aminoterminus, is a key regulator of neutrophil migration and adhesion.

Molecular Mechanisms of Neutrophil Migration

by
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BIOGRAPHY

Rachael Eckert was born on June 19, 1977 in Kingston, New York and lived in Milton, NY with her parents, Sally and Frederick Eckert, sister, Laura, and brother, David, before moving to Wilson, NC in 1987. She attended James B. Hunt, Jr. High School for two years, before acceptance into the North Carolina School of Science and Mathematics (NCSSM) in Durham, NC in 1993. She graduated from NCSSM in 1995 and completed three years of undergraduate courses at North Carolina State University (NCSU) before fulfilling requirements for acceptance into the NCSU College of Veterinary Medicine in 1998. She graduated from NCSU with a Doctorate of Veterinary Medicine in May 2002 and began work as an equine veterinarian at a two-doctor private practice in Greensboro, NC. After a year in practice she returned to NCSU in August 2003 to begin a PhD program in Immunology under the direction of Samuel L. Jones.

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CHAPTER I. LITERATURE REVIEW

OVERVIEW OF MOLECULAR MECHANISMS OF NEUTROPHIL MIGRATION

Introduction

Inflammation is a central component in the pathophysiology of disease. The body responds to pathogens with a vast arsenal of immune cells and attempts to resolve the invasion with a rapid return to *status quo*. Preferably, a disease condition comes and goes and there is scarce remaining evidence of the battle. Indeed, inflammation is required for protection against colonization by infectious pathogens and in the clearing of traumatically damaged tissues, which is made clear by the shortened lifespan of animals with primary immunodeficiencies such as leukocyte adhesion disorders (LAD) [1,2], affecting innate immunity, or severe-combined immunodeficiencies (SCID), affecting adaptive immunity [3,4]. Unfortunately, robust inflammation can also result in collateral damage to civilian tissues. The immune system has an intricate system in place for down-regulating inflammation to limit unnecessary damage. As the enemy threat retreats and the forces are spent, apoptotic neutrophils are contained and cleared by macrophages [5]. Lipid mediator production switches from the chemotactic and pro-inflammatory leukotrienes to anti-inflammatory lipoxins. This conversion serves to limit the further transmigration of neutrophils [6], promote the infiltration of monocytes [7] and enhance macrophage phagocytosis of apoptotic cells [8] which transforms activated macrophages into a reparative phenotype necessary for the resolution phase of acute inflammation [9-12]. To concurrently stimulate the termination of active inflammation, the production of resolvins (resolution-phase interaction products) and protectins from omega-3 polyunsaturated fatty acids is initiated [11,13-15]. Resolvins and protectins promote chemokine removal [16] and reduce

the transmigration of leukocytes to areas of resolving inflammation [13,15,17]. Despite these measures, inflammation can still potentially become dysregulated and the host requires outside allied forces to reverse the offensive strike.

Neutrophils are the immune system's first line of defense against infection and typically comprise 60% of the mammalian circulating white blood cell pool and can increase to 80-90% in acute bacterial infection [18]. These first-responders kill bacteria by either phagocytosis [19], which contains the toxic metabolites and microorganisms within an intracytoplasmic phagosome [19,20], or, when particles are too large to ingest, through respiratory burst which results in a release of reactive oxygen intermediates (ROI) and proteases into the surrounding tissue [20,21]. In the case of bacterial invasion, this resulting tissue destruction helps to trap the microorganisms in a localized reservoir of digested tissue and by-products of inflammation, known as pus [22]. But, there is a thin and dangerous line that separates beneficial from detrimental tissue destruction in inflammation. Unfortunately, a major component of the clinical syndrome of certain noninfectious diseases is excess neutrophilic inflammation. Neutrophils can overwhelmingly flood a tissue due to immune complex deposition, as seen in glomerulonephritis, due to acute ischemic tissue injury, as seen during strangulating gastrointestinal disorders, or due to multi-factorial causes, as seen in ulcerative colitis, chronic obstructive pulmonary disease, and multi-organ failure in sepsis [23-32]. These diseases affect a large segment of the veterinary population and there remains a search for beneficial therapeutics for the resulting dysregulated inflammation. A deeper

understanding of how neutrophils move into these tissues at the molecular level will pave the way for the development of more effective therapeutic strategies to limit tissue destruction.

Neutrophils are initially pulled out of the circulation through interactions between the fucose-containing sialyl-Lewis^x residues on neutrophils [33,34] and selectins expressed on an activated endothelium [35]. Through this weak endothelial-neutrophil binding, neutrophils escape the high flow circulation and begin to roll along the endothelial membrane, able to sample the local environment. After encountering a gradient of chemoattractant, chemical messengers derived from pathogens or secreted from neighboring cells, neutrophils are recruited to the site of inflammation. As a specialized eukaryotic chemotactic cell, the neutrophil can sense as little as a 5-10% difference in concentration of chemoattractant between the ends of the cell [36,37]. A few of the more important chemoattractants for neutrophils include the complement protein C5a [38], platelet activating factor (PAF) [39], leukotriene B_4 (LTB_4) [40], interleukin-8 (IL-8), and the formylated bacterial peptide, formyl-Met-Leu-Phe (fMLF) [36]. Neutrophil chemoattractant receptors are seven transmembrane G-protein coupled receptors (GPCR) linked to several key signaling pathways [41] and binding results in activation of the cell. Once activated, the neutrophil firmly adheres to the endothelium through heterodimeric β2 integrin binding, mostly CD18/CD11b, to endothelial cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1). Once firmly bound, the neutrophil undergoes spreading followed by migration across the endothelium into the tissue space. In the tissues the neutrophil becomes polarized and exhibits a distinctive shape change. In the polarizing neutrophil, the edge

closest to the largest concentration of chemoattractant, called the leading edge, reorganizes its actin cytoskeleton and forms a rounded protrusion called the pseudopod. The opposite pole of the cell is the trailing edge, which forms a thinner, blunted appendage called the uropod. As the leading edge extends, with strong β2 integrin adhesion, and the trailing edge detaches, with weaker β2 integrin adhesion, the cell treadmills along towards the site of inflammation. The focus of this review is to highlight important molecular mechanisms of neutrophil migration as potential targets for anti-inflammatory therapy.

Actin Polymerization

In order for a cell to migrate, a force must be generated intracellularly to physically move the plasma membrane; this is accomplished through ATP-dependent actin polymerization. Actin was originally discovered as a major protein in muscle that converts chemical energy, from the hydrolysis of ATP, into contractile movement, through interaction with myosin [42-44]. Actin was further recognized as a highly conserved protein present in most eukaryotic cells [44] and a key component of the cytoskeleton [45,46]. In the cytosol of resting neutrophils is a rich supply of β actin in monomeric form [47], G-actin, which is prevented from polymerizing due to association with actin-binding proteins such as profilin and thymosin [48-51]. ATP-bound G-actin monomers near the plasma membrane polymerize to form filaments, called F-actin [44]. When the cell is stimulated to alter its cytoskeleton, nucleation promoting factors, such as the Wiskott-Aldrich syndrome protein (WASP) or

WAVE/SCAR proteins, activate the seven-member Arp2/3 complex to initiate a new filament [52-56].

An actin filament is inherently polar with one where polymerization takes place, the barbed end, and one end where depolymerization occurs, the pointed end [57]. The elongating barbed end, named for the arrowhead appearance formed from myosin units bound to the filament [58,59], is continuously lengthened by the addition of ATP-actin monomers [60]. As the filament grows, ATP is irreversibly hydrolyzed [61,62], which consequently increases the rate of dissociation of actin from the filament, and ADP-actin is released from the pointed end [60,63]. Profilin acts as a nucleotide exchange factor by binding free actin-ADP and catalyzing the exchange for ATP, and thus provides a pool of free ATP-bound G-actin monomers ready for polymerization [64,65], while the actin-binding protein β-thymosin simply sequesters G-actin [50,51]. Capping proteins, such as gelsolin, terminate continuous filament elongation and help maintain short, strong filaments [64]. In addition to *de novo* actin nucleation by Arp2/3, new sites for actin polymerization can occur due to the uncapping or severing of filaments. The process of uncapping is poorly understood, but involves removal of capping proteins from the barbed end and has been suggested to involve phosphoinositide signaling [66-68]. The creation of free barbed ends through the severing of existing filaments occurs by actin-depolymerization factor (ADF)/cofilin activity [69,70]. ADF/cofilin also initiates depolymerization at the pointed end, which, in turn, provides free actin monomers for further polymerization [70].

A long, thin filament would be similar to the fiberglass poles used in pole vaulting—it would bend against a pushing force. In order to distribute the force over a larger area, the cytoskeleton of a rapidly advancing leading edge of a motile neutrophil is composed of a highly branched latticework of actin [64,71]. To create these branches the Arp2/3 complex binds the existing filament and a new 'daughter' filament is initiated at a 70º angle, growing in the barbed direction [72-74]. There are currently two theories as to how the activated Arp2/3 complex initiates a branch. The side-branching model, the heavily favored theory, suggests that the Arp2/3 complex binds the side of a filament with Arp2 and Arp3 forming the first two new subunits and nucleation continuing in the barbed direction [72]. The end-branching model proposes that the activated Arp2/3 complex competes with capping proteins at the end of an existing filament [75,76]. In this model, Arp2 remains in line with the existing filament and is the base for the addition of monomers for elongation, while Arp3 is positioned on the outside and is the base for the formation of the new branch [75,76]. Extensive branching provides strength to the actin in the pseudopod, but the actual force that physically displaces the cell membrane is thought to be due to the "Brownian ratchet" model in which stored thermal energy causes the lengthening filament to bend and exhibit Brownian motion; as actin monomers continue to intercalate and the filament eventually straightens against the leading edge, a propulsive force is generated and physically moves the membrane [64,77]. The unbending actin filament cannot generate enough force to extend the cell membrane unless the polymerized actin has first been stabilized by cross-linking proteins, such as filamen or α-actinin, or anchored to the underlying substratum through localized

adhesion sites [64]. Actin filament bundles, called stress fibers, end at sites of focal contact of the plasma membrane and provide additional stability to sites of cytoskeletal-substratum adhesion [64].

Polarization

The establishment of a leading edge and trailing edge is essential for polarization and directional migration. Neutrophils have the ability to quickly redirect their orientation and travel in the opposite direction when the source of chemoattractant changes location [36,78]. In a resting neutrophil, the chemoattractant GPCRs are distributed evenly over the surface of the cell [79-81]. Upon stimulation, there is a slight increase in the Gβγ subunits of the GPCR at the front of the cell as opposed to the rear, which may increase sensitivity, but the difference is not enough to create a sufficiently dynamic signaling event for polarization [82]. To accomplish two seemingly opposing phenomenon in the same cell, lamellipodial extension and uropod retraction, there exists an intricate system of interconnected signaling pathways. Phopshatidylinositol kinase (PI3K) and the Rho family GTPases have been shown to be instrumental in orchestrating membrane protrusion and retraction in neutrophils [37,83,84]. The Rho-family GTPases have been shown to activate the nucleation promoting factor WAVE/Scar, one activator of the Arp2/3 complex, and are suggested to be an important switch capable of activating multiple signaling pathways simultaneously to achieve spatial control of the cytoskeleton [85]. The most prominent Rho GTPases in neutrophils are of the Rac subfamily, Cdc42 and Rac [86], and are capable of promoting actin

polymerization [87,88]. After chemoattractant GPCR stimulation, $G\alpha_i$ activation leads to the accumulation of lipid products of PI3K, such as phosphatidylinositol-3,4,5-triphosphate (PIP3), at the leading edge and the activation of Cdc42 and Rac, which promotes further PI3K accumulation and actin polymerization, all in a positive feedback loop with F-actin; for further amplification of the signal, the target of Cdc42, p21-activated kinase –1 (PAK1), can feed back and activate Cdc42 [83,84,89-91].

The positive feedback loop between PIP3 and the GTPases Cdc42 and Rac responsible for promoting membrane protrusion must be localized to the leading edge to enable polarity of the cell. To accomplish this, PIP3 accumulation is inhibited at the sides and rear of the cell through the localization and activity of PIP3-degrating enzymes including phosphatase and tensin homologue (PTEN) and 5-phosphatases [92,93]. Work in *Dictyostelium discoideum*, simple amoeboa that have similar aptitudes for chemotactic sensitivity as neutrophils, has indeed shown that as the cell is polarizing towards the gradient of chemoattractant, there is an internal gradient of PIP3 created by localization of PI3K at the leading edge and PTEN at the sides and rear of the cell [37,92,94,95]. The 5-phosphatases, including the most characterized member SHIP, constitute over 90% of the PIP3 phosphatase activity in neutrophils and are probably responsible for most of the PIP3 degradation [78,96].

When a GPCR is activated by chemoattractant, $G\alpha_i$ directs the PI3K signaling responsible for membrane protrusion at the leading edge while $G\alpha_{12}$ and $G\alpha_{13}$ stimulate a Rho-dependent pathway that directs the rear of the cell to inhibit lamellipodial formation and retract the uropod to enable forward migration [86,97]. Upon stimulation by fMLP, RhoA,

myosin light chain, and myosin heavy chain has been shown to translocate to the trailing edge of neutrophil-like differentiated HL-60 cells and subsequent uropod formation depends on both Rho-stimulated kinase (ROCK) and myosin II [86]. Myosin II function is regulated through phosphorylation of myosin light chain (MLC) by myosin light chain kinase (MLCK), which is regulated further upstream by Ca^{2+} and ROCK [37,86]. Myosin filament formation and activation at the sides and rear of the cell are responsible for the inhibition of membrane protrusion and mechanical retraction of the uropod [37,98]. Overexpression of RhoA inhibits polarity, F-actin reorganization, PIP3 accumulation, and Rac activation in chemoattractant-stimulated neutrophils, highlighting the ability of the signaling in the rear of the cell to simultaneously inhibit the front [86]. Likewise, Rac activity in the pseudopod *locally* suppresses Rho activity, but Rac activity, in turn, is required *globally* for Rho- and myosin-associated uropod regulation [86,99].

In vitro inhibition of PI3K inhibits cell polarity while, alternatively, the addition of cell-permeant PIP3 in the absence of chemoattractant induces polarity and migration in neutrophils, highlighting the involvement of the PI3K pathway in chemotaxis [83,100-107]. PI3K is a heterodimer composed of a 110-kD and 85-kD subunit [108-111]. In neutrophils, chemoattractant GPCRs activate class 1A and class 1B PI3K [112-114], yet studies in transgenic mice have shown that activation of AKT and neutrophil chemotaxis requires only p110γ of the class 1B PI3Ks [105,107,110]. The neutrophils of PI3Kγ knockout mice have the ability to polymerize actin and migrate, but demonstrate severe defects in both [105,110], yet produce no PIP3 after chemoattractant stimulation, suggesting that PI3K is important for

the internal compass of directed migration. The signaling pathways that link PI3K to the activation of the small GTPases are poorly understood. Binding of PIP3 *in vitro* enhances the guanine exchange factor (GEF) activity of Vav1 [115] and members of the Vav family have been shown to be GEFs for RhoA, RhoG, Rac and Cdc42 [116]. Vav1/3 proteins are required for β2 integrin-dependent adhesion, spreading, and β2 activation of Cdc42, Rac1, and RhoA in neutrophils [116]. Yet, experiments with Vav1/3 knockout neutrophils have shown that Vav1 does not appear to be required for chemotaxis, as visualized by video microscopy, or chemoattractant-induced activation of Rac1, Rac2, or Cdc42 [116]. Although not involved in GPCR signaling of neutrophils, Vav does seem to be critical for β2 integrin-dependent Rho GTPase activation and signaling in neutrophils [116]. P-Rex is a GEF that activates the GTPase Rac upon regulation by PIP3 and the Gβγ subunit of GPCRs [117-120]. P-Rex1 is highly expressed in neutrophils and has been shown to be involved in C5a-stimulated reactive oxygen species production [119]. Because P-Rex functions downstream of Gβγ proteins in neutrophils and is both directly and synergistically activated by PI3K [119], it is an attractive candidate as an important GEF in the spatial regulation of chemotaxis. Further work needs to be done to define the individual GEFs important for PI3K signaling in the establishment of neutrophil polarity.

Locomotion

In order to migrate, the neutrophil must first spread and adhere to the ECM as polarization begins, then achieve firm adhesion of the leading edge pseudopod to the

substratum, and finally contract the rear uropod to move the cell body forward. Spreading is an active process characterized by an increase in the surface area, reduction in height, and a decrease in the distance from the neutrophil to the substrate along with increased adhesion [121]. As early as 10 seconds after fMLF stimulation, neutrophils begin to spread in an anisotrophic manner as polarization initiates [121]. As the cell is activated to migrate, the adhesive state of the β2 integrins is important for the necessary signaling and tractional events required for chemotaxis. Regulation of the adhesion and transmigration of leukocytes in veterinary diseases has been extensively reviewed by *Radi et al* and will be briefly discussed here as it pertains to neutrophil migration [122]. The predominant β2 integrin on the surface of neutrophils is CD11b/CD18 [123], also called Mac-1, which mediates neutrophil adhesion to activated endothelium, fibrinogen, and other matrix proteins derived from plasma, enabling chemotaxis and emigration [124]. For migration to cease, for example when cells arrive at their destination of inflammation, cells develop focal adhesions and firm adhesion. A focal adhesion site consists of a cluster of activated integrins on the plasma membrane, bound to the cytoskeleton via adaptor proteins and co-localized with other focal adhesion proteins, creating an F-actin rich site of stable attachment to the extracellular matrix (ECM) [125-127]. As highly motile cells, neutrophils generally have smaller areas of concentrated adhesion, called podosomes, where transient adhesion to the ECM provides the traction necessary for migration [128-130]. Activated neutrophils can increase their adhesiveness, as necessary, in several ways. Upon activation, CD11b/CD18 is upregulated through translocation to the plasma membrane from intracellular secretory vesicles [50,131].

In general, integrins on the surface of resting neutrophils, and those in focal adhesion, are fixed and evenly distributed across the cell membrane [132,133]. Chemoattractant activation of neutrophils induces a clustering of the surface β2 integrins and a change from a low to an intermediate affinity state to enable stable adhesion to tissue substrates [134,135]. To achieve localized adhesion in the pseudopod, integrins, as transmembrane receptors, become laterally mobile in the plasma membrane and there is a continuous recycling of integrins to the leading edge [132,136].

Myosin II, an actin based motor protein, is responsible for the final mechanical force necessary to retract the rear of the cell to allow forward movement of the cell body [98]. Myosin II is composed of two heavy chains, which comprise the head and tail components, and four light chains [137]. The head binds F-actin and, powered by the hydrolysis of ATP, moves along the filament while the tail binds other myosin filaments [138]. As previously described, the serine/threonine kinase, myosin light chain kinase (MLCK), activated by Ca^{2+}/calmodulin and ROCK, phosphorylates serine 19 on the myosin light chain, allowing myosin to bind actin and enabling contraction [139].

Conclusions

Neutrophils are the "Green Berets" of the immune system. They are impressive in their recruitment, zealous in their actions, and Kamikaze in their missions. No mammal can survive the war on pathogens without the mighty neutrophil. On the other hand, when they become rogue warriors and inflammation becomes perpetual, neutrophils can be difficult to

restrain. Glucocorticoids, one of our biggest arsenals against inflammation in veterinary medicine, are virtually powerless against limiting neutrophils migration. Understanding the intercellular machinery and biochemistry behind neutrophil migration will enable future research to focus therapy at the molecular level. The following work consists of three chapters, each of which explores a different signaling pathway important for neutrophil migration downstream of chemoattractant stimulation. Chapter II examines the role of p38 MAPK in equine neutrophil polarization and migration. Chapter III characterizes the chemoattractant-induced phosphorylation of the PKA substrate and cytoskeletal protein Vasodilator-stimulated Phosphoprotein (VASP). Chapter IV explores the role of the N-terminal region of the actin cross-linking protein Myristoylated Alanine-Rich C-kinase Substrate (MARCKS).

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CHAPTER II

p38 MITOGEN-ACTIVATED KINASE (MAPK) IS ESSENTIAL FOR EQUINE NEUTROPHIL MIGRATION

Submitted to *Veterinary Immunology and Immunopathology*

Abstract

Equine laminar tissues do not contain resident neutrophils and have less superoxide dismutase (SOD) activity as compared to other equine tissues, which makes them inherently more vulnerable to neutrophil ROS production. In the advanced clinical stages of acute laminitis, affected feet show a breakdown in the basement membrane, neutrophil infiltration, and platelet-neutrophil aggregates in laminar dermal veins, highlighting the contribution of neutrophils to the pathophysiology of the disease. The aim of this study was to determine the role of p38 MAPK in the mechanism underlying equine neutrophil migration to potentially reveal therapeutic targets that will limit lamellar damage from the neutrophil influx seen in acute laminitis. Our results show that the endogenous chemoattractant LTB_4 transiently activated p38 MAPK and induced chemotaxis of equine primary neutrophils. Inhibition with the p38 MAPK specific inhibitor SB203580 reduced LTB_4-induced migration in a dose-dependent manner with an IC_{50} of 2.8μM. We next examined the potential mechanisms behind the ability of SB203580 to abolish migration. We found that inhibition of p38 MAPK with 10 μM SB203580 disrupted the ability of neutrophils to polarize in response to LTB_4 and PAF. In contrast, p38 did not appear to be required for chemoattractant- or PKC-induced β2-integrin adhesion or chemoattractant-induced upregulation of surface β2 integrins, but was required for TNFα-induced adhesion. These findings support a function for p38 MAPK in equine neutrophil migration and suggest the potential for the ability of p38 MAPK inhibition to limit neutrophilic inflammation in the laminae during acute laminitis.

Abbreviations: LPS, lipopolysaccharide; TLR, toll-like receptor; COX-2, cyclooxygenase 2; IL1-β, Interleukin 1-β; IL-6, interleukin-6; DRD, differential mRNA display; MAIL, Molecule possessing Ankyrin-repeats Induced by Lipopolysaccharide; MMPs, matrix metalloproteinase enzymes; ROS, reactive oxygen species; MAPK, Map Kinase; PGE_2, prostaglandin E_2; PAF, Platelet-activating factor; ICAM, intercellular adhesion molecule; Erk, extracellular-signal regulated kinase; PI3K, phosphoinositide 3-kinase; cAMP, cyclic-AMP; keratinocyte-derived cytokine (KC); MK, MAPK-activated protein kinase; LSP1, leukocyte-specific protein 1; TxA_2, thromboxane; 5-HT, serotonin

Introduction

Laminitis is a painful and aggressive disease process that is responsible for considerable morbidity and mortality in horses throughout the world [1,2]. Acute laminitis is difficult to treat; by the time of first observation of clinical signs, notable foot pain, the momentous cascade of pathology has already begun. With ongoing research, laminitis is increasingly being recognized as an inflammatory disease with systemic mechanisms and identifying the lamellar tissues as being the ultimate and final indicator of the process [3-6].

Although laminitis can occur secondarily to a range of causes, including black walnut toxicity [7], carbohydrate overload [8] and excessive weight bearing after an injury to a contralateral limb [9], the most significant risk factor for hospitalized horses has recently been confirmed in a retrospective study as endotoxemia [10]. Endotoxemia occurs after the absorption of lipopolysaccharide (LPS) of gram negative bacterial cell walls into the bloodstream from an area of mucosal damage such as ischemic intestine in colic or necrotic endometrium in retained placenta of mares [11,12]. LPS transmits a "danger signal" to the immune system through CD14/TLR-4 signaling on cells and strongly activates leukocytes [13-16], platelets [17,18] and endothelial cells [19-22] initiating an overwhelming cascade of dysregulated inflammation that can lead to hypotensive shock, organ failure, and death [11,12,23,24].

Experimental models of laminitis, such as black walnut toxicosis and carbohydrate overload models, have allowed for investigation into the pathophysiology that precedes the observed clinical signs that accompany lamellar inflammation. Studies using oral starches to

induce acute laminitis in ponies have shown the presence of platelet-neutrophil aggregates in laminar dermal veins of affected feet and corresponding prevention of laminitis with pre-treatment of ponies with platelet aggregation inhibitors [25,26]. Altered circulating neutrophil dynamics [27] and interleukin 1-β (IL1-β), interleukin-6 (IL-6), and cyclooxygenase-2 (COX-2) upregulation have been shown in the laminae during the prodromal stages of black-walnut induced laminitis [4,28,29], suggesting that a systemic inflammatory response and subsequent tissue response in the lamina are important in the pathophysiology of laminitis. Additionally, differential mRNA display (DRD) has revealed an increase in 'Molecule possessing Ankyrin-repeats Induced by Lipopolysaccharide' (MAIL), a signaling molecule important for the regulation of inflammatory cytokines [30] in lamellar tissues [4]. The presence of these pro-inflammatory proteins ignites the insidious lamellar inflammation and orchestrates the damage to come.

In the later clinical stages of laminitis, pathological manifestations in affected feet include a breakdown in the basement membrane [31], neutrophil infiltration, and a loss of collagen and laminin [32], with a positive correlation between lameness level and laminin cleavage [33]. This proteolytic breakdown in the basement membrane leads to laminar separation and is thought to be due to matrix metalloproteinase enzymes (MMPs) [34]. MMP-2, secreted by basal cells, and MMP-9, secreted by neutrophils, have been found in the lamellar tissues in acutely laminitic horses [32]. Once secreted, MMPs remain in an inactive form until activation by proteases or reactive oxygen species (ROS) [35]. Recently a positive

correlation between MMP-9 and neutrophils was found in laminae of acutely laminitic horses, suggesting a role for neutrophils in the structural damage that transpires [36].

Equine laminar tissues do not normally contain resident neutrophils [3,36], but in both the early stages and developmental stages of black-walnut-induced laminitis a significant increase in neutrophils are found around laminar venules [3]. Because the laminae have inherently less superoxide dismutase (SOD) activity as compared to other equine tissues [36], they are likely more susceptible to the effects of neutrophil ROS production during activation. Our goal is to reveal potential new therapeutic targets to limit neutrophil migration during acute laminitis and limit the destruction that occurs due to MMPs, ROS production and the escalating inflammatory cell influx following chemokine secretion.

The signaling molecule p38 Map Kinase (MAPK) that has been shown to be involved in signaling during inflammation, the cell cycle, and cell differentiation, is stimulated by growth factors, cytokines and chemoattractants and is central to the induction of inflammation [37,38]. Our previous work has shown that p38 MAPK is essential for COX-2 expression and PGE_2 production in LPS-stimulated equine peripheral blood leukocytes [39]. To further characterize the role of p38 MAPK in inflammation in horses, we investigated p38 MAPK in the mechanism regulating equine neutrophil migration as a potential target for pharmacologic intervention to limit tissue destruction as seen in laminitis. The p38 MAPK pathway has been shown to be essential for neutrophil chemotaxis in response to a variety of chemoattractants through utilization of the specific p38 MAPK inhibitor SB203580 in

primary human neutrophils [40-42] and through experiments with neutrophils from mice deficient in the major downstream substrate of p38, MAPK-activated protein kinase 2 (MK2) [43]. We hypothesized that p38 MAPK is required for equine neutrophil migration in response to endogenous chemoattractants LTB_4 and PAF.

Materials and methods

Reagents

Dimethyl sulfoxide (Me_2SO), fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ sheep anti-mouse immunoglobulin G (IgG) antibody, phorbol 12-myristate 13-acetate (PMA), ovalbumin (OVA), gelatin, o-phenylenediamine dihydrochloride, Triton X-100, paraformaldehyde, piperazine bis-2-ethane sulfonic acid (PIPES), and HEPES were from Sigma Chemical Co. (St. Louis, MO). Powdered phosphate-buffered saline (PBS) and Hanks' balanced salt solution (HBSS) were from Life Technologies (Grand Island, NY). Ethylenediamine tetraacetate dihydrate (EDTA) was from Fisher Scientific (Atlanta, GA). Ficoll-Paque was obtained from Amersham Biosciences (Piscataway, NJ). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Recombinant human TNFα was from R & D Systems, Inc. (Minneapolis, MN). Leukotriene B_4 (LTB_4) and 1-O-Hexadecyl-(7,7,8,8-d_4)-2-O-acetyl-s*n*-glycerol-3-phosphorylcholine (PAF) were from Cayman Chemical (Ann Arbor, MI). Rhodamine-conjugated phalloidin and calcein were obtained from Molecular Probes (Eugene, OR). Monoclonal phospho-p38 MAPK (Thr180/Tyr182) Ab and total p38 MAPK Ab were obtained from Cell Signaling Technology, Inc. (Beverly, MA). HRP-conjugated rabbit anti-mouse IgG secondary antibody was obtained from Pierce (Rockford, IL). Diisopropylfluorophosphate (DFP) was from BD Biosciences (San Diego, CA). Ninety-six-well Immulon 2 plates were from Dynatech (Chantilly, VA). Ninety-six-well ChemoTx chemotaxis chambers were from Neuroprobe (Gaithersburg, MD). Monoclonal antibody (mAb) IB4 (anti-β2, CD18 (Wright et al., 1983)) was purified.

Neutrophil isolation

Fresh heparinized equine whole blood was collected from healthy horses between the ages of five and fifteen years old using a protocol approved by the NCSU Institutional Animal Care and Use Committee (IACUC). Blood samples were transferred to sterile 15 ml conical tubes at room temperature and allowed to settle for 45 min. The leukocyte rich plasma (LRP) supernatant was collected and neutrophils were isolated. Approximately 6 mls of plasma was layered on 5 mls of sterile, endotoxin-free Ficoll-Paque solution and spun at 1800 rpm for 20 minutes. Remaining red blood cells were lysed by hypotonic lysis and neutrophils were washed once with HBSS. Neutrophils were used if they demonstrated greater than 98% viability, as determined by exclusion of trypan blue dye incorporation. Cells were re-suspended in HBSS with 20mM HEPES, 8.9 mM sodium bicarbonate, 1mM Ca^{2+} and 1mM Mg^{2+} prior to assays ($HBSS^{++}$).

Western blotting

Cells were lysed in 2X lysis buffer (1% Triton X-100, 50mM Tris, 0.9% NaCl, 50mM NaF, 50mM Na pyrophosphate, and 1% deoxycholate) containing protease inhibitors (10µg/ml aprotinin/leupeptin, 0.1mg/ml pepstatin, 1mM iodoacetamide, and 5mM diisopropylfluorophosphate) and placed on ice for 20 minutes with agitation. Samples were then clarified by microcentrifugation for 10 minutes at 14,000 rpm. Total protein in the lysates was determined by BCA (Pierce). Sample aliquots were mixed with an equal volume of 2X sample buffer with 2-ME, boiled for 5 minutes, and stored at -20ºC until SDS-PAGE

analysis. Equal protein concentrations were analyzed by 10% SDS-PAGE. Resolved samples were transferred to Immobilon-P PVDF transfer membrane (Millipore, Billerica, MA) and blocked for 1 hour with blocking buffer (5% non-fat milk in TBS-T (5M NaCl, 1M Tris-HCl and 0.1% Tween 20)) before overnight incubation with monoclonal phospho-p38 MAPK (Thr180/Tyr182) Ab (1:500 in TBS-T with 5% BSA). Membranes were washed three times for five minutes each with TBS-T. Membranes were then incubated with HRP-conjugated anti-rabbit Ab for 1 hour at room temperature with agitation (1:2000 in blocking buffer). Membranes underwent a final wash with TBS-T (three times for five minutes) before detection of phospho-p38 using enhanced chemiluminescence (ECL, Amersham Biosciences) following manufacturers protocol. Membranes were re-probed for total p38 expression by first incubating in a stripping buffer (1% SDS, 2M Tris-HCl (pH 6.7), and 0.8% 2-mercaptoethanol) for 1 hour with agitation at 55ºC. Membranes were then rinsed two times for three minutes each with PBS, then incubated for 1 hour at room temperature in blocking buffer before blotting for total p38 protein (1:1000 in TBS-T with 5% BSA). Exposed radiographic films were developed, electronically scanned, and the density of protein bands of interest was determined using densitometry software (ZERO-Dscan 1.1, Scanalytics). Data are presented from representative experiments (immunoblots) as mean fold change over vehicle control (± SEM) for three horses.

Migration assay

Purified equine neutrophils were pre-treated with 10 μM SB203580 or vehicle control for 30 minutes at 37º C before labeling with the fluorescent dye calcein (2μg/ml) for 30 minutes at room temperature. Cells were then washed and resuspended in a chemotaxis buffer containing $HBSS^{++}$ with 2% FCS. 1 x 10^4 cells were placed on a 96-well filter with a 2μm pore size membrane on a ChemoTx® plate (Neuro Probe, Inc., Gaithersburg, MD). Lower wells of the plate were filled with chemotaxis buffer to measure background migration or buffer with 10 nM PAF or 10 nM LTB_4 to measure chemotaxis. Standard wells contained 1 x 10^4 labeled cells. Cells were allowed to migrate across the membrane for 1 hour at 37 ˚C. After incubation, cells on the top of the filter were washed away with PBS. 0.5 mM EDTA was added to the top of the filter for 5 min to detach adherent cells. The plate was then centrifuged at 1000 rpm for 1 min. The filter was removed and the fluorescence was measured in the lower wells (485 nm excitation, 530 nm emission wavelengths) using an fMax fluorescence plate reader (Molecular Devices). Percent migration was determined by dividing the fluorescence of each well by the fluorescence of the standard wells containing 1 x 10^4 labeled cells.

Actin detection

Purified neutrophils suspended in $HBSS^{++}$ ($3x10^5$) were pre-treated with 10 μM SB203580 or vehicle control for 30 minutes at 37º C. Cells were then added to wells of a 12-well cell-culture plate (Corning Inc., Corning, NY) containing 12 mm glass coverslips pre-

coated with FBS. Cells were stimulated by inserting a 200-µl micropipette tip filled with 100 µl 1% solidified low-melt agarose in PBS with or without 10 nM LTB_4 or 10 nM PAF for 1 minute at 37°C. The tips and buffer were removed from the wells, and the cells were fixed for 20 min at RT with fixation buffer (25 mM PIPES, 50 mM KCl, 10 mM MgSO4, 5 mM EGTA, and 3% paraformaldehyde, pH 7). The fixation buffer was removed, and the cells were then permeabilized with ice-cold Triton buffer (0.5% Triton X-100, 10 mM PIPES, 300 mM sucrose, 100 mM KCl, 3 mM MgCl2, and 10 mM EGTA, pH 6.8) for 10 min on ice. Coverslips were washed twice with ice-cold protein solution (0.2% gelatin, 0.2% azide, 0.1% OVA in PBS) and then incubated with rhodamine phalloidin in PBS (1:20) for 20 min at RT. Coverslips were washed twice with PBS and once with o-phenylenediamine dihydrochloride (1 mg/ml) in glycerol and mounted on glass slides. Cells were examined using an Olympus VANOX AHS-3 photomicroscope. Polarity of the actin cytoskeleton was determined from the distribution of the rhodamine phalloidin staining as described [44,45]. The total number and the number of polarized cells were determined in 5 high-power fields (hpf) for each coverslip. Data were expressed as the percentage of cells with F-actin distribution typical of polarized cells in 5 hpf.

Adhesion assay

Purified equine neutrophils were pre-treated with vehicle control or 10 µM SB203580 at 37º C for 30 minutes. Cells (1 x 10^7/mL) were then resuspended in HBSS and incubated with calcein 2µg/mL for 30 minutes at RT. Cells were washed and resuspended in $HBSS^{++}$

to a final concentration of 2 x 10^6/mL. Cells ($1x10^5$) were added to FCS-coated wells of a 96-well Immulon 2 plate (Dynatech, Chantilly, VA). After a 10 minute incubation at 37° C, 10 ng/ml PMA, 10 nM LTB_4, 10 nM PAF, or 100 ng/ml TNFα was added before a final incubation at 37° C for either 3 minutes (LTB_4 and PAF) or 30 minutes (PMA and TNFα) for maximum adhesion. The fluorescence was measured (485 nm excitation, 530 nm emission wavelengths) using an fMax fluorescence plate reader (Molecular Devices) before and after subsequent washes with 150 mL PBS to dislodge non-adherent cells. Fluorescence after washing was divided by the fluorescence before washing to calculate percent adhesion.

CD18 detection

Neutrophils were isolated and suspended in HBSS with 1 mM Ca^{2+} to a concentration of $4x10^6$/ml. Cells were then unstimulated or stimulated with 50 nM LTB_4 or 50 nM PAF for 5 minutes at 37ºC. Samples were placed on ice and washed once with wash buffer [1% FBS, 0.1% Na Azide in PBS], centrifuged at 1200rpm for 10 minutes at 4ºC, before incubation with 10 μg/ml IB4 antibody (α-CD18) in 200μl wash buffer for 40 minutes on ice. Cells were then washed twice in wash buffer before the cell pellet was resuspended in 200μl of 1:50 dilution of FITC-labeled sheep-α-mouse IgG for 20 minutes on ice. Cells underwent two final washes before resuspension in 1ml of sterile PBS and immediate analysis. The relative fluorescence of $1x10^4$gated neutrophils was measured using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The mean fluorescence of each neutrophil

population was normalized to the mean fluorescence of unstimulated neutrophils treated with vehicle control.

Statistical analysis

Statistical differences were determined using the Student's two-tailed t test, paired sample for means or two-sample assuming equal variance. A p value<0.05 was considered statistically significant.

Results

LTB_4 activates p38 MAPK in equine neutrophils

To confirm that chemoattractant induces p38 MAPK activation in equine neutrophils, as previously shown in other species [40,42,46], we stimulated purified neutrophils with 100nM LTB_4, a potent endogenous chemoattractant for neutrophils that results from the oxygenation and enzymatic conversion of arachidonic acid by lipoxygenases [47,48] and measured levels of activation-associated phospho-p38 protein levels in cell lysates with Western blot analysis as described in *Section 2.3*. Membranes were then stripped and reblotted for total p38 protein content to ensure that any increase in phospho-p38 was due to increased phosphorylation of the protein rather than increased total protein levels. LTB_4 induced a rapid increase in the phosphorylation of p38 MAPK to levels 2.5 times that of vehicle control within 1 minute after stimulation (Figure 1). Levels of phospho-p38 remained significantly increased for up to 10 minutes after stimulation but returned to

baseline levels by 20 minutes. In contrast, levels of total p-38 protein remained unchanged after stimulus throughout the incubation.

Inhibition of p38 MAPK abolishes chemotaxis in equine neutrophils

To determine whether p38 MAPK was required for neutrophil migration, we pre-treated neutrophils with the specific p38 inhibitor SB203580 (10 μM), a concentration we have previously shown to abolish LPS-induced p38 activation-associated phosphorylation and COX-2 expression in equine leukocytes [39], before evaluating chemotaxis of cells across a membrane into wells containing chemoattractant. Approximately 52% and 42% of vehicle control treated cells migrated towards LTB_4 or platelet-activating factor (PAF), respectively (Figure 2), while only 5% of cells migrated towards buffer alone. When p38 activity was blocked through pre-incubation with 10 μM SB203580, only 5% of cells were able to migrate into the lower well, regardless of chemoattractant, which was a significant reduction when compared to vehicle control treated cells. We concluded from these data that p38 is important for directed migration in equine neutrophils.

SB203580 inhibits LTB4-induced migration in a dose-dependent manner

We next evaluated the ability of SB203580 to inhibit LTB_4-induced migration over a range of concentrations. We found a significant reduction in migration of cells pre-treated with 5, 10, or 25μM SB203580 as compared to cells pre-treated with vehicle control (Figure 3). SB203580 pre-treatment had no effect on random migration as treated cells had no significant change in % migration towards buffer alone ('Control'). To determine the IC_{50}, we

plotted % Inhibition, defined as the % reduction in migration of SB203580 treated cells as compared to vehicle control treated cells, against log of SB203580 concentration in μM. We found the IC_{50} for LTB_4-induced migration of equine neutrophils to be 2.8μM.

Inhibition of p38 MAPK diminishes chemoattractant-induced actin polarization

In the immediate stages following receptor ligation of chemoattractant, neutrophils establish polarity in order to achieve directed migration towards a gradient of chemokine. A clear F-actin rich leading edge pseudopod and trailing edge uropod are established and the cell shape changes from symmetrical and circular to asymmetrical and tear drop [49]. To begin to uncover the mechanism behind the ability of SB203580 to inhibit migration, we examined the role of p38 MAPK in the chemoattractant-induced polarization of neutrophils. Primary equine neutrophils were plated in wells containing FCS-coated glass coverslips before stimulation with chemoattractant. Cells were then fixed and permeabilized, F-actin was stained with rhodamine-phalloidin as described in *Materials and Methods* and stained cells were examined using fluorescence microscopy. Polarity of the actin cytoskeleton was determined from the distribution of the rhodamine-phalloidin staining and shape changes of the neutrophils as described previously [44,45]. Stimulation with 10 nM LTB_4 or 10 nM PAF resulted in 50% polarization of vehicle control treated cells, as compared to only 10% of unstimulated cells (Figure 4). There was a significant reduction in % polarization in cells pre-treated with 10 μM SB203580 as only 18% and 12% of cells were able to polarize in response to LTB_4 or PAF, respectively. We concluded from these data that p38 MAPK

activity is required for chemoattractant-induced polarization of equine neutrophils and may be important for establishing direction in migrating cells.

p38 MAPK is not required for chemoattractant-induced integrin mediated adhesion

Before neutrophils can actively migrate they must first adhere to cellular and tissue substrates via β2 integrin ligation [50,51]. The predominant β2 integrin on the surface of neutrophils is CD11b/CD18 [52], also called Mac-1, which mediates neutrophil adhesion to activated endothelium, fibrinogen, and other matrix proteins derived from plasma, enabling chemotaxis and emigration [53,54]. We hypothesized that blockade of p38 was disrupting cellular polarization and chemotaxis through affects on β2 integrin-dependent cell adhesion. To investigate the role of p38 in β2 integrin-dependent adhesion, we stimulated SB203580 pre-treated neutrophils with chemoattractants LTB_4 or PAF, the pro-inflammatory mediator TNFα, or the PKC activator PMA and measured % adhesion as described in *Section 2.6* after serial PBS washes to dislodge non-adherent cells. All stimulants induced a significant increase in % adhesion; 50-60% of cells were adherent, compared to less than 10% of unstimulated cells ('Control') (Figure 5). Pre-incubation with 1 or 10 μM SB203580 significantly reduced TNFα-induced adhesion from 50% to 32% and 22%, respectively. In contrast, pre-treatment with SB203580 had no affect on adhesion induced by chemoattractants LTB_4 or PAF nor from adhesion secondary to PKC activation by PMA. From these data we concluded that p38 is not required for chemoattractant or PMA-induced adhesion, but does play a role in TNFα-induced adhesion.

Inhibition of p38 does not affect chemoattractant-induced upregulation of surface β2-integrin expression

As circulating neutrophils encounter inflammatory mediators, surface L-selectin, responsible for their initial rolling and contact with endothelial cells, is shed and CD11b/CD18 is upregulated through translocation to the plasma membrane from intracellular secretory vesicles [55-57]. Activation of neutrophils with chemoattractant also induces a clustering of the surface β2 integrins and a change from a low to an intermediate affinity state which results in stable adhesion to tissue substrates, enabling the cell to polarize and establish directed migration towards a source gradient of chemoattractant [50,51]. To further define the mechanism behind the requirement for p38 in chemotaxis, we evaluated the role of p38MAPK in the upregulation of surface β2 integrin expression on chemoattractant-stimulated neutrophils. Purified neutrophils were pretreated with 10 μM SB203580 before stimulation with 50 nM LTB_4 or 50 nM PAF for 5 minutes. Cells were then placed on ice and washed before incubation with α-CD18 antibody, subsequent staining with FITC-labeled sheep-α-mouse antibody and analysis as described in *Materials and Methods*. Stimulation with LTB_4 or PAF resulted in a significant increase in surface expression of CD18 when compared to unstimulated cells, although this upregulation was not affected by pre-treatment with SB203580 (Figure 6). We concluded from these data that p38 is not essential for the chemoattractant-induced upregulation of surface β2 integrins and the role of p38 MAPK in migration likely occurs downstream of β2-integrin adhesion.

Discussion

We have shown that p38 MAPK is essential for chemotaxis in equine neutrophils. To reveal the mechanism behind the requirement for p38, we have shown that inhibition of p38 reduces chemoattractant-induced actin polarization, but has no effect on chemoattractant-induced adhesion or upregulation of surface β2 integrins. Additionally, we found that p38 MAPK is required for TNFα-induced adhesion, but not for PKC-induced adhesion, highlighting the intricate nature of cellular signaling in the highly motile neutrophil. The internal signaling that occurs after ligation of chemoattractant receptors on neutrophils involves the activation of multiple signaling molecules including the MAPKs p38 and extracellular-signal regulated kinase (Erk) [40,58-60], phosphoinositide 3-kinase (PI3K) [58,61], Ca^{2+} [62], and cAMP [63] with specificity of signaling for each stimulus involving cross-talk pathways to allow for a hierarchy in the face of multiple stimuli [64,65]. In fact, it has been shown in human neutrophils that the PI3K pathway predominates in chemotaxis towards the intermediate endogenous chemoattractants LTB_4 and PAF, while p38 MAPK predominates in migration towards the bacterial formylated peptide fMLP and complement fragment C5a [65]. In contrast to human neutrophils, our data show that inhibition of p38 MAPK abolished migration towards LTB_4 and PAF suggesting that equine neutrophils have a different signaling hierarchy for chemoattractant-induced migration. Before we can predict the migration preference of equine neutrophils in an inflamed tissue bathed in both chemokines and bacterial products and further gain the capability to pharmacologically modulate neutrophilic inflammation, a deeper understanding of the signaling mechanisms in

response to an array of activators will be required. Our finding that p38 MAPK has no effect on β2-integrin upregulation or chemoattractant-induced adhesion suggests that p38 is affecting migration downstream of β2-integrin adhesion. In support, intravital microscopy has shown that systemic treatment of mice with p38 inhibitors SB203580 and SKF86002 does not affect neutrophil adhesion or emigration in response to keratinocyte-derived cytokine (KC), but does impair chemotaxis [60]. In contrast to the dynamic adhesion seen in neutrophils after chemoattractant stimulation, TNFα induces sustained adhesion with a high affinity state of the β2 integrin receptors resulting in a non-motile, firmly adhered cell [66]. TNFα levels are often high in states of chronic inflammation, which is thought to contribute to prolonged tissue retention of neutrophils [66]. Our data have shown that inhibition of p38 significantly reduces TNFα-induced adhesion (Figure 5). Previous work in human cells has shown that TNFα-induced neutrophil function is critically dependent on p38 activity including IL-8 production, CD11b upregulation, adhesion, and superoxide generation [40,66-68]. Studies using the black walnut or oligosaccharide models of acute laminitis have failed to show increases in TNFα expression in equine vascular smooth muscle cells or lamellae [5,69], yet plasma levels of TNFα are significantly increased during equine endotoxemia [70,71]. The potential role of TNFα in neutrophil function in the laminae during acute and chronic laminitis has yet to be determined.

We have shown that inhibition of p38 MAPK disrupted the ability of equine neutrophils to properly polarize in response to the endogenous lipid chemoattractants LTB_4

and PAF (Figure 4) and abolished subsequent migration (Figure 2). The chemotaxis assay used here evaluated the ability of a cell to migrate from a well containing buffer through a 2μm pore in a membrane towards a well with chemoattractant and measured net chemotaxis. Therefore, we have not investigated chemokinesis, or non-directional migration ability and can only hypothesize about how p38 is regulating the mechanism of neutrophil migration and disrupting chemotaxis. Our finding that SB203580 interrupts polarization suggests that p38 has a role in coordinating the actin cytoskeleton and establishing a committed direction for migration, most likely through activation of a downstream signaling target. One specific downstream target of p38 [72] that is rapidly activated in neutrophils after stimulation with chemoattractants PAF or fMLP is MAPK-activated protein kinase (MK) 2 [40,43,46,59]. Intracellular staining of fMLP-polarized murine neutrophils has revealed activated phospho-MK2 colocalized with F-actin in the leading edge lamellipodium [43], indicating the involvement of MK2 in cytoskeletal rearrangement. Furthermore, time-lapse video microscopy of $MK2^{-/-}$ murine neutrophils has shown that cells lacking MK2 have altered cell morphology and increased migrational velocities during chemotaxis, but a loss in their directionality towards fMLP [43,73]. A likely specific downstream target mediating the effects of MK2 is the actin binding protein leukocyte-specific protein 1 (LSP1) [74,75]. Recent studies have shown that MK2 phosphorylates LSP1 in a p38-dependent manner and phosphorylated-LSP1 colocalizes with F-actin in the lamellipodium orienting towards an fMLP gradient [73]. These findings further dissect the potential mechanism behind the role

of p38 in neutrophil migration and suggest downstream pathways to similarly explore in equine cells.

Systemic inhibition of p38 in mouse and human models of endotoxemia results in reduced inflammation and limits the pathophysiology of disease [76-80]. Although we have previously shown *in vitro* that SB203580 reduces equine peripheral leukocyte COX-2 protein and mRNA expression, PGE_2 production, and cytokine expression [39; unpublished data] there are currently no published studies of *in vivo* use of p38 inhibitors in horses. Further investigation is warranted to evaluate the effects of systemic p38 inhibition on other tissues, the potential to disrupt the p38-dependent anti-inflammatory effects of IL-10 and the possible suppression of other immune cells in equines. The effect of p38 inhibition in the face of ongoing inflammation is also unknown. Because the earliest signs of endotoxemia and acute laminitis often go undetected, it may be difficult to stifle the systemic inflammation before the cascade of lamellar destruction begins. When p38 inhibitors were given to mice 12 hours after exposure to aerosolized LPS, serum levels of TNFα were unchanged when compared to controls, but the migration of neutrophils to the airspaces was significantly reduced [81]. This result suggests that giving a p38 inhibitor in the face of clinically apparent acute laminitis has the potential to similarly reduce neutrophil accumulation and limit the ongoing laminar damage and merits further study.

Histological examination of laminitic lamellar tissues [82] as well as *in vivo* Laser Doppler flowmetry [83,84] provides evidence for ischemia-reperfusion injury characterized by hypoperfusion of the digit although provides no definitive cause for the damage [34].

When neutrophils encounter thrombin-stimulated, fibrinogen-bound platelets at sites of injured endothelium in the microcirculation, arrest and β2-integrin adhesion occurs resulting in a platelet-neutrophil aggregate and co-stimulation [85,86]. Although there does not appear to be systemic coagulation during acute laminitis [87,88], microthrombi and platelet-neutrophil aggregates can be found in the digital circulation [25,89] and inhibition of platelet aggregation prevents the onset of disease in carbohydrate overload models in ponies [26]. It has been shown that endotoxin stimulation results in the release of the vasoconstrictors thromboxane (TxA_2) and serotonin (5-HT) from activated equine platelets and leukocytes [18,71]. Recent evidence has also shown that LPS-induced platelet production of TxA_2 is p38 MAPK-dependent and can be attenuated with pre-treatment with SB203580. Additional investigation into the characteristics of neutrophil-platelet interactions in the equine digit microcirculation may lead to a better understanding of the dynamics of the process and providing further support for the use of p38 inhibitors in the treatment for acute laminitis.

Figure 1. LTB_4 rapidly activates p38 MAPK in equine neutrophils. Purified neutrophils were stimulated with 100nM LTB_4 or vehicle control for the indicated periods of time during a 20 minute incubation at 37 ºC before lysis and western blot analysis for phospho-p38. Membranes were then stripped and reprobed for total p38 levels. Immunoblots were electronically scanned and the density of each band was quantified. Data are the mean ± SEM presented as the fold change in band density over vehicle control for A) phospho-p38 protein expression or B) total p38 protein expression. LTB_4 induced a rapid increase in the phosphorylation of p38 to levels 2.5 times that of vehicle control within 1 minute. Levels of phospho-p38 remained significantly increased for up to 10 minutes after stimulation and returned to baseline levels by 20 minutes. In contrast, levels of total p-38 protein remain unchanged after stimulus throughout the incubation. Asterisks denote a significant increase in phospho-p38 levels as compared to vehicle control ($p<0.05$). Data are representative of three independent experiments from three separate donors.

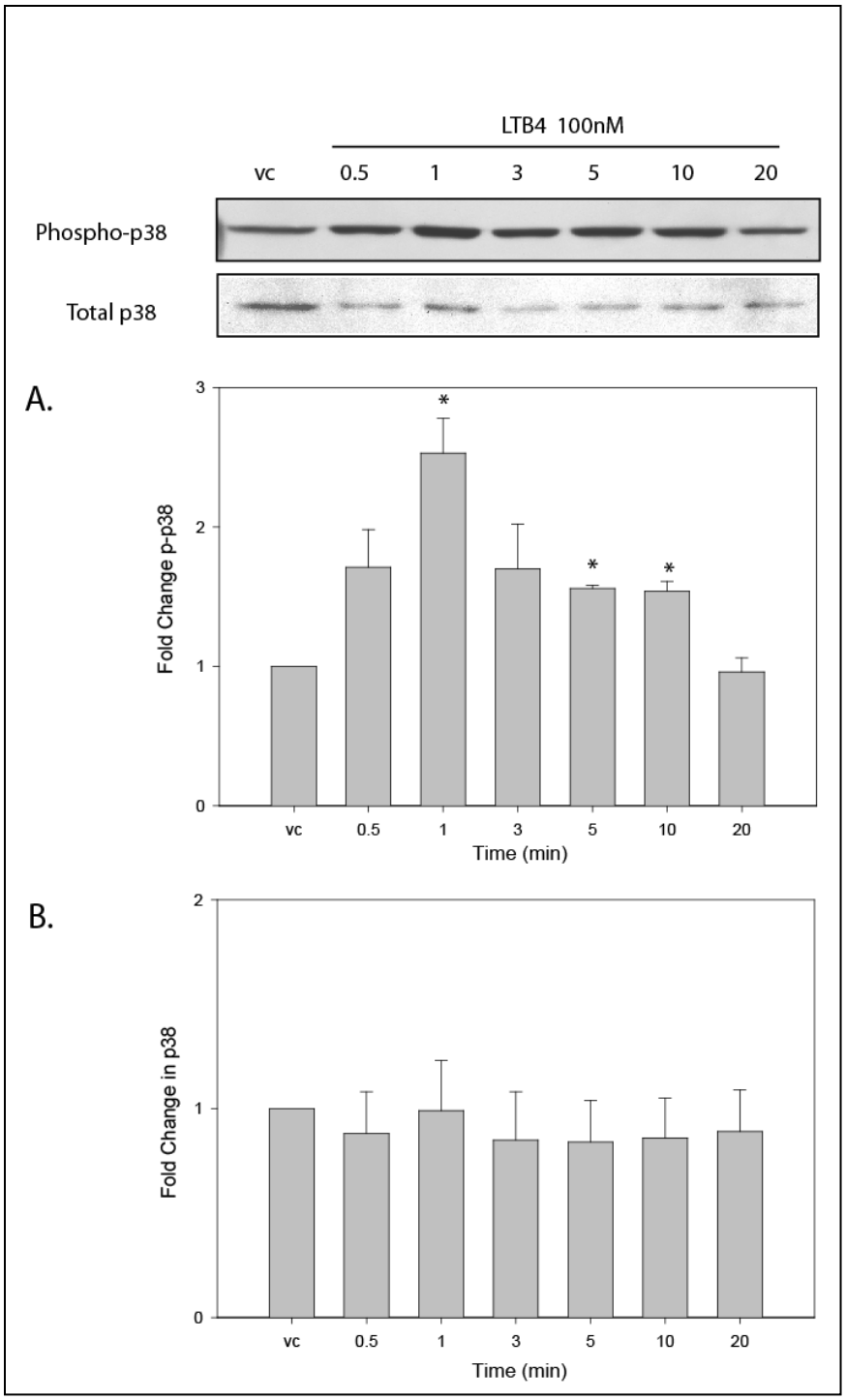

LTB4 100nM
vc
0.5
1
3
5
10
20
Phospho-p38
Total p38
A.
Fold Change p-p38
Time (min)
B.
Fold Change in p38
Time (min)

Figure 2. Inhibition of p38 MAPK abolishes chemotaxis in equine neutrophils. Purified equine neutrophils were pre-treated with vehicle control (Vehicle) or 10 μM SB203580 (SB) for 30 minutes at 37º C before labeling with calcein for use in a chemotaxis assay as described in *Materials and Methods*. Briefly, 1 x 10^4 cells from each group were plated on a 2μm membrane of a ChemoTx® plate overlying wells containing HBSS, 10 nM LTB_4, or 10 nM PAF. Standard wells contained 1 x 10^4 labeled cells, representing 100% migration. After 1 hour of incubation, the fluorescence was measured in the lower wells, as described in *Materials and Methods*, and percent migration was calculated by dividing the fluorescence of each well divided by the fluorescence of standard wells and is presented as the mean ± SEM. Chemoattractants LTB_4 and PAF induced a significant increase in migration (55% and 45%, respectively) of vehicle control cells as compared to buffer alone (Control) (*, $p<0.05$). Pre-treatment with 10 μM SB203580 significantly reduced the LTB_4- and PAF-induced migration when compared to vehicle control treated cells (▲, $p<0.05$). Data are representative of three independent experiments using separate donors.

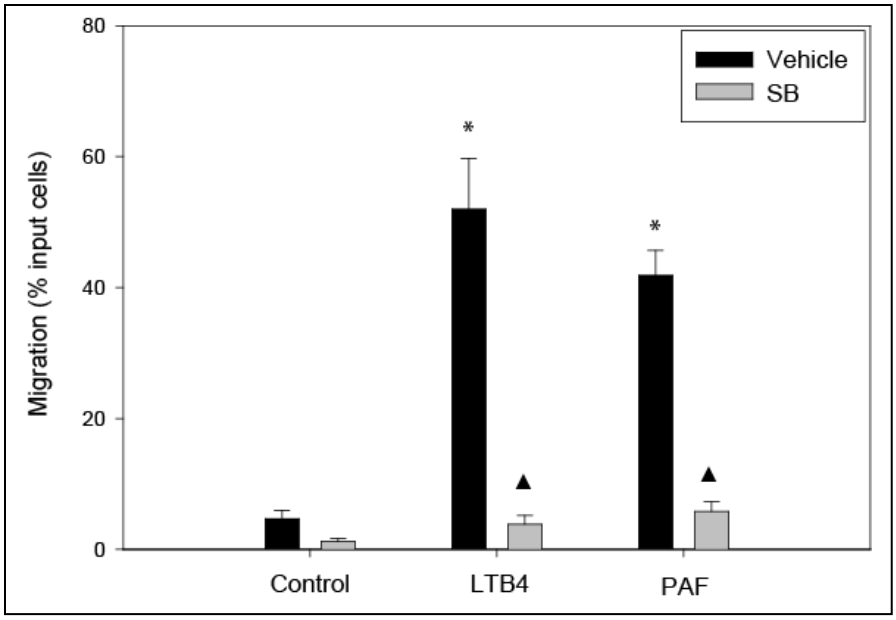

80
60
40
20
0
Migration (% input cells)
Vehicle
SB
*
*
▲
▲
Control
LTB4
PAF

Figure 3. SB203580 inhibits LTB_4-induced migration in a dose-dependent manner with an IC_{50} of 2.8μM. Purified equine neutrophils were pre-treated with vehicle control ('0 μM SB') or increasing concentrations of SB203580 before calcein labeling and use in a chemotaxis assay as described for Figure 2 using 1 nM LTB_4 as the chemoattractant. Pre-treatment with 5 μM or greater of SB203580 significantly reduced migration when compared to vehicle control treated cells ('0 μM SB') (*, $p<0.05$). SB203580 did not increase background migration at any concentration. To determine the IC_{50}, % Inhibition was first calculated by subtracting % Migration after each concentration (μM) of SB20350 from % Migration of control cells ('0 μM SB') and dividing by % Migration of control cells (representing 100% Migration). % Inhibition was then plotted against log (SB203580 concentration μM) and a Sigmoidal curve was fit to the data. The concentration of SB203580 that resulted in a 50% inhibition of migration, as compared to vehicle control treated cells, was 2.8μM. Data are representative of three independent experiments using separate donors.

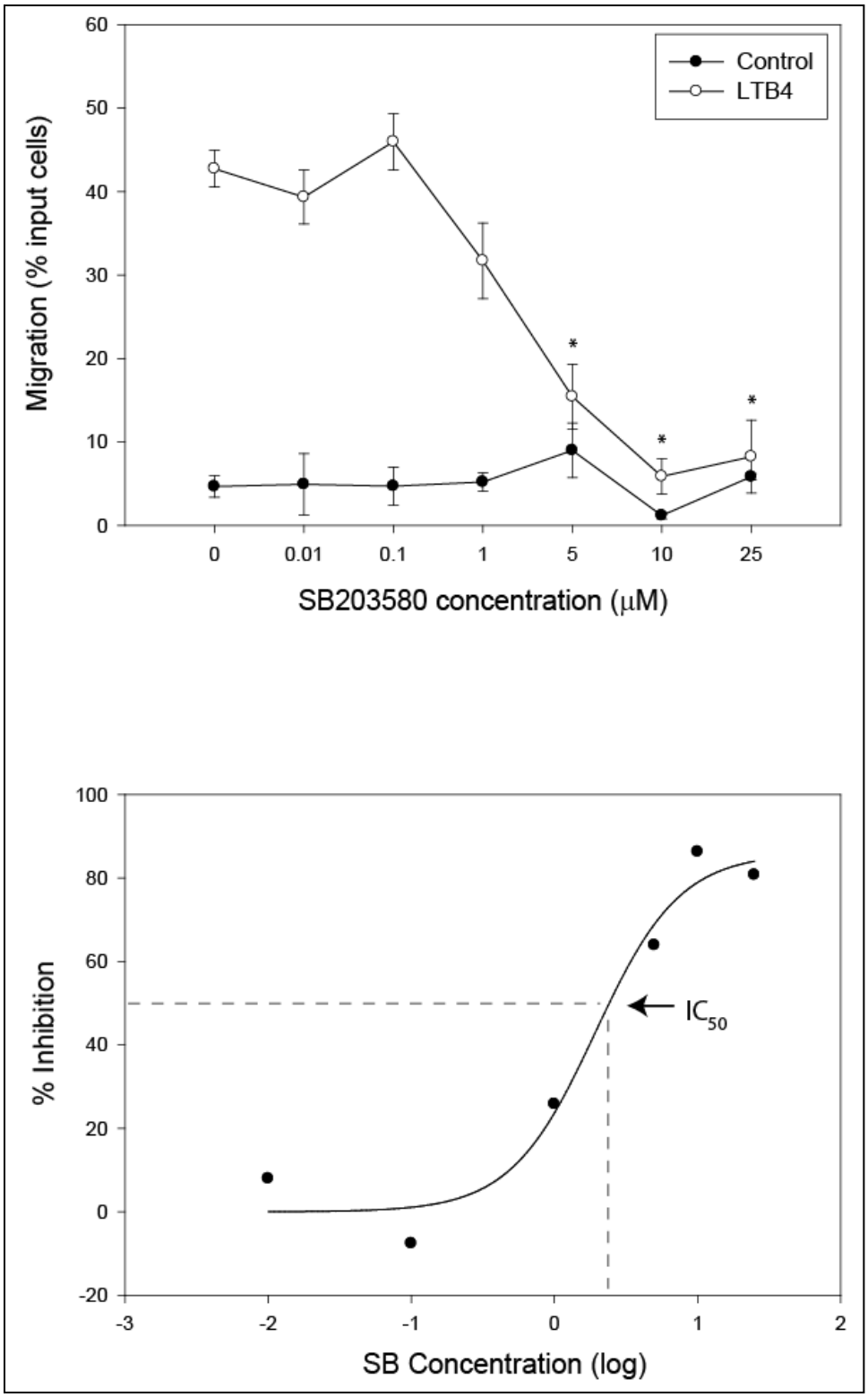

Migration (% input cells)
60
50
40
30
20
10
0
Control
LTB4
*
*
*
0
0.01
0.1
1
5
10
25
SB203580 concentration (μM)
% Inhibition
100
80
60
40
20
0
-20
IC50
-3
-2
-1
0
1
2
SB Concentration (log)

Figure 4. Inhibition of p38 MAPK diminishes chemoattractant-induced actin polarization. Purified neutrophils were pre-treated with 10 μM SB203580 or vehicle control for 30 minutes at 37º C, then added to wells of a 12-well cell-culture plate containing 12 mm glass coverslips pre-coated with FBS. Cells were left resting or stimulated with 10 nM LTB_4 or 10 nM PAF for 1 minute at 37°C before fixation, permeabilization, and staining for F-actin with rhodamine-phalloidin as described in *Materials and Methods*. Polarity of the actin cytoskeleton was determined from the distribution of the rhodamine-phalloidin staining as described [44,45]. The total number of polarized cells was determined in 5 high-power fields (hpf) for each coverslip and data were expressed as the percentage of cells with F-actin distribution typical of polarized cells. Data shown are mean % Polarization ± SEM from three independent experiments using three separate donors. LTB_4 and PAF induced a significant increase in polarity (50% polarized) of the cells as compared to unstimulated cells (10% polarized) treated with vehicle control (*, $p<0.05$). Cells pre-treated with SB203580 had only a minimal increase in polarity (12% after PAF and 18% after LTB_4), which was significantly reduced from vehicle control treated cells (▲, $p<0.05$).

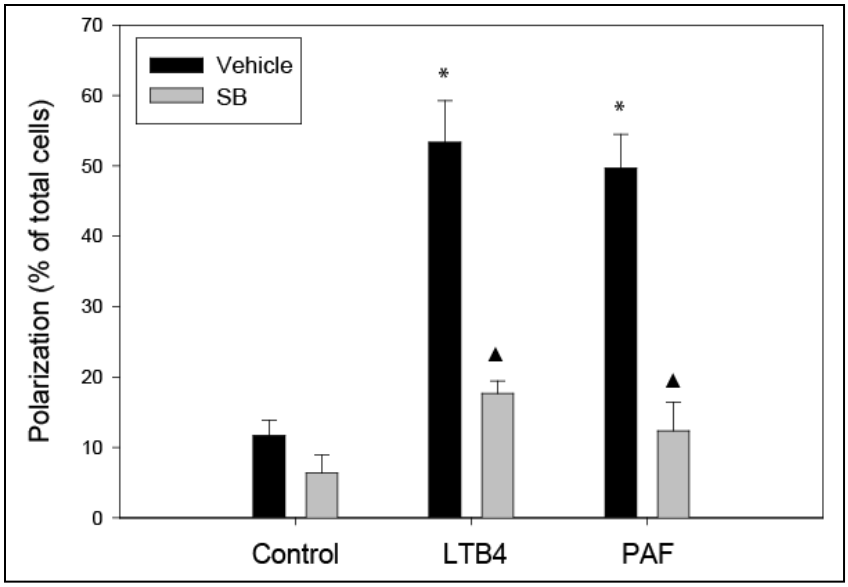

Vehicle
SB
Polarization (% of total cells)
70
60
50
40
30
20
10
0
*
*
▲
▲
Control
LTB4
PAF

Figure 5. p38 MAPK is not required for chemoattractant-induced integrin mediated adhesion. Purified equine neutrophils were pre-treated with vehicle control or 10 μM SB203580 at 37º C for 30 minutes before calcein labeling and use in an adhesion assay as described in *Materials and Methods*. Adhesion was induced through stimulation with 10 ng/ml PMA, 10 nM LTB_4, 10 nM PAF, or 100 ng/ml TNFα for either 3 minutes (LTB_4 and PAF) or 30 minutes (PMA and TNFα). The fluorescence was measured (485 nm excitation, 530 nm emission wavelengths) using an fMax fluorescence plate reader (Molecular Devices) before and after subsequent washes with 150 mL PBS to dislodge non-adherent cells. Fluorescence after washing was divided by the fluorescence before washing to calculate percent adhesion. Data are presented as mean % adhesion ± SEM and representative of three independent experiments using three separate donors. Cells treated with LTB_4, PAF, TNFα, or PMA exhibited significantly increased % adhesion as compared to unstimulated control cells (*, $p<0.05$). Pre-treatment with 1 μM or 10 μM SB203580 resulted in a significant decrease in TNFα-induced adhesion as compared to cells treated with vehicle control (▲, $p<0.05$), but did not affect LTB_4-, PAF-, or PMA-induced adhesion.

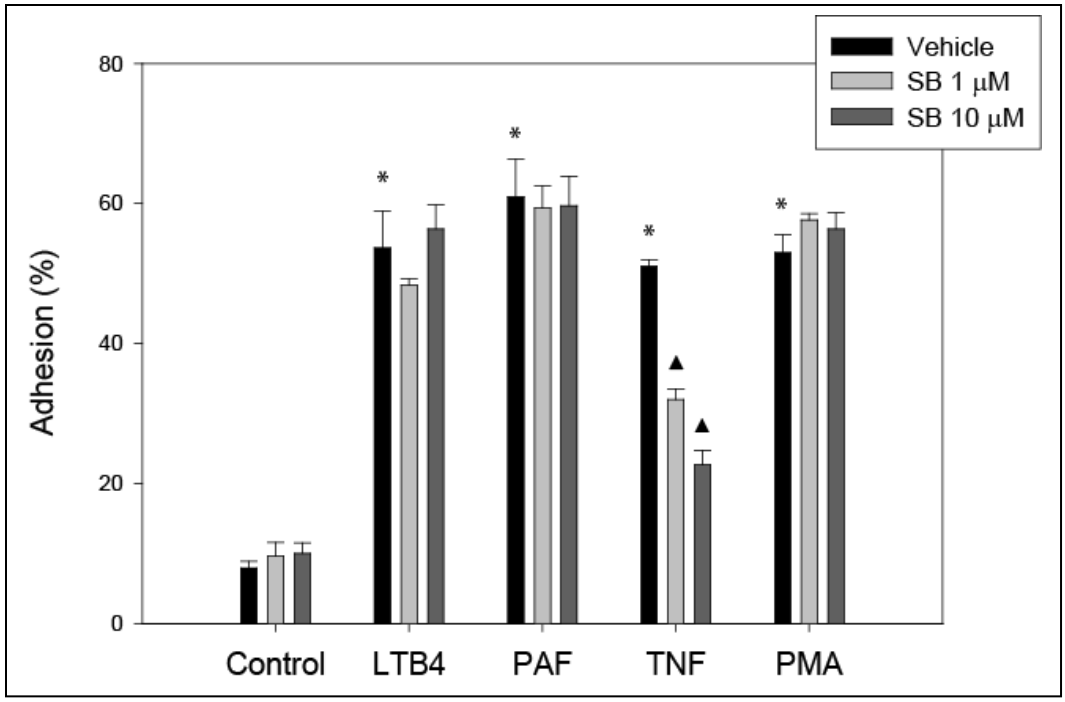
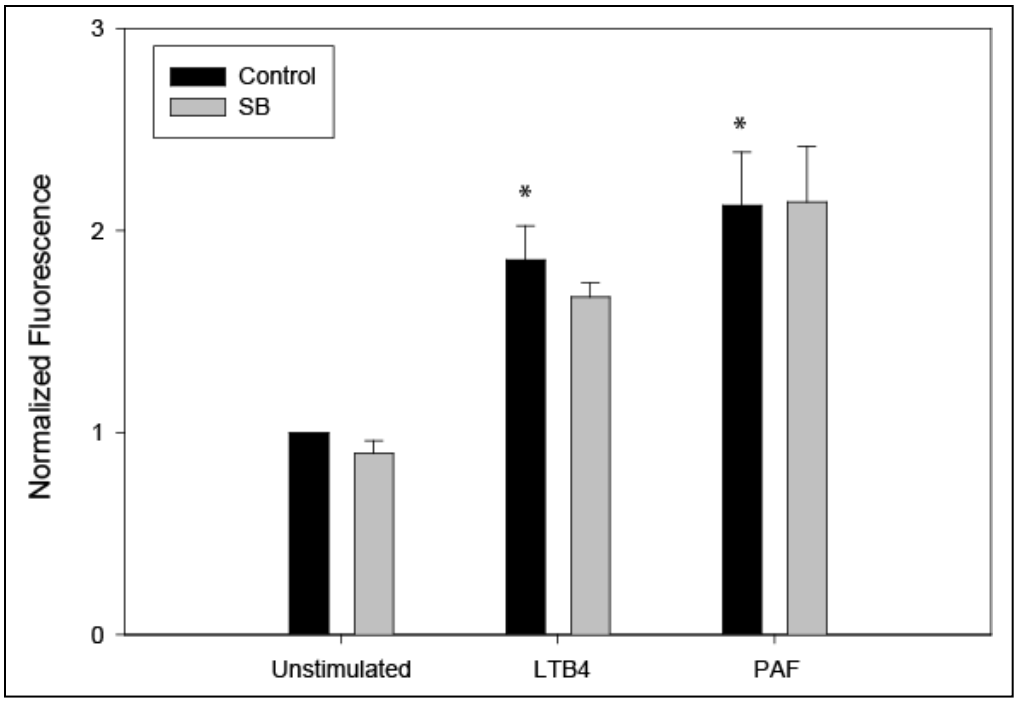

Vehicle
SB 1 μM
SB 10 μM
Adhesion (%)
80
60
40
20
0
Control
LTB4
PAF
TNF
PMA

Figure 6. Inhibition of p38 does not affect chemoattractant-induced upregulation of surface β2-integrin expression. Purified neutrophils were pretreated with 10 μM SB203580 for 30 minutes at 37ºC before stimulation with 50 nM LTB_4 or 50 nM PAF for 5 minutes. After stimulation, samples were immediately placed on ice and washed once before incubation with 10 μg/ml IB4 antibody (α-CD18). Cells were then washed twice and resuspended with FITC-labeled sheep-α-mouse antibody. Cells underwent two final washes before suspension in 1ml of sterile PBS and immediate FACS analysis as described in *Materials and Methods*. The mean fluorescence of each neutrophil population was normalized to the mean fluorescence of unstimulated neutrophils treated with vehicle control. Data is representative of three independent experiments using cells from different donors. Stimulation with LTB_4 or PAF resulted in a significant increase in surface expression of CD18 when compared to unstimulated cells (*, $p<0.05$), and this upregulation was not affected by pre-treatment with SB203580.


Control
SB
Normalized Fluorescence
3
2
1
0
*
*
Unstimulated
LTB4
PAF

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CHAPTER III.

REGULATION OF VASP SERINE 157 PHOSPHORYLATION IN HUMAN NEUTROPHILS AFTER STIMULATION BY A CHEMOATTRACTANT

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Abstract

Vasodilator-stimulated phosphoprotein (VASP) is a cAMP-dependent protein kinase (PKA) substrate that links cellular signaling to cytoskeletal organization and cellular movement. VASP is phosphorylated by PKA on serine 157, which is required for VASP function in platelet adhesion and fibroblast motility. Our hypothesis is that PKA regulates neutrophil migration through VASP Ser 157 phosphorylation. The objective of this study was to characterize VASP Ser 157 phosphorylation in chemoattractant-stimulated neutrophils. Formylated Met-Leu-Phe (fMLF), IL8, leukotriene B_4 (LTB_4), or platelet-activating factor (PAF) stimulation resulted in an initial increase in VASP Ser 157 phosphorylation that was maximal by 30s, and was followed by a return to baseline Ser 157 phosphorylation by 10 min. In contrast, stimulation with the non-chemoattractant pro-inflammatory cytokine TNFα did not affect Ser 157 phosphorylation. The kinetics of fMLF-induced VASP Ser 157 phosphorylation levels closely matched the kinetics of the fold-change in F-actin levels in fMLF-stimulated neutrophils. fMLF-induced Ser 157 phosphorylation was abolished by pretreatment with the PKA inhibitor H89 and the adenylyl cyclase inhibitor SQ22536. In contrast, fMLF-induced Ser 157 phosphorylation was unaffected by the PKC inhibitors calphostin and staurosporine, the PKG inhibitors Rp-8-pCPT-cGMP and KT5823, and the CamKII inhibitor KN-62. Inhibition of adhesion with either EDTA or the anti-β2 integrin antibody IB4 did not alter fMLF-induced VASP phosphorylation or dephosphorylation. These data show that chemoattractant stimulation of human neutrophils induces a rapid and transient PKA-dependent VASP Ser 157

phosphorylation. Adhesion does not appear to be an important regulator of the state of VASP Ser 157 phosphorylation in chemoattractant-stimulated neutrophils.

Abbreviations: cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase; VASP, vasodilator-stimulated phosphoprotein; PKG, cGMP-dependent protein kinase; fMLF, formyl-met-leu-phe; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; HBSS, Hank's balanced salt solution; LTB_4, leukotriene B_4; PAF, platelet activating factor; IBMX, 3-isobutyl-1-methylxanthine; EDTA, ethylenediamine tetraacetate dihydrate; FCS, fetal calf serum; DFP, diisopropylfluorophosphate; IL-8, interleukin-8; 7-TM, 7-transmembrane; HLA, human leukocyte antigen; vc, vehicle control; Ro, Ro-20-1724; 4-MB, 4-{[3',4'-(Methylenedioxy)benzyl]amino}-6-methoxyquinazoline.

Introduction

Uncovering the signaling mechanisms that control neutrophil migration can lead to pharmacological therapies to reduce inappropriate neutrophil activity and limit tissue damage during inflammation. Neutrophil migration is regulated through integrin-mediated adhesion to endothelial surfaces, followed by membrane protrusion at the leading edge and de-adhesion at the trailing edge of the motile cell [1,2]. Membrane protrusions at the leading edge pseudopod are formed by the assembly of branched arrangements of actin filaments that polymerize and produce physical movement of the plasma membrane of the cell [1,2]. Thus, precise coordination of integrin-mediated adhesion and cytoskeletal organization must occur to enable migration. The mechanisms coordinating these events are not well characterized. Cyclic AMP (cAMP), via activation of cAMP-dependent protein kinase (PKA), generally inhibits neutrophil activation [3-5]. Our lab has determined that PKA is a key regulator of integrin activation and adhesion as well as cytoskeletal organization in polarizing cells and, ultimately, directed migration [4,5]. Thus, PKA is a key organizer of adhesion and cytoskeletal polarity during migration in neutrophils. We are now defining the effectors of PKA in the mechanism regulating neutrophil adhesion and cytoskeletal organization during migration.

The PKA substrate vasodilator-stimulated phosphoprotein (VASP) is an member of the Ena/VASP family of F-actin-associated proteins that is a key effector of PKA in cytoskeletal organization, integrin adhesion, and migration [6,7]. In fibroblasts, VASP localizes to the actin cytoskeleton at adhesion sites by binding the focal adhesion proteins

zyxin and vinculin [8-11] and at the leading edge lamellipodium by binding the scaffolding protein lamellipodin [12]. VASP binds to its partners at these sites via an N-terminal pleckstrin homology-like EVH1 domain that binds to FPPPP motifs in zyxin, vinculin, and lamellipodin [6,12-14]. The C terminal EVH2 domain contains an F-actin binding site and a coiled-coil motif important for VASP oligomerization [13,15]. VASP also contains a central proline-rich region with binding sites for the actin-monomer binding protein profilin [16] and SH3 and WW domain-containing proteins [6,13] suggesting that VASP is a key element of signaling module formation at sites of cytoskeletal organization. VASP promotes actin polymerization by nucleating actin filaments and by delivering profilin-G-actin complexes to the barbed end. VASP is phosphorylated by PKA, PKG, and PKC on serine 157, and by PKA and PKG on serine 239 and threonine 278 [17,18]. Ser 157 is the preferred site for PKA-induced phosphorylation [18,19]. Phosphorylation by PKA reduces VASP's capacity to bind, bundle, and nucleate actin filaments [6,20,21] and, thus, is a crucial post-translational modification.

VASP is essential for normal fibroblast lamellipodial dynamics and cellular migration [22-24]. The ability of the Ena/VASP family member Mena to regulate migration requires Ser 236, which corresponds to VASP Ser 157 [25]. VASP Ser 157 is also required for the ability of cAMP to inhibit platelet integrin-mediated adhesion [26-28]. Together, these data demonstrate that VASP Ser 157 and its phosphorylation are important for regulating cellular adhesion and migration. However, little is known about VASP and its phosphorylation in neutrophil migration. We hypothesized that PKA mediates its effects on neutrophil migration

by regulating VASP Ser 157 phosphorylation. We began testing this hypothesis by characterizing VASP Ser 157 phosphorylation in neutrophils stimulated by chemoattractants.

Materials and Methods

Reagents

Ficoll-Paque Plus, and Dextran T500 were from Amersham Biosciences (Piscataway, NJ). Dimethyl sulfoxide (Me_2SO), fMLF, phorbol 12-myristate 13-acetate (PMA), Triton-X 100, pepstatin, HEPES, and poly-l-lysine were from Sigma Chemical Co. (St. Louis, MO). Powdered phosphate-buffered saline (PBS) and Hank's balanced salt solution (HBSS) were from Life Technologies (Grand Island, NY). Recombinant human TNFα was from R & D Systems, Inc. (Minneapolis, MN). Leukotriene B_4 (LTB_4) and 1-O-Hexadecyl-(7,7,8,8-d_4)-2-O-acetyl-*sn*-glycerol-3-phosphorylcholine (PAF) were from Cayman Chemical (Ann Arbor, MI). KT5720, H-89, staurosporine, and 3-isobutyl-1-methylxanthine (IBMX) were from Alexis (San Diego, CA). Rp-8-pCPT-cGMPS was from Biolog Life Science Institute (Bremen, Germany). Ethylenediamine tetraacetate dihydrate (EDTA) was from Fisher Scientific (Atlanta, GA). KN-62 was from Biomol International (Plymouth Meeting, PA). KT5823, SQ22536 (9-(Tetrahydro-2'-furyl)adenine), Ro-20-1724 (4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone), and 4-{[3',4'-(Methylenedioxy)benzyl]amino}-6-methoxyquinazoline (4-MB) were from Calbiochem (EMD Biosciences; San Diego, CA). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT). Calcein was obtained from Molecular Probes (Eugene, OR). Alexa Fluor 546 phalloidin was from Invitrogen (Carlsbad,

CA). HRP-conjugated goat anti-mouse IgG1(γ) secondary antibody was from Caltag Laboratories (Burlingame, CA). HRP-conjugated rabbit anti-mouse IgG secondary antibody was obtained from Pierce (Rockford, IL). Mouse IgG1 anti-VASP monoclonal antibody, diisopropylfluorophosphate (DFP), and human recombinant interleukin-8 (IL-8) were from BD Biosciences (San Diego, CA). Monoclonal antibodies IB4 (anti β2, CD18) [29] and W6/32 [anti-class I human leukocyte antigen (HLA)][30] were prepared as described. Phospho-VASP (Ser157) mAb and anti-rabbit HRP-conjugated secondary antibody were obtained from Cell Signaling (Beverly, MA). Anti-Lpl mAb Lpl4A.1 (IgG1) was prepared as described previously [31] and used as purified IgG. Anti-phospho-LplI4E11 was a kind gift of Dr. Eric Brown (University of California, San Francisco) and used as a tissue culture supernatant.

Preparation of neutrophils

Human leukocyte rich plasma was separated from whole blood using dextran sedimentation. Neutrophils were isolated using a Ficoll gradient. In brief, approximately 6mls of plasma was layered on 5mls of sterile, endotoxin-free Ficoll-Paque solution and spun at 1800 rpm for 20 minutes. Neutrophils were used if they demonstrated greater than 98% viability, as determined by exclusion of trypan blue dye incorporation. Red blood cells were lysed by hypotonic lysis and remaining neutrophils were washed once with HBSS. Cells were re-suspended in HBSS with 20mM HEPES, 8.9 mM sodium bicarbonate, 1mM Ca^{2+}

and 1mM Mg^{2+} prior to assays ($HBSS^{++}$). Cells pre-treated with EDTA were suspended in HBSS, rather than $HBSS^{++}$, to prevent Ca^{2+}- and Mg^{2+}-dependent integrin ligand binding.

Western Blotting

For preparation of lysates, purified neutrophils ($25x10^6$/mL) were treated with inhibitors in microcentrifuge tubes and incubated at 37° C. Cells ($2.5x10^6$) were then added to 5% FCS-coated wells of a 24-well tissue culture plate (Costar, Cambridge, MA) and allowed to adhere for 5 minutes at 37° C. Cells were then stimulated with 100nM fMLF, 100ng/mL TNFα, 10ng/mL PMA, or appropriate vehicle control (VC) for the indicated time periods during incubation at 37° C. After 20 total minutes, 2X Triton lysis buffer [2% Triton-X, 50mM Na Fluoride, 2.5mM Na pyrophosphate, 100mM TRIS, 5mM DFP, 100μg/mL pepstatin, 1mM iodoacetamide, 1mM PMSF, and 10μg/mL aprotinin/leupeptin] was added to wells and plates were incubated on ice with agitation for 20 minutes. After lysis, cell solutions were transferred to microcentrifuge tubes and spun at 14,000 rpm for 8 minutes. Supernatants were collected and protein concentrations were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL). Samples were mixed with 2X sample buffer [125mM Tris, pH 6.7, 5% SDS w/v, 20% glycerol v/v, 0.01% bromophenol blue w/v, 0.01% 2-mercaptoethanol v/v in di H_2O] and boiled for 5 minutes. Equal protein concentrations were analyzed by 10% SDS-PAGE. Resolved samples were transferred to Immobilon-P PVDF transfer membrane (Millipore, Billerica, MA) and blocked for 1 hour with Superblock Blocking Buffer (Pierce, Rockford, IL) before overnight incubation with the indicated

primary antibody at 4° C. Membranes were washed for 30 minutes before 1 hour incubation with appropriate HRP-conjugated secondary antibody. Membranes were again washed before development with enhanced chemiluminescence (ECL WB Substrate, Pierce, Rockford, IL) and radiography (Biomax scientific imaging film, Kodak). Films were scanned and the density of the bands was measured with densitometric software (Scanalytics, Fairfax, VA).

Chemotaxis Assay

Human neutrophils were isolated and labeled with the fluorescent dye calcein for 30 minutes at room temperature. Cells were then washed and resuspended in a chemotaxis buffer containing $HBSS^{++}$ with 2% FCS. Cells were treated with the indicated inhibitors for 30 minutes at 37 ˚C prior to the placement of 1×10^4 cells on a 2μm pore size membrane on a ChemoTx® plate (Neuro Probe, Inc., Gaithersburg, MD). Lower wells of the plate were filled with chemotaxis buffer with or without 100nM fMLF. Standard wells contained 1×10^4 labeled cells. Cells were allowed to migrate for 1 hour at 37 ˚C. After incubation, cells on the top of the filter were washed away with PBS. 0.5 mM EDTA was added to the top of the filter for 5 min to detach adherent cells. The plate was then centrifuged at 1000 rpm for 1 min. The filter was removed and the fluorescence was measured in the lower wells (485 nm excitation, 530 nm emission wavelengths) using an fMax fluorescence plate reader (Molecular Devices). Percent migration was determined by dividing the fluorescence of each well by the fluorescence of the standard wells containing 1×10^4 labeled cells.

Actin Polymerization Assay

Human neutrophils were isolated as previously described, washed once in Buffer A [140mM NaCl, 1mM KH_2PO_4, 5mM Na_2PO_4, 1.5mM $CaCl_2$, 0.3mM $MgSO_4$, 1mM $MgCl_2$, 10mM HEPES, pH7.4] before final resuspension in Buffer A at a concentration of 5 x 10^6/ml. 98µl of cell suspension was placed in sterile BD Falcon™ 5ml polystyrene round-bottom tubes (BD Biosciences, Bedford, MA). Cells were stimulated with 100nM fMLF for indicated lengths of time before fixation with 100µl 3% formaldehyde and 0.1% BSA in PBS for 20 minutes. Cells were then washed once in Buffer A before permeabilization with 0.1% Triton and 1 unit per reaction of Alexa Fluor 546 phalloidin in 100µl Buffer A for 30 minutes on ice. 400µl of PBS was added to the sample before transfer to a fresh tube for FACS analysis.

Actin Staining

Freshly isolated human neutrophils (0.5 x 10^6) in HBSS++ with 1% BSA were added to FCS-coated glass coverslips in a 24-well tissue culture plate. Cells were allowed to settle onto the glass before 100nM fMLF or vehicle control was added for indicated periods of time of incubation at 37°C. The supernatant was removed and cells were fixed with 0.5ml 3% paraformaldehyde solution [25mM PIPES, pH 7, 50mM KCl, 3mM MgCl, 3% paraformaldehyde w/v, 10mM EGTA in sterile water] for 20 minutes. Coverslips were then washed with PBS three times before extraction with 0.5ml cold Triton lysis buffer [10mM PIPES, pH 6.8, 0.5% Triton-X 100 v/v, 300mM sucrose, 100mM KCl, 3mM $MgCl_2$, in

sterile water]. Cells were washed again before staining with Alexa-phalloidin 546 diluted 1:20 in PBS for 30 minutes at room temperature. Fluorescence of Alexa-phalloidin-546-stained F-actin was viewed with a Nikon TE-200 inverted epifluorescence microscope using a digital camera (SPOT, Diagnostics Instruments Inc.) and associated software for image capture.

Coating of Plates with Immune Complexes

Each well of a 24-well sterile tissue culture plate was coated with 200µL of 100µg/ml poly-l-lysine in PBS and incubated at room temperature for 45 minutes. Wells were then washed 3 times with PBS before adding 200µl fresh 1% gluteraldehyde in PBS at room temperature for 15 minutes. Wells were again washed 3 times with PBS before the addition of 200µl of 100µg/ml BSA in PBS for 4 hours at room temperature. Wells were dumped, then blocked with 200µl of 1% human albumin serum w/v and 0.1 M glycine, pH 6.8 in PBS overnight at 4 ºC. Wells were washed again 3 times with PBS, before addition of 200µl of 1:50 α-BSA antiserum for 2 hours at room temperature. Wells were washed 3 final times with PBS before use in experiments.

Statistical Analysis

Data are reported as mean ± SE. Data were analyzed by student's two-sample t-test assuming equal variance or paired two-sample for means. A $p<0.05$ was considered statistically significant.

Results

Effects of chemoattractant stimulation on VASP serine 157 phosphorylation

Formyl-Met-Leu-Phe (fMLF) is a formylated peptide that acts as a potent chemoattractant for neutrophils [32]. The neutrophil fMLF receptor is a 7-transmembrane (7-TM) G-protein coupled receptor linked to several key signal transduction pathways including the cAMP/PKA pathway [2,33]. Other important 7-TM neutrophil chemoattractant receptors include IL8 [34], LTB_4 [35], and platelet-activating factor (PAF) [36]. To begin to uncover the role for Ser 157 in neutrophil migration, we first sought to determine the effect of chemoattractant stimulation on VASP Ser 157 phosphorylation. Phosphorylation of VASP on serine 157 results in a change in electrophoretic mobility that can be detected by Western blot analysis as a doublet band. The upper 50kD band corresponds specifically to Ser 157 phospho-VASP and the lower 46kD band corresponds to Ser 157 dephospho-VASP [17, 18]. Thus, densitometry can be used to calculate the percent VASP phosphorylation as a ratio of the density of the upper phosphoprotein band to the density of both bands combined, representing total VASP.

Approximately 40% of VASP was phosphorylated on Ser 157 in unstimulated neutrophils (Figure 1). Stimulation with fMLF induced rapid and transient Ser 157 phosphorylation. Maximal phosphorylation was observed by 30 seconds, with 60% of VASP phosphorylated (Figure 1). VASP phosphorylation then decreased, returning to baseline levels by 10 minutes following stimulation. Stimulation of cells with the chemoattractants PAF, LTB_4, or IL8 resulted in a similar pattern of rapid phosphorylation that peaked by 30 seconds and waned within 10 minutes (Figure 2). To determine whether the fMLF-induced phosphorylation pattern was specific for VASP, we evaluated the fMLF-induced phosphorylation response of another important leukocyte-specific cytoskeletal protein, the actin bundling protein L-plastin. L-plastin phosphorylation increased after 1 minute of stimulation with fMLF and sustained for at least 20 minutes (Figure 1). The prolonged fMLF-induced phosphorylation of L-plastin is in stark contrast to the rapid phosphorylation and dephosphorylation event of VASP. To determine whether the characteristic pattern of VASP phosphorylation was unique to chemoattractant stimulation, we also stimulated cells with two non-chemoattractant activators of neutrophil adhesion: the pro-inflammatory cytokine TNFα and the PKC activator PMA. Stimulation of cells with TNFα at concentrations sufficient to induce strong adhesion (data not shown) did not result in a change in the phosphorylation state of VASP Ser 157 as compared to vehicle control at any time point we examined (Figure 3). Alternatively, stimulation of cells with PMA (10ng/ml) resulted in rapid and sustained VASP Ser 157 phosphorylation for the incubation period of 30 minutes (Figure 3). Because fMLF induces a dependable and rapid phosphorylation and

dephosphorylation event of Ser 157 of VASP, we used it as a representative chemoattractant for the remainder of our studies.

Ser 157 phosphorylation of VASP after fMLF stimulation corresponds with polarization and maximum F-actin levels

To begin to investigate the function of serine 157 phosphorylation of VASP in migration, we fixed neutrophils at each time point after fMLF stimulation to examine the actin cytoskeleton. Cells were stained for F-actin using Alexa Fluor 546-conjugated phalloidin to either visualize polarization and actin distibution in fMLF-stimulated adherent cells or measure changes in total F-actin content in polarizing cells in suspension using FACS. At 30 seconds post fMLF stimulation, neutrophils began to polarize and develop an F-actin rich leading edge and a trailing edge uropod (Figure 4A). In contrast, vehicle control cells remained spherical with diffuse distribution of F-actin. From 60 seconds to 20 minutes of fMLF stimulation, cells continued to polarize, as evidenced by membrane ruffles and further development of the leading edge, in preparation for migration. When total F-actin content was measured in cells in suspension, vehicle control treated cells had similar levels of F-actin at all time points examined, except for a significantly reduced amount (0.78) at 20 minutes as compared to "time zero" (Figure 4B). In contrast, cells stimulated with fMLF had a significant 2.5 fold increase in F-actin levels within 30 seconds. By 60 seconds, F-actin levels decreased to 2-fold over "time zero," and continued to decline towards basal levels. Total F-actin levels after fMLF stimulation mirrored the increase and decrease of Ser 157

phosphorylation levels seen previously. Taken together, the point of maximal VASP Ser 157 phosphorylation, 30 seconds post fMLF stimulation, coordinates functionally with maximal increases in F-actin content in the cell and morphologically with maximal polarization and reorganization of the cytoskeleton.

Phosphorylation of Ser 157 of VASP is PKA dependent

Because PKA, PKC, and PKG have all been shown to phosphorylate Ser 157 of VASP [18, 19, 37], we sought to determine which kinases are responsible for the fMLF-induced Ser 157 phosphorylation in neutrophils. We pretreated cells with kinase-specific inhibitors at a range of concentrations prior to 60 seconds of fMLF stimulation. fMLF-induced VASP Ser 157 phosphorylation was abolished by the PKA-specific inhibitor H89, but not by KT5720, a structurally unrelated and less-specific PKA inhibitor [38] (Figure 5), although both KT5720 and H89 were able to inhibit PKA-dependent immune complex-induced phosphorylation of L-plastin in neutrophils (Figure 9). Because H89 also potently inhibits Cam Kinase II, which could account for the discrepancy between H89 and KT5720 to inhibit fMLF-induced VASP phosphorylation, we pretreated cells with the CamKII inhibitor KN-62 (25μM), but did not see an affect on fMLF-induced VASP Ser 157 phosphorylation (Figure 5). VASP Ser 157 phosphorylation was abolished by pre-treatment with 3mM SQ22536, a cell-permeable adenylyl cyclase inhibitor (Figure 5), further supporting a role for PKA in the mechanism of VASP Ser 157 phosphorylation in response to fMLF stimulation. fMLF-induced VASP Ser 157 phosphorylation was not affected by the

PKG inhibitors Rp-8-pCPT-cGMP (50μM) or KT5823 (2.5μM), nor the PKC inhibitors staurosporine (100nM) or calphostin C (10μM) (Figure 5), ruling out a role for PKG or PKC in this mechanism.

To examine further whether PKA was responsible for the fMLF-induced phosphorylation of VASP, we prolonged PKA activity by elevating intracellular cAMP concentrations with Ro-20-1724, an inhibitor for the cAMP-specific PDE IV, or prolonged PKG activity by treatment with the cGMP-specific PDE-V inhibitor, 4-(methylenedioxy)-benzylamino-6-methoxyquinazoline (4-MB), or both with the broad PDE inhibitor IBMX, and examined phosphorylation levels of VASP after 20 minutes of fMLF stimulation, when VASP Ser 157 is dephosphorylated to baseline levels. 4-MB-treated cells contained similar amounts of phosphorylated VASP (30%) as vehicle control cells after 20 minutes of fMLF stimulation (Figure 6). In contrast, both Ro-20-1724- and IBMX-treated cells had significantly more phosphorylated VASP than control cells. This level of phosphorylation (60% and 50%, respectively) was analogous to the maximum level seen immediately after fMLF-stimulation, before the rapid dephosphorylation event. From these data we conclude that down-regulation of cAMP, and thus PKA activity, is critical for the dephosphorylation of VASP Ser 157 following fMLF stimulation.

Alteration of PKA Activity Decreases fMLF-induced Migration

Next we examined the requirement for VASP Ser 157 phosphorylation and dephosphorylation in the mechanism of neutrophil migration in response to fMLF

stimulation, assessing both chemotaxis and chemokinesis. Our approach was to inhibit phosphorylation with 25μM H89, or inhibit the dephosphorylation event with 400μM IBMX and determine the effect on fMLF-induced migration. Pre-treatment with H89 resulted in a significant reduction in directed migration towards fMLF when compared to untreated control cells ('HBSS/fMLF,' Figure 7). When PKA activity was prolonged with IBMX, there was a significant reduction in fMLF-induced migration. Additionally, there was a significant reduction in chemokinesis after both H89 and IBMX treatment ('fMLF/HBSS,' and 'fMLF/fMLF,' Figure 7). We conclude from these results that alteration in PKA activity, which corresponds to altered VASP Ser 157 phosphorylation/dephosphorylation, results in reduced neutrophil chemokinesis and chemotaxis.

Adhesion Does Not Regulate VASP Ser 157 Phosphorylation in Neutrophils Stimulated by fMLF

Adhesion is an important regulator of VASP Ser 157 phosphorylation in fibroblasts [39]. Moreover, many neutrophil functions are dependent on adhesion for optimal activation. To determine whether adhesion regulates neutrophil activation through modification of VASP Ser 157 phosphorylation, we examined whether inhibition of adhesion affects fMLF-induced VASP Ser 157 phosphorylation. We found no difference in the kinetics or extent of VASP phosphorylation after fMLF stimulation in cells attached to a surface as compared to those in suspension (unpublished results). Treatment with 1mM EDTA, to block adhesion by inhibiting Ca^{2+}- and Mg^{2+}-dependent integrin ligand binding, slightly reduced the magnitude

of fMLF-induced VASP phosphorylation for up to 180 seconds (Figure 7). By 5 minutes, EDTA treated cells maintained similar levels of phospho-VASP as control cells. To alternatively block adhesion, we pre-treated cells with the β2 integrin (CD 18) specific monoclonal antibody IB4 [29] or an isotype matched control antibody, W6/32, specific for class I human leukocyte antigen (HLA) [30]. Pretreatment with IB4 at a concentration that maximally inhibited adhesion to β2 integrin substrates (unpublished results) did not affect fMLF-induced phosphorylation or dephosphorylation of VASP as compared to cells treated with control antibody at any time point (Figure 8). We conclude that neither fMLF-induced phosphorylation nor subsequent dephosphorylation of VASP Ser 157 is β2-integrin adhesion dependent.

IC-induced Ser 157 phosphorylation of VASP is PKA-dependent

Immune complex-induced activation of neutrophils, through ligation of Fcγ receptors, results in integrin activation and initiates intracellular signaling necessary for the induction of an inflammatory response. IC-induced activation is dependent on both PKA-dependent signaling, and PI 3-kinase pathways [40,41]. Ligation of Fcγ receptors results in a transient increase in intracellular cAMP with a peak at 5 minutes and return to baseline by 15 minutes, and this increase is blocked by inhibition of PI 3-kinase [40]. Sustained adhesion to immune complexes, over 30 minutes, results in PKA-dependent phosphorylation of L-plastin (Lpl) [40,42]. To determine whether VASP potentially has a role in IC-induced integrin activation in neutrophils, we examined levels of phospho-VASP in IC-activated neutrophils. After

adherence to FCS-coated wells, there was no appreciable phosphorylation of L-plastin or VASP (Figure 9). In contrast, IC-induced adhesion resulted in phosphorylation of both Lpl and VASP, and this phosphorylation was abolished by pre-treatment with the PKA inhibitors KT5720 or H89. We can conclude from these data that IC stimulation of both VASP Ser 157 phosphorylation and Lpl phosphorylation are PKA-dependent.

Discussion

Our data demonstrate that chemoattractant stimulation induces rapid phosphorylation and subsequent dephosphorylation of Ser 157 of VASP in human neutrophils. This rapid phosphorylation is PKA-dependent, while dephosphorylation requires the downregulation of PKA. Unlike in fibroblasts [39], integrin mediated adhesion does not appear to play a role in regulating the state of VASP phosphorylation in chemoattractant-stimulated neutrophils, although, IC-activation of neutrophil integrins results in PKA-dependent VASP phosphorylation. Inhibition or prolonged activation of PKA results in decreased fMLF-directed migration. Our data suggest that VASP and its phosphorylation on Ser 157 is an important element in the mechanism of human neutrophil migration.

It is attractive to speculate that VASP may regulate neutrophil migration by affecting the organization of the actin cytoskeleton. Similar to the role of VASP in other cells, VASP phosphorylation may have a negative effect on actin polymerization in neutrophils. The phosphorylation of VASP by PKA has been shown to inhibit F-actin binding and reduce actin nucleation [20,43]. Our data show that after fMLF stimulation of neutrophils, there is

rapid polymerization of actin that is maximal by 30 seconds and the cells begin to exhibit a shape change with focal distribution of F-actin to the leading edge in preparation for directed migration (Figure 4) [44]. Our data show that VASP is coordinately maximally phosphorylated at 30 seconds after fMLF stimulation during this phase of rapid actin polymerization and maximal increase in F-actin content, with a return to baseline phosphorylation levels by 10 minutes. The beginning of VASP Ser 157 dephosphorylation coincides with a decrease in F-actin content and reorganization of F-actin in the cell as the cell body condenses (Figure 4). Our data suggest that transient phosphorylation of VASP may be important for initiating actin polymerization and the shape change required for adhesion and, ultimately, polarization of chemoattractant-stimulated neutrophils. Because Ser 157 phosphorylation reduces actin binding, it is likely that this rapid phosphorylation of VASP releases it from the cytoskeleton. This may in turn help destabilize the somewhat rigid cortical actin cytoskeleton of resting neutrophils, which may be necessary to ultimately enable polymerization of new F-actin and reorganization of the existing cytoskeletal structure.

This proposed model for the role of VASP Ser 157 phosphorylation in the regulation of F-actin dynamics and shape changes in migrating neutrophils is consistent with the observation that actin polymerization is inhibited by PKA-mediated phosphorylation of VASP Ser 157 [20]. Moreover, studies in platelets have shown that polymerization cannot occur until dephosphorylation occurs, most likely by protein phosphatases PP2A, PP2B, and PP2C (*in vitro*) [18,27]. Global activation of PKA with cAMP elevating agents in neutrophils

inhibits F-actin polymerization and organization [45,46], adhesion [4,47], and migration [3,48,49]. Our data suggest that this may at least in part be due to reduced dephosphorylation of VASP.

Another way that Ser 157 phosphorylation could be regulating cell motility is through effects on VASP's affinity for binding partners. VASP binds via its EVH1 domain to several proteins including vinculin, zyxin, and lamellipodin. Although it has been shown that Ser 157 phosphorylation does not affect VASP EVH1-mediated binding to vinculin or zyxin [50,51], there are likely alternate EVH1 domain-binding proteins whose binding is affected by VASP Ser 157 phosphorylation levels. Ser 157 is located in the proline-rich region near the $(GP_5)_3$ motif that binds the actin-binding protein profilin which promotes the addition of actin monomers to barbed ends of actin filaments [52,53]. Surprisingly, though, VASP Ser 157 phosphorylation does not directly affect profilin binding. However, profilin competes with the signaling molecule Abl tyrosine kinase (Abl) for binding to the proline rich motif of VASP via their shared SH3 domains, and Abl's interaction with VASP is reduced by Ser 157 phosphorylation [39]. Interestingly, the SH3 domains of the tyrosine kinases Fyn, Lyn, and Src bind the Ena/VASP family member EVL, although phosphorylation of EVL Ser 157 inhibits only Src SH3 binding [54]. Perhaps Ser 157 phosphorylation uncovers profilin binding sites on VASP, thus indirectly increasing the association of profilin-actin monomer complexes with VASP. In this scenario, VASP dephosphorylation may then be necessary for re-localization of VASP-profilin-actin monomer complexes to sites of F-actin polymerization. Uncovering how the phosphorylation of Ser 157 of VASP in neutrophils

affects assembly of binding partner complexes will help clarify its role in cytoskeletal polarity and chemotaxis.

The association between VASP and integrins in neutrophils remains elusive. On one hand, VASP appears to be critical for integrin function, at least in platelets. Indeed, the ability of VASP to regulate platelet integrins is dependent on Ser 157 [26]. On the other hand, integrin engagement has a profound effect on VASP Ser 157 phosphorylation in fibroblasts. Fibroblast detachment increases PKA-dependent VASP Ser 157 phosphorylation and reattachment rapidly decreases phosphorylation, yet Ser 157 becomes re-phosphorylated to an intermediate level during cell spreading [39]. Previous work has shown that β2 integrins down-regulate PKA activity during neutrophil adhesion [55], suggesting that a similar mechanism to that in fibroblasts for regulating VASP phosphorylation may exist. In light of this data, we sought to determine whether integrin-mediated adhesion was required for dephosphorylation of VASP in chemoattractant-stimulated neutrophils. We found no role for adhesion in the pattern of VASP phosphorylation in response to fMLF stimulation. We found no difference in fMLF-induced VASP phosphorylation or dephosphorylation when comparing cells in suspension to cells adherent to β2 integrin substrate fetal calf serum. Inhibition of β2 integrin engagement, through depletion of Ca^{2+} and Mg^{2+} or through antibody blockade, did not affect the rapid phosphorylation and dephosphorylation of VASP in response to fMLF. On the other hand, activation of Fc receptor and β2 integrin ligation with immune complexes resulted in VASP Ser 157 phosphorylation, and this response was PKA-dependent. Our data suggest that VASP Ser 157 phosphorylation and subsequent

dephosphorylation occurs prior to or independently of β2 integrin signaling, and that stable IC-induced adhesion alone is sufficient to induce phosphorylation. Thus, VASP Ser 157 phosphorylation serves an important, yet incompletely understood, role in integrin-mediated cell adhesion and migration and further studies need to be done to dissect the sequence of events that occur during neutrophil adhesion and integrin engagement.

At the center of the regulatory control of neutrophil migration is PKA. PKA regulates β2 integrin avidity and adhesion in neutrophils [4] and directs migration through activation of its many effectors, including VASP. Our lab has recently shown that asymmetrical PKA activity is required for polarization of the actin cytoskeleton and neutrophil migration [5]. Indeed, a gradient of PKA inhibitor, expected to asymmetrically decrease PKA activity resulting in low PKA activity at the leading edge and high PKA activity at the opposite pole, is sufficient to induce migration and polarize the actin cytoskeleton. This suggests that PKA activity must be precisely and spatially regulated to enable polarization and migration. When we presumably destroyed the spatial control of PKA activity with the use of H89, thereby inhibiting all activity, or IBMX, and flooding the cell with cyclic nucleotides and destroying the ability of the cell to maintain local pockets of activity through localized phosphodiesterase activity, there was a significant reduction in both chemokinesis and chemotaxis (Figure 7). We can correlate this data with the knowledge that PKA induces VASP phosphorylation, and that the down-regulation of PKA is required for the dephosphorylation, and therefore conclude that altered VASP phosphorylation results in reduction in neutrophil migration. Until we are able to genetically modify VASP

phosphorylation levels in primary neutrophils, as previously accomplished in fibroblasts [23], we cannot fully define the impact and function of VASP serine 157 phosphorylation on neutrophil migration.

We have shown that VASP Ser 157 phosphorylation in response to fMLF is PKA-dependent. In support, the kinetics of VASP Ser 157 phosphorylation closely matches that of adenylyl cyclase activity. In neutrophils, chemoattractants fMLF, LTB_4, C5a, and IL8 induce a rapid and transient stimulation of adenylyl cyclase activity that peaks by 1 minute and returns to baseline by 8 minutes [56]. Pre-incubation of neutrophils with H89 or the adenylyl cyclase inhibitor SQ22536 resulted in abolishment of fMLF-induced VASP phosphorylation. Yet, pre-incubation with KT5720 did not affect phosphorylation. Although KT5720 is regarded as a PKA inhibitor, it in fact inhibits many other protein kinases more potently [38]. As a promiscuous inhibitor, perhaps KT5720 is also inhibiting an alternate kinase responsible for the down-regulation of fMLF-induced PKA activity, resulting in a net intermediate level in the cell and no apparent reduction in VASP phosphorylation levels as visualized by Western Blotting. Alternatively, pre-incubation with KT5720 before IC stimulation resulted in the abolishment of VASP phosphorylation and PKA-dependent L-plastin phosphorylation. One explanation for the discrepancy between H89 and KT5720 to inhibit VASP Ser 157 phosphorylation after fMLF stimulation could be explained by differences in their ability to penetrate the membrane or, once cytosolic, various subcellular regions of the cell. KT5720 has been shown to inhibit PKA-dependent endothelin-stimulated neutrophil migration, but only after cells were electroporated [57], which suggests that membrane permeation of

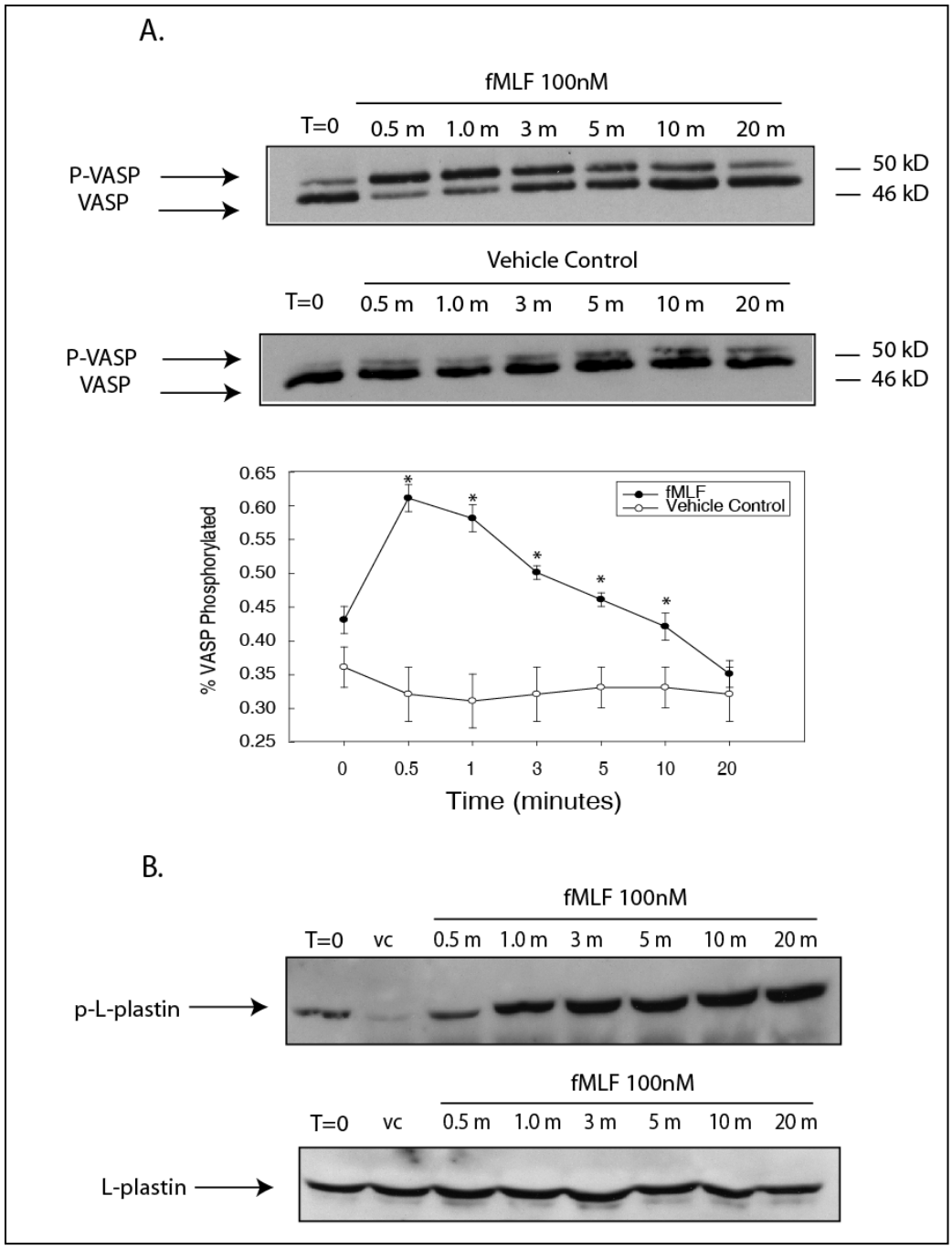
KT5720, structurally unrelated to H89, may be unpredictable in neutrophils. The various isoforms of PKA have a unique spatial restriction and ability to be active in precise "micropockets" in the cytosol because of the assortment of specific A kinase anchoring proteins (AKAPs) that help localize the enzyme [58].

Because neutrophils have an impressive 50,000 membrane fMLF receptors with a dissociation constant of only 20nmol/L [59], we can hypothesize that potent and geographically specific inhibition of PKA would be necessary prior to stimulation in order to see an appreciable reduction in cAMP-dependent VASP phosphorylation levels. Perhaps different isoforms of PKA are responsible for IC- versus fMLF-induced VASP-phosphorylation to fMLF. The relative potencies of H89 and KT5720 for the two different isoforms, and their splice variants, of the PKA catalytic subunits are unknown [58,60]. Because increases in cAMP, using a PDE-IV-specific inhibitor, prolonged fMLF-induced VASP Ser 157 phosphorylation, and increases in cGMP, using a PDE-V-specific inhibitor, had no effect, we conclude that fMLF-induced VASP Ser 157 phosphorylation is indeed cAMP-dependent. We have considered the possibility that cAMP is signaling in a PKA-independent fashion: one recently identified signaling target being exchange protein directly activated by cAMP (Epac). Epac is a signaling molecule that links cAMP to the small GTPase Rap1 and has been implicated in the pathways for cell adhesion [61,62], insulin secretion [63, 64], exocytosis [63,65], and as an inhibitor of the ERK5 pathway in cardiomyocytes [63,66]. Because H89 does not inhibit Epac and Epac protein is not

expressed in fMLF-stimulated neutrophils [67], we do not consider Epac a likely candidate for cAMP-activated VASP phosphorylation.

In conclusion, we have shown in this paper that pre-treatment of cells with the PKA inhibitor H89, which abolishes VASP phosphorylation in response to fMLF, or a PKA activator IBMX, which prolongs VASP phosphorylation in response to fMLF, results in a decrease in fMLF-directed migration. VASP appears to be an important effector of PKA in the mechanism of neutrophil migration. In our model of neutrophil migration, we expect VASP to be predominately phosphorylated on Ser 157 at the trailing edge and predominately dephosphorylated at the leading edge pseudopod. This model is consistent with our proposed mechanism by which VASP control of F-actin polymerization and structure may be regulated by Ser 157 phosphorylation. However, our hypothesis is in contrast to recent studies in fibroblasts that demonstrate that PKA activity and Ser 157 phospho-VASP is enriched in fibroblast lamellipodia [68]. Moreover, spatial restriction of PKA to the fibroblast leading edge and subsequent phosphorylation of α4 integrins is required for migration suggesting that the role for PKA in regulating fibroblast and neutrophil polarity and migration is fundamentally different. Hence, a unique role for PKA substrates such as VASP may exist for neutrophils and other cells that must rapidly respond to chemoattractant signals and initiate migration more quickly than fibroblasts.

Figure 1. VASP serine 157 and L-plastin phosphorylation in response to fMLF. *A*, Purified neutrophils were stimulated with 100nM fMLF or vehicle control (Me_2SO) for the indicated time periods during a 20 minute incubation at 37 ºC after adherence to FCS-coated wells as described in Materials and Methods. Equal protein amounts at each time point were analyzed for VASP by Western Blot analysis. Densitometry was performed and the density of the upper 50 kD and lower 46 kD bands were measured. "% VASP Phosphorylated" was calculated by dividing the upper phosphorylated band by the total amount of VASP (upper 50kD band + lower 46kD band) and presented as the mean ± SE. Stimulation of cells resulted in rapid phosphorylation and dephosphorylation of serine 157 of VASP. The % of phosphorylated VASP was significantly greater than vehicle control (vc) at the time points from 0.5 to 10 minutes (*, $p<0.05$) after fMLF stimulation. Data are representative of at least four separate experiments (n=6 fMLF, n=4 vc) using neutrophils from different donors. *B*, Purified neutrophils were stimulated as above. Equal protein amounts at each time point were analyzed for L-plastin and phospho-L-plastin by Western Blot. Stimulation with 100nM fMLF did not alter the amount of total L-plastin in the cells. fMLF stimulation resulted in increased levels of phospho-L-plastin by 1 minute, and this level of phosphorylation was sustained for at least 20 minutes.


A.
fMLF 100nM
T=0
0.5 m
1.0 m
3 m
5 m
10 m
20 m
P-VASP
VASP
50 kD
46 kD
Vehicle Control
T=0
0.5 m
1.0 m
3 m
5 m
10 m
20 m
P-VASP
VASP
50 kD
46 kD
% VASP Phosphorylated
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
fMLF
Vehicle Control
0
0.5
1
3
5
10
20
Time (minutes)
B.
fMLF 100nM
T=0
vc
0.5 m
1.0 m
3 m
5 m
10 m
20 m
p-L-plastin
fMLF 100nM
T=0
vc
0.5 m
1.0 m
3 m
5 m
10 m
20 m
L-plastin

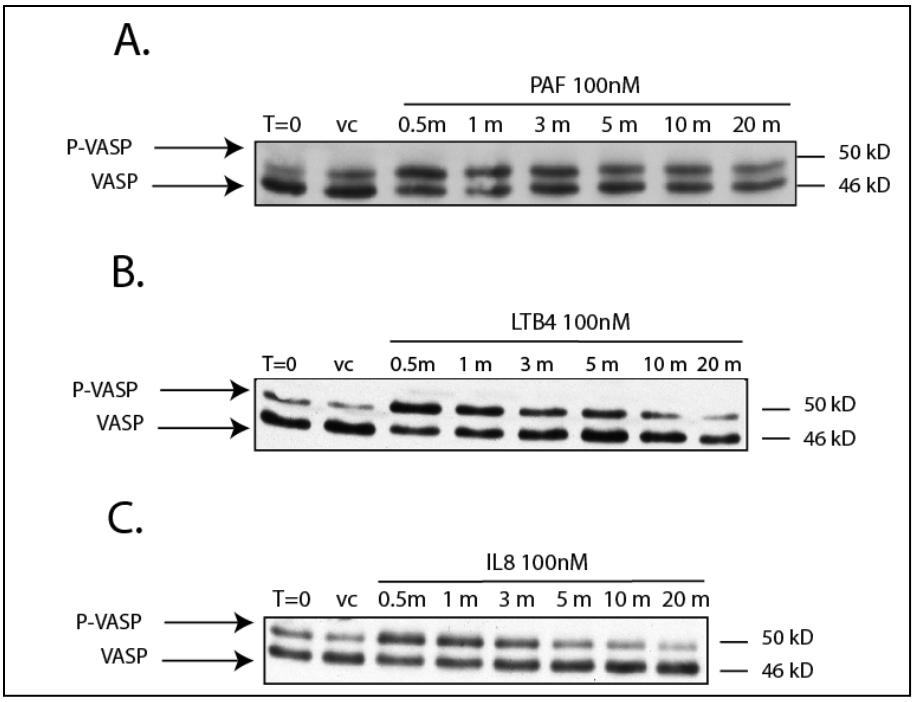


Figure 2. VASP serine 157 phosphorylation in response to chemoattractants PAF, LTB_4, and IL8. Purified neutrophils were stimulated with *A*, 100nM PAF, *B*, 100nM LTB_4, *C*, 100nM IL8, or vehicle control (Me_2SO) for the indicated time periods during a 20 minute incubation at 37 ºC after adherence to FCS-coated wells as described in Materials and Methods. Equal protein amounts at each time point were analyzed for VASP by Western Blot analysis. Stimulation with PAF, LTB_4, or IL8 resulted in a similar pattern of rapid phosphorylation and dephosphorylation of serine 157 of VASP as seen after fMLF stimulation. Data are representative of at least 3 separate experiments using neutrophils from different donors.

Figure 3. Neutrophil activators TNFα and PMA do not induce the rapid and transient serine 157 phosphorylation of VASP that is seen with chemoattractants. *A*, Purified neutrophils were stimulated with 100ng/ml TNFα, 100nM fMLF, or vehicle control (Me_2SO) for the indicated time periods during a 20 minute incubation at 37 ºC after adherence to FCS-coated wells as described in Materials and Methods. Equal protein amounts at each time point were analyzed for VASP by Western Blot analysis. Stimulation of cells with TNFα resulted in no significant change in the phosphorylation state of VASP as compared to vehicle control *B*, Purified neutrophils were stimulated with 10ng/ml PMA, or vehicle control (Me_2SO) for the indicated time periods during a 30 minute incubation at 37 ºC after adherence to FCS-coated wells as described in Materials and Methods. Stimulation of cells with the PKC activator PMA resulted in phosphorylation of Ser 157 of VASP that was sustained for at least 30 minutes. Data are representative of least three separate experiments using neutrophils from different donors.

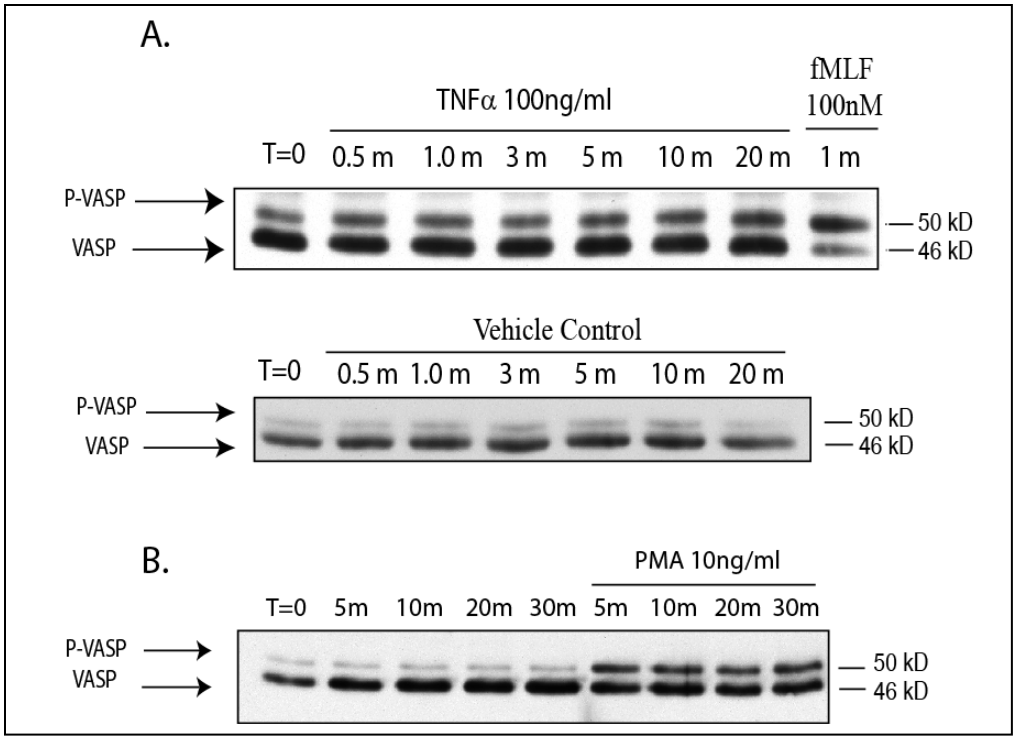

A.
TNFα 100ng/ml
fMLF
100nM
T=0
0.5 m
1.0 m
3 m
5 m
10 m
20 m
1 m
P-VASP
VASP
50 kD
46 kD
Vehicle Control
T=0
0.5 m
1.0 m
3 m
5 m
10 m
20 m
P-VASP
VASP
50 kD
46 kD
B.
PMA 10ng/ml
T=0
5m
10m
20m
30m
5m
10m
20m
30m
P-VASP
VASP
50 kD
46 kD

Figure 4. The rapid phosphorylation and dephosphorylation of Ser 157 of VASP after fMLF stimulation corresponds with polarization and F-actin levels in neutrophils. *A*, Purified neutrophils were allowed to adhere to FCS-coated glass coverslips prior to 100nM fMLF or vehicle control (Me_2SO) stimulation for the indicated periods of time before permeabilization, fixation, and staining of F-actin with phalloidin. At 30 seconds, fMLF-stimulated neutrophils began to polarize and develop an F-actin rich leading edge and a trailing edge uropod. In contrast, vehicle control cells remained spherical with diffuse distribution of F-actin. From 60 seconds to 20 minutes of fMLF stimulation, cells continued to polarize, as evidenced by membrane ruffles and further development of the leading edge, in preparation for migration. *B*, Purified neutrophils were stimulated with vehicle control (Me_2SO) or 100nM fMLF for 0, 30s, 60s, 180s, or 300s before fixation, permeabilization, and staining with Alexa Fluor 546 phalloidin, as described in Materials and Methods, and FACS analysis. The reported Mean Fluorescence Intensity (MFI) for each sample was divided by a "time zero" unstimulated control to determine the fold change in F-actin levels for each treatment group. Vehicle control treated cells had similar levels of F-actin at all time points, except a significantly reduced amount (0.78) at 20 minutes as compared to "time zero" (*, $p<0.05$). In contrast, cells stimulated with fMLF had a 2.5 fold increase in F-actin levels within 30 seconds. By 60 seconds, F-actin levels decreased to 2-fold over "time zero," and continued to decline towards basal levels. Cells stimulated with fMLF for 30s, 60s, and 180s had significantly increased levels of F-actin when compared to vehicle control cells at the same time points (■, $p<0.05$). Data is representative of three independent experiments.

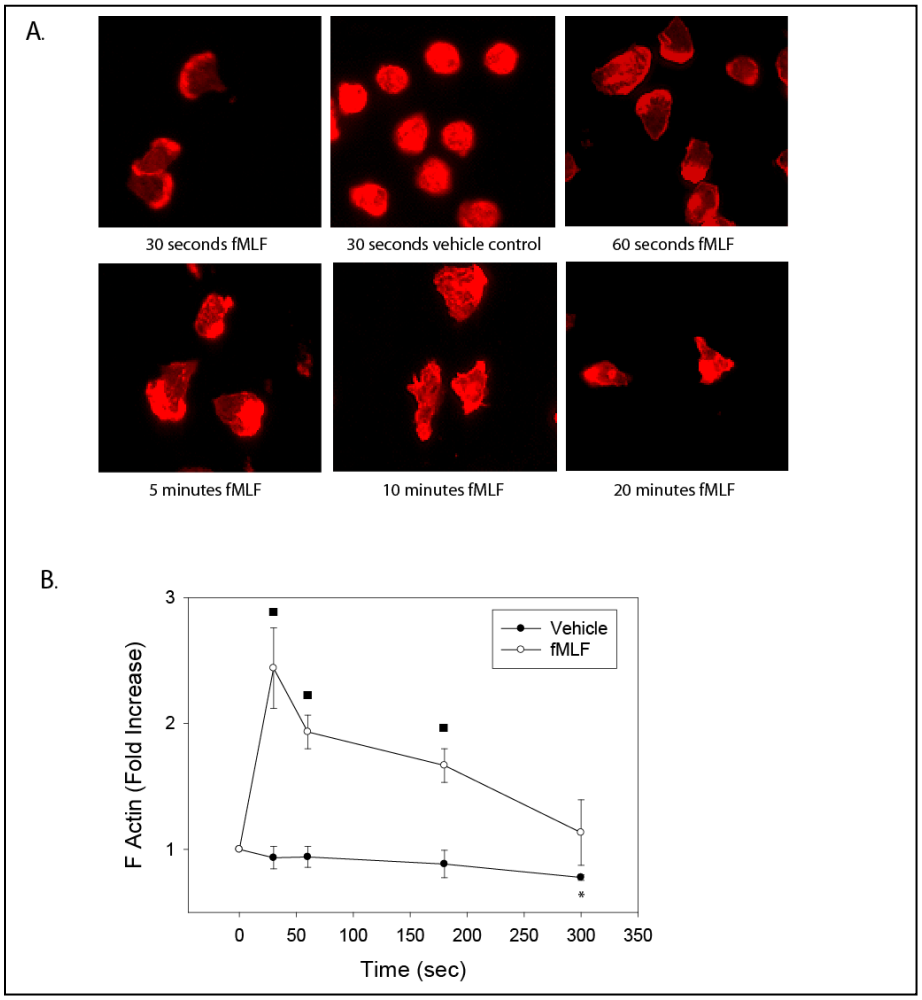

A.
30 seconds fMLF
30 seconds vehicle control
60 seconds fMLF
5 minutes fMLF
10 minutes fMLF
20 minutes fMLF
B.
F Actin (Fold Increase)
Time (sec)
Vehicle
fMLF
0
50
100
150
200
250
300
350
1
2
3
*

Figure 5. VASP fMLF-induced serine 157 phosphorylation is PKA-dependent. *A*, Purified neutrophils were pre-treated with a range of concentrations of the PKA-inhibitors H-89 or KT5720, the adenylyl cyclase inhibitor SQ22536, or vehicle control (Me_2SO) before stimulation with 100nM fMLF for 60 seconds at 37 ºC after adherence to FCS-coated wells as described in Materials and Methods. Equal protein amounts were analyzed by Western Blot analysis using an anti-whole VASP specific antibody. Densitomety was performed as previously described for the maximum concentrations of each inhibitor. KT5720 (25μM) pre-treatment did not reduce levels of fMLF-induced VASP Ser157 phosphorylation as compared to vehicle control (vc). In contrast, H89 (25μM) or SQ22536 (3mM) pre-treatment resulted in significantly reduced levels of fMLF-induced VASP Ser 157 phosphorylation, similar to those of untreated cells (*, $p<0.05$). *B*, Cells were pre-treated as in '*A*' with a range of concentrations the indicated inhibitors before stimulation with 100nM fMLF. Inhibition of PKC (Staurosporine and Calphostin C), PKG (KT5823 and Rp-8-pCPT-cGMP), or Cam Kinase II (KN-62) did not reduce fMLF-induced VASP Ser 157 phosphorylation levels. Data are representative of at least 3 separate experiments using neutrophils from different donors.

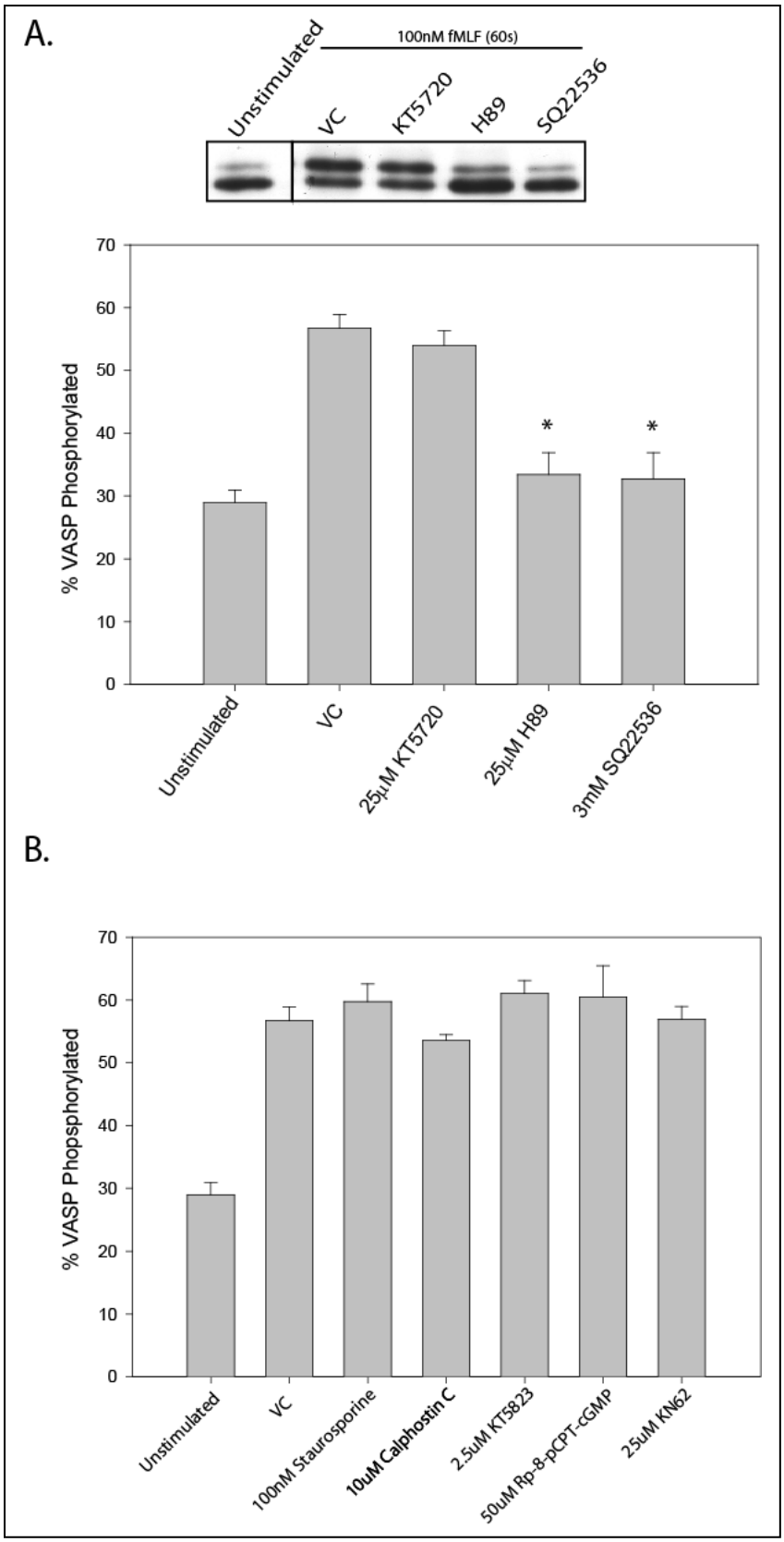

A.
100nM fMLF (60s)
Unstimulated
VC
KT5720
H89
SQ22536
% VASP Phosphorylated
70
60
50
40
30
20
10
0
*
*
Unstimulated
VC
25μM KT5720
25μM H89
3mM SQ22536
B.
% VASP Phopsphorylated
70
60
50
40
30
20
10
0
Unstimulated
VC
100nM Staurosporine
10uM Calphostin C
2.5uM KT5823
50uM Rp-8-pCPT-cGMP
25uM KN62

Figure 6. Dephosphorylation of VASP requires downregulation of PKA. Purified neutrophils were pre-treated with the indicated concentrations of the PDEIV-specific inhibitor Ro-20-1724 (Ro), to reduce degradation of cAMP, or the PDEV-specific inhibitor 4-{[3',4'-(Methylenedioxy)benzyl]amino}-6-methoxyquinazoline (4-MB), to reduce cGMP degradation. Cells were alternatively treated with IBMX to reduce degradation of both cAMP and cGMP. Cells were then stimulated with 100nM fMLF for 20 minutes at 37 ºC after adherence to FCS-coated wells as described in Materials and Methods. Equal protein amounts at each time point were analyzed for VASP by Western Blotting and densitometric analysis was performed as previously described. IBMX and Ro treatment resulted in a significant increase in the amount of phosphorylated VASP when compared to vehicle control treated cells (*, $p<0.05$). Treatment with 4-MB did not result in increased phosphorylation levels of VASP when compared to vehicle control. Data are representative of at least 3 separate experiments using neutrophils from different donors.

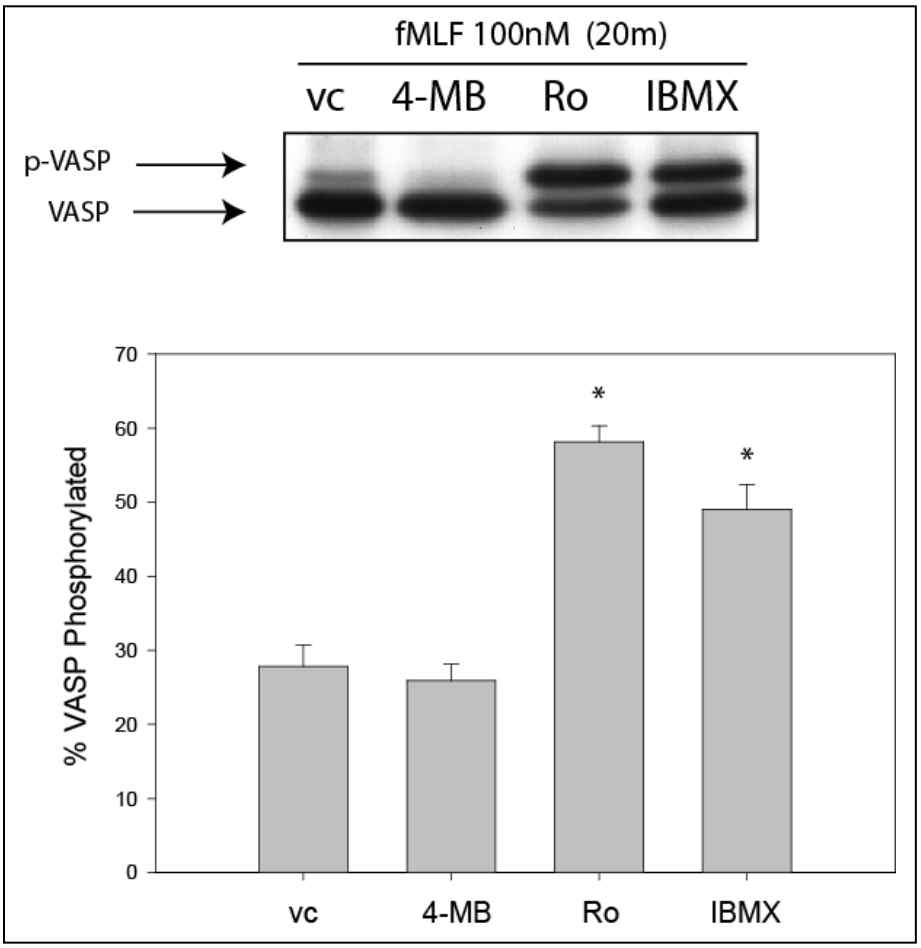

fMLF 100nM (20m)
vc
4-MB
Ro
IBMX
p-VASP
VASP
% VASP Phosphorylated
70
60
50
40
30
20
10
0
*
*
vc
4-MB
Ro
IBMX

Figure 7. Effect of altered PKA activity on fMLF-induced migration. Purified calcein-labeled neutrophils were pre-treated with the indicated inhibitor prior to placement of 1 x 10^4 cells on a 2μM membrane of a 96-well ChemoTx® plate. The lower wells contained chemotaxis buffer with or without 100nM fMLF and cells on the top of the filter were in $HBSS^{++}$ or $HBSS^{++}$ with 100nM fMLF suspension. After 1 hour of incubation at 37 ºC, non-migrated cells were scraped off the top of the filter. 0.5mM EDTA was added prior to centrifugation to release the cells from the lower side of the filter. The fluorescence was measured in the lower wells. Percent migration was calculated by dividing the fluorescence of the wells by the fluorescence of standard wells containing 1 x 10^4 labeled cells and is presented as the mean ± SE. Less than 10% of untreated cells exhibited random migration into wells without chemoattractant (HBSS/HBSS). In contrast, 100nM fMLF induced a significant increase in directed migration (HBSS/fMLF) of untreated cells into the lower chamber. Pre-treatment with H89 resulted in a significant reduction in directed migration towards fMLF when compared to untreated control cells. When PKA activity was prolonged with IBMX, there was also a significant reduction in fMLF-induced migration. There was a significant reduction in chemokinesis after both H89 and IBMX treatment (fMLF/HBSS and fMLF/fMLF) (*, $p<0.05$).

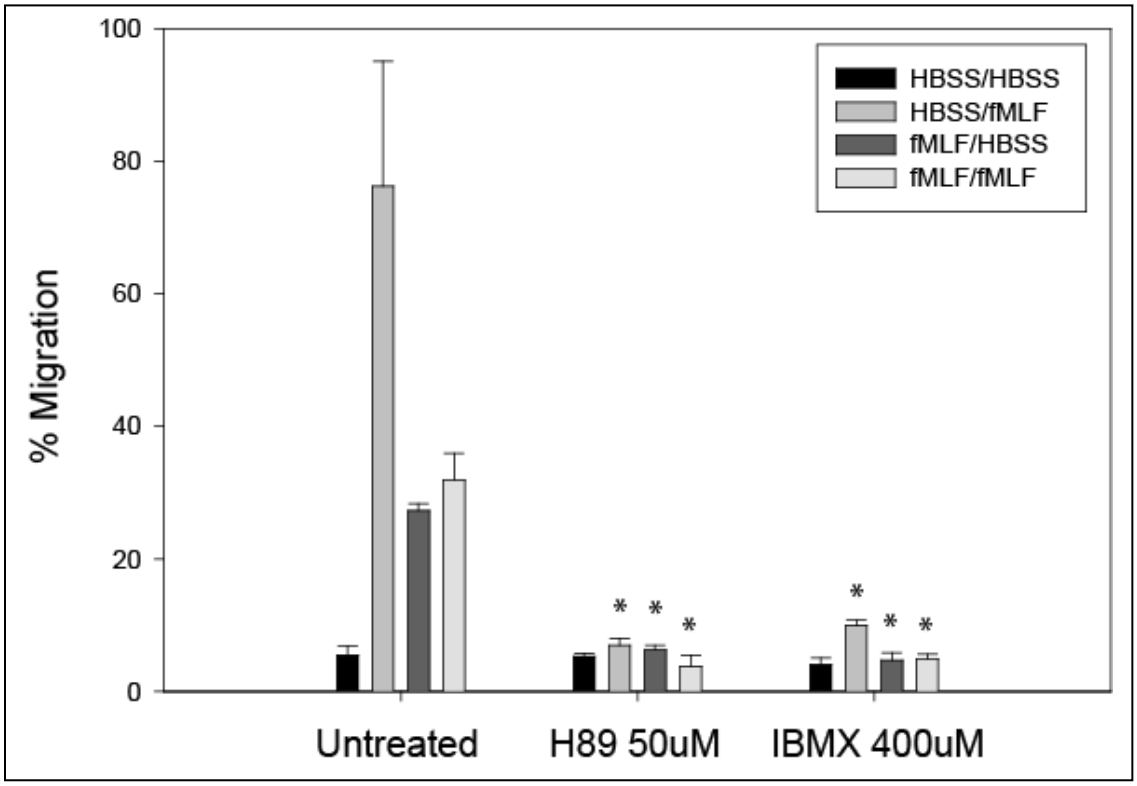
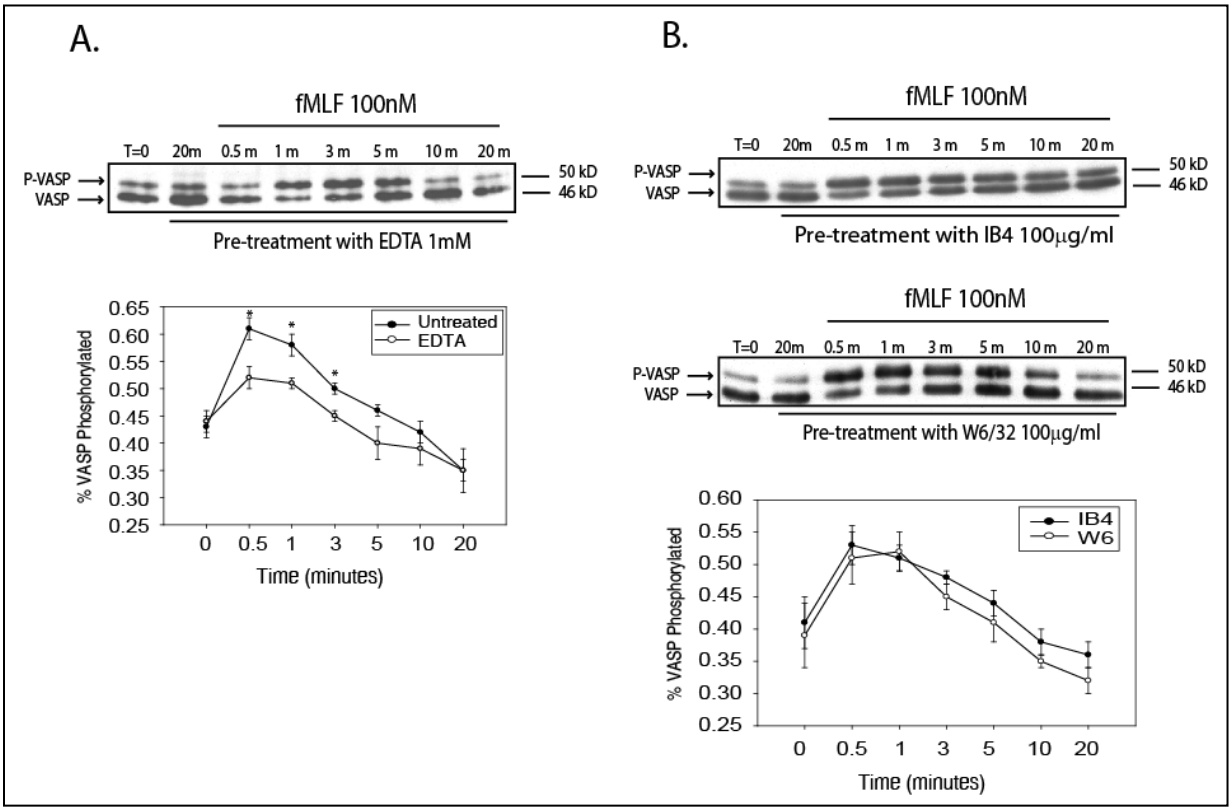

100
80
60
40
20
0
% Migration
HBSS/HBSS
HBSS/fMLF
fMLF/HBSS
fMLF/fMLF
*
*
*
*
*
*
Untreated
H89 50uM
IBMX 400uM

Figure 8. Adhesion does not regulate fMLF-induced serine 157 VASP phosphorylation. *A*, Purified neutrophils were pre-treated with 1mM EDTA to prevent adhesion. Cells were then unstimulated or stimulated with 100nM fMLF for the indicated time periods during a 20 minute incubation at 37 ºC after plating on FCS-coated wells as described in Materials and Methods. Equal protein amounts at each time point were analyzed for VASP by Western Blot analysis. Densitometry was performed and the density of the upper 50 kD and lower 46 kD bands were measured. "% VASP Phosphorylated" was calculated by dividing the upper phosphorylated band by the total amount of VASP (upper 50kD band + lower 46kD band) and presented as the mean ± SE. EDTA pre-treatment significantly reduced the amount of fMLF-induced VASP phosphorylation at the 30s, 60s, and 3 minute time point (*, $p<0.05$), but did not affect the overall pattern of rapid phosphorylation followed by rapid dephosphorylation. *B*, Purified neutrophils were pre-treated with 100μg/ml of IB4 or W632 antibody, prior to stimulation with fMLF as in *A*. There was no difference in serine 157 phosphorylation after IB4 blockade of β2 integrins when compared to cells treated with the control antibody W6/32. Data are representative of at least 3 separate experiments using neutrophils from different donors.


A.
fMLF 100nM
T=0
20m
0.5 m
1 m
3 m
5 m
10 m
20 m
P-VASP
VASP
50 kD
46 kD
Pre-treatment with EDTA 1mM
% VASP Phosphorylated
Untreated
EDTA
Time (minutes)
B.
fMLF 100nM
Pre-treatment with IB4 100μg/ml
fMLF 100nM
Pre-treatment with W6/32 100μg/ml
IB4
W6
% VASP Phosphorylated
Time (minutes)

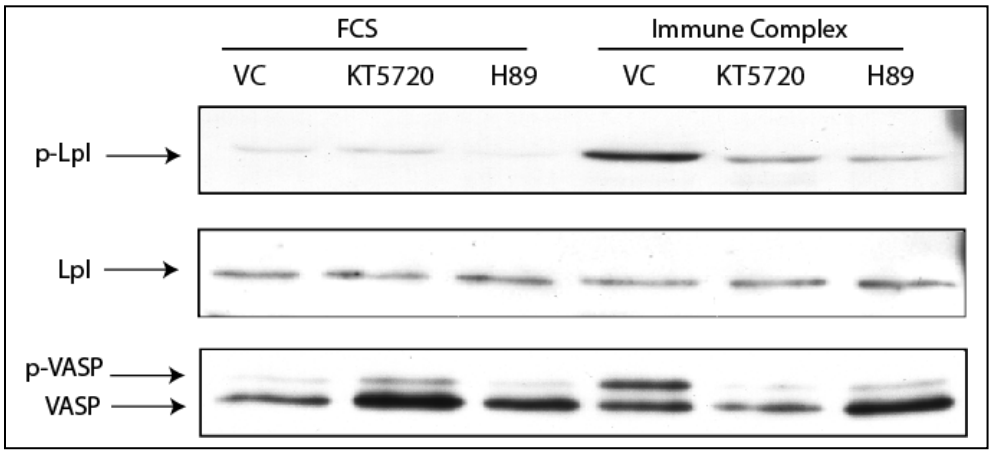


Figure 9. Immune complex activation of neutrophils results in PKA-dependent Ser 157 VASP phosphorylation. Purified neutrophils were pre-incubated with 25μM KT5720, 25μM H89, or vehicle control for 30 minutes at 37º C. 2 x10^6 cells were then added to FCS- or immune complex (IC)-coated wells of a 24-well tissue culture plate (as described in *Materials and Methods*). Cells were allowed to adhere for 30 minutes before Triton lysis. Equal protein amounts were evaluated by Western Blot analysis. Phospho-L-plastin (p-Lpl) blots were stripped and reprobed for total L-plastin (Lpl). There was no appreciable phosphorylation of Lpl or VASP in cells that were allowed to adhere to FCS. In contrast, IC-induced adhesion resulted in phosphorylation of both Lpl and VASP, and this phosphorylation was abolished by pre-treatment with KT5720 or H89. Blots are representative of 3 separate experiments using neutrophils from 3 separate donors.

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CHAPTER IV.

MARCKS PROTEIN REGULATION OF HUMAN NEUTROPHIL MIGRATION *IN VITRO*

Abstract

Neutrophils migrate into infected tissues where they are essential for host defense. However, the products of activated neutrophils can be quite damaging to host cells. Thus, the mechanism of neutrophil migration is a target of anti-inflammatory drug development. Neutrophils must re-organize the actin cytoskeleton to establish a leading edge pseudopod and a trailing edge uropod in order to migrate. PKC is required for neutrophil migration as is the regulatory molecule calmodulin. The actin binding protein Myristoylated Alanine-Rich C-kinase Substrate (MARCKS), which is phosphorylated by PKC and binds calmodulin, is a potential point of convergence of these two regulatory mechanisms. Stimulation of neutrophils with the chemoattractant peptide fMLF results in rapid (30-60 sec) MARCKS phosphorylation, which correlates with its dissociation from the cell membrane. The hypothesis that MARCKS has a role in the regulation of neutrophil migration was tested using a cell permeant peptide derived from the MARCKS myristoylated aminoterminus (MANS peptide).Treatment of isolated human neutrophils with MANS (50 μM) significantly inhibited their migration and adhesion in response to fMLF, IL8, or LTB_4. In contrast, a missense peptide (RNS peptide) did not affect migration or adhesion. MANS significantly reduced the F-actin content in neutrophils 30s after fMLF-induced polymerization, although the peptide did not alter the ability of cells to polarize or spread. MANS did not alter fMLF-induced increases in surface expression of β2 integrins. Our data suggest that MARCKS, via its myristoylated aminoterminus, is a key regulator of neutrophil migration and adhesion.

Abbreviations: MARCKS, Myristoylated Alanine-Rich C-kinase Substrate; fMLF, formylated-Met-Leu-Phe; IL8, interleukin-8; LTB_4, leukotriene B_4; F-actin, filamentous actin; PMA, phorbol 12-myristate 13-acetate; IC, immune complex; PLC, phospholipase C (PLC); PIP_2, phosphatidyl inositol biphosphate; IP_3, inositol triphosphate; DAG, diacylglycerol; ECM, extracellular matrix

Introduction

Neutrophils are first responders of the immune system and rapidly migrate into developing sites of inflammation to phagocytose bacteria and clear tissue debris. Neutrophils are initially pulled out of the circulation through interactions between the fucose-containing sialyl-Lewis^x residues on neutrophils [1,2] and selectins expressed on an activated endothelium [3]. Through weak endothelial-neutrophil binding, neutrophils escape the high flow circulation and roll along the endothelial membrane, able to sample the local environment. After encountering a gradient of chemoattractant, neutrophils are recruited to the site of inflammation. Once activated, the neutrophil firmly adheres to the endothelium through β2 integrin binding to endothelial cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1). Once firmly bound, the neutrophil undergoes spreading followed by migration across the endothelium into the tissue space. Although required in the tissues for adequate host defense against a range of infectious pathogens, a major component of the clinical syndrome of certain noninfectious diseases is excessive neutrophilic inflammation. Further definition of the molecular mechanisms of neutrophil migration will lead to the development of therapeutics to limit the tissue migration and resulting destruction seen from neutrophils in inflammatory disorders.

Stimulation of chemoattractant receptors activates numerous responses in neutrophils including migration, adhesion, phagocytosis, and respiratory burst, all of which require reorganization of the cytoskeleton [4-7]. Multiple signaling pathways coordinate to precisely direct the neutrophil and enable controlled activation. One pathway that has been shown to

promote migration is mediated by protein kinase C (PKC). PKC is a serine threonine kinase activated by chemoattractant stimulation involved in many cytoskeletal-dependent processes of neutrophils including actin assembly [8-11], degranulation [12], oxidative burst [13-15], adhesion [16,17], and migration [17-20]. Chemoattractant receptor stimulation also activates the hydrolytic phophodiesterase enzyme phospholipase C (PLC) at the plasma membrane resulting in hydrolysis of phosphatidyl inositol biphosphate (PIP_2) and release of secondary messengers inositol triphosphate (IP_3), leading to Ca^{2+} release into the cytosol from the endoplasmic reticulum, and diacylglycerol (DAG) which are responsible for propagation of the signal and activation of PKC, calmodulin and other downstream messengers [21-24] . Neutrophils contain a range of PKC isoforms including the classical Ca^{2+}-sensitive PKCβs and PKCα, the predominant isoforms expressed, the novel Ca^{2+}-independent PKCδ, and the atypical Ca^{2+}- and DAG-independent PKCζ [25].

Through use of specific inhibitors and activators, the PKC pathway has shown to be instrumental in neutrophil migration [17-20], yet the cytoskeletal target mediating the activities of PKC has yet to be elucidated. One potential candidate is the actin binding protein Myristoylated Alanine-Rich C-kinase Substrate (MARCKS). Initially membrane bound, myristoylated membrane-bound MARCKS is rapidly phosphorylated by PKC and as a result translocates to the cytosol following chemoattractant stimulation of neutrophils [26]. Plasma membrane-association of MARCKS can also be disrupted by binding of Ca^{2+}-activated calmodulin, another signaling molecule shown to be required for neutrophil chemotaxis [27]. These mutually exclusive pathways [28] direct the subcellular localization and presumably

the involvement of MARCKS in regulation of the actin cytoskeleton. Although the mechanisms involved in MARCKS-mediated cytoskeletal rearrangement are unknown, there is compelling evidence to suggest an association. MARCKS has been shown to bind and cross-link actin in a variety of cell types [29-31]. Peptides analogous to the effector domain of MARCKS are able to induce actin polymerization and bundle existing filaments [29,32,33] In fibroblasts, MARCKS co-localizes with F-actin in lamellipodia suggesting a close relationship of MARCKS with the cytoskeletal machinery responsible for directed migration [34]. We hypothesized that MARCKS, via its myristoylated aminoterminus, is a key regulator of neutrophil migration and adhesion.

We have utilized a cell-permeant, myristoylated peptide (MANS) identical to the first 24 amino acids of the MARCKS protein to investigate the requirement for the N-terminal region of MARCKS in neutrophil migration. MANS has been previously shown to disrupt MARCKS association with mucin granule membranes [35] and inhibit mucin secretion from epithelial cells and inhibit granule release from leukocytes [31,35-37]. Our results using primary human neutrophils show that MANS peptide, but not RNS, inhibits chemoattractant-induced neutrophil migration and β2 integrin-dependent adhesion *in vitro*. In exploration of the mechanism behind MANS-mediated inhibition of migration, we have shown that MANS reduces F-actin content at 30s after chemoattractant stimulation, but not at later time points as cells are rapidly polarizing. Additionally, we found that MANS does not affect β2 integrin surface expression nor ability of PMA- or fMLF-stimulated neutrophils to polarize or spread.

Our data suggest that MARCKS, via its myristoylated aminoterminus, is a key regulator of neutrophil migration and adhesion.

Materials and Methods

Reagents

Ficoll-Paque Plus, and Dextran T500 were from Amersham Biosciences (Piscataway, NJ). Dimethyl sulfoxide (Me_2SO), fMLF, phorbol 12-myristate 13-acetate (PMA), Triton-X 100, pepstatin, HEPES, o-phenylenediamine (OPD) dihydrochloride, and poly-l-lysine were from Sigma Chemical Co. (St. Louis, MO). Powdered phosphate-buffered saline (PBS) and Hank's balanced salt solution (HBSS) were from Life Technologies (Grand Island, NY). Ethylenediamine tetraacetate dihydrate (EDTA) was from Fisher Scientific (Atlanta, GA). Leukotriene B_4 (LTB_4) was from Cayman Chemical (Ann Arbor, MI). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT). Calcein and Alexa-Fluor-546-phalloidin was obtained from Molecular Probes (Invitrogen; Carlsbad, CA). Diisopropylfluorophosphate (DFP) and human recombinant interleukin-8 (IL-8) were from BD Biosciences (San Diego, CA). Monoclonal antibody IB4 (anti β2, CD18) was prepared as described [38]. FITC-labeled sheep-α-mouse antibody was from eBioscience (San Diego, CA).

Peptides

Synthesis of the MNS and RNS peptides was performed by Genemed Synthesis, Inc. (San Francisco, CA). The MANS peptide is identical to the first 24 amino acids of the MARCKS protein: myristic acid-GAQFSKTAAKGEAAAERPGEAAVA. The RNS peptide is a randomly scrambled control: myristic acid -GTAPAAEGAGAEVKRASAEAKQAF. Both peptides have been previously described [31]. Viability of cells was validated after peptide treatment through trypan blue dye exclusion.

Preparation of neutrophils

Human leukocyte rich plasma was separated from whole blood using dextran sedimentation. Neutrophils were isolated from plasma using a Ficoll gradient. In brief, approximately 6mls of plasma was layered on 5mls of sterile, endotoxin-free Ficoll-Paque solution and spun at 1800 rpm for 20 minutes. Neutrophils were used if they demonstrated greater than 98% viability, as determined by exclusion of trypan blue dye incorporation. Red blood cells were lysed by hypotonic lysis and remaining neutrophils were washed once with HBSS. Cells were re-suspended in HBSS with 20mM HEPES, 8.9 mM sodium bicarbonate, 1mM Ca^{2+} and 1mM Mg^{2+} prior to assays ($HBSS^{++}$).

Chemotaxis Assay

Human neutrophils were isolated and labeled with the cell-permeant fluorescent dye calcein for 30 minutes at room temperature. Cells were then washed and resuspended in a chemotaxis buffer containing $HBSS^{++}$ with 2% FCS. Cells were treated with the indicated

concentration of peptide for 30 minutes at 37 ˚C prior to the placement of 1 x 10^4 cells on a 2uM pore size membrane on a ChemoTx® plate (Neuro Probe, Inc., Gaithersburg, MD). Lower wells of the plate were filled with chemotaxis buffer with or without chemoattractant. Standard wells contained 1 x 10^4 labeled cells. Cells were allowed to migrate for 1 hour at 37˚C. After incubation, cells on the top of the filter were washed away with PBS. 0.5 mM EDTA was added to the top of the filter for 5 min to detach adherent cells. The plate was then centrifuged at 1000 rpm for 1 min. The filter was removed and the fluorescence was measured in the lower wells (485 nm excitation, 530 nm emission wavelengths) using an fMax fluorescence plate reader (Molecular Devices). Percent migration was determined by dividing the fluorescence of each well by the fluorescence of the standard wells containing 1 x 10^4 labeled cells.

Adhesion Assay

Purified human neutrophils (1 x 10^7/mL) were suspended in HBSS and incubated with calcein 1μg/mL for 30 minutes at RT. Cells were washed and resuspended in $HBSS^{++}$ to a final concentration of 2 x 10^6/mL. Cells were treated with varying concentrations (25-50μM) of MANS, RNS, or PBS for 30 minutes at 37° C. Cells from the various treatment groups ($1x10^5$) were added in triplicate to Immulon 2 plates (Dynatech, Chantilly, VA) coated with 5% FCS in PBS or with immune complexes (IC). After incubation at 37° C for 10 minutes, adhesion was stimulated with 100nM fMLF for 3 minutes or 10ng/ml PMA for 30 minutes at 37° C. IC-stimulated groups were incubated for 30 minutes at 37° C. The fluorescence was

measured (485 nm excitation, 530 nm emission wavelengths) using an fMax fluorescence plate reader (Molecular Devices) before and after serial washes with 150 μL PBS to dislodge non-adherent cells. Fluorescence after washing was divided by the fluorescence before washing to calculate percent adhesion.

Coating of Plates with Immune Complexes

Each well of a 24-well sterile tissue culture plate was coated with 200μL of 100μg/ml poly-l-lysine in PBS and incubated at room temperature for 45 minutes. Wells were then washed 3 times with PBS before adding 200μl fresh 1% gluteraldehyde in PBS at room temperature for 15 minutes. Well were again washed 3 times with PBS before 200μl of 100μg/ml BSA in PBS for 4 hours at room temperature. Wells were dumped, then blocked with 200ml of 1% human albumin serum/0.1 M glycine, pH 6.8 in PBS was added overnight at 4 ºC. Wells were washed again 3 times with PBS, before adding 200μl of 1:50 α-BSA antiserum for 2 hours at room temperature. Wells were washed 3 times with PBS before use in experiments.

Actin Polymerization Assay

Human neutrophils were isolated as previously described, washed once in Buffer A [140mM NaCl, 1mM KH_2PO_4, 5mM Na_2PO_4, 1.5mM $CaCl_2$, 0.3mM $MgSO_4$, 1mM $MgCl_2$, 10mM HEPES, pH7.4] before final resuspension in Buffer A at a concentration of 5 x 10^6/ml. 98ul of cell suspension was placed in sterile BD Falcon™ 5ml polystyrene round-bottom tubes (BD Biosciences, Bedford, MA). Cells were stimulated with 100nM fMLF for indicated

lengths of time before fixation with 100ul 3% formaldehyde and 0.1% BSA in PBS for 20 minutes. Cells were then washed once in Buffer A before permeabilization with 0.1% Triton and 1 unit per reaction of Alexa-Fluor-546 phalloidin in 100ul Buffer A for 30 minutes on ice. 400ul of PBS was added to the sample before transfer to a fresh tube for FACS analysis.

CD18 Detection

Neutrophils were isolated and suspended in HBSS with 1mM Ca2+ to a concentration of $4x10^6$/ml. Cell were pretreated with 50uM MANS, RNS, or PBS control for 30 minutes at 37ºC. Cells were then stimulated with 50ng/ml PMA for 15 minutes or fMLF 100nM for 3 minutes at 37ºC. Samples were placed on ice and washed once with wash buffer [1% FBS, 0.1% Na Azide in PBS], centrifuged at 1200rpm for 10 minutes at 4ºC, before incubation with 10ug/ml IB4 antibody (α-CD18) in 200ul wash buffer for 40 minutes on ice. Cells were then washed twice in wash buffer before the cell pellet was resuspended in 200ul of 1:50 dilution of FITC-labeled sheep-α-mouse antibody for 20 minutes on ice. Cells underwent two final washes before resuspension in 1ml of sterile PBS and immediate analysis using a FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The mean fluorescence of each neutrophil population was normalized to the mean fluorescence of vehicle control-treated neutrophils.

Actin Detection

Freshly isolated human neutrophils (0.5×10^6) in $HBSS^{++}$ with 1% BSA were pretreated with 25uM MANS, RNS, or PBS control for 30 minutes at 37ºC. After peptide treatment, cells from each group were added to FCS-coated glass coverslips in wells of a 24-well tissue culture plate. Cells were allowed to adhere to the coverslips before stimulation with 100nM fMLF for 60 seconds or 10ng/ml PMA for 15 minutes at 37°C. The supernatant was removed and cells were fixed with 0.5ml 3% paraformaldehyde solution [25mM PIPES, pH 7, 50mM KCl, 3mM MgCl, 3% paraformaldehyde w/v, 10mM EGTA in sterile water] for 20 minutes. Coverslips were then washed gently with PBS twice and PBS-protein [0.2% gelatin, 0.2% azide, 0.1% ovoalbumin in PBS] twice before extraction with 0.5ml cold Triton lysis buffer [10mM PIPES, pH 6.8, 0.5% Triton-X 100 v/v, 300mM sucrose, 100mM KCl, 3mM $MgCl_2$, in sterile water]. Cells were washed again before staining with Alexa-phalloidin-546 diluted 1:50 in PBS-protein for 30 minutes at room temperature. Coverslips were mounted with a thin film of OPD-glycerol [1mg/ml OPD in glycerol] and Cytoseal™ 60 (Richard-Allen Scientific; Kalamazoo, MI). Fluorescence of Alexa-phalloidin-546-stained F-actin was viewed with a Nikon TE-200 inverted epifluorescence microscope using a digital camera (SPOT, Diagnostics Instruments Inc.) and associated software for image capture. Cells were evaluated for PMA-induced spreading and fMLF-induced polarization. Treatment groups were blinded to the independent slide reader. In the PMA-treated cells, 100 total cells were counted and scored as "spread," as evident by increased surface area, membrane ruffling, and increased formation of F-actin, or "not spread," if they were inactivated, small, round resting

neutrophils. In the fMLF-treated cells, 100 total cells were counted and scored as "unpolarized," if they were inactivated resting cells, "intermediate polarized," evident by increased F-actin and shape change, with no clear leading edge, or "polarized," as evident by redistribution of F-actin to membrane and a shape change with the clear formation of an F-actin rich leading edge.

Statistical Analysis

Data are reported as mean ± SE. Data were analyzed by Student's two-sample t-test assuming equal variance or one-way ANOVA. A $p<0.05$ was considered statistically significant.

Results

MANS peptide significantly reduces fMLF-induced migration in a dose-dependent manner

To investigate the role of MARCKS in neutrophil migration, we pre-treated purified calcein-labeled neutrophils with a range of concentrations (5μM to 50μM) of MANS or control peptide, RNS, for 30 minutes at 37º C before use in a chemotaxis assay to evaluate directed migration as described in *Materials and Methods*. Approximately 55% of cells pre-treated with vehicle control (VC) alone ('0 μM') migrated to lower wells containing the chemoattractant formylated-Met-Leu-Phe (fMLF) (Figure 1). Only 3% of VC-treated cells exhibited random migration by migrating into a lower well containing HBSS buffer alone.

Cells pre-treated with concentrations of 10μM or more of MANS peptide had a significant dose-dependent decrease in percent migration, with complete reduction to background levels at a concentration of 50μM. There was no effect on migration after treatment with concentrations of up to 50μM of the RNS control peptide. Interestingly, although background migration never exceeded 5% in any group, MANS peptide significantly increased background migration as compared to RNS peptide at doses of 10μM or above, although this result was not repeated in subsequent assays. To calculate the IC_{50} we plotted % inhibition, defined as the % reduction in migration of MANS treated cells as compared to vehicle control treated cells against MANS concentration in μM. We determined that the concentration of MANS that inhibits 50% of fMLF-induced migration is 17.1 μM (data not shown).

MANS reduces migration towards alternate chemoattractants

We next evaluated the diversity of the ability of MANS to reduce neutrophil migration and tested the effect on chemotaxis induced by endogenous chemoattractants interleukin-8 (IL8) or leukotriene B_4 (LTB_4). Neutrophils were pre-treated with 50μM of MANS, 50μM of RNS, or vehicle control before use in a chemotaxis assay. Similar to vehicle control treated cells, RNS-treated cells attained 60% migration towards fMLF and 80% migration towards IL8 or LTB_4 (Figure 2). Alternatively, MANS treatment diminished migration towards fMLF, IL8, and LTB_4; the percentage of cells able to migrate into the

lower wells was reduced to 10-12%. Background migration was similar among the treatment groups.

MANS significantly reduces fMLF-induced adhesion

To begin to explore the mechanisms responsible for the impressive ability of MANS to abolish chemotaxis in neutrophils, and ultimately gain insight into the function of the N-terminus of MARCKS, we questioned whether MANS interferes with β2 integrin-dependent adhesion. Before neutrophils can actively migrate they must first adhere to cellular and tissue substrates via β2 integrin ligation [39]. The predominant β2 integrin on the surface of neutrophils is CD11b/CD18 [40], also called Mac-1, which mediates neutrophil adhesion to activated endothelium, fibrinogen, and other matrix proteins derived from plasma, enabling chemotaxis and emigration [41]. We hypothesized that MANS was disrupting neutrophil chemotaxis by interfering with β2-integrin-dependent adhesion and, hence, the ability of cells to initiate migration. Primary neutrophils were pre-treated with 25 or 50 μM of MANS or RNS before use in an adhesion assay as described in *Materials and Methods*. Approximately 50% of vehicle control treated cells ('0 μM') exhibited adhesion in response to fMLF (Figure 3). There was no reduction in fMLF-induced adhesion in cells pre-treated with 25μM or 50μM of RNS. In contrast, there was significant reduction in percent adhesion of cells pre-treated with MANS peptide. Concentrations of 25μM and 50μM MANS resulted in the reduction in adhesion to 24% and 16%, respectively. To calculate the IC_{50} we plotted

% inhibition, defined as the % reduction in adhesion of MANS treated cells as compared to vehicle control treated cells, against MANS concentration in μM. We determined that the concentration of MANS that inhibits 50% of fMLF-induced adhesion is 12.5 μM (data not shown).

MANS reduces both PMA- and IC-induced adhesion

We next questioned whether the effect of MANS was specific to the transient adhesion event induced by chemoattractant activation or, instead, was a more general effect and would also impede the stable adhesion induced by alternate neutrophil activators. To address this question we induced stable adhesion with phorbol 12-myristate 13-acetate (PMA) or through activation with immune complexes (IC). PMA, a potent activator of PKC, induces β2 integrin activation and firm adhesion of neutrophils [17]. Immune complex-induced activation of neutrophils occurs through ligation of Fcγ receptors and similarly results in integrin activation and stable adhesion. Peptide-treated cells were added to FCS-coated wells and subsequently stimulated with PMA, or added to wells coated with stable immune complexes, before serial washes and determination of percent adhesion as described in *Materials and Methods*. RNS-treated cells had similar levels of adhesion as control treated cells ('RNS 0μM') after both PMA and IC stimulation, with both groups exhibiting 35-40% adhesion (Figure 4). Alternatively, MANS-treated cells exhibited a significant reduction in adhesion. Concentrations of 25μM and 50μM of MANS resulted in only 24% and 18%

adhesion after PMA stimulation, respectively. Similarly, 25μM and 50μM of MANS peptide reduced IC-induced adhesion to 20% and 12%, respectively. These data show that MANS peptide has an inherent ability to disrupt β2 integrin-dependent adhesion of neutrophils, independent of the stimulus.

MANS does not affect ability of neutrophils to spread

Neutrophils spread on the ECM before active migration [42]. Spreading is an active process characterized by an increase in the surface area, a reduction in cell height, and a decrease of the distance from the neutrophil to the substratum [42]. Spreading and adhesion work together to provide substantial traction to enable locomotion to be generated by the actin cytoskeletal machinery [42-44]. MARCKS has been shown to play a role in the regulation of cell spreading in cardiomyocytes [45]. Work in fibroblasts has shown that expression of an amino-terminal mutant of MARCKS that disrupts MARCKS localization prevents cell spreading and adhesion [34]. To perhaps reveal a defect in spreading in MANS-treated neutrophils to explain the effect of MANS on migration, we examined the morphology of peptide-treated neutrophils induced to spread through stimulation with PMA. PBS- or peptide-treated cells were plated on FCS-coated glass coverslips, stimulated with PMA for 10 minutes, then fixed, permeabilized and stained with Alexa-phalloidin-546 to stain F-actin. Cells were examined using fluorescence microscopy and scored as "spread" or "not spread" according to the criteria outlined in *Materials and Methods*. Approximately 90-95% of cells were fully spread after 10 minutes of PMA stimulation and there were no

differences between the treatment groups (Figure 5). Although MANS decreased neutrophil β2 integrin-dependent adhesion in a dose-dependent manner (Figure 3), it does not appear to be due to effects on the ability of the cells to spread.

MANS does not affect fMLF-induced surface β2 integrin expression

As circulating neutrophils encounter inflammatory mediators, surface L-selectin, responsible for the initial rolling and contact with endothelial cells, is shed and CD11b/CD18 is upregulated through translocation to the plasma membrane from intracellular secretory vesicles [46,47]. Activation of neutrophils with chemoattractant also induces a clustering of the surface β2 integrins and a change from a low to an intermediate affinity state which results in stable adhesion to tissue substrates [39,48]. Because MANS has been shown to inhibit exocytic processes such as myeloperoxidase release from neutrophils [36] and mucin release from airway epithelial cells [35], we hypothesized that MANS was disrupting adhesion through interference with the ability of neutrophils to upregulate surface β2 integrins. Purified neutrophils were pretreated with 50μM MANS, 50μM RNS, or PBS before stimulation with PMA or fMLF. Surface β2-integrin expression was evaluated using IB4 antibody (α-CD18) and flow cytometric analysis as described in *Materials and Methods*. Both PMA and fMLF induced a significant 1.5-fold increase in surface CD18 in PBS control cells as compared to unstimulated cells ('T=0') (Figure 6). RNS-treated cells and MANS-treated cells exhibited a similar increase in CD18 after both PMA and fMLF stimulation with

both groups expressing a 1.5-fold increase over respective peptide-treated unstimulated cells ('T=0'). From these data we concluded that MANS does not affect the upregulation of the surface β2 integrins and is disrupting adhesion through an alternate mechanism.

MANS blunts the initial fMLF-induced rise in F-actin in the polymerizing neutrophil

In the cytosol of resting neutrophils is a rich supply of actin in monomeric form, G-actin, that is prevented from polymerizing due to its binding to actin-binding proteins such as profilin and thymosin [49-53]. When stimulated by chemoattractants, rapid intracellular signaling results in polymerization of actin and polarization of the neutrophil as it establishes a leading edge in the direction of migration. MARCKS has been shown to bind and cross-link actin *in vitro* [30] and co-localize with F-actin in the leading edge lamellipodium in fibroblasts [34], suggesting a role for MARCKS in dynamic cytoskeletal rearrangement. To determine whether the effect of MANS on migration was due to disruption of chemoattractant-induced actin polymerization, we stimulated peptide-treated cells in suspension with fMLF before fixation, permeabilization, and staining with Alexa-Fluor-546-phalloidin and FACS analysis to quantify F-actin content in the cell, as described in *Materials and Methods*. Stimulation with fMLF resulted in a 2.5 fold increase in F-actin after 30 seconds in PBS-treated control neutrophils, with a rapid return to resting levels by 300s (Figure 7). Cells pre-treated with RNS peptide had similar levels of F-actin as control cells at all time points. In contrast, cells pre-treated with MANS had less than a 2-fold increase in F-actin levels at 30 seconds after fMLF stimulation, which was significantly different from

PBS- and RNS-treated cells, yet had similar levels at 60s, 180s, and 300s where F-actin content continued to decline towards basal levels. We concluded that MANS may interfere with the early immediate polymerization event after chemoattractant stimulation, although, as cells are fully polarizing, MANS-treated cells appear to overcome the effect and achieve equivalent levels of F-actin.

MANS does not affect the ability of cells to polarize

To further explore the effect of MANS in the early polymerization phase of neutrophils, we stimulated neutrophils with fMLF for 60s, the time point that cells achieve maximum polarization and F-actin content in our assay (Figure 6) [54] and examined the morphology of the cells. We scored the extent of polarization in PBS-, RNS-, and MANS-treated cells as "unpolarized," "intermediate polarized," or "fully polarized," as described in *Materials and Methods*. In all treatment groups, less than 10% of cells were "unpolarized" after 60s of fMLF stimulation (Figure 8). There were no notable differences between groups in ability to polarize as 55-65% of cells and 30-40% of cells were "fully polarized" or "intermediately polarized," respectively, after fMLF stimulation. We concluded that although MANS inhibits chemotaxis, it does not appear to affect the ability of neutrophils to initially polarize and establish a direction in response to chemoattractants.

Discussion

We have shown that a cell permeant peptide derived from the MARCKS myristoylated aminoterminus (MANS peptide) inhibits chemotaxis and adhesion in primary human neutrophils with an IC_{50} of 17.1 and 12.5 μM, respectively (Figures 1-4). MANS reduced the fold change in F-actin at 30s after chemoattractant stimulation, but did not affect F-actin at later time points during polarization nor the ability to upregulate surface β2 integrin expression (Figures 6-7). Treatment with MANS peptide did not appear to affect the ability of neutrophils to polarize or spread when visualized by microscopy (Figures 5,8). These data suggest that the N-terminus of MARCKS is essential for neutrophil migration, through a yet unknown mechanism. MARCKS is localized to the plasma membrane in resting cells through hydrophobic interaction between its N-terminal myristoyl moiety and membrane phospholipids and though electrostatic interaction between the basic effector domain and acidic lipid bilayer of the cell membrane [55-57]. Stimulation of neutrophils with fMLF results in rapid phosphorylation of MARCKS, with maximal phosphorylation by 40 seconds, and translocation to the cytosol before dephosphorylation by 4 minutes and a subsequent return to the plasma membrane [26]. We can speculate that MANS is acting in a competitive manner by occupying MARCKS binding sites on the plasma membrane thus preventing endogenous MARCKS from reassociating with the membrane after phosphorylation. MANS has been shown to inhibit MARCKS binding to mucin granule membranes of human bronchial epithelial cells, while not affecting phosphorylation levels [35]. Mutation of the N-terminal myristolylation site of MARCKS reduces membrane

binding [58-60] allowing only weak binding via the PSD domain [57] resulting in mostly cytosolic localization of the protein, that is still able to be phosphorylated by PKC [59]. From these data, we can presume that MANS treatment of neutrophils similarly results in cytosolic localization of the dephosphorylated form of MARCKS in the early stages of migration, and, thus, MARCKS has an integral role at the plasma membrane during the initiation of locomotion and active migration.

The transient pattern of PKC-mediated phosphorylation of MARCKS tightly corresponds to the polymerization of actin in chemoattractant-stimulated neutrophils (Figure 7). At 30s after fMLF-stimulation, the point of maximal F-actin content in the cell during polarization, MARCKS is maximally phosphorylated and redistributed to the cytosolic fraction [26]. As F-actin content returns to baseline levels at 3 minutes post-stimulation, MARCKS becomes dephosphorylated and returns to the membrane [26]. Our data show that MANS peptide reduced F-actin levels at 30 seconds after fMLF-stimulation but did not affect F-actin content at later time points during polarization (Figure 7). MARCKS binds F-actin in the phosphorylation site domain (PSD) and, through alteration of the basic charge of the PSD, PKC-dependent phosphorylation negatively affects the F-actin binding and cross-linking activity of MARCKS [29,30]. Perhaps, in the immediate stages after chemoattractant stimulation when a globally pliable cell membrane is required to achieve a dynamic shape change, PKC-dependent phosphorylation of MARCKS serves to transiently decrease crosslinking and reduce the rigidity of the cell membrane to allow remodeling. As actin continues to polymerize during the shape change, MARCKS returns to the membrane and

resumes crosslinking activity to give the cell membrane the stiffness and architecture it requires for migration.

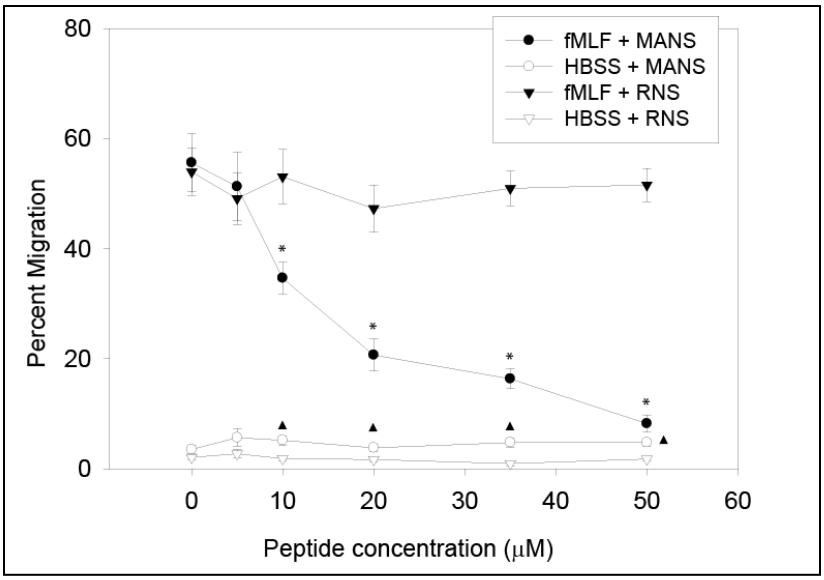
MARCKS has been shown to localize to the leading edge lamellipodium and co-localize with F-actin [34] and PKC [61-64], suggesting that MARCKS is an effector of PKC in migration. Each PKC isoform has different molecular characteristics to determine the subcellular localization and activity of the enzyme in response to a variety of stimuli [65-67]. Work in CHO K1 cells has shown that, in contrast to phorbol esters that result in membrane localization of PKCδ, treatment with hydrogen peroxide results in activation of PKCδ without translocation, highlighting stimulus-dependent control of PKC signaling [59,67]. Interestingly, in the same study, while WT-MARCKS was not phosphorylated after treatment of cells with hydrogen peroxide, a cytosolically-located unmyristoylated mutant was, suggesting that subcellular targeting of PKC may play an important role in regulating the activity of MARCKS [59]. Neutrophils contain PKCβ, PKCα, PKCδ, and the atypical Ca^{2+}- and DAG-independent PKCζ [25]. With the use of specific inhibitors and inhibitory synthetic peptides, the atypical PKCζ has been shown to be responsible for chemoattractant-induced actin assembly, chemotaxis, and β2 integrin-dependent adhesion in neutrophils [17]. But, several studies have failed to show any ability of PKCζ to phosphorylate MARCKS [61,68,69]. By potentially displacing MARCKS to the cytosol, MANS may be inhibiting migration by shuffling MARCKS to an alternate subcellular compartment, limiting further signaling from PKC or other kinases. Further exploration into the PKC regulation of

MARCKS in neutrophil signaling is warranted to understand the requirement for the subcellular localization of PKC and how it may direct the activity of MARCKS in cytoskeletal reorganization.

Along with its role in the cross-linking and bundling of actin [30,33], MARCKS has been implicated in stabilizing focal adhesions [70,71]. A focal adhesion site consists of a cluster of activated integrins on the plasma membrane, bound to the cytoskeleton via adaptor proteins, colocalized with other focal adhesion proteins creating an F-actin rich site of stable attachment to the extracellular matrix (ECM) [72-74]. As highly motile cells, neutrophils have smaller areas of concentrated adhesion, called podosomes, where transient adhesion to the ECM provides the traction necessary for migration [75-77]. In general, integrins on the surface of resting neutrophils, and those in focal adhesion, are fixed and evenly distributed across the cell membrane [78,79]. To achieve localized adhesion, integrins, as transmembrane receptors, are laterally mobile in the plasma membrane and rapidly turnover at the leading edge to provide the necessary "pull" for migration [78,80]. MARCKS has been shown to colocalize with adhesion sites in cultures human macrophage and fibrosarcoma cell lines [64,71]. Overexpression of MARCKS in tumor-derived choroidal melanoma cells (OCM-1) results in increased spreading and formation of focal contacts, [81] while nonphosphorylatable MARCKS mutants increased F-actin cluster mobility in dendritic spines [82] and in growth cones [70]. We have shown that MANS decreases β2 integrin-dependent adhesion (Figures 3 and 4), without altering the ability of cells to spread in response to PMA on FBS (Figure 5) or upregulate surface CD18 expression (Figure 6).

Perhaps MANS is disrupting the ability of MARCKS to regulate integrin mobility in the membrane and preventing strong adhesion, thereby limiting the necessary asymmetry of adhesion for directed migration. Our data support evidence for the stabilization of adhesion sites by MARCKS; MANS-induced disruption of MARCKS function reduces strength of adhesion and abolishes directed migration *in vitro*. Further characterization of MARCKS' association with neutrophil adhesion sites and effects on integrin mobility and affinity may reveal the functional role of MARCKS in neutrophil migration.

Figure 1. MANS peptide significantly reduces fMLF-induced migration in a dose-dependent manner. Purified calcein-labeled neutrophils were pre-treated with the indicated concentrations of MANS or control peptide, RNS, for 30 minutes at 37º C before use in a chemotaxis assay. 1 x 10^4 cells from each group were plated on a 2μm membrane of a ChemoTx® plate overlying wells containing HBSS with or without 100nM fMLF. Standard wells contained 1 x 10^4 cells, representing 100% migration. After 1 hour of incubation, the fluorescence was measured in the lower wells, as described in *Materials and Methods*, and percent migration was calculated by dividing the fluorescence of each well divided by the fluorescence of standard wells and is presented as the mean ± SE. There was no affect on migration after treatment with concentrations up to 50μM of the RNS control peptide. In contrast, there was a dose-dependent decrease in percent migration after treatment with concentrations of MANS greater than 10μM, and a complete reduction to background levels at a concentration of 50μM. Asterisks denote a significant reduction in migration of MANS-treated cells as compared to RNS at the same concentration of peptide ($p<0.05$). The background migration of MANS-treated cells was significantly increased as compared to RNS-treated cells. Triangles denote a significant difference in background migration in MANS-treated cells as compared to RNS at the same concentration of peptide (▲<0.05). Data is representative of three independent experiments.


fMLF + MANS
HBSS + MANS
fMLF + RNS
HBSS + RNS
Percent Migration
Peptide concentration (μM)
0
20
40
60
80
0
10
20
30
40
50
60
*
*
*
*

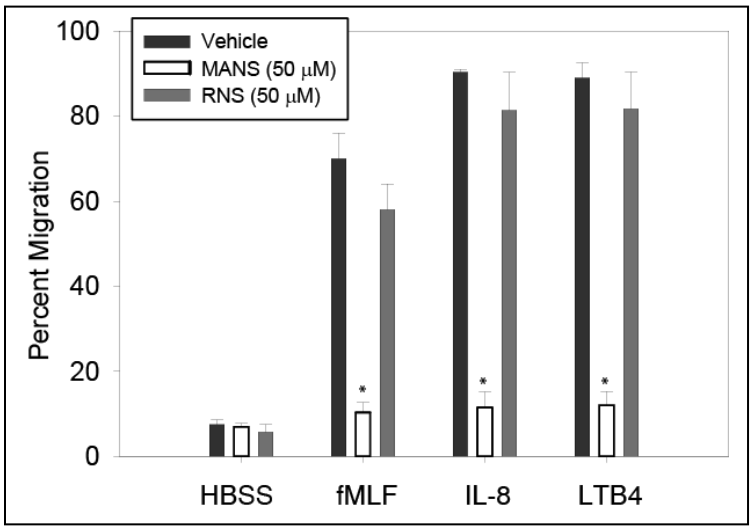


Figure 2. MANS reduces migration towards alternate chemoattractants. Purified calcein-labeled neutrophils were pre-treated with 50μM of MANS, 50μM RNS, or vehicle control for 30 minutes at 37º C before use in a chemotaxis assay as previously described for Figure 1. RNS-treated cells migrated similarly to those treated with vehicle control and attained 60% migration towards fMLF and 80% migration towards IL8 or LTB_4. MANS treatment diminished migration towards fMLF, IL8, and LTB_4 to levels comparable to background migration (HBSS). Asterisks denote a significant decrease in percent migration of cells treated with MANS as compared to RNS ($p<0.05$). Data is representative of three independent experiments.

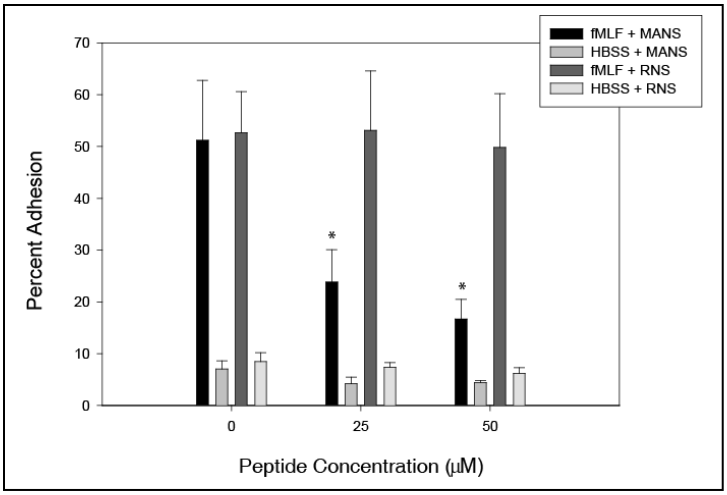


Figure 3. MANS significantly reduces fMLF-induced adhesion. $1x10^5$ purified calcein-labeled neutrophils were pre-treated with the indicated concentrations of MANS or RNS peptide for 30 minutes at 37º C, then plated in triplicate in FCS-coated wells of an Immulon 2 plate. Cells were incubated for 10 minutes at 37 ºC before stimulation with 100nM fMLF or HBSS for 3 minutes. Fluorescence was measured as described in *Materials and Methods* in the wells before and after serial washes with PBS to remove non-adherent cells. Percent adhesion is calculated as the fluorescence of the wells after washes divided by the fluorescence of the wells before washes and data are presented as the mean ± SE. There was no reduction in adhesion after treatment of cells with 25μM or 50μM of RNS peptide as compared to control treated cells ('0'). In contrast, there was significant reduction of adhesion of cells pre-treated with 25μM or 50μM of MANS peptide. Asterisks indicate a significant difference in adhesion in MANS-treated cells as compared to RNS-treated cells at the same concentration of peptide ($p<0.05$). Data is representative of three independent experiments.

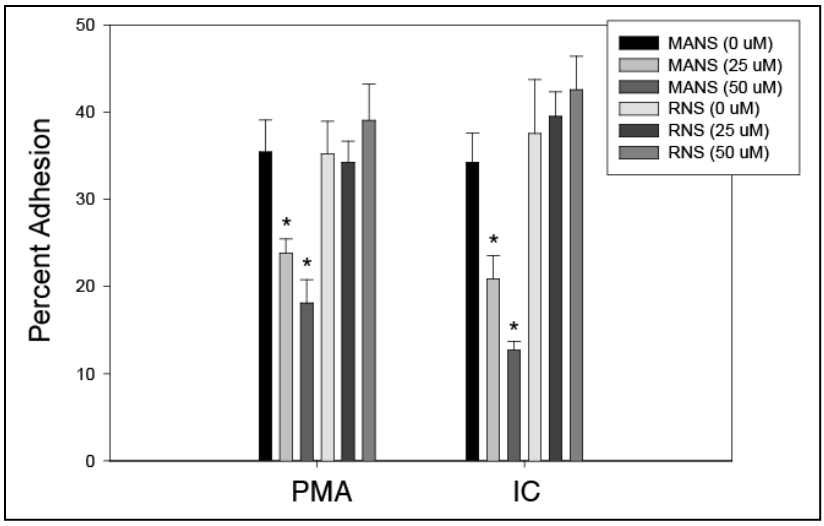


Figure 4. MANS reduces both PMA- and IC-induced adhesion. $1x10^5$ purified calcein-labeled neutrophils were plated in triplicate in FCS- or IC-coated wells of an Immulon 2 plate and incubated for 10 minutes at 37 ºC before stimulation with 10ng/ml PMA or HBSS, respectively, for 30 minutes. Percent adhesion was calculated as described for Figure 3 and data are presented as mean ± SE. RNS-treated cells had similar levels of adhesion as control treated cells ('RNS 0μM') after both PMA and IC stimulation. Alternatively, MANS-treated cells exhibited a significant reduction in adhesion. Asterisks indicate a significant difference between MANS-treated and RNS-treated cells at the same concentration of peptide ($p<0.05$). Data is representative of three independent experiments.

Figure 5. MANS does not affect PMA-induced spreading. Peptide- or PBS-treated cells were plated on FCS-coated glass coverslips, stimulated with PMA for 10 minutes, then fixed, permeabilized, stained with Alexa-phalloidin-546 for F-actin and visualized using fluorescence microscopy. 100 total cells were counted and scored as "spread," as evident by increased surface area, membrane ruffling, and increased formation of F-actin, or "not spread," if they were inactivated, small, round, resting neutrophils. *A*, Representative "spread" neutrophils after peptide or PBS treatment at 40X magnification. *B*, Percentage of total cells spread was calculated and data shown are mean±SE. Approximately 90-95% of cells were fully spread after 10 minutes of PMA stimulation, and there were no differences between the treatment groups. The scoring of cells was blinded to the independent slide reader and results are representative of three independent experiments using separate donors.

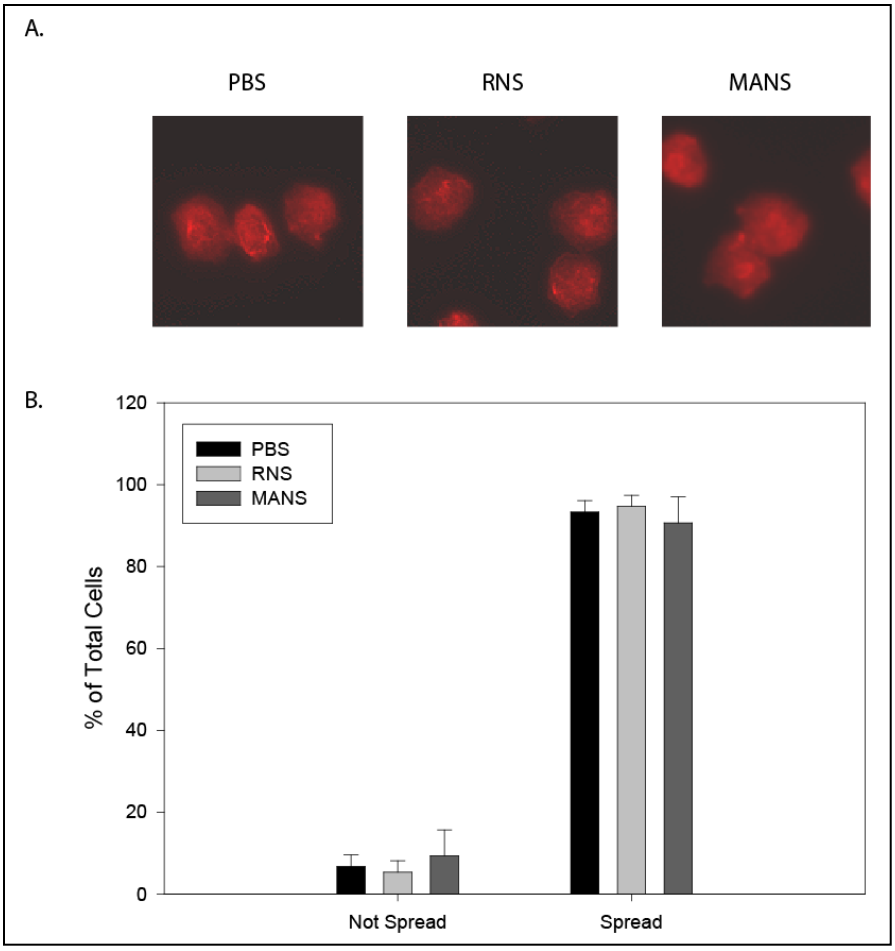

A.
PBS
RNS
MANS
B.
120
100
80
60
40
20
0
% of Total Cells
PBS
RNS
MANS
Not Spread
Spread

Figure 6. MANS does not affect fMLF-induced surface β2 integrin expression. Purified neutrophils were pretreated with 50μM MANS, 50μM RNS, or PBS control for 30 minutes at 37ºC before stimulation with 50ng/ml PMA for 15 minutes or fMLF 100nM for 3 minutes at 37ºC. Samples were placed on ice and washed once before incubation with 10μg/ml IB4 antibody (α-CD18). Cells were then washed twice and resuspended with FITC-labeled sheep-α-mouse antibody. Cells underwent two final washes before suspension in 1ml of sterile PBS and immediate FACS analysis. Both PMA and fMLF induced a significant 1.5-fold increase in CD18 on PBS control cells as compared to unstimulated cells ('T=0') (*$p<0.05$). RNS-treated cells (■$p<0.05$) and MANS-treated cells (▲$p<0.05$) exhibited a similar increase in CD18 after both PMA and fMLF stimulation with both groups expressing a 1.5-fold increase over respective peptide-treated unstimulated cells ('T=0'). Data is representative of three independent experiments using cells from different donors.

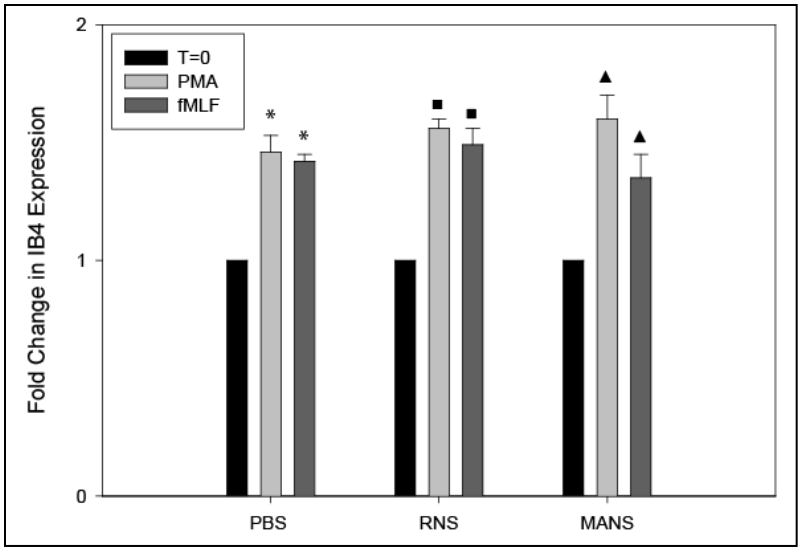

Fold Change in IB4 Expression
2
1
0
T=0
PMA
fMLF
*
*
■
■
▲
▲
PBS
RNS
MANS

Figure 7. MANS blunts the initial fMLF-induced rise in F-actin in the polymerizing neutrophil. Purified neutrophils were pre-treated with 50μM MANS, 50μM RNS, or PBS for 30 minutes at 37º C. Cells from each group were then stimulated with 100nM fMLF for 0, 30s, 60s, 180s, or 300s before fixation, permeabilization, and staining with Alexa-Fluor-546-phalloidin and FACS analysis, as described in *Materials and Methods*. The reported Mean Fluorescence Intensity (MFI) for each sample was divided by the respective "T=0" unstimulated control to determine the fold change in F-actin levels. fMLF stimulation of PBS control cells resulted in a 2.5-fold increase in F-actin content over unstimulated cells ('T=0') at 30 seconds, with a return to resting levels by 300 seconds. RNS-treated cells had similar levels of F-actin at all time points as compared to PBS control. In contrast, cells pre-treated with MANS had a significant decrease in F-actin levels at 30 seconds after fMLF stimulation compared to PBS- and RNS-treated cells, but not at 60s, 180s, or 300s where F-actin content continued to decline towards basal levels. Asterisks indicate a significant difference between fold-change in F-actin of MANS-treated cells as compared to RNS-treated cells ($p<0.05$). Data is representative of three independent experiments.

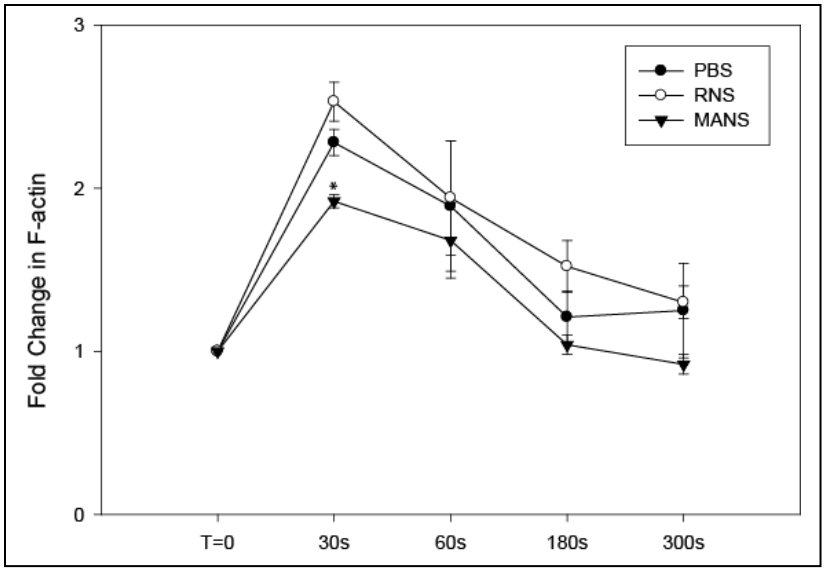
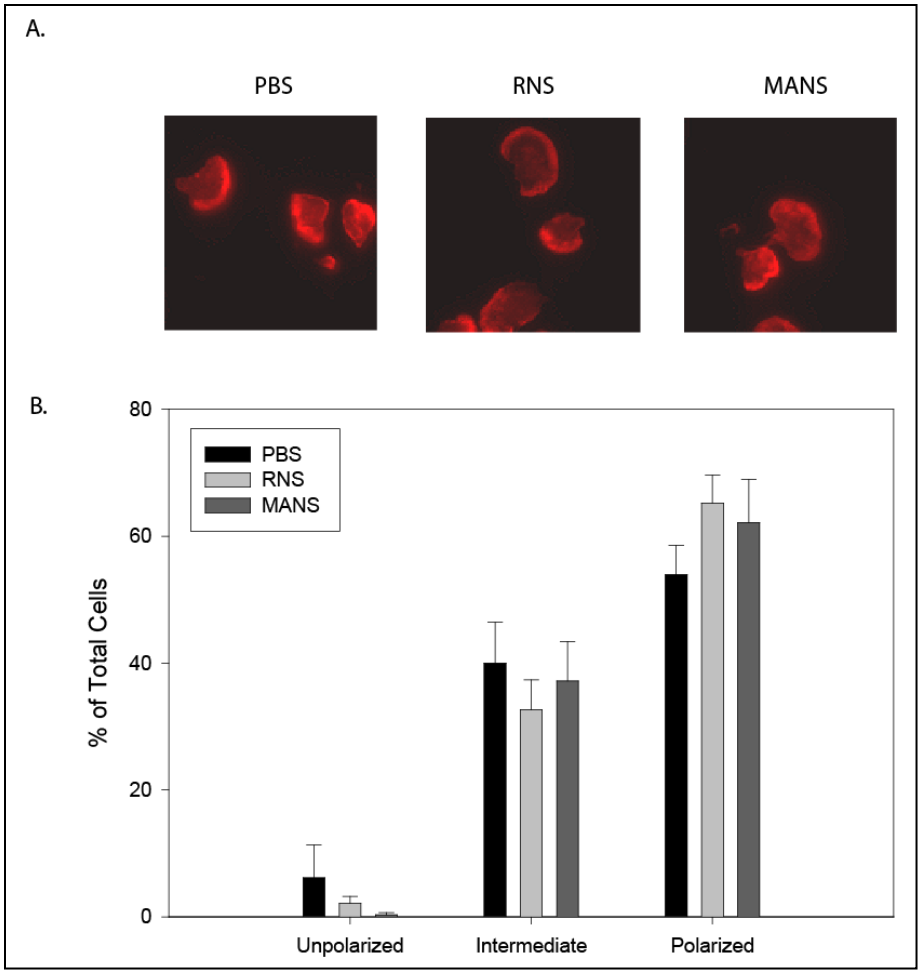

Fold Change in F-actin
3
2
1
0
T=0
30s
60s
180s
300s
PBS
RNS
MANS
*

Figure 8. MANS does not affect fMLF-induced polarization. Peptide- or PBS-treated cells were plated on FCS-coated glass coverslips, stimulated with fMLF for 60s, then fixed, permeabilized, stained with Alexa-phalloidin-546 for F-actin and visualized using fluorescence microscopy. 100 total cells were counted and scored as "unpolarized," if they were inactivated resting cells, "intermediate polarized," evident by increased F-actin and shape change, with no clear leading edge, or "polarized," as evident by redistribution of F-actin to the membrane and a shape change with the clear formation of an F-actin rich leading edge. *A*, Representative "fully polarized" neutrophils after peptide or PBS treatment at 40X magnification. *B*, Percentage of cells "unpolarized," "intermediate polarized," and "fully polarized" was calculated and data shown are mean±SE. In all treatment groups, less than 10% of cells were “unpolarized” after 60s of fMLF stimulation. There were no notable differences between groups in ability to polarize as 55-65% of cells and 30-40% of cells were “fully polarized” or “intermediate polarized,” respectively, after fMLF stimulation The scoring of cells was blinded to the independent slide reader and results are representative of three independent experiments using separate donors.


A.
PBS
RNS
MANS
B.
80
60
40
20
0
% of Total Cells
PBS
RNS
MANS
Unpolarized
Intermediate
Polarized

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CHAPTER V.

DISSERTATION SUMMARY

This thesis encompassed work performed over four years at the NCSU College of Veterinary Medicine in the laboratory of Samuel L. Jones. Chapter I reviewed the molecular mechanisms and signaling events that occur during neutrophil migration. Chapter II explored the requirement of p38 MAPK activity for directed equine neutrophil migration. We have previously shown that p38MAPK is required for LPS-induced COX2 expression, PGE_2 production, and inflammatory cytokine production in equine peripheral blood leukocytes [1, unpublished data]. As inhibition of p38 shows promise as a treatment for endotoxemia in horses, we explored whether inhibition of p38 would limit aberrant neutrophil migration, such as in laminitis. Our finding that p38 is required for chemoattractant-induced migration and polarity of equine neutrophils is novel and provides substantial evidence for investigation into the use of p38 inhibitors in horses *in vivo*.

Chapter III characterized the chemoattractant-induced phosphorylation of serine 157 of Vasodilator-stimulated Phosphoprotein (VASP) in human neutrophils. We found that chemoattractant stimulation results in a PKA-dependent, transient and rapid phosphorylation and dephosphorylation event of serine 157 of VASP. Previous work in our lab has shown that asymmetrical PKA activity is required for neutrophil migration with low PKA activity in the leading edge and high PKA activity in the trailing edge; this is in contrast to findings in fibroblasts where high PKA activity is found in leading edge lamellipodia [2,3]. Further determination of the intracellular localization of VASP during chemoattractant stimulation

and migration will lead to insight into the role of VASP in cytoskeletal rearrangement in neutrophils.

Chapter IV investigated the role of the N-terminal region of the cytoskeletal protein Myristoylated Alanine-Rich C-kinase Substrate (MARCKS) using a cell-permeant peptide identical to the first 24 amino acids of the protein (MANS). Previous work in epithelial cells has shown that MANS displaces MARCKS from reassociation with the granule membrane after phosphorylation and cytosolic localization, presumably by occupying binding sites [4]. Treatment of primary human neutrophils with MANS abolished migration and adhesion, but did not affect polarity or upregulation of β2 integrin expression. Our next step will be to determine the subcellular localization of MARCKS and phosphorylated MARCKS in the neutrophil during chemoattractant-induced cytoskeletal rearrangement using immunofluoresence. Future directions also include using the MANS peptide in a murine air pouch inflammation model to answer the question of whether MANS can inhibit neutrophil chemotaxis *in vivo*.

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